BIOCHEMICAL STUDIES ON THE EGG FORMATION IN THE DOMESTIC FOWL I. ON THE OCCURRENCE OF PHOSPHOPROTEIN AND THE DETERMINATION OF NUCLEIC ACIDS IN LIVER AND SERUM OF LAYING HENS

BIOCHEMICAL STUDIES ON THE EGG FORMATION IN THE DOMESTIC FOWL

I. ON THE OCCURRENCE OF PHOSPHOPROTEIN AND THE DETERMINATION OF NUCLEIC ACIDS IN LIVER AND SERUM OF LAYING HENS

By

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A complete knowledge of egg-formation would include not only an understanding of the chemical constituents of the egg, but also the exact information of the manner and order in which these constituents are laid down in the ovary during the egg-production. Since Warner (1) and Lawrence and Riddle (2) noted the increase of lipid in relation to the ovarian activity, many studies have been carried out demonstrating the significant changes in calcium, lipids and certain organic phosphorus fractions preceding and during egg-laying. However, there had been little evidence on the characteristic serum protein, until Sasaki (3) found the existence of a serological specific substance in the laying serum. Later Roepke *et al.* (4.5) and Laskowsky (6.7) brought forward considerable proof that the material was serum vittelin, a kind of phosphoprotein similar to the major protein of egg yolk, by the serological and analytical means.

So far as we know, few attempts have been made to clarify the site in which the phosphoprotein was synthesized. This paper is devoted primarily to the study of the occurrence of phosphoprotein, and also to determine the nucleic acids content and the crude protein concentration in tissues and serum of the domestic fowl with reference to the site of phosphoprotein formation. In a series of studies of which this is the first one, we propose to secure the evidence that the phosphoprotein appears in the liver and that a close relationship exists between the occurrence of phosphoprotein and the contents of nucleic acids in the liver of normal laying hens.

* This experiment was accomplished while the author was at the Laboratory of Zootechnical Science (of Professor K. Sasaki), Faculty of Agriculture, Tokyo University, Tokyo (1950).

Materials and Methods

This experiment was conducted on twenty-six fowls of following breeds; White Leghorn, Barred Plymouth Rock and Rhode Island Red. They were divided into four groups; eight laying hens, six non-laying, hens six cocks and six newly hatched (one or two days old) chicks. The samples of the laying hens and cocks were taken from March to July, and those of non-laying hens in September and October, namely the moulting season. We found either a shelled egg in the oviduct or a matured follicle (3.3~3.8 cm in diameter) in the ovary of every laying hens; while in the non-laying hens there were several immatured ova (less than 0.5 cm in diameter) in the ovary.

Preparation of the crude protein fraction: Immediately after death by bleeding, specimens of serum and of various organs (*e.g.* liver, kidney, spleen, small intestine and brain) were removed to prepare the crude protein fraction. But in this experiment the analysis of chicks' serum was omitted due to the defficiency of its quantity for examination The preparation procedure of the crude protein fraction was as follows:

Tissue, immediately after taken is minced in the cold motor, or
Serum + cold 10 per cent trichlor acetic acid (TCA) in an equal volume
↓ centrifuge
wash, with cold 5 per cent TCA, twice
↓
wash, with ethanol
↓
boil, for 30 minutes to extract lipid with Bloor's mixture, three times
↓
dry, in a desiccater
↓
weigh, as Crude Protein

We employed Schmidt and Thannhauser's method (8) with slight modification for the analysis of phosphoprotein-and nucleic acids-phosphorus as follows:

Sample, 50 mg crude protein
↓
+2.5 cc n-KOH, incubate in a paraffin tube for 15 hours at 30°C
↓
+2.5 cc water
↓ centrifuge
+5.0 cc → 1 cc supernatent (1/5).....Total Phosphrus.....A
↓
4.0 cc supert.
↓
+0.8 cc 6n-HCl, neutralize
+0.4 cc 5 per cent TCA
↓ ↓
B.....DNA-P..... precipitant (4/5) supert.→1/2 (2/5).....Prot.-P + RNA-P C
↓
1/2 (2/5)
※

