Electrophoretic and Immunologic Studies on the Chicken Serum and Egg Yolk. (26385)

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The serum of laying hens can be distinguished from that of cocks immunologically (1,2,3), chemically(4,5) and electrophoretically(6). The similarity(2,5,7) and difference(8) were noted between the phosphoprotein in the serum of laying hens and that in egg yolk. Hosoda *et al.*(9) using immunologic technics determined the content of the specific substance in the serum of laying hens and found that its titer correlates well with the ovarian activity of hens. Recently, Urist *et al.*(10) did chemical and electrophoretic studies on the serum of estrogenized cocks with aid of the ultracentrifuge.

The present work was initiated to elucidate the chemical nature of the antigens in specific immunologic reaction to the serum of laying hens or egg yolk, and the relationship between the specific substance in the serum of laying hens and that in egg yolk.

Materials and methods. Fifteen White Leghorn laying hens and cocks were sacrificed, and the serum and yolk in the follicles obtained from them were centrifuged in a Hitachi ultracentrifuge, at 40,000 rpm (105,400 $\times$ G) at 5°C for 8 hrs. Three partitions of the serum of laying hens, top layer (LSI) middle partition (LSII) bottom sediment (LSIII), 3 partitions of the serum of cocks, top layer (CSI middle partition (CSII) bottom sediment (CSIII), and 2 partitions of yolk, supernatant solution (YI) sediment (YII), were separated visually by ultracentrifugation. Partition YII was washed once with isotonic saline and ultracentrifuged again for 2 hours. Fractionation of phosphorus was carried out by Schneider's method(11). Phosphorus content of each fraction was determined by the method of Gomori(12). Nitrogen content was determined by the Kjeldahl method. Antisera was prepared by immunizing rabbits with the serum of laying hens, or yolk or the 5 ultracentrifuged partitions obtained from the serum of laying hens. Cock serum was added to these antisera to absorb species-specific antibodies. Sodium cyanide was added in a concentration of 0.1% to all the antigens and antisera for preservation. Interfacial precipitin reaction test was employed to measure antigenic potency, which was expressed as the highest dilution of antigen giving a positive reaction with each antiserum after 1 hr of incubation at 25°C. One cc of each antiserum previously absorbed with cock serum was mixed with 0.1-0.2 cc of a heterologous ultracentrifuged partition of the serum of laying hens or egg yolk. The antiserum-antigen mixtures were incubated at 37°C for 2 hr, and kept at 3-5°C overnight. The precipitates formed by the absorbing antigens were removed by centrifugation at 3,000 rpm for 10 min. Complete disappearance of the antigen was confirmed by the interfacial precipitin reaction test. Single diffusion precipitin test in agar-antiserum gel, according to the technic of Oudin(13) was used for the analysis of each partition. Test tubes of 4 mm diameter were used. Concentration of the antisera in the gel was 50%. 0.3 cc of the antigen diluted with the same amount of saline were placed upon the antiserum gel. The migration of precipitin zones was observed after 48 hr of incubation at 37°C. Filter paper electrophoresis was carried out by the method of McKinley *et al.*(14). Electropherograms of serum and yolk of the chicken and each ultracentrifuged partition of both the serum and yolk were made in barbital buffer, pH 8.6, containing 20% of methanol instead of water. Electropherograms were performed at 0.3 miliamperes per cm of filter paper for 7 hr, and stained with Amidoschwarz 10 B and Sudan III. Stained radioautographs were also made from the electropherograms of the serum and yolk, obtained from laying hens 24 hr after injection of 800 μc P^{32} as Na_2HPO_4, and of each ultracentrifuged par-

TABLE I. Distribution of Phosphorus, Nitrogen and Antigenic Potency among Partitions of Chicken Sera and Yolk Obtained by Ultracentrifugation.

Partitions	Acid soluble phosphorus	Lipid phosphorus	Protein phosphorus	Total nitrogen	Total protein	Antigenic potency*	Volume ratio
	μg/cc			mg/cc			
Laying hen serum							
Total (LST)	40	117	101	7.6	46.6	640	10
I (LSI)	0 (0)	530 (53)	0 (0)	3.1 (.3)	17.9 (1.8)	5,120	1
II (LSII)	36 (29)	67 (53)	5 (4)	4.1 (3.2)	25.1 (20.1)	80	8
III (LSIII)	116 (12)	200 (20)	899 (90)	46.5 (4.6)	290.6 (29.1)	5,120	1
Cock serum							
Total (CST)	60	64	0	4.1	24.8	0	10
I (CSI)	153 (23)	0 (0)	0 (0)	1.5 (.2)	8.4 (1.3)	0	1.5
II (CSII)	33 (41)	91 (75)	0 (0)	3.8 (3.1)	23.5 (18.4)	0	8.2
III (CSIII)	0 (0)	132 (4)	0 (0)	2.9 (.6)	18.4 (3.9)	0	.3
Yolk	μg/g			mg/g			
Total (YT)	250	5,000	1,045	27.7	164.9	20,480	10
I (YI)	244 (188)	5,247 (4,040)	52 (40)	21.9 (16.8)	129.0 (99.3)	10,240	7.7
II (YII)	261 (60)	1,957 (450)	4,126 (949)	40.5 (9.3)	253.3 (58.3)	40,960	2.3

