PREPARATION OF COMPONENT I (PREALBUMIN) OF THE BLOOD SERUM OF DIETHYLSTILBESTROL-TREATED COCKERELS

by

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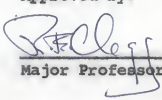

Major Professor

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INTRODUCTION

The serum of the laying hen and the diethylstilbestrol-treated cockerel contains two additional electrophoretic components not evident in the sera of non-laying hens and normal cockerels. These two additional components are related to significant increases in the calcium, phosphorus and total protein content. One of these components moves in front of the albumin and has been designated as prealbumin or Component I. The other component appears in the β-globulin area of the electrophoretic picture and has been designated as Component 6. Component I was found to be present in the bottom portion of the centrifuge tube after ultracentrifugation, whereas Component 6 was found in the top portion (28).

The fractionation and purification of Component I has been carried out. An electrophoretically homogeneous Component I was obtained by salt fractionation and LKB column electrophoresis (6). However, only a small amount of the sample was obtained by this method. The main purpose of this investigation was to overcome this problem of sample size. By using the combination of ultracentrifugation, density gradient centrifugation and DEAE ion-exchange chromatography; a relatively large amount of the purified Component I was obtained. In addition, an attempt was made to carry out a preliminary characterization of Component I.

LITERATURE REVIEW

In the early part of the twentieth century, investigators noticed that the calcium, phosphorus and nitrogen content of the laying hen's serum was somewhat different from that of non-laying hens or cockerels. The pioneers in this field were Laskowski (19) and Hess (14). They reported separately that the total phosphorus content in laying hen serum was higher than the phosphorus in the sera of non-laying hens or cockerels. Hughes (16) confirmed their results. McDonald and Riddle (23) showed further that there were no significant differences in the filtrable calcium, filtrable inorganic phosphorus and non-protein nitrogen between the sera of the laying hens and non-laying hens or cockerels. However, an increase in the non-filtrable calcium, non-filtrable inorganic phosphorus, lipid phosphorus and protein phosphorus was shown. A similar increase in the plasma protein nitrogen of the pigeon was also noted when estrogens were administered.

Deutsch and Goodloe (11) found that the plasma from the female chicken produced electrophoretic patterns which differed considerably from the patterns obtained from males. In comparing these two kinds of patterns, they found that in the female pattern, a component was found in front of the albumin, and the size of the β-globulin fraction was increased. Moore (29) confirmed their

results and further indicated that the differences in the two patterns appeared only after the beginning of sexual maturity and became more significant with age. Kibrick and Blonstein (17) reported that the total protein and the β-globulin fraction of the sera increased considerably with age. Brandt et al. (3) confirmed these results and indicated that, in general, the total protein, α-globulin fraction and β-globulin fraction increased with age, while the albumin and the γ-globulin fraction remained unaltered. At the same time, they noted that a fast moving fraction was present in the electrophoretic pattern of the sera of laying hens but absent from the sera of the non-laying hens and cockerels. The fast moving component appeared at the start of egg production and indicated that there may be some relationship between these additional components and egg production. Sendroy and Collison (40) indicated that the serum of female pigeons during the egg laying period showed a significant increase in the β-globulin area, and a fast moving component was present on the electrophoretic pattern.

Moore (30) found that the electrophoretic pattern of the female hormone treated cockerel serum was similar to the laying hen pattern, Clegg et al. (9) confirmed these results and indicated that injection of diethylstilbestrol into cockerels brought about changes in the serum protein of these birds which were similar to that of laying hens. The fast moving fraction was designated as

Component I, and the component which caused the increased size of the β-globulin area was designated as Component 6.

In comparing the electrophoretic patterns of chicken sera before and after ether extraction, Moore (30) showed that much of the lipid had been removed from the sera. The ether extraction method of McFarlane (24) reduced the area represented by several electrophoretic components and caused some of them to disappear completely, and at the same time, the nitrogen content was decreased. Ericson (12) showed that the electrophoretic pattern of cockerel blood serum was not affected by extraction with cold ether, but that on ether extraction, the pattern of diethylstilbestrol-treated cockerels changed in the same manner as the pattern of the laying hens which showed a decrease in the size of the β-globulin and a disappearance of the prealbumin. This indicated that Components I and 6 probably were lipoproteins.

Calcium exists in the blood in two forms, namely, filtrable calcium and non-filtrable calcium. By the method of Nicholas (32), Correll and Hughes (10) reported that the total calcium content in serum of the laying hen increased a great deal while the filtrable calcium remained the same. McDonald and Riddle (23) indicated that injection of larger doses of estrogens markedly increased the calcium and phosphorus content in normal, parathyroidectomized, and hypophysectomized pigeons. They indicated also that the phospho-

protein may be involved in the calcium binding. Hein and Clegg (13) reported that the increase of non-filtrable and total calcium as associated with the appearance of Components I and 6 in laying hen serum and diethylstilbestrol-treated cockerel serum. Clegg et al. (7) indicated further that the presence of calcium in the buffer did not change the number and order of the electrophoretic components of the normal cockerel serum but reduced the mobility of the fast moving Component I of the laying hen serum and of the diethylstilbestrol-treated cockerel serum. In this case, Component I migrated in the area considered as β-globulin instead of in front of the albumin. This related the non-filtrable calcium to Component I. They concluded that most of the calcium binding ability was the property of Component I.

In order to determine the P^{32} activity associated with the blood serum and egg yolk proteins after the administration of radioactive phosphorus, Clegg and Hein (8) employed moving boundary electrophoresis and radioactive tracer techniques and found that the electrophoretic Components I and 6 of the diethylstilbestrol-treated rooster blood serum exhibited a very high phosphorus activity. A similar result was obtained from the laying hen serum. Ericson (12) in his study on the phosphorus distribution in the electrophoretic components of the diethylstilbestrol-injected cockerel and laying hen found that maximum activity of

P^{32} was established in the blood serum of the chicken after five days of daily doses of P^{32}. He confirmed the results of Clegg and Hein (8) that Component 6 contained the largest percentage of the total serum P^{32} associated with the serum protein, although Component I exhibited the highest P^{32} activity, (P/N ratio). According to Chargaff (5), phosphorus may play an important role in the binding of lipid to protein. Since these components increased in amount when the chicken began egg production (3), it is apparent that they may be related to the high phosphorus containing proteins of the egg yolk.