※
+0.2 cc conc. ammonia, neutr.
↓
+0.2 cc 20 per cent $CaCl_2$
+1.0 cc 0.5 per cent $MgCO_3$
| centrif., after shaking,
↓ stand for 30 min.
ppt (2/5).....Prot.-P .....D

Ashing of each fractions (A, B, C and D) was carried out with perchloric acid in a water bate, occasionally adding a small amount of concentrated hydrogen peroxide (phosphate free) to stimulate the process. Inorganic phosphorus was determined by Fiske and Subbarow's method (9) taking care of the concomitance of exogenous phosphorus, and the color intensity was measured by Pulflich's Stufenphotometer.

Results

The experimental results obtained for the amounts of crude protein, nucleic acids and phosphoprotein-phosphorus of serum and the various organs are shown in Tables 1 and 2. The ratio of desoxyribonucleic acid to ribonucleic acid (RNA/DNA) was also calculated and presented in the table 1.

1. Crude protein : The average crude protein content of the laying hens' liver was higher than that of the baby chicks ($a=0.05$), but scarcely showed difference from that of non-laying hens and cocks. Whereas the crude protein content of laying hens' serum was richer than that of non-laying hens ($a=0.05$) and cocks ($a=0.01$).

2. Phosphoprotein-phosphorus : The phosphoprotein-phosphorus was found in the liver and serum of the laying hens only, while it could not be detected either in non-laying hens, cocks and newly hatched chicks (Table 1), or in the other organs of the laying hens (Table 2). Although there were some fluctuations in the phosphoprotein-phosphorus content of the liver and serum, there were average contents of phosphoprotein of 19.5 mg /g fresh tissue and 15 mg/cc serum respectively, if the conception that the phosphoprotein of liver and serum contain about 1 per cent of phosphorus is valid (4. 6. 10. 11).

3. Nucleic acids : In the course of the determination of phosphoprotein-phosphorus, an determination of phosphorus of DNA and RNA was made. Though some variations of nucleic acids content existed within the same group, it was noted that the average DNA content of the laying hens' liver was higher than that of chicks ($a=0.05$), and that the average RNA of the laying hens' liver was greater than that of non-laying hens ($a=0.01$) and the cocks ($a=0.01$). The average of the RNA/DNA value of the liver was higher in the order of the baby chicks, laying hens, cocks and non-laying hens. The

Table 1. The crude protein, nucleic acids- and phosphoprotein-phosphorus content of liver and serum in the fowl.

No.	Liver				Serum		Liver				Serum	
	Crude 1 protein	DNA3 -P	RNA3 -P	Prot.3 -P	Crude 2 prot.	Prot.3 -P	Crude 1 prot.	DNA3 -P	RNA3 -P	Prot.3 -P	Crude 2 Prot.	Prot.3 -P
i) Laying hens							iii) Cocks1					
1	192	230	480	80	—	—	167	200	400	0	24	0
2	190	230	480	170	32	320	177	200	420	0	33	0
3	145	220	480	100	50	320	210	200	440	0	29	0
4	168	160	440	120	48	200	157	165	390	0	34	0
5	201	140	460	100	60	280	189	140	390	0	27	0
6	145	180	500	100	45	400	149	160	400	0	35	0
7	145	200	500	100	49	200						
8	162	200	500	160	62	400						
M	168	195	480	116	49	303	175	178	407**	0	30**	0
sd	23.2	33.0	21.4	32.1	10.0	82.8	22.3	26.0	19.7	—	4.4	—
RNA/DNA :	2.46±0.39						: 2.29±0.30					
ii) Non-laying hens							iv) Newly hatched chicks					
1	116	230	480	0	25	0	120	190	500	0		
2	177	260	440	0	34	0	145	170	550	0		
3	154	260	440	0	37	0	160	160	520	0		
4	130	215	420	0	25	0	110	150	470	0		
5	143	150	360	0	—	—	105	160	520	0		
6	133	180	400	0	—	—	140	130	497	0		
M	142	216	423**	0	32*	0	130*	160*	497	0		
sd	21.3	44.1	40.9	—	6.5	—	21.7	16.8	38.3	—		
RNA/DNA :	1.96±0.19*						: 3.10±0.38*					

