Studies on the Protein Patterns and Free Amino Acids in the Serum of Laying Hen

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The serum or plasma protein patterns of laying hen has been studied by several workers in recent years. A. Rako[9] reported that total serum protein showed a positive and significant correlation with the production of eggs. F. K. Kristjansson et al[4]., using starch gel electrophoresis, found that prealbumin B was present in the serum of laying hens, but not present in the serum of males, immature females or mature females which are out of laying. Also, from the investigation with starch gel electrophoresis technique, I. E. Lush[5] found that characteristic changes in five plasma proteins occurred in egg-laying and moulting behaviour.

There exists a considerable amount of literature on electrophoretical research for blood proteins of birds, but little work has been done in order to investigate the blood protein patterns chromatographically.

The present study was conducted to investigate the serum protein patterns of laying hen under various feeding conditions such as semi-purified diet, formula feed and fasting, by means of liquid chromatography and paper electrophoresis. In addition, the contents of serum proteins and free amino acids were also measured.

MATERIALS AND METHODS

1. Test hens and keeping

Four 2-year-old Single Comb White Leghorn hens (one strain) were used in this study. Two hens (No. 1 and No. 2) were used for serum protein analysis, and four hens (No. 1–No. 4) were used for free amino acid analysis of serum. The hens were caged individually and reared with formula feed. The experiments of semi-purified diet were carried out for 7–12 days after the fasting period of 48 hours. The composition of the semi-purified diets used in these experiments is shown in Table 1. Feeds and water were provided ad libitum.

2. Sampling of blood and preparation of sera for analysis

Samples of blood (3–5 ml) were drawn into vaccum blood collecting tubes with specific needles at 9 : 30 a.m. from the wing vein, and were deposited at 38°C

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Table 1. Composition of semi-purified diets (%)

Ingredients	Diet A	Diet B
Egg albumen*	26	13
Dextrine	4	4
Corn starch	30	40.2
Fiber	5	5
Lime stone	5	5
Dicalcium phosphate	1	1
Mineral mix.[1)]	5	5
Corn oil	5	5
Choline chloride	0.15	0.15
Glucose	18.4	21.2
Vitamin B mix.[2)]	0.30	0.30
Vitamin A, D[3)]	0.15	0.15
Total	100.0	100.0
Protein level	20	10

* About 78 % protein (N × 6.25)

1) Composition of this mixture, $CaCO_3$ 5.6%, $Ca_3(PO_4)_2$ 52.4%, K_2HPO_4 16.8%, $MgSO_4$ $7H_2O$ 4.7%, $Fe(C_6H_5O_7)_2$ $6H_2O$ 2.6%, $ZnCl_2$ 0.05%, KI 0.07%, $CuSO_4$ $5H_2O$ 0.05%, H_3BO_3 0.02%, $CoSO_4$ $7H_2O$ 0.002%, $MnSO_4$ 1.2%, NaCl 16.5%.

2) Containing 440 mg of riboflavin, 440 mg of Ca-pantothenate, 440 mg of nicotinic acid, 2,200 mg of choline chloride and 13.2 mg of folic acid per 100 grams.

3) Containing 10,000 I.U. of vitamin A and 1,500 I.C.U. of vitamin D_3 per one gram of supplement.

for 30 minutes, then stored at 2°C for one or two days. The samples were centrifuged at 3500 r.p.m. for 10 minutes to collect serum. The serum (about one gram) was weighed into a small cellophane bag, and dialized against an acetic acid ammonium acetate buffer solution (M/10 acetic acid 8 : M/10 ammonium acetate 2, pH 3.9) for two days at 2°C.

3. Fractionation of serum proteins with CMC[7)11)]

The dialized serum was chromatographed on 2–4 g of carboxy-methylcellulose ion exchanger (CMC, Serva Co. 0.95 meq/g) in a column 15 cm long and 2.2 cm wide. The elution was accomplished by changing pH of eluting buffer from 3.9 to 11.0 gradually, and the eluting volume was about 2,000 ml in each case. Fractions (8–10 ml) were collected with an automatic collector, and estimated by measuring the optical densities at 280 mμ with a spectrophotometer (Hitachi, EPU-2A).

Except the above-mentioned buffer solution, sodium phosphate (M/5 sodium phosphate monobasic 39 : M/5 sodium phosphate dibasic 61, pH 7.0), and sodium carbonate (M/10 sodium carbonate pH 11.0) were employed in this study.