By dilution, Laskowski (20) precipitated a phosphoprotein fraction from laying hen serum. McKinley et al. (26) (27), on the other hand obtained a precipitate from estrogenized pullets. By using an isoelectric precipitation method, McIndoe (25) isolated two kinds of phospholipoprotein and designated them as "light" phospholipoprotein and "dense" phospholipoprotein. Schjeide and Urist (38) had found two new components in the estrone-injected rooster serum by the ultracentrifugation method. One of the newly formed components was designated as Component X_1 which was a phosphoprotein while the other component was designated as Component X_2 which was phospholipoprotein. They (39) indicated that these components were responsible for the calcium binding. Misra (28) prepared an electrophoretically and ultracentrifugally

homogeneous Component 6 from the laying hen serum and the diethylstilbestrol-treated cockerel serum by using the combined method of salt fractionation, ultracentrifugation and electrophoresis. Malik (21) determined the amino acids present and the NH_2-terminal amino acids of Component 6. He indicated the whole Component 6 contained alanine and isoleucine as the NH_2-terminal amino acids, whereas delipidized Component 6 had threonine as an NH_2-terminal amino acid in addition to the alanine and isoleucine.

Component I was found mainly in the "Bottom Fraction" of the laying hens sera and the sera obtained from the diethylstilbestrol-treated cockerels after ultracentrifugation. By the methods of moving boundary electrophoresis, salt fractionation and LKB column electrophoresis; Chen (6) prepared a rather pure Component I which was electrophoretically homogeneous.

This investigation was intended to develop a method for obtaining larger amounts of purified Component I from the diethylstilbestrol-treated cockerels.

MATERIALS AND METHODS

Sample Preparation

Non-laying hens, laying hens and cockerels were used as the experimental animals in this investigation. They were from one to two years old and were housed at the Kansas State University Poultry Farm. The cockerels and non-laying hens were injected daily intramuscularly with 7.5 mg. of diethylstilbestrol in 0.5 ml. of propylene glycol for seven days, and were sacrificed on the eighth day. The blood was collected in 50 ml. centrifuge tubes (1" x 4"). The blood of normal cockerels and of the laying hens used for comparison was taken from the wing vein (about 10 ml. for each bird). The blood was clotted at 38°C. for two or three hours and then centrifuged at 3,000 r.p.m. for 20 min. at 0°C. The serum was collected and stored at 5°C. overnight.

Preparation of the Bottom Fraction

In order to obtain a preliminary separation of the components of the whole serum, ultracentrifugation was employed. A Spinco Preparative Ultracentrifuge Model L, manufactured by Beckman Instruments Inc., Spinco Division, Palo Alto, California, was used.

Lusteroid cellulose tubes (5/8" x 3") were filled fully with

the serum and capped tightly with aluminum caps. The filled tubes were placed in the No. 40 Rotor which had been previously cooled, and the sample was then centrifuged at 38,000 r.p.m. for 16 hours at 0°C. Three fractions were apparent after centrifugation. These three fractions were designated as "Top Fraction", "Middle Fraction" and "Bottom Fraction". Misra (28) and Malik (21) had prepared a homogeneous Component 6 from the "Top Fraction", while Chen (6) had found that the "Bottom Fraction" contained a high concentration of Component I. This "Bottom Fraction" was used as the starting material in this investigation

Buffers

According to the results of Brandt and Clegg (3), a borate chloride buffer of pH 8.6 was suitable for the separation of Component I from the blood serum in electrophoretic studies. The borate chloride buffer had the following composition: 0.05 M boric acid; 0.05 M potassium chloride and 0.012M sodium hydroxide. The pH of the buffer was adjusted with sodium hydroxide to pH 8.6.

The calcium containing buffer was prepared as follows: 340 mg. of calcium carbonate was dissolved in the borate chloride buffer by bringing the buffer to pH 3.0 with hydrochloric acid and then adjusted to pH 8.6 again by using sodium hydroxide solution.

Moving Boundary Electrophoresis

The Aminco Portable Electrophoresis Apparatus, manufactured by the American Instrument Company Inc., Silver Springs, Maryland, was used in this investigation. The technique had been described by McColloch (22), Stanley (44), Brandt (4) and Ericson (12).

Before electrophoresis, the sample was diluted with and dialyzed overnight against a suitable buffer at 5°C. to give a final concentration of approximately 1% protein. The electrophoresis was conducted at 20 ma. and 0°C. The ascending boundaries were photographed by means of the Schlieren Lens System with Kodak Contrast Panchromatic Sheet Film.

Density Gradient Centrifugation

Density gradient centrifugation is a method for the separation of particles into zones by centrifugation. A solution of the particles is layered on the top of a density gradient column prepared from some solvent and solute in which the particles are soluble. The steep positive density gradient supports the layer of particles and prevents sedimentation in the liquid and at the same time insures that the particles will migrate at a steady rate. When the material is thus arranged, the particles will separate into zones as they migrate through the column under the influence of centrifugal force. Each zone will consist of one type of molecule,

moving through the gradient at its characteristic sedimentation velocity. If the centrifuge is stopped while the molecules are still sedimenting, the molecules will then be separated according to the differences in their sedimentation rates.

The ideal gradient-forming materials (43) would be chemically inert to the molecules being studied, non-toxic, readily soluble in water and salt solution of high density and of high molecular weight. The high density is required to form a steep gradient and the high molecular weight to reduce the osmotic pressure of the gradient in concentrated solutions. Along with these, the material should be of low viscosity in order to permit rapid sedimentation and ease of fractionation. If the particles of interest are later to be analyzed for total protein content, the gradient material should not contain any nitrogen.

In this investigation, the density gradient centrifugation was conducted by the swinging-bucket rotor, i.e., "25-D Rotor", manufactured by Beckman Instruments, Inc. Sucrose solution was used as the gradient-forming material. The concentrations of the sucrose solutions were: 10%; 20%; 30%; 40%; and 50%.in the borate chloride buffer solution (pH 8.6). Five ml. portions of each of the sucrose solutions were introduced into the centrifuge tubes (1" x 3") by means of a pipette in the order of their increasing density. After the gradient was formed, 5 ml. of the sample

(the borate chloride solution of the "Bottom Fraction" obtained after ultracentrifugation) was added to the top of the gradients. The tube was covered tightly with the aluminum cap and fixed on the 25-D Rotor. The centrifugation was carried out at 15,000 r.p.m. for 16 hours. In addition to certain fractions visible as layers in the sucrose solution, a precipitate was found at the end of centrifugation. This precipitate was analyzed by moving boundary electrophoresis, and a further fractionation of it was carried out by means of ion-exchange chromatography.

Ion-Exchange Chromatography

A group of cellulose ion-exchangers, developed by Sober et al. (41) possessed a high capacity for the adsorption of proteins and released the adsorbed material completely under mild conditions. The adsorption capacity of these substances was greatest at low salt concentration and desorption was favored by increased salt concentration and/or change of pH.

