Biochemical Studies on Egg Yolk Formation in the Domestic Fowl

II. Fractionation of Serum Phosphoproteins

Shigeru TOKUE, Kazunobu ONO and Kazue ASO

(Research Center, Nihon Nosan Kogyo Co., Ltd.)

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It has been reported that the serum of laying hen is different from that of non-laying hen in many chemical properties. Marked differences between both sera consist in the contents of calcium, phospholipid, and protein. Especially, the serum of laying hen is characterized by the presence of phosphoprotein, which is demonstrated by ultra-centrifugation or a serological method.

LASKOWSKI[1] obtained the phosphoprotein fraction of serum as a precipitate by diluting laying-hen plasma. ROEPKE and BUSHNELL[2] observed the precipitation of phosphoprotein in the dialysis of hen serum. MCKINLEY and associates[3,4] presented an evidence for the occurrence of phosphoprotein in the precipitate from the serum of pullets treated with estrogen, on dilution. MCINDOE[5] reported the chemical constitution of the material precipitated, by dilution with water, from the plasma of laying hen. BLOCK et al.[6] separated the chicken serum into 18 fractions by ion-exchange cellulose.

The present paper is concerned with the differences among protein, lipid, and protein-bound phosphorus in the sera of laying hen, non-laying hen, and cockerel by using the chromatographic technique.

Materials and Methods

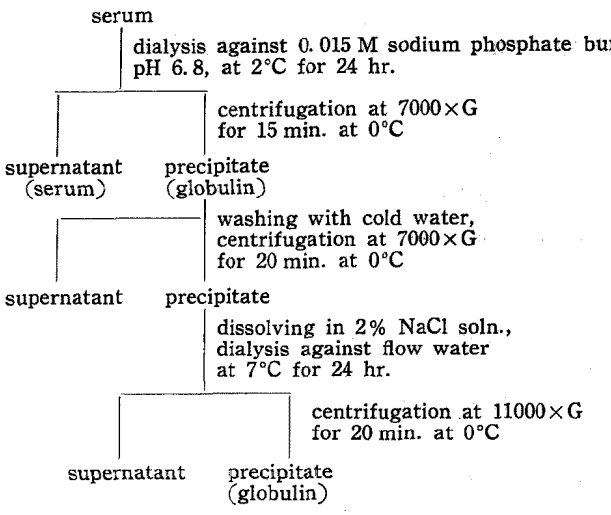
Crossbred laying hens (White Leghorn ♂ × Rhode Island Red ♀, 2 years old) were used. They had been kept in individual laying cages and fed a practical-type diet. The composition of the diet was the same as that described in the previous paper[7]. Hormone-treated and non-treated White Leghorn cockerels (4 months old) were used. They had been raised in the same conditions as mentioned above.

Hormone treatment was carried out by intramuscular injection of synthetic estrogen (p,p'-dicarboethoxy-oxytrans-α, β-diethylstilbene with the proprietary name Euvestin, a product of the TAKEDA Pharmaceutical Industries, Ltd.) in a daily dose of 4 mg for 10 days.

Birds weighing 1.9～2.1 kg were selected at random and killed by decapitation and bleeding. Blood obtained by bleeding was incubated at 37°C for 2 hours. Then serum was separated and used for experiments. The method of BLOCK et al.[6] was applid to sample preparation and chromatography.

Serum was dialyzed for 24 hours against 0.015 M sodium phosphate buffer at pH 6.8. The dialyzed serum, from which the precipitate formed had been removed, was applied to the column for chromatographic analysis. The precipitate formed, globulin, was washed in water, dissolved in 2% NaCl solution, and dialyzed against flow water. Then it was lyophilized and stored in a refrigerator for further chromatographic analysis.

These procedures are illustrated in the following diagram.



Column

A column, 1×20 cm, was filled with a suspension of DEAE-cellulose, which had been prepared from cellulose powder, produced by L. LIGHT & Co., Ltd., England, and from cotton cellulose powder, produced by the TOYO ROSHI Co., Japan, in 0.015 M sodium phosphate buffer, pH 6.8. Its content was compressed with air to a constant height.

Development of Chromatography

Four milliliters of the dialyzed serum, or about 0.1 g of the precipitated globulin, was loaded on the column and washed with the starting buffer. The protein fractions were eluted from the column by using several buffers with successively increasing concentrations of sodium chloride.