* Expressed as the highest dilution giving a positive reaction with anti-laying hen serum absorbed with cock serum.

Figures in parentheses expressed in μg of partitions from 1 cc of serum or from 1 g of yolk.

tition of the serum and yolk. The corresponding fractions on unstained radioautograms were separated by cutting the paper, and extracted by dipping in saline overnight for detection of positive reaction on immunologic interfacial precipitin tests.

Results. Distribution of phosphorus, nitrogen, protein and antigenic potency (as determined by titrations with anti-laying hen serum absorbed with cock serum) among the ultracentrifugal partitions of the sera of laying hens, the sera of cocks, and the yolks are tabulated in Table I. LSI and SCI were yellow or white, LSII and CSII were the color of serum, and contained most parts of the total serum. LSIII and CSIII were a sediment and soluble in isotonic saline. YI was a clear yellow supernatant and soluble in isotonic saline. YII was a white sediment of yolk and insoluble in isotonic saline, but soluble in hypertonic (more than 8%) saline. Concentration of lipid P was very high in LSI and low in LSII and LSIII. Almost all protein P was sedimented in LSIII, and none was found in LSI. Concentration of nitrogen in LSI was low, and very high in LSIII. Antigenic potency with anti-laying hen serum absorbed with cock serum was high in LSI and LSIII and very low in LSII. Antigenic potency per unit protein was highest in LSI, high in LSIII and lowest in LSII. In the serum of cocks, concentration of lipid P was low compared with that in the serum of laying hens, and no lipid P was found in CSI. No protein P was found in the serum of cocks and immunologic reaction with antiserum was negative. Concentration of lipid P was high in YI and low in YII. Protein P was almost completely sedimented in YII and was scarcely found in YI. Concentration of nitrogen in YII was twice as high as in YI. Antigenic potency with antiserum was higher in YII though high potency was still found in YI. Antigenic potency per unit protein was twice as high in YII as in YI.

Electropherograms and radioautograms of the serum of laying hens and antigenic potency of each fraction are illustrated in Fig. 1. In the electrophoretic pattern of LSI only one slow moving component was found. Staining with Sudan III revealed it was a lipoprotein. The strong antigenic potency was noted in this low-density lipoprotein, which is considered a phospholipo-P-free protein. This specific antigenic substance in this reaction was completely destroyed by incubation at 80°C for 30 min. LSII contained a large amount of albumin. Antigenic potency was not found in any fractions

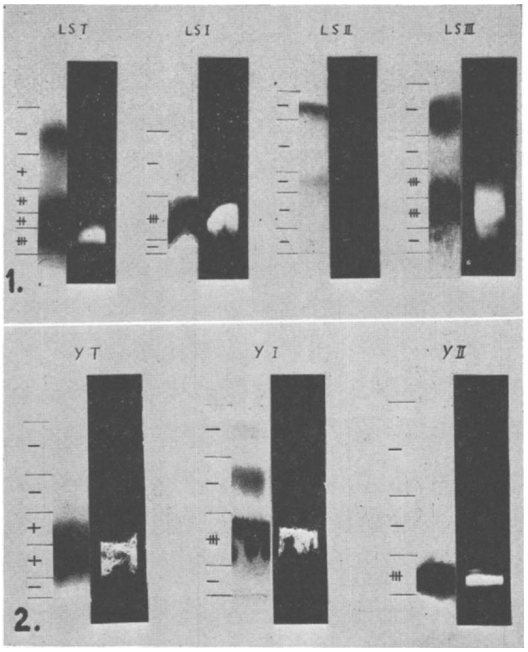

FIG. 1. Electropherograms of serum of laying hen (LST) and its ultracentrifuged partitions (LSI, LSII, LSIII), these radioautographs, and antigenic potency with anti-laying hens serum absorbed with cock serum shown by —, +, ++, and +++, of each fraction of electropherograms.