According to the different affinity of the adsorbent and eluant, proteins were separated by increasing the salt concentration and/or changing the pH of the eluant carried out in continuous or stepwise manner. Sober and Peterson (37) had carried out the chromatography of human serum and horse serum. Misra (28) had accomplished the fractionation of chicken whole serum, and a

separation of Component 6 also had been carried out by this process. The present investigation employed the DEAE-SF anion-exchanger as the adsorbant to separate the precipitate obtained from the density gradient centrifugation in order to prepare Component I.

To pack the column, the dry powder of DEAE-SF anion-exchanger was suspended in a suitable amount of distilled water, and was stirred and mixed thoroughly. The water was removed by means of filtration. The wet DEAE-SF was then put into a large amount of buffer solution and was stirred and suspended in the buffer solution. The DEAE-SF buffer suspension was then stored overnight at room temperature. The column was first filled with buffer solution, and the flow rate was adjusted by means of the screw on the rubber tube attached to the outlet tube. The DEAE-SF buffer suspension was poured on the top of the buffer filled column at a slow rate. After the column was filled with the ion-exchanger, a piece of filter paper was put on the top of the ion-exchanger and the column stored in the cold room (7°C.) overnight.

Before putting the sample on the ion-exchanger column, the packed column was saturated with borate chloride buffer and adjusted to a constant flow rate. The sample solution (the borate chloride buffer solution of the precipitate obtained from the density gradient centrifugation) was then introduced into the top of the column by means of a syringe. A separation was effected

stepwise by gradient elution, by using various salt concentrations (0.00 M to 0.35 M sodium chloride solution).

The eluate was collected in a fraction collector at a flow rate of 10 ml./30 min. Five milliliters of the eluate were collected in each tube, and the position of the protein in the various tubes was determined at 280 mu. in a Beckman Spectrophotometer, Model D U.

Concentration of Samples

In the concentration of a protein solution, the main difficulty is to keep the salt and hydrogen ion concentration in the solution constant and to maintain the biological activity of the protein. Freeze-drying and Vacuum-distillation are not suitable for this purpose. Ultrafiltration (35) has been widely used in the concentration of protein solutions. It solves the difficulties of maintaining salt and hydrogen ion concentrations of the protein solution, but it has the disadvantage of the long period of time required for the concentration process.

The use of high water-absorbing agents has made possible a rapid method of concentrating protein solutions. Koln (18) used polyethylene-glycol (Carbowax' 20 M) as the absorption agent in the concentration of protein solutions. Plamstierna (36) used Cellugel Super (3000), a carboxy-methyl-cellulose derivative, as the absorption agent. The solution to be concentrated was

enclosed in a dialysis bag, and the bag placed on a bed of dry Cellugel and covered with the drying agent. A high speed of concentration was obtained. In this investigation, Biodryex, manufactured by Lövadalens Industri Aktiebolag, Centralpalatset, Stockholm C., Sweden., was used as the absorption agent. The solution to be concentrated was put in a dialysis tube and then put on a bed of the Biodryex and covered with the Biodryex. It gave a desirable concentration rate and one hundred milliliters of the protein solution was concentrated to 20 ml. overnight at 5°C.

The concentrated protein solution then was dialyzed against distilled water for 24 hours to remove the excess salt. The solution was then subjected to lyophilization in a Virtis Freeze-Mobile manufactured by Vir Tis Co. Inc., Gardiner., N.Y.

Sodium Dextran Sulfate Treatment

A simple and rapid method for removing the lipoprotein from human serum had been reported by Oncley (34) and Bernfeld (1). This method is based on the interaction between lipoprotein and macromolecular sulfated polysaccharides. Homma (15) indicated that the lipoprotein of laying hen serum and diethylstilbestrol-treated non-laying hen serum could be removed as a precipitate by the addition of the sodium salt of dextran sulfate. In this

investigation, Sodium Dextran Sulfate (from dextran with M.W. 2×10^6), manufactured by Pharmacia, Uppsala, Sweden., was used as the precipitating agent. Ten ml. of the sample was added to 20 mg. of the sodium dextran sulfate, and a yellowish-white precipitate was produced. The mixture was then centrifuged at 5,000 r.p.m. for 30 min. at 0°C. At the end of the centrifugation, the dextran-lipoprotein complex was precipitated. The supernatant was collected and dialyzed against the borate chloride buffer (pH 8.6) for 24 hours, and the differences of the electrophoretic components of the sample before and after the treatment of dextran sulfate were indicated by electrophoretic analysis.

Delipidization

The protein was delipidized according to the method of Nelson and Freeman (31). The protein was transferred into a 150 ml. beaker and treated with 83 ml. of absolute methanol with constant stirring for 30 min. Eighty-three milliliters of chloroform were added, and the contents were transferred to a 250 ml. volumetric flask which was kept in a water bath at 60°C. for 15 min. The flask then was cooled and the volume was made up to 250 ml. with chloroform. The precipitated protein was separated from the solvent by means of a Buchner funnel and dried in an oven at 105°C. for a half hour and weighed.

The starting substances were obtained by lyophilization.

ANALYTICAL PROCEDURES

I. Nitrogen Determination

A Micro-Kjeldahl method (33) was used for the nitrogen determination of Component I. About one mg. of the dry powder and delipidized Component I was weighed into the Kjeldahl flask, and to this flask was added 1 ml. of Conc. H_2SO_4 and a few mg. of catalyst consisting of one part of K_2SO_4 and three parts of $CuSO_4$. The flask was heated on the micro-electric heater until the contents were digested, and a few drops of 30% H_2O_2 were added to the flask after it was cold. The flask then was heated again until the contents became colorless.

The flask was cooled, and the digest was transferred quantitatively to the micro-Kjeldahl distillation apparatus. Seven milliliters of 30% sodium hydroxide were added to it, and the digest was steam distillated. The distillate was collected in 5 ml. of 4% boric acid solution containing a few drops of a mixed indicator composed of 100 ml. of 0.03% methyl-red and 15 ml. of 0.1% methylene blue, then titrated with 0.01 N H_2SO_4. A standard solution containing 0.6023 mg. of nitrogen per ml. was prepared and treated in the same way as the unknown sample. Along with these, a blank was also prepared.