A flow rate of 20 to 30 m*l* per hour was achieved with nitrogen gas pressure. All operations were performed at 7~9°C. Each 4 m*l* of effluent was collected in a tube by a fraction collector. Each fraction was read for protein concentration with the spectrophotometer in the ultraviolet absorption at 280 mμ.

Buffer

Buffer No.	NaCl concentration
Starting buffer	0.015 M sodium phosphate buffer, pH 6.8
No. 1	0.020 M NaCl in 0.013 M sodium phosphate buffer, pH 6.8
2	0.027 M 〃
3	0.053 M 〃
4	0.073 M 〃
5	0.093 M 〃
6	0.11 M 〃
7	0.13 M 〃

8	0.21 M NaCl in 0.013 M sodium phosphate buffer, pH 6.8
9	0.27 M "
9A	0.30 M "
9B	0.40 M "
9C	0.50 M "
9D	0.80 M "
10	2.0 M NaCl in 0.1 M monobasic sodium phosphate
11	2.0 M NaCl in 0.1 M dibasic sodium phosphate
12	0.2 N NaOH
13	0.5 N NaOH

Methods of Analysis

On the specific fractions obtained in globulin chromatography, the effluent comprising each protein peak was pooled, concentrated to about half of the original volume by pervoration, and dialyzed against flow water for 48 hours. The precipitate formed by dialysis was collected by centrifugation, dried, and weighed. This dried precipitate was treated three times with ethanol-ether (3:1, v/v), and finally with aceton, dried, and weighed. The protein-bound phosphorus in it was determined by Allen's method (Nakamura's modification)[8]. Nitrogen determination was made by the micro-Kjeldahl method. Lipid content was determined by the method of McIndoe[5].

Results and Discussion

In figs. 1~3 are shown the elution diagrams obtained when the dialyzed serum was chromatographed on the anion exchanger, DEAE-cellulose. The elution diagrams of the laying hen (LH), immature pullet and cockerel are similar and contain no specific fractions, although there are some differences in the peak area among the birds. The fraction eluted

Fractionation of Serum Protein

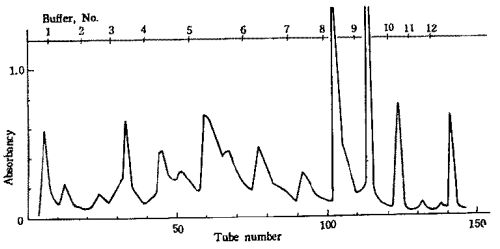


Fig. 1. Cockerel

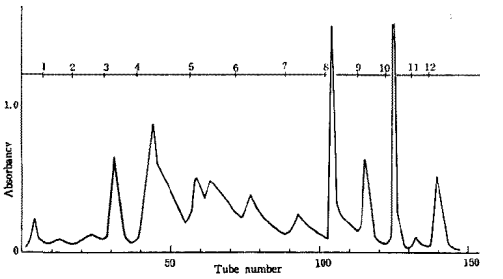


Fig. 2. Immature Pullet

with buffer No. 8 gave a large peak. Further experiment indicated that the fraction was a mixture which was separated to several fractions by elution with buffers containing intermediate concentration of buffer Nos. 7~9.

The results of chromatography on the

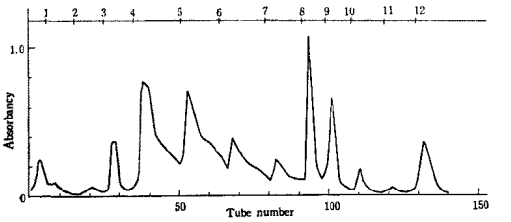


Fig. 3. Laing Hen

dialyzed sera of the estrogen-treated cockerel (TC) and non-treated cockerel (NC), as shown in figs. 4 and 5, indicate no marked difference between them. There are, however, some small peaks eluted with buffer Nos. 9, 10, and 11, which do not appear in the sera of the control (fig. 4) and LH (fig. 3). So these small peaks do not seem to present any significant fractions.