4. Electrophoretical analysis[8)] and optical measurement of serum proteins

Serum (0.0008 ml) was spotted on the position of one-third from the anode of

cellulose acetate paper (Fuji Separax) with a micropipette. Beronal buffer solution (0.007 M pH 8.6) was used. Electrophoresis was carried out for 50–60 min. at 0.8 mA/cm. The stripes were stained in Ponceau 3R (6% trichloroacetic acid solution) and destained with 1% glacial acetic acid. Each fraction was measured by the indirect method.

The percentage of protein in serum was measured with Serum Protein Refractometer (Atago Inst.) optically.

5. Paper chromatographical analysis of free amino acids in the serum

Free amino acids in the serum were qualitalively analyzed by a method of the two-dimensional ascending paper chromatography. Serum proteins which precipitated by adding 0.1–0.4 ml of 2/3 N H_2SO_4, 0.1–0.3 ml of 10% sodium tungstate and 0.3 ml of H_2O were removed by centrifugation. The whole transparent liquid was spotted in one corner of the paper (Toyo No. 51 filter paper, 40 × 40 cm) for analysis of free amino acids. As the first running solvent n-butanol: acetic acid: water (60 : 15 : 20) and as the second solvent phenol : water (4 : 1) were used. A small beaker containing a sodium cyanide solution was placed inside the dish to prevent oxidation of phenol. 0.1% ninhydrin (W/V) in n-butanol was sprayed to locate the free amino acid. Known standards were run in the same system to identify the spots. A rough evaluation of spots of free amino acids was carried out by the method of Reid et al[10].

RESULTS AND DISCUSSION

1. Analysis of serum proteins with liquid chromatography

About thirteen fractions could be demonstrated in serum samples in this study. But not any sera had all fractions. Fig. 1 shows the effluent diagrams of hen's serum on various conditions. The isoelectric points of human plasma proteins have been reported in the range of pH from 3.9 to 7.3[1], but that of hen was not clarified. Details of the individual fractions showing remarkable changes through condition are as follows.

Fraction A

The protein of this fraction decreased strikingly through fasting in this study, and it also tended to decrease in the case of laying. Considering the eluting pH of this fraction, it seems to involve mucoproteins. The influence of the bodily reserve of energy, therefore, may instantly appear upon this fraction of the serum proteins. Therefore, it seems that this fraction has a considerable relation to egg-laying.