Calculation: The mg. of nitrogen per mg. of total sample weight was calculated as follows:

$$\text{mg. of nitrogen per mg. of sample} = \frac{\text{ml. of } H_2SO_4 \text{ for sample} \times 0.6023}{\text{ml. of } H_2SO_4 \text{ for standard} \times \text{sample wt.}}$$

II. Phosphorus Determination

The method of Sperry (42) was employed in this investigation. One milligram of the dry Component I was weighed in the digesting flask. One ml. of 70% perchloric acid was added to the flask. The flask was placed on the micro-electric heater and digested for two hours. After digestion, the flask was cooled, and five ml. of distilled water were added. The sample was then subjected to color development. Four milliliters of dilute aminonaphtholsulfonic acid-sulfite solution (stock solution diluted 1:100 with sulfite solution) were added to each sample, followed by 2 ml. of 1.25% ammonium molybdate solution. The sample was shaken, and the absorbance was read after 20 minutes. The Klett-Summerson Photoelectric Colorimeter, manufactured by Klett MFG. Co., N.Y. was employed with No. 40 and No. 42 filters. A series of standard phosphorus stock solutions were treated in the same way as the unknown samples. The colorimeter was adjusted to zero against distilled water, and the reading of the blank was substrated from all of the readings of the samples and standards.

Calculation: Milligrams of phosphorus per mg. of sample weight was calculated as follows:

$$\text{Mg. of phosphorus per mg. of sample} = \frac{\text{phosphorus present according to calibration curve}}{\text{Sample weight}}$$

EXPERIMENTAL AND RESULTS

An increase of the protein, phosphorus and lipid content of the blood serum as the chicken starts egg production has been observed. The same change was noted in the blood sera of cockerels injected with diethylstilbestrol. From the electrophoretic studies, two new components were found in the sera. One of these components moved in front of the albumin and has been designated as pre-albumin or Component I. The other moved in the area of the β-globulin and has been designated as Component 6.

Chen (6) found that Component I was present mainly in the "Bottom Portion" of serum of laying hens or diethylstilbestrol-treated cockerels after ultracentrifugation. Therefore the "Bottom Portion" of the diethylstilbestrol-treated cockerel blood serum after ultracentrifugation was used as the starting material for the preparation of Component I.

Characterization of the Sera. The differences of the electrophoretic patterns of the sera of laying hens, non-laying hens, normal cockerels and the diethylstilbestrol-treated cockerels have been indicated by many authors (3,9,12,13,7,8,28).

For the purpose of reference, moving boundary electrophoresis of the different samples were carried out, and the characteristic electrophoretic patterns are shown in Plate I. As shown in A and

B of Plate I, the electrophoretic pattern of non-laying hen serum was almost identical to that of the normal cockerel serum. On the other hand, the electrophoretic pattern of the laying hen serum was approximately similar to the pattern of the diethylstilbestrol-treated cockerel serum (C and D of Plate I). The differences in electrophoretic patterns were shown clearly in the increasing size of the β-globulin area and the extra peak appearing in front of the albumin area. The component responsible for the increase of the β-globulin size has been designated as Component 6, while the component responsible for the extra peak in front of the albumin has been designated as Component I, the prealbumin.

The electrophoreses were conducted at a current of 20 ma. for 60 min. at 0°C. The electrophoretic patterns were photographed on Kodak Contrast Process Panchromatic Sheet Film.

Fractionation by Ultracentrifugation. Ultracentrifugation was employed for the separation of the "Bottom Fraction" from the whole serum of the diethylstilbestrol-treated cockerels or non-laying hens. For each experiment, six of the diethylstilbestrol-treated chickens were sacrificed, and the ultracentrifugation was carried out as previously described.

After ultracentrifugation, three distinct layers were found in the centrifuge tube as shown in Plate II. The "Top Fraction" was a pale yellow waxy substance, which was easily removed by

EXPLANATION OF PLATE I

A comparison of the electrophoretic patterns of:

(A) Whole serum obtained from the non-laying hens.

(B) Whole serum obtained from the normal cockerels.

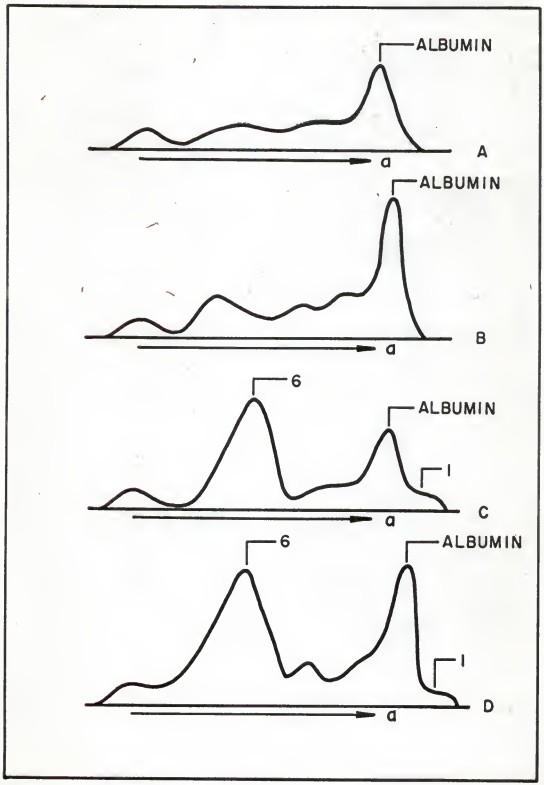
(C) Whole serum obtained from the laying-hens.

(D) Whole serum obtained from the diethylstilbestrol-treated cockerels.

The electrophoretic analyses were conducted under the condition of:

Buffer:	Borate chloride buffer (0.05 M. pH 8.6)
Time:	60 Minutes
Temperature:	0°C.
Current:	20 ma.

PLATE I



means of a spatula. The "Middle Fraction" was a slight yellowish liquid, which was removed by decantation. And the "Bottom Fraction" was a reddish, semi-solid substance which remained in the centrifuge tube.

The "Bottom Fraction" obtained from the ultracentrifugation was dissolved in a minimum amount of the borate chloride buffer (pH 8.6). Five milliliters of the "Bottom Fraction" solution were diluted to 20 ml. with the same buffer. In order to run the moving boundary electrophoretic analysis, this solution was divided into two equal portions. One was dialyzed against the borate chloride buffer solution (pH 8.6); while the other portion of it was dialyzed against the calcium containing borate chloride buffer solution (pH 8.6). After 24 hours dialysis, the electrophoretic analyses were carried out. The results are shown in Plate III.

When the pattern of "Bottom Fraction" (A of Plate I) is compared with the whole serum (D of Plate I), an increase in the size of Component I and a decrease in the size of Component 6 were evident. This indicated that the "Bottom Fraction" contained a higher percentage of Component I and a lower percentage of Component 6 than the whole serum.