Fractionation of Serum Protein

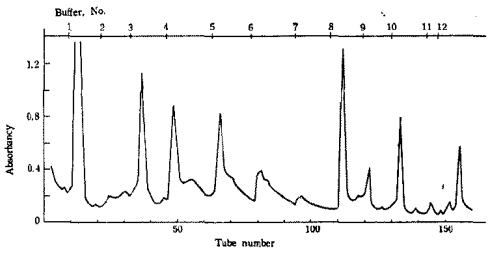


Fig. 4. Estrogen-treated Cockerel

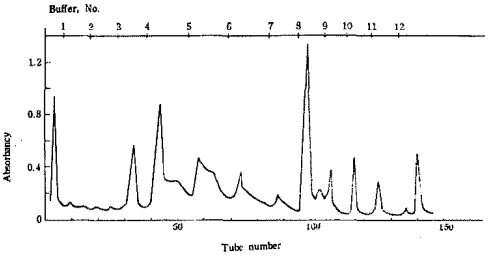


Fig. 5. Non-estrogen-treated Cockerel

In figs. 6~8 are shown the elution diagrams of the precipitated globulin. In these diagrams, differences are recognized among three elution curves. These differences, presenting four fractions which were eluted with buffer Nos. 9 A, 9 B, 9 C, and 11 (0.3, 0.4, 0.5, and 2.0 M NaCl solution), are not found in the globulin of the NC. The protein concentration of fraction No. 9 B increased by injection of estrogen. These four fractions seemed to form a phosphoprotein fraction. In figs. 7 and 8, a specific fration which had been contained only in cock's globulin was eluted in buffer No. 9 D. This fact was ascertained in five analysis.

Fractionation of Serum Globulin

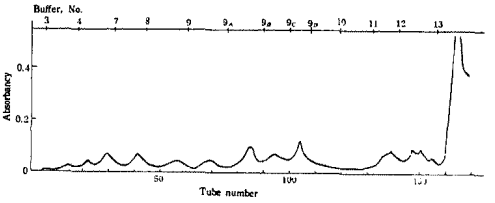


Fig. 6. Laying Hen

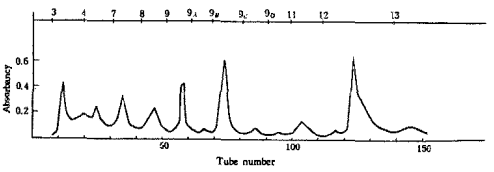


Fig. 7. Estrogen-treated Cockerel

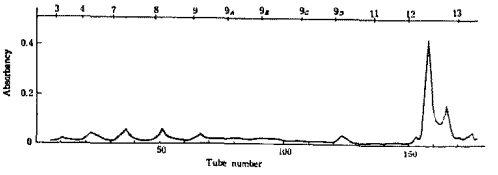


Fig. 8. Non-estrogen-treated Cockerel

It is a well-known fact that much lipid is contained in the sera of the LH and TC. It is assumed from the observation of serum conditions that lipid exists in the precipitated globulin in a very great proportion, gives a deep yellow color to it, and sometimes makes it gelatinous. Table 1 shows the lipid concentration and protein-bound phosphorus in the precipitated serum globulin. The results of determination of lipid and protein-bound phosphorus in the four fractions, which seem to be phosphoprotein, are given in Table 2.

Table 1. Concentration and percentage composition of lipid and protein-bound phosphorus in precipitated serum globulin

	Sample	Lipid	Lipid percentage	Protein-bound phosphorus
	mg	mg	%	%
Estrogen-treated cockerel (TC)	53.9	35.45	65.77	0.95
Laying hen (LH)	50.0	34.65	69.30	0.69

Table 2. Concentration and percentage composition of lipid and protein-bound phosphorus in four fractions

Fraction		Sample	Lipid	Lipid percentage	Protein-bound phosphorus
LH	0.3 M	9.20 mg	1.8 mg	19.6%	6.7%
	0.4 M	24.65	2.25	9.1	2.4
	0.5 M	4.10	0.2	4.9	2.7
	2.0 M	23.25	2.65	11.4	16.0
Total		61.20	Precipitated globulin loaded		962 mg
TC	0.3 M	6.60	1.2	18.2	6.1
	0.4 M	32.20	6.0	18.6	3.6
	0.5 M	6.35	2.3	36.2	7.0
	0.0 M	6.40	2.0	25.0	17.1
Total		51.55	Precipitated globulin loaded		1,880 mg

Remarks: The values given in Tables 1 and 2 are mean values of three experiments.

The percentage of the total phosphoprotein fractions to the used globulin is about 6.3% LH and 2.7% in TC (Table 2). In LH, As the precipitated globulin is contained in the resum at about 2%, the ratio of the phosphoprotein fraction to the serum is only about 0.12% (Table 3). Of course, it is assumed that these concentrations are variable among birds, but this figure seems to be very small.