Fraction C

Being a major component, the fraction was found in all the serum. It seemed

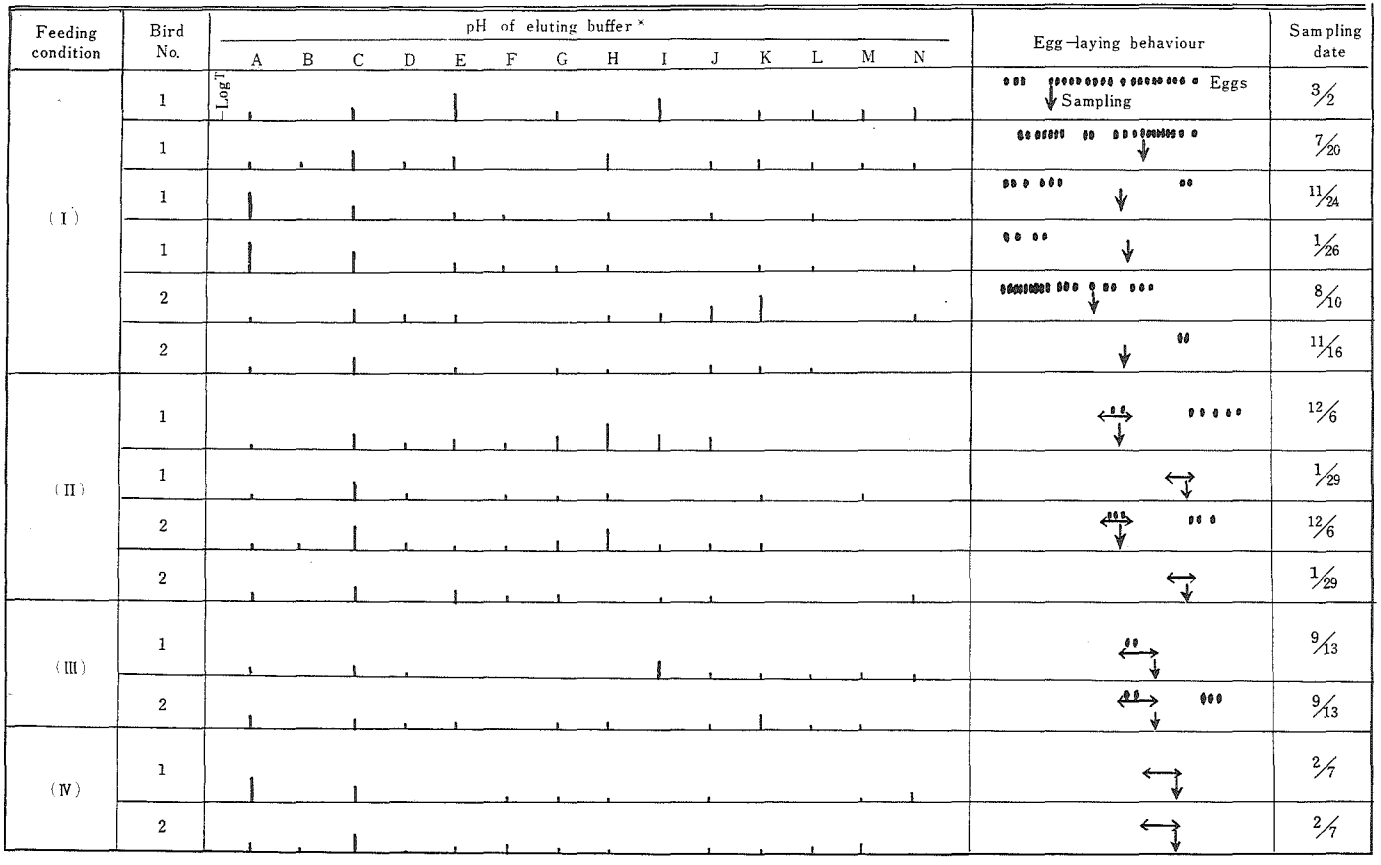

Fig. 1. Fractionation of serum proteins on various feeding conditions and egg-laying behaviour
(I) Formula feed (II) Fasting (III) 20% protein semi-purified diet (IV) 10% protein semi-purified diet
* A (3.8–4.2) B (4.3–4.7) C (4.8–4.9) D (5.0–5.3) E (5.4–6.1) F (6.2–6.6) G (6.7–7.2) H (7.3–7.9) I (8.0–8.6) J (8.7–9.2) K (9.3–9.7) L (9.8–10.2) M (10.3–10.6) N (10.7) ↔ : Test period

to involve serum albumin judging from the eluting pH range. Generally, low protein diets, non-protein diets or fasting bring about decrease of serum albumin[13]. P. W. Waldroup[15] reported that the serum alpha and beta globulin component of the hen fed with low protein diets dropped significantly and the decrease of the total protein was caused by this drop. But fraction C which seemed to involve serum albumin was not so changeable in the serum of low protein diet fed hens in the study.

Fraction D

This fraction appeared in almost all the serum of laying, and it seemed to be lipoprotein which is the most characteristic component in the serum of laying hen as reported early[3]. There was, however, no certain evidence for this fraction being lipoprotein.

Fraction F

This fraction had a tendency to disappear in the serum of egg-laying hen within a few days before or after sampling of blood. I. E. Lush[5] reported that fraction 8 appeared as each hen went out of laying and disappeared a few days before laying. It suggested that 'Fraction F' might be the same component as Lush's fraction 8. But in the fasting state, it appeared in the serum of egg-laying hen exceptionally.

Although the other fractions were fairly changeable, proteins in the range of pH 6.7–9.3 (from G to J) showed a considerable increase in relation to the egg-laying. But it may be impossible to account for details of these proteins at this stage.

2. Determinations of the total protein level with refractometer

The results of measurement are presented in Fig. 2, and it shows a fair difference among individuals and considerable alterations in each hen. It has been reported that the reduction of plasma proteins occurred through fasting or lowering the protein level of diet, and it resulted in hypoproteinamia[6]. On the other hand, A. Rako[9] reported that the serum protein level of hens was higher during the egg-laying period than it was in the unproductive phase. P. D. Sturkie[14] described, however, there was no significant difference between the total plasma protein level of laying and non-laying hens, but there was a considerable variation among groups of laying birds.