A comparison of the patterns A and B of Plate III indicated that the Component I in pattern B moved in the β-globulin area

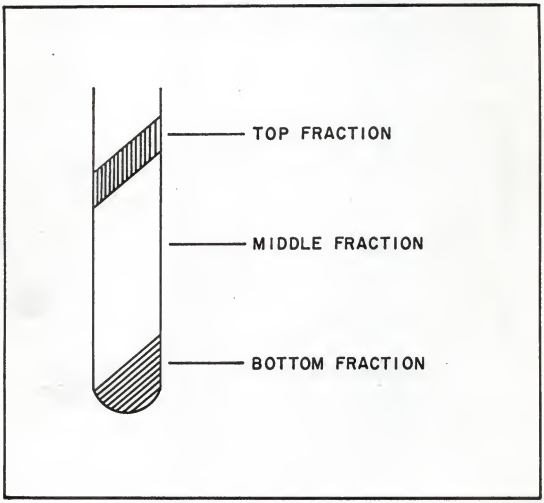
EXPLANATION OF PLATE II

A schematic diagram of diethylstilbestrol-treated cockerel serum fractions obtained upon centrifugation at 38,000 r.p.m. at 0°C. for 16 hours in a Spinco Ultracentifuge Model L.

PLATE II

DIETHYLSTILBESTROL-INJECTED COCKEREL

SERUM FRACTIONS



EXPLANATION OF PLATE III

Electrophoretic patterns of:

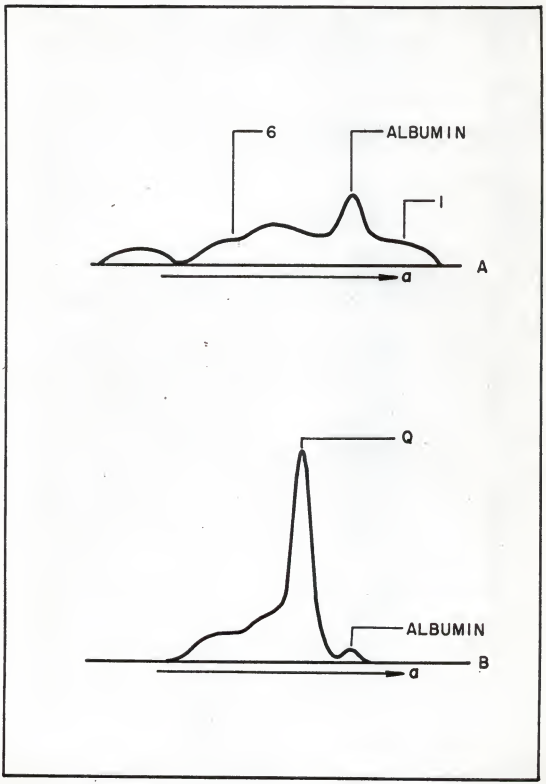
(A) "Bottom Fraction" on dialysis against borate chloride buffer at pH 8.6

(B) "Bottom Fraction" on dialysis against calcium containing borate chloride buffer at pH 8.6

The "Bottom Fraction" was obtained from the whole sera of the diethylstilbestrol-treated cockerels by means of centrifugation at 38,000 r.p.m. for 16 hours by removing of the "Top Fraction" and the "Middle Fraction". The "Bottom Fraction" remained in the tube. The electrophoretic analyses were conducted under the condition of:

Buffer	Borate chloride buffer at pH 8.6 with or without calcium ion.
Time	60 min.
Temperature	0°C.
Current	20 ma.

PLATE III



designated as "Q" instead of as a prealbumin. This agreed with the observation of Clegg (7) that the Component I moved in the β-globulin area when sufficient calcium ion was present. The change in the mobility of Component I was a good method of indicating its presence in a fraction.

Application of Density Gradient Centrifugation. Since Brakke (2) had employed the density gradient centrifugation method in his study of the separation of potato yellow-drawf virus, this method has been widely used in the separation of viruses, cell particules, lipoproteins and human serum. In this investigation, an attempt was made to separate Component I from the diethylstilbestrol-treated cockerel serum by using the method of density gradient centrifugation.

The "Bottom Fraction" obtained from the ultracentrifugation of the diethylstilbestrol-treated cockerel serum was dissolved in a minimum amount of the borate chloride buffer (pH 8.6) and clarified by mild centrifugation at 5,000 r.p.m. for 20 min. at 0°C. The supernatant was then used as the starting material in the density gradient centrifugation.

Sucrose was used as the density gradient forming agent, and the centrifugation was carried out as previously described. A series of preliminary studies indicated that Component I was concentrated in the precipitate formed during the density gradient centrifugation.

The precipitate was obtained by pouring off the supernatant. It was a pale yellow semi-solid substance. The precipitate was dissolved in a minimum amount of the borate chloride buffer (pH 8.6), and three milliliters of this solution were diluted to 10 ml. with the same buffer. One half of this was dialyzed against the calcium-containing borate chloride buffer at pH 8.6 while the other half was dialyzed against the borate chloride buffer at pH 8.6. After 24 hours dialysis, the electrophoretic analyses were carried out. The results are shown in Plate IV.

The precipitate was an electrophoretically homogeneous component as shown in A of Plate IV, and it possessed the characteristic of Component I in that its mobility was decreased markedly in the presence of the calcium ion (as shown in B of Plate IV). In addition, it appeared as a prealbumin when it was mixed with normal cockerel serum (C of Plate IV). Further purification of this "precipitate" was carried out by the next step.

Application of Ion-Exchange Chromatography. The starting material in this process was the precipitate obtained from the density gradient centrifugation of the "Bottom Fraction" of the diethylstilbestrol-treated cockerel serum after ultracentrifugation. The precipitate was dissolved in the minimum amount of the borate chloride buffer and clarified with mild centrifugation at 5,000 r.p.m. for 20 min. It then was dialyzed against the borate chloride buffer (pH 8.6) for 24 hours. Ten milliliters of

EXPLANATION OF PLATE IV

Electrophoretic patterns of:

(A) "Precipitate" on dialysis against the borate chloride buffer pH 8.6

(B) "Precipitate" on dialysis against the calcium-containing borate chloride buffer pH 8.6

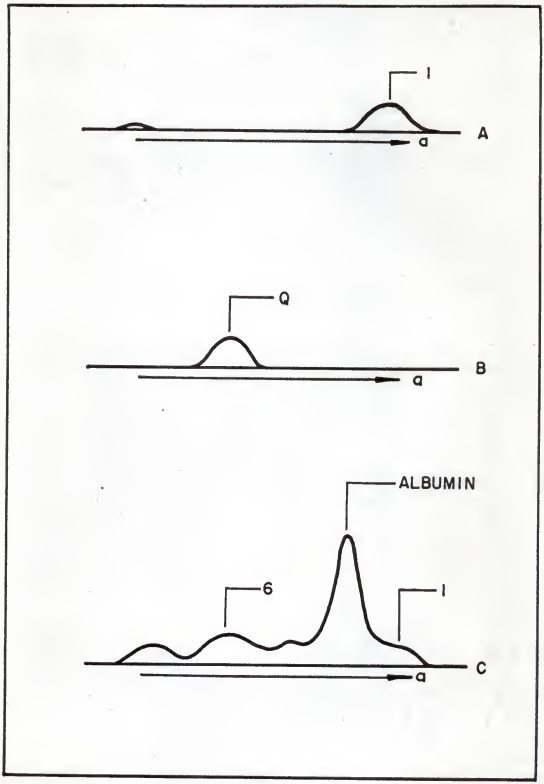
(C) Combined mixture of "Precipitate" and "normal cockerel whole serum" on dialysis against the borate chloride buffer pH 8.6

"Precipitate" was obtained from the "Bottom Fraction" of the whole diethylstilbestrol-treated cockerel serum after density gradient centrifugation.