1. Dry matter weight mg/g fresh tissue.
2. Dry matter weight mg/cc serum.
3. Phosphorus μg/100mg crude protein.

* $a=0.05$, ** $a=0.01$ Comparing with the value of the laying hens, significant at the level of

Table 2. The crude protein, nucleic acids-and phosphoprotein-phosphorus content of various organs in the laying hens.

No.	Crude prot.	DNA-P	RNA-P	Protein -P.	Crude prot.	DNA-P	RNA-P	Protein -P.
i) Kidney					iii) Spleen			
2	150	180	340	0	148	780	580	0
4	126	220	380	0	147	600	540	0
5	140	180	380	0	141	800	480	0
6	110	260	580	0	133	800	800	0
7	146	220	620	0	138	800	800	0
8	162	220	580	0	157	800	760	0
M	141	213	480	0	144	763	667	0
sd	19.9	30.2	142.5	—	8.5	80.4	152.6	—
ii) Small intestine					iv) Brain			
3	100	240	360	0	100	80	240	0

differences between the laying hens and the non-laying hens ($a=0.05$), and between baby chicks and laying hens ($a=0.05$) were significant.

Upon close inspection we detect no nucleic acids in the serum.

Discussion

Among a considerable number of works dealing with the drastic changes in the blood serum and tissue of the fowl during the egg-laying, few paid particular attention to the problem of the site of phosphoprotein (vittelin) production. Much interest has been manifested in the study as to where the phosphoprotein was formed in the laying hens. The results of the present experiment definitely indicated that there was an occurrence of the phosphoprotein only in the liver. This was detected before in the serum of the laying hens (5. 6). We also noted RNA and RNA/DNA of laying hens' liver were tein formation than those of the other adult fowls, indicating that a rapid promuch higher was taking place (Table 1).

It was well known that a similar phenomena — the formation and mobilization of the building materials readv for egg-formation, as mentioned above,— appeared experimentally by the administration of estrogen (12–17) or of gonadotropic hormone (18) to birds. Many studies were carried on with these estrogenized birds to show biochemical evidences of egg-formation, as well as normal laying hens. However, the identity of the natures of these natural and estrogenized phenomena seemed to be a matter of speculation.

A dominant increase of phosphoprotein was reproted by Mandel and Madel (19) in the liver of the estrogenized pigeon from 0.012 mg (no treatment) to 0.070 mg (treated with 0.25 mg estradiol dipropionate for 7 days)/100 mg crude protein. Recently Hosoda *et al.* (20) conducted a hindering experiment of vittelin production by para-nitro-orthotoluidine administration to the laying hens and claimed the liver as the locus. Our findings that phosphoprotein (vittelin) appeared in a notable amount in the liver of laying hens and that this protein would be formed there was conclusively identical with that of Mandel and Mandel and Hosoda *et al.* However, there were some discrepancies in phosphoprotein-phosphorus content from the result of Mandel and Mandel. These discrepancies might be ascribed to the use of different species (pigeon) or to the treatment of birds with estrogen in the experiment of Mandel *et al.*

The existence of phosphoprotein in the serum (plasma) of laying hens has been demonstrated by several authors, as described above, and it was further confirmed (21–24) by isotopical or electrophoretical investigations that the serum vittelin were located in the *r*-globulin fraction of laying serum. The average phosphoprotein-phosphorus content in the laying serum was 0.150 (0.100–0.248) mg/cc serum in the present experiment. This value was higher

than that reproted by Roepke *et al.* (5), Laskowsky (6) and Hevesey (25) (e.g. 0.041, 0.089 and 0.094 mg/cc serum, respectively). But the result of Laskowsky's analysis also indicated that there were some fluctuations in the protein-phosphorus content from 0.041 to 0.150 mg/cc serum. Therefore, the possibility might be admitted that a variable amount of phosphoprotein exists in the laying hens' serum depending on the rate of ovum growth in the ovary.