In the present study, as shown in Fig. 2, the serum protein level of hens was strikingly reduced through fasting, and it also showed a slight increase in the serum of laying compared with that of non-laying.

From the fact that the hens laid normal eggs in the severe state such as fasting, it was suggested that the proteins for laying might be reserved for a few days.

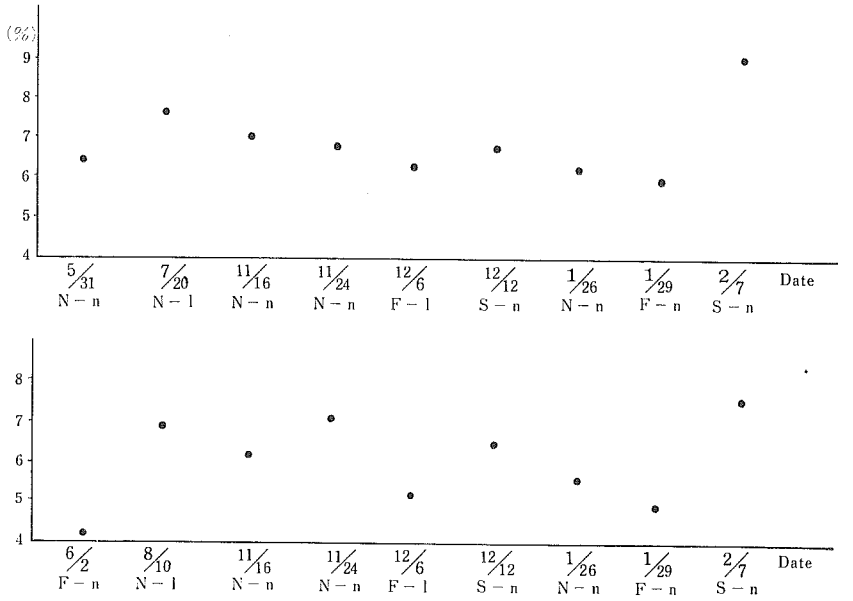

Fig. 2. Measurements of the total serum proteins with refractometer
N : Formula feed S : Semi-purified diet F : Fasting 1 : Laying n : Non-laying

It is impossible to clarify the reason why the serum protein levels of the both No. 1 and No. 2 hens arose abnormally when the semi-purified diet of 10% protein level was provided.

3. Paper electrophoretic analysis

The bands of five components; albumin, alpha 1, alpha 2, beta and gamma globulin were observed. In the previous study, a considerable increase of the ratio of albumin to globulin (A/G ratio) was observed in the serum of fasting. This increase might be especially due to the reduction of gamma globulin, rather than to the rise of albumin (Table 2). By some previous investigations into the relations between serum protein components and egg production, it is recognized that the A/G ratio of laying hens was lower than that of non-laying[12]. In this study the No. 1 hen showed such a tendency. It is interesting to note that when the hens were fed with the 20% protein level semi-purified diet, their A/G ratio reduced considerably as the result of decrease of albumin.

Finally, the reasons why the A/G ratio arose strikingly in the case of fasting, and why the negative correlation was observed between A/G ratio and the protein level in the serum, were not obvious.

4. Free amino acids of serum

Table 3. summarizes the results from the paper chromatograms of the free amino acids of serum. It was possible to observe eleven amino acids in this

Table 2. Serum protein components influenced by feeding conditions and egg-laying behaviour

Condition and behaviour	Percent of component					A/G ratio	Total protein (%)
	Albumin	Globulin					
		alpha 1	alpha 2	beta	gamma		
No. 1							
N-1	40.4	9.6	11.5	13.5	25.0	0.68	7.0
F-1	40.8	8.2	10.2	10.2	30.6	0.69	6.3
S-1	41.5	6.2	7.7	7.7	36.9	0.56	6.7
N-n	43.8	1.4	2.8	8.4	43.8	0.78	6.2
F-n	58.0	14.0		7.0	20.9	1.39	6.0
No. 2							
F-1	44.6	17.2		10.3	25.9	0.87	5.2
S-n	34.5	8.0	6.9	9.2	41.4	0.53	6.5
N-n	44.4	5.6	9.3	11.1	29.6	0.80	5.6
F-n	51.5	3.0	12.1	9.1	24.2	1.06	5.0