The electrophoretic analyses were conducted under the condition of:

Buffer	Borate chloride buffer with or without calcium ion pH 8.6
Time	60 min.
Temperature	0°C.
Current	20 ma.

PLATE IV



this solution were introduced into the chromatographic column.

Before introducing the sample, the buffer solution on the top of the filter paper was removed through the outlet tube, the sample was then introduced to the top of the DEAE-SF anion-exchange column by using a syringe. A reservior of the eluant was then connected to the top of the chromatographic column, and the eluant was allowed to flow slowly into the tube. The elution was then started. The flow rate was kept constant at 10 ml./30 min.

Borate chloride buffer containing various concentrations of sodium chloride was used as the eluant. The volumes employed were as follows:

110 ml. of 0.05 M Borate Chloride Buffer (pH 8.6)

275 ml. of 0.25 M sodium chloride in 0.05 M Borate Chloride Buffer. (pH 8.6)

150 ml.of 0.35 M sodium chloride in 0.05 M Borate Chloride Buffer. (pH 8.6)

Five milliliters of the eluate were collected in each of the tubes in the fraction collector. The protein in each tube was indicated at 280 mμ. The results are shown in Plate V and Table I.

As shown in Plate V, there was major peak accompanied by a minor peak. The major fraction was eluted with 0.25 M sodium chloride in borate chloride buffer (pH 8.6).

Application of Concentration Method. Plamstierna (36) and

Koln (18) had reported their successful results in the concentration of protein solutions by using the cellulose derivatives. Biodryex was used at this stage for the concentration of the Component I solution.

The starting material of this process was the collected samples of high protein content from the ion-exchange chromatographic tubes No. 35 through No. 50 as shown in Plate V. The high protein solution was put into a dialysis tube and laid on the bed of the pre-cooled Biodryex, and the dialysis tube was covered with it. The Biodryex containing beaker was then covered completely with the tin foil and set in the refrigerator. After eight hours of the concentration process, the water-saturated Biodryex was removed from the surface of the dialysis tube, and new Biodryex was added, and the process continued for 24 hours. One hundred milliliters of the sample solution were concentrated to twenty milliliters by this treatment. The concentrated solution was then dialyzed against distilled water overnight at 5° to 7°C. in order to remove the extra salt in the concentrated protein solution.

The resulting solution was lyophilized. Twenty milliliters of the Component I solution were completely dried after six hours of freeze drying. A white powder was obtained and was designated as P_f.

EXPLANATION OF PLATE V

Elution diagram of "Precipitate" obtained from the "Bottom Fraction" of the diethylstilbestrol-treated cockerel serum after density gradient centrifugation on DEAE-SF anion exchange chromatography. The "Precipitate" was dialyzed against 0.05 M borate chloride buffer (pH 8.6) for 24 hours before putting on the column.

Ten milliliter fractions/30 minutes were collected and analyzed for protein concentration at 280 mμ.

(a) 0.25 M NaCl in 0.05 M borate chloride buffer pH 8.6 introduced at this point.

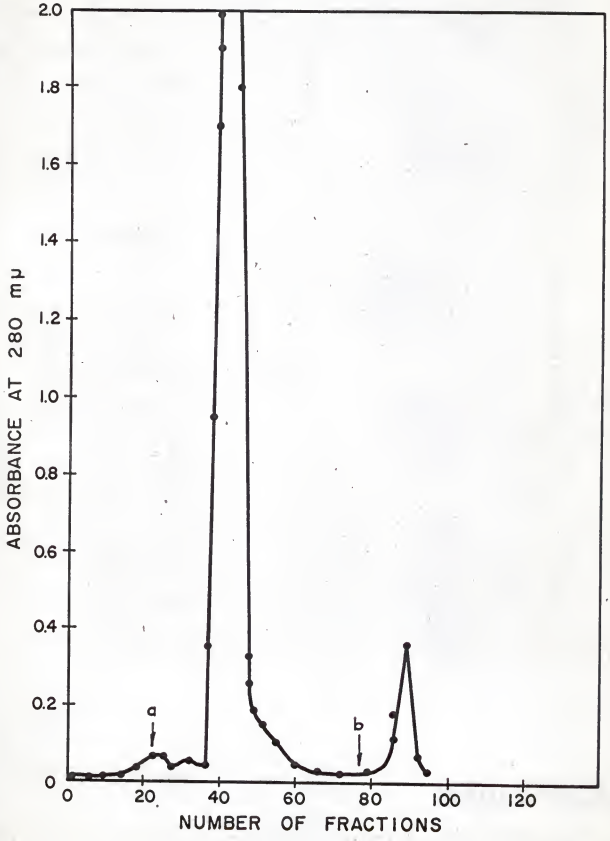
(b) 0.35 M NaCl in 0.05 M borate chloride buffer pH 8.6 introduced at this point.

Buffers used for elution:

110 ml.	0.05 M borate chloride buffer pH 8.6
275 ml.	0.25 M NaCl in 0.05 M borate chloride buffer pH 8.6
150 ml.	0.35 M NaCl in 0.05 M borate chloride buffer pH 8.6

Five milliliters of eluant were collected in one tube. The diagram was prepared from the result shown in Table I.

PLATE V


2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
ABSORBANCE AT 280 mμ
a
b
0
20
40
60
80
100
120
NUMBER OF FRACTIONS

A fraction of the solution prepared from the density gradient centrifugation was similarly lyophilized, and the end product was designated as P_s.