In view of the evidences descibed above, it is likely that phosphoprotein would be formed in the liver and carried to the ovary by the blood stream and finally deposited in the ovum, similar to the case of phosphatides production in laying hens (25–27).

Now let us consider the problem of nucleic acids content. Generally speaking, it was well established that nucleic acids played an important and essential part in the cell; DNA played a basic role in the cell division, and its content presented the cell number in a certain amount of tissue, whereas RNA was abundant in the cell in which rapid protein synthesis was occurring to produce the main bulk of cytoplasmic protein for growth or to perform a large amount of secretion. It has hitherto been remarked that RNA content and the RNA/DNA oflaying hens' liver are greater than that of the non-laying hens and cocks. At the same time the DNA content of the chicks' liver was less than that ofthe laying hens, and then the RNA/DNA appeared somewhat higher than that of the laying hens' liver. Consequently, the conclusion was drawn that the significant preparation of protein was occurring in the liver of both the laying hens and baby chicks, particularly in laying hens' liver where the phosphoprotein might be produced at a great rate.

Common *et al.* (28, 29) and Mandel *et al.* (19, 30) examined the effects of gonadal hormones on nucleic acids of liver and serum, and showed that estrogen evoked at apparent increase of weight, crude protein and total RNA, but only a slight increase of DNA in the liver. According to Common *et al.* (29) the RNA/DNA was relatively higher in younger chick but declined a little during its growth. When they reached their reproductive stages the value of ratio began to increase in the liver of the female but did not show a simllar tendency in the male. Hence they attributed these sexual differences in nucleic acids content to the role of endogenous estrogen.

Information as to the existence of nucleic acids in the blood serum had been obscure on account of its low density, if they occur at all. We could not find any measurable nucleic acids, even in the laying serum, whereas Mandel *et al.* and Common *et al.* noticed the increase of the nucleic acids content in the estrogenized birds.

There was no significant differences among the crude protein content of the adult fowls' liver. But the crude protein concentration of the serum was higher in the laying hens than the cocks and non-laying hens. Sturkies *et*

el. (31, 32) stated that the plasma protein concentration of the male (4 per cent) was lower than that of the female without regard to egg-production (4.6–5.3 per cent). It was also apparent that the estrogen caused the increase of plasma protein content, while the thyroxine prevented this effect. It was accordingly conceivable that the lowness of serum protein in non-laying hens compared with laying hens might be ascrbed to the fact that the non-laying hens were just moulting.

The present studies, therefore, presented several fresh problems to be resolved. There are three main subjects as follows : i) The properties of serum phosphoprotein (vittelin), ii) the mechanism of the phosphoprotein synthesis in the liver and iii) the mode of vittelin deposition into the ovary. Further experiments on the natures of serum vittelin and the phosphoprotein formation with the liver slice *in vitro* are in progress.

Summary

1. An experiment was made to identify the site of the phosphoprotein (vittelin) formation in the laying hens.

2. The determination of phosphoprotein of several organs was carried out, and the occurrence of phosphoprotein was detected both in the liver as well as in the serum.

3. The difference in the RNA and also in the RNA/DNA existed between laying hens and other adult fowls. This fact was discussed in relation to the phosphoprotein formation in the liver of laying hens. No nucleic acids could be founnd in the serum, even in that of the laying hens.

4. From those findings it was considered that the liver was to be considered as the site of phosphoprotein (vittelin) formation.

5. The crude protein content in the serum was higher in laying nens than those of cocks and non-laying hens.

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