N : Normal (formula feed) S : Semi-purified diet (20% protein level) F : Fasting
1 : Laying n : Non-laying

Table 3. Free amino acids in blood serum

	No. 1		No. 2		No. 3		No. 4	
	N	F	N	F	N	F	N	F
Cystine	++++	++++	++++	++++	++++	++++	++	++++
Glycine	++++	++++	++++	++++	++++	+	++++	
Glutamic acid			++++				++++	+
Serine	++	++++	++++	++++	++++	++++	++++	++++
Histidine				++	++	+		++
Arginine	++++	++++	++	++	++	++++	++++	++++
Alanine		++	++++	++++	+			
Methionine (Valine)		+			++	++	+	+
Leucine (Isoleucine)		+		+	++	++	+	++
Lysine	++++	++++	++++	++++		++++		++++
Aspartic acid								+

N : Formula feed F : Fasting
++++>+++>++>+

experiment. Among these amino acids, leucine (isoleucine), cystine, serine and histidine seemed to be somewhat high on fasting for 48 hours compared with the normal state. In contrast, glutamic acid and glycine tended to decrease slightly. The amounts of other amino acids showed little change. Hill et al[2]., reported that lysine and threonine in the plasma of chicks highly increased on removal of diet for 12–48 hours. Since the amounts of lysine and threonine in the serum on fasting for 48 hours did not differ so much from that of the normal feeding condition, thus it would be supposed that body tissue proteins would not work so much as the energy source under the starved condition for two days.

SUMMARY

1. Using liquid chromatography with CMC and paper electrophoresis the variances of the laying hen's serum protein patterns influenced by feeding conditions; fasting, formula ration and semi-purified diets (10 % and 20 % protein levels) feeding were investigated and discussed.

2. Thirteen fractions were demonstrated by means of liquid chromatography. Fraction A, which seemed to involve mucoprotein, strikingly reduced through fasting. Fraction D seemed to be lipoprotein, and showed characteristic variances on laying. Fraction F seemed to involve the characteristic protein for egg production. Other fractions fairly changed through feeding conditions and egg-laying.

3. According to the electrophoretic analysis, fasting brought about a considerable increase of the A/G ratio as the result of a progressive decrease in the gamma globulin fraction. Moreover, when hens were fed with the 20 % protein level semi-purified diet, reduction of albumin, in the both analyses, electrophoresis and liquid chromatography, was observed.

4. Free amino acid analysis of serum by paper chromatography showed some increase of leucine (isoleucine), cystine, serine and histidine on fasting for 48 hours, but glutamic acid and glycine showed a tendency to decrease slightly.

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産卵鶏における血清蛋白質ならびに遊離アミノ酸含量の変動について

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要約

種々の飼料条件が産卵鶏の血清蛋白質のパターンならびに遊離アミノ酸含量におよぼす影響を調査する目的で本実験を行なった。供試鶏としては白レグ産卵鶏を用い，これの絶食時ならびに慣用配合飼料，半精製飼料（蛋白質源として卵白粉を用い蛋白質含量を10%および20%の2種類に調整した）給与時における血清蛋白質パターンを濃度勾配法による液体クロマトグラフィーおよび電気泳動法により，また遊離アミノ酸は二次元ペーパークロマトグラフィーによりそれぞれ調査した。その結果は以下のごとくである。

1. 血清蛋白質は液体クロマトグラフィーにより13のフラクション（AからNまで）に分割され，そのパターンは飼料条件や産卵状況によりかなり異なった。すなわち絶食により，フラクションAが著しく減少したが，産卵中にも同様な傾向が伺われた。フラクションCは血清アルブミンが主体と考えられるが，条件による変動は少なかった。フラクションDは産卵中の血清において多くみられることから産卵に関係する蛋白質と考えられた。フラクションFは逆に産卵中において多く消失した。

2. 絶食によりA/G比（アルブミンとグロブリンの比率）はかなり増加した。これはγグロブリンの減少によるものと考えられる。なお半精製飼料（20%蛋白質レベル）給与時においてはアルブミンの減少がみられた。

3. 48時間の絶食によりロイシン（イソロイシン），シスチン，セリン，およびヒスチヂンがやや増加したが，グルタミン酸とグリシンは逆に幾分減少した。

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