Effect of Dextran Sulfate. The sera of the laying hen, the diethylstilbestrol-treated cockerel and the precipitate solution obtained from the density gradient centrifugation of the "Bottom Fraction" of the diethylstilbestrol-treated cockerel serum after ultracentrifugation were used as the samples in the sodium dextran sulfate precipitation process previously described. Electrophoretic analyses of each sample before and after the sodium dextran sulfate treatment were carried out, and the results are shown in Plate VI. In comparing the electrophoretic patterns of the whole laying hen serum before and after the dextran treatment, (as shown in C of Plate I and A of Plate VI), it appeared clearly that the size of Component 6 (β-globulin site) decreased, and the size of Component I remained unaltered. Similar results were obtained with the diethylstilbestrol-treated cockerel serum. In addition, the patterns of the precipitate solution before and after the dextran treatment were completely identical (as shown in A of Plate IV and C of Plate VI). This indicated that Component 6 can form a complex with the sodium dextran sulfate, whereas Component I does not form a complex with the dextran molecule.

EXPLANATION OF PLATE VI

Electrophoretic patterns of samples after the treatment of sodium dextran sulfate:

(A) Whole serum obtained from the laying hens

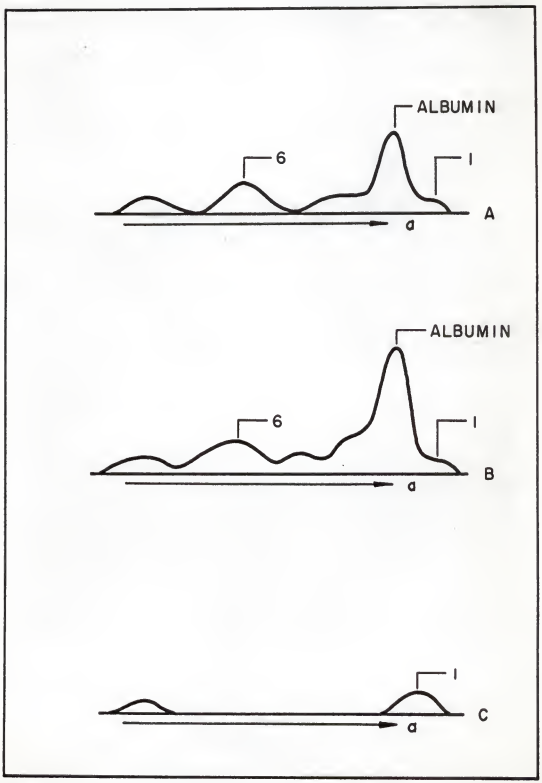
(B) Whole serum obtained from the diethylstilbestrol-treated cockerels.

(C) Precipitate solution obtained from the density gradient centrifugation of the "Bottom Fraction" of the diethylstilbestrol-treated cockerel serum after ultra-centrifugation.

The electrophoreses were conducted in the conditions of:

Buffer	Borate chloride buffer pH 8.6
Time	60 min.
Temperature	0°C.
Current	20 ma.

PLATE VI



Delipidization. P_s and P_f were the starting samples. By treating with absolute methanol and chloroform as previously described, the samples were delipidized. The delipidized P_s was designated as $^{d}P_s$, while the delipidized P_f was designated as $^{d}P_f$.

The Nitrogen and Phosphorus Content of Purified Component I.

The nitrogen content of the samples was determined by the micro-Kjeldahl method (33), while the phosphorus content of the samples was determined by the colorimetric method of Sperry (42). The samples used in the nitrogen and phosphorus determination were P_s, P_f, $^{d}P_s$, and $^{d}P_f$. The results are shown in Table II. The purified Component I, as indicated in Table II, contained a rather high percentage of phosphorus.

TABLE II. Nitrogen and Phosphorus Contents and the P/N Values

Sample	Nitrogen % *	Phosphorus % *	P/N	No. of Determination
(1) P_f	9.12±0.22	2.80± 0.15	0.308	8
(2) ^dP_f	10.03±0.09	2.62± 0.034	0.262	4
(3) P_s	13.44±0.18	3.39± 0.006	0.265	8
(4) ^dP_s	12.74±0.02	2.97± 0.038	0.233	4

* Standard Deviation

P_f: The electrophoretic and chromatographic homogeneous Component I

^dP_f: The delipidized P_f

P_s: The electrophoretic homogeneous Component I

^dP_s: The delipidized P_s

DISCUSSION

The composition of the serum of the chicken varies with sex. When a chicken starts egg production, new electrophoretic components appear in the blood serum along with a great increase of the phosphorus and lipid content of the serum (11). Diethylstilbestrol-treated cockerel serum has the same general characteristics as the serum of the laying hen. Two additional electrophoretic components have been observed in the laying hen serum and the diethylstilbestrol-treated cockerel serum as compared with the electrophoretic patterns of the non-laying hen serum and the normal cockerel serum. One of the newly formed proteins has been designated as Component I and appears in the electrophoretic pattern as prealbumin, while the other has been designated as Component 6 and appears in the electrophoretic pattern as a β-globulin (9).

Since the appearance of Component I and Component 6 has been associated with egg formation, investigators have attempted to isolate and purify Components I and 6, and then to determine their characteristics. For various reasons, none of these reports a satisfactory result. The biggest problem has been isolating a reasonable amount of a homogeneous sample.

Component 6 has been isolated by Misra (28) from laying hen serum and purified so that it was homogeneous to electrophoretic

and ultracentrifuge analyses, although slight heterogeneity was observed in ion-exchange chromatography. Further, Malik (21) determined the amino acid content of the Component 6, and at the same time determined the N-terminal amino acids of Component 6.

Component I was found by Chen (6) to be present in both the "Mixed Fraction" and the "Bottom Fraction" of the laying hen serum and the diethylstilbestrol-treated cockerel serum after ultracentrifugation.

By using moving boundary electrophoresis, Chen (6) found that the "Bottom Fraction" was a better source of Component I. A slight heterogenic Component I was obtained by the method of salt fractionation, and an electrophoretically homogeneous Component I was isolated by the use of column preparative electrophoresis, but only small amounts of sample were isolated by this procedure.

In the present investigation, the "Bottom Fraction" of the diethylstilbestrol-treated cockerel serum after ultracentrifugation was used as the starting substance. A precipitate was obtained by employing density gradient centrifugation. As a result of moving boundary electrophoretic analysis, the electrophoretic pattern of the precipitate indicated only one peak which could be identified as prealbumin of the original serum. This indicated that the precipitate was essentially

Component I. In comparing the electrophoretic patterns of the precipitate and the patterns of "Precipitate II" of Chen (6), these two appear to be essentially the same. It is believed that the precipitate from the density gradient centrifugation and the "Precipitate II" from salt fractionation are similar.

The electrophoretically homogeneous Component I was further fractionated by means of the DEAE-SF anion exchange chromatography. The results were two fractions, a major fraction and a relatively minor fraction. The major fraction was homogeneous to the electrophoretic and chromatographic analysis and was considered to be the purified Component I.