FIG. 2. Electropherograms of serum of the laying hen (YI) and its ultracentrifuged partitions (YI, YII), these radioautographs, and antigenic potency with anti-laying hen serum absorbed with cock serum, shown by —, +, ++, and +++, of each fraction on electropherograms.

of LSII. LSIII contained at least 3 components; albumin, gamma globulin and phosphoprotein. The positive antigenic reaction was found in a wide range of slow moving components. This specific antigenic substance(s) was also completely destroyed at 80°C. Electropherograms of the partitions of the serum of cocks obtained by ultracentrifugation, showed a complete absence of the lipoprotein component in CSI and absence of one of the slow-moving components, phosphoprotein, in CSIII. Electropherograms and radioautograms of yolk and antigenic potency of each fraction are shown in Fig. 2. YI contained 3 components. The slow moving one of these components was phospholipo-P-free protein, as shown by the stain with Sudan III and the radioautograph with P^{32}. The strong antigenic potency was found in this component. YII contained phosphoprotein which gave a positive reaction with anti-laying hen serum absorbed with cock serum. These specific antigenic substances in both fractions were completely destroyed at 80°C.

Results of the interfacial precipitin reaction tests with various antisera absorbed with cock serum are given in Table II. Antigenic titer with anti-LSI serum was high in LSI and YI, but negligible in LSIII and YII. The titer with anti-LSIII serum was high in LSIII and YII, but negligible in LSI and YI. While anti-LSIII, anti-YI or anti-YII serum reacted with all antigens, anti-Y serum reacted stronger with LSI and YI than with the other partitions, and anti-YII serum reacted stronger with LSIII and YII. In cross absorption test, the antibodies contained in anti-LSI serum and anti-YI serum were not absorbed with partition of LSIII and the antibody to LSIII was not absorbed with either LSI or YI. The residual antigenic potency to homologous antigen was still observed after the absorption with heterologous antigen. When anti-YI serum and anti-LSI serum were absorbed with YII, no precipitin reaction was observed.

Results of agar-antiserum gel single diffusion tests are illustrated in Fig. 3. When antigen LSI and YI were placed upon the gel prepared by mixing anti-LSI serum, a strong positive precipitin zone was observed, and a faint zone was observed in the case of antigen YII. This weak zone disappeared when the antiserum was absorbed with LSIII, while the stronger zone remained. No appreciable precipitin zone was observed in the gel with anti-LSII serum, though very faint

TABLE II. Precipitin Reaction between Partitions of Serum of Laying Hens and Yolk by Ultracentrifugation and Antisera to These Partitions.

Antiserum* to partition	Precipitin titer *vs* antigen				
	LSI	LSII	LSIII	YI	YII
LSI	5.120†	0	0	10.240	0
LSII	5.120	80	10.240	5.120	20.480
LSIII	0	0	10.240	0	40.960
YI	5.120	160	2.560	10.240	5.120
YII	1.280	80	10.240	320	40.960

* Absorbed with cock serum, and diluted 1:3.
† Expressed as the highest dilution of antigen giving a positive reaction with each antiserum.

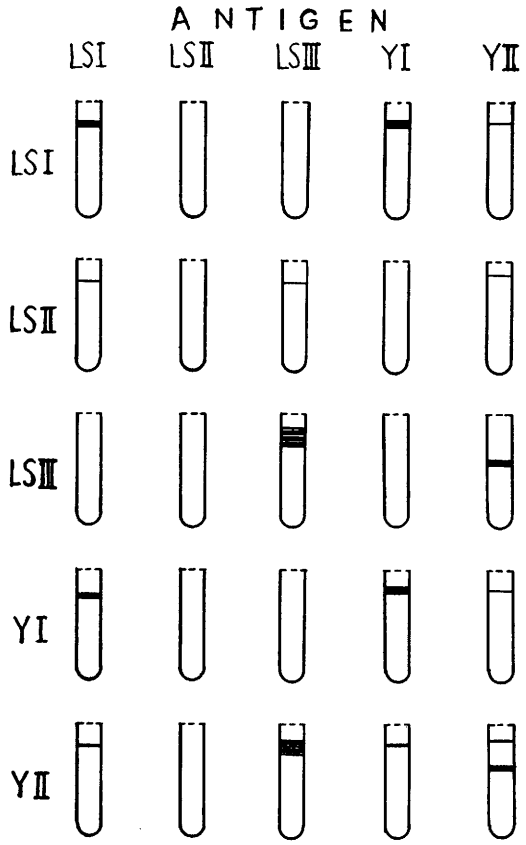

FIG. 3. Agar gel diffusion precipitin reactions among partitions of serum of laying hens and of yolk obtained by ultracentrifugation, and antisera to those substances. --- boundary between antigen and agar-antiserum gel. ___ strong perecipitin zone; —— weak one.