Table 3. Protein concentration in resum

Sample*	Total protein in serum	Serum protein after dialysis	Precipitated globulin
TC 2 mg/day	7.20%	4.30	2.90%
TC 2 mg/day	8.21	4.75	3.46
NC	3.75	2.81	0.94
NC	4.08	3.12	0.96
LH	5.08	3.34	1.74
LH	5.69	3.64	2.05
LH	6.20	4.30	1.90
LH	5.55	3.19	2.36
LH	5.34	3.41	1.93

* TC: estrogen-treated cockerel, NC: non-treated cockerel, and LH: laying hen.

McINDOE[5] reported that plasma phosphoprotein contained 80% lipid. In this experiment, serum globulin contained much lipid, as shown in Table 1. Especially, lipid content in the serum was higher in TC than in LH (Table 2). HONMA et al.[9] also observed an increase of low-density lipoprotein level in LH. Judging from a large quantity of lipid contained in egg yolk, it may be natural that plenty of lipid is contained in the blood of LH.

When the four fractions were compared between LH and TC, the content of protein-bound phosphorus did not vary so much between them (Table 2). These results suggest that the phosphoprotein synthesized by TC may have the same properties as that by LH.

Summary

The blood sera of the laying hen, estrogen-treated and non-treated cockerels were dialyzed against 0.015 M sodium phosphate buffer, pH 6.8, at 2°C for 24 hours. The resulting precipitated globulin was separated by centrifugation, re-dialyzed, and lyophilized. Four milliliters of supernatant serum and about 0.1 g of lyophilized globulin were applied separately to a column which had been filled with a suspension of DEAE-cellulose in 0.015 M sodium phosphate buffer, pH 6.8, and the content of which had been compressed with air to a constant height 1×20 cm.

Stepwise elution was carried out by 0.013 M sodium phosphate buffer, pH 6.8, containing various concentrations of sodium chloride. Each protein fraction was read for protein concentration with a spectrophotometer in the ultraviolet at 280 mμ.

The chromatography of globulin of the laying hen and that of the estrogen-treated cockerel showed four fractions, which were not observed in globulin of the non-treated cockerel. These four fractions were eluted by 0.3, 0.4, 0.5, and 2.0 M sodium chloride containing buffer and found to have 6.7, 2.4, 2.7, and 16.0% protein-bound phosphorus in the laying hen and 6.1, 3.6, 7.0, and 17.1% in the estrogen-treated cockerel, respectively. The ratio of the total amount of the four fractions to the precipitated globulin was 6.3% in the laying hen and 2.7% in the estrogen-treated cockerel. These four fractions seemed to be phosphoprotein.

Acknowledgments

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卵黄生成に関する生化学的研究

II. 血清燐蛋白の分離

徳江　茂・小野和延・麻生和衛

（日本農産工業株式会社　中央研究所）

要　約

産卵鶏，雄鶏およびエストロジェン処理雄鶏の血清を，pH 6.8 の 0.015 M 燐酸ソーダ緩衝液に対して，2°C で 24 時間透析を行なつた．その結果，沈殿したグロブリン部分と，上澄血清部分について，DEAE-セルローズによるカラム・クロマトグラフィーを行ない，溶出液はフラクション・コレクターで 4 cc ずつ分取し，紫外部 280 mμ における吸光度を測つて，蛋白含量を調べた．

その結果，血清部分には，別に著しい差が見られなかつたが，沈殿したグロブリン部分から，雄にはない特異的な 4 画分を得た．

この 4 画分は，0.3 M，0.4 M，0.5 M および 2.0 M 食塩含有燐酸ソーダ緩衝液で溶出され，蛋白性燐を含有し，その量は，それぞれ産卵鶏で 6.7%，2.4%，2.7%，16.0%，エストロジン処理雄鶏で 6.1%，3.6%，7.0%，17.1% であつた．その他，脂質も相当量含有され，エストロジエン処理雄鶏では 18～36% であり，産卵鶏の 4.9～19% より著しく多かつた．また雄鶏に特有と思われる蛋白質が，0.8 M 食塩含有緩衝液で溶出された．

上記の 4 画分合計量が使用グロブリン量に対する割合は産卵鶏で 6.3%，処理雄鶏で 2.7% であつた．