Anionic polysaccharides are the specific reagents for precipitation of low density lipoproteins of human serum (1) (34). This is true also in the laying hen and estrogenated non-laying hen (15) or diethylstilbestrol-treated cockerel serum. Lipoprotein has the characteristic property of combining these anionic macromolecules, and this appears to be a good method to distinguish the lipoprotein from other kinds of proteins in the serum. The size of Component 6 in the electrophoretic pattern of the supernatant was decreased after the addition of sodium dextran sulfate. This is evidence that Component 6 is a lipoprotein, whereas Component I which was isolated from the diethylstilbestrol-treated cockerel serum was not affected by the dextran treatment.

This treatment indicated that Component I is probably not a lipoprotein even though it contains a relatively large amount of phosphorus.

The concentrated electrophoretic homogeneous Component I (P_s) and the concentrated chromatographic and electrophoretic homogeneous Component I(P_f) were lyophilized. Both of them are phosphorus-containing substances but with different P/N values. P_f has the P/N value of 0.306, while P_s has the P/N value of 0.265. The phosphorus content of P_f is higher than that of P_s. This is an indication that the ion-exchange chromatography was effective in concentrating the component.

The phosphorus content of P_f and P_s was decreased by the delipidiration process. The P/N value of ^dP_f is 0.262, while the P/N value of ^dP_s is 0.233. This indicated that a portion of the phosphorus was removed along with the lipid when treated with organic solvents.

Based on the present results of electrophoretic and chromatographic analyses along with the P/N value determination, P_f is a purified Component I. The method for the preparation of Component I developed in this investigation has the advantage of shortening the preparation time and of making available larger quantities of purified Component I.

Several problems for further study seem of interest on the basis of the present investigation. Since a large quantity of purified Component I may be obtained by the combined technique of ultracentrifugation, density gradient centrifugation and ion-exchange chromatography, the chemical composition of Component I may be investigated.

SUMMARY

(1) The "Bottom Fraction" of the diethylstilbestrol-treated cockerel serum after ultracentrifugation showed a high content of Component I and low content of Component 6 and other components. It was used as the starting substance in this investigation.

(2) A precipitate was isolated by employing density gradient centrifugation from the "Bottom Fraction" of the serum. Sucrose was used as the density forming agent. The precipitate appeared to be electrophoretically homogeneous Component I.

(3) An electrophoretically and chromatographically homogeneous Component I was isolated by the application of DEAE-SF anion exchange chromatography.

(4) The purified Component I did not possess the property of precipitating with the anionic polysaccharide molecules. This indicates that Component I was not a lipoprotein.

(5) The P/N value of the purified Component I was 0.308. This indicated that the phosphorus content of Component I was very high, and the Component I may belong to the phosphoprotein category.

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APPENDIX

TABLE I

Protein concentration of five ml. fractions in terms of optical density at 280 mμ. as determined with a Beckman spectrophotometer model DU.

These fractions were obtained from the precipitate solution of the density gradient centrifugation after dialysis against the borate chloride buffer pH = 8.6 and carried out in the DEAE-SF anion exchange chromatography with the elution of 0.00 M to 0.35 M sodium chloride-borate chloride buffer pH 8.6 solution.

No. of Fractions	Optical density at 280 mμ.	No. of Fractions	Optical density at 280 mμ.	No. of Fractions	Optical density at 280 mμ.	No. of Fractions	Optical density at 280 mμ.	No. of Fractions	Optical density at 280 mμ.
1	0.013	21	0.073	41	2.00	61	0.061	81	0.022
2	0.035	22	0.097	42	∝	62	0.039	82	0.030
3	0.012	23	0.090	43	∝	63	0.045	83	0.430
4	0.008	24	0.070	44	∝	64	0.058	84	0.458
5	0.042	25	0.057	45	1.80	65	0.022	85	0.180
6	0.013	26	0.043	46	0.335	66	0.035	86	0.115
7	0.001	27	0.066	47	0.336	67	0.041	87	0.130
8	0.025	28	0.058	48	0.264	68	0.018	88	0.136
9	0.012	29	0.044	49	0.185	69	0.022	89	0.365
10	0.027	30	0.044	50	0.185	70	0.050	90	0.103
11	0.037	31	0.073	51	0.152	71	0.023	91	0.065
12	0.027	32	0.054	52	0.140	72	0.024	92	0.043
13	0.037	33	0.031	53	0.145	73	0.050	93	0.033
14	0.023	34	0.048	54	0.127	74	0.046	94	0.062
15	0.021	35	0.031	55	0.115	75	0.045	95	0.225
16	0.059	36	0.047	56	0.123	76	0.037	96	0.214
17	0.043	37	0.360	57	0.090	77	0.032	97	0.137
18	0.041	38	0.950	58	0.064	78	0.035	98	0.105
19	0.058	39	1.70	59	0.037	79	0.044	99	0.098
20	0.037	40	1.90	60	0.055	80	0.015	100	0.117

PREPARATION OF COMPONENT I (PREALBUMIN) OF THE BLOOD SERUM OF DIETHYLSTILBESTROL TREATED COCKERELS

by

YEE PIN WU

B.S., Taiwan Provincial College of Agriculture, 1958

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Biochemistry

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1963

The sera of laying hens and diethylstilbestrol-treated cockerels contain two additional electrophoretic components not appearing in the sera of non-laying hens or cockerels. One of the two components which has been designated as Component I moves in front of the albumin, while the other component which has been designated as Component 6 moves with the β-globulin. The increase of the phosphorus, calcium and nitrogen in the sera of laying hens and diethylstilbestrol-treated cockerels has been associated with the appearance of these two components.

According to an electrophoretic study, the "Bottom Fraction" of diethylstilbestrol-treated cockerel serum after ultracentrifugation has a high content of Component I. This fraction is a good source for the preparation of Component I.

The"Bottom Fraction" of the diethylstilbestrol-treated cockerel serum after ultracentrifugation was used as the starting substance in this investigation. An electrophoretic homogeneous Component I was isolated in the precipitate form by means of density gradient centrifugation. A further fractionation of the precipitate was carried out by means of DEAE-SF anion exchange chromatography, and resulted in a high purified Component I which was homogeneous to electrophoretic and chromatographic analyses.

Component I, isolated from the diethylstilbestrol-treated cockerel serum, did not form a precipitate with anionic poly-

saccharide macromolecules. This indicated that Component I may not be a lipoprotein. The P/N value of the purified Component I was 0.308. Component I was found to be high in phosphorus, and this indicated that Component I is a phosphoprotein.