reaction was still found in cases of antigens LSI, LSIII and YII. Several zones were observed in the gel containing anti-LSIII serum when LSIII was used as antigen, but only one zone was observed when antigen YII was used. These zones were still observed after the antiserum was absorbed with LSI. The precipitin zone in the gel containing anti-YI serum was similar to that in the gel containing anti-LSI serum. When anti-YI serum absorbed with YII was used in a gel, no precipitin zone was found with antigen YI or LSI. In the reaction of anti-YII serum against each antigen, a zone was observed in both case of antigen LSI and YI, which disappeared when anti-YII serum was absorbed with YI, and 2 zones were found in the case of antigen YII, one of which disappeared when anti-YII serum was absorbed with YI. As antiserum to YII reacts with almost all the partitions of the serum and yolk except LSII and the immunologic reaction completely disappeared when the antisera to any partitions were absorbed with YII, YII may consist of at least 2 antigenic substances, one of which is also present in YI.

Discussion. The present work demonstrates that at least 2 specific antigenic substances are present in the serum of laying hens. They react with the antiserum to laying hen serum absorbed with cock serum prepared so as to react only with the serum of laying hens, the serum of estrogenized cockerels and pullets, or a solution of egg yolk. One of these substances is a low density phospholipo-P-free protein which is present in the top layer obtained by ultracentrifugation, the other is a phosphoprotein or a phosphoprotein complex which is present in the sediment obtained by ultracentrifugation. Similarly, 2 specific substances are present in egg yolk, one in the supernatant solution and the other in the sediment obtained by ultracentrifugation. These 2 specific substances are present in the serum of laying hens and are not found in the serum of cocks, and are capable of producing the specific antibodies by immunizing rabbits. The specific antibody or antibodies to one specific substance can not be absorbed with the other substance.

The present work also shows that approximately 90% of the phospholipides are not associated with phosphoprotein either in the serum of laying hens or in the yolk. A similar result was noted by Schmidt *et al.*(15) in the egg yolk. Low density phospholipo-P-free protein in the top layer of the serum of laying hens may be almost identical with that in the supernatant solution of yolk as shown by electrophoresis, N/P ratios, solubility in saline, and pattern of precipitin zone in agar-antiserum gel. This material may be a single substance because only one precipitin zone was found in the reaction. Phosphoprotein or phosphoprotein complex contained in the sediment of egg yolk is quite insoluble in isotonic saline and soluble in hypertonic

(more than 8%) saline, differing from that contained in the serum of laying hens which is soluble in isotonic saline. The N/P ratio in the sediment of the serum of laying hens is much higher than that in the sediment of egg yolk. Furthermore, the antigenic substances in the sediment of laying hens are not a single substance, because, when antiserum to the sediment of the serum of laying hens was used, several precipitin zones formed with the sediment of the serum, but only one zone formed with the sediment of yolk. These results show a difference between the chemical structure of phosphoprotein or phosphoprotein complex of the serum of laying hens and that of egg yolk. The substance in the blood of laying hen may be transformed into substance in egg yolk during the passage through the granulosa of the follicle.

Summary. Electrophoretic and immunologic studies on the partitions of the sera of laying hens and of cocks and egg yolk obtained by ultracentrifugation showed the presence of 2 specialized components, (a low density phospholipo-P-free protein and a phosphoprotein or a phosphoprotein complex) either in the serum of laying hens or in egg yolk, which were not found in the serum of cocks. Each component was capable of producing the specific antibody or antibodies. The specific antibody or antibodies to one specialized component could not be exhausted by absorption with the other component of the serum. Low density phospholipo-P-free protein, found in the top layer of the serum of laying hens, obtained by ultracentrifugation is almost identical with that found in the supernatant solution of egg yolk obtained by ultracentrifugation. This protein is a single antigenic substance as shown by the appearance of only one precipitin zone in the agar-antiserum gel. Phosphoprotein or phosphoprotein complex, found in the sediment of the serum of laying hens, is not identical with that found in the sediment of egg yolk, since the former is soluble in isotonic saline, while the latter is quite insoluble in isotonic saline. The pattern of precipitin reaction zone to the material in sediment of serum in agar-antiserum gel, which showed several zones, differed from that to the material in sediment of yolk.

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