Comparative Chemical Studies on the Proteins in the Quail and Chicken Egg Whites

(II) On the Ovalbumin

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INTRODUCTION

As to the Japanese quail ovalbumin, under the comparison with the chicken ovalbumin, the following facts were noticed in the previous report[1], by one of the authors. In spite of the crystallization repeated several times with sodium sulfate, no revelation of a single electrophoretic pattern was brought forth by the quail ovalbumin. In the ovalbumin was contained a small amount of other protein than that pattern. In both phosphate buffer (*pH* 7.00) and carbonate buffer (*pH* 9.80) the electrophoretic mobility of the quail ovalbumin was ascertained to be smaller than that of the chicken ovalbumin. Comparing with the *pH* value of the chicken ovalbumin eluted from the CM-cellulose column, that of the quail ovalbumin was slightly higher. These suggest the isoelectric point of the quail ovalbumin is higher than that of the chicken ovalbumin.

When the quail ovalbumin aqueous solution was released from the frozen-state kept at $-20°C$ for one year, no variation was visible in the solution, while the chicken ovalbumin solution produced a small amount of precipitate being a denatured protein.

In the present paper, some descriptions were made concerning the purification process, molecular weight, amino acid and carbohydrate compositions of the Japanese quail ovalbumin.

MATERIALS AND METHODS

Materials——Fertile eggs of the Japanese quail (*Coturnix coturnix japonica*) were secured from the Laboratory of Animal Breeding, Faculty of Agriculture, Kagoshima University, within 24 hr after being laid.

Preparation of ovalbumin—— According to the common method, ovalbumin was separated from the homogenized egg white by the crystallization repeated five times with sodium sulfate. As indicated in the previous paper[1], although the chicken ovalbumin was purified only by the salting-out procedure, the quail ovalbumin was left unrefined even by the same treatment owing to the inclusion of a small amount of other protein. This ovalbumin was therefore chromatographed on a column of CM-cellulose (2.3 × 25 *cm*) and the fractions corresponding to the albumin peak were collected, and

then dialyzed against 20 % polyethylene glycol for concentrating. After being dialyzed the solution against the water, and subsequently 0.1 *M* acetate buffer *pH* 4.50, the ovalbumin solution (0.93 %, 4 *ml.*) was applied on Sephadex G-100 column (2.2×52 *cm*) and collected in 5 *ml.* fractions. The protein and neutral sugar amounts in each fraction were determined, separately. Sugar content was measured by the phenol-sulfuric acid method[2] on the basis of the calibration curve for mannose. The combination of ovalbumin fractions obtained by multiple gel filtrations was dialyzed against the water, and the ovalbumin aqueous solution was used as the sample for carbohydrate and amino acid analysis.

Estimation of the molecular weight of quail ovalbumin and duck ovalbumin by Sephadex gel-filtration —— Estimation of the molecular weight of ovalbumin was done, referring to the convenient Sephadex gel-filtration method proposed by WHITAKER[3] and ANDREWS[4]. Several authentic proteins of known molecular weights were in turn applied on to a 2.64×64 *cm*. Sephadex G-100 column (Sephadex from Pharmacia, Uppsala, Sweden) and eluted with 0.05 *M* carbonate buffer, *pH* 10.0, (ionic strength 0.10) to measure each elution volume, V. The void volume, V_0 was measured by eluting the blue dextran (Mol. Wt.; approximate 200×10^4). The concentration of blue dextran was estimated by the optical density at 610 *nm*. The column was obtained from Shoei Works Co. Ltd. One *ml.* of the respective protein solution applied contained 4 *mg* of protein. Fraction size was taken to be 3 *ml.*

Authentic proteins used were as follows: human *r*-globulin (M. W., 160000), bovine serum albumin (M. W., 67000), chicken ovalbumin (M. W., 45000), bovine pancreas chymotrypsinogen A (M. W., 23000), whale skeletal muscle myoglobin (M. W., 17800), horse heart cytochrome c (M. W., 12400). These proteins were obtained from Miles Lab., Inc., Kankakee, U. S. A., except for chicken ovalbumin crystallized five times in our laboratory. After drawing of the estimation curve for molecular weight, the refined quail ovalbumin and duck ovalbumin were eluted through the same column, respectively.

Identification and determination of neutral sugar in the quail ovalbumin —— The analysis of neutral sugar was carried out by gas-liquid chromatography, using Shimazu GC 4B Gas chromatograph. The sample for the chromatography was prepared according to the procedure described by BOLTON et al.[5] and SWEELEY et al.[6]. Twenty *mg* of quail ovalbumin was hydrolyzed with 0.5 *N* HCl in dry methanol (15 *ml.*) in sealed tube for 24 *hr* at 75–80°*C*., and after concentration under the reduced pressure, it was placed in a vacuum desiccator over KOH pellets for 24 *hr* to dryness. The resultant methyl glycosides were treated with a solution of acetic anhydride (1.4 *ml.*) in methanol (4 *ml.*) containing twenty *mg* of silver acetate for 24 *hr* at room temperature to acetylate. The filtrate was evaporated to dryness and kept in a vacuum desiccator for 24 *hr* over KOH pellets. The material was dissolved in 0.6 *ml.* of pyridine, and then 0.05 *ml.* of hexamethyldisilazane and 0.05 ml. of trimethyl-chlorosilane were added to 0.25 *ml.* of this solution, and shaken vigorously. The sample of trimethylsilylated reaction mixture was injected on to a glass column (4 *mm*×2 *m*) packed with 3.1 % Silicone GE SE-30 on Supelcoport (80–100 *mesh*), from Nihon Chromato Works, Ltd. After being kept at 155°*C* for five minutes, the column was programmed at 4°*C*/*min*. to a maximum of 215°*C*. The temperatures of an injection part and a detector (hydrogen flame ionization detector) were 180°*C* and 250°*C*, respectively. Carrier gas was nitrogen with a flow

of 50 *ml/min.*. Peaks were identified by comparison with the authentic materials. A known amount of the authentic glucose was employed as the internal standard.

Identification and determination of hexosamine in the quail ovalbumin——The identification and determination of hexosamine in the quail ovalbumin were performed according to the Pearson's procedure[7] and the Elson-Morgan method modified by BOAS[8]. Ten *ml.* of 8 *N* HCl was added on the ovalbumin aqueous solution (2 %, 10 *ml.*) and the mixture was hydrolyzed in a sealed tube for 7 *hr* at 100°*C*. The filtrate was concentrated to a small volume under the reduced pressure, and then evaporated in a vacuum desiccator over sodium hydroxide pellets, being kept for one week. The dried hydrolysate was dissolved and diluted to be 25 *ml.* with 0.3 *N* HCl. This solution (2 *ml.*) was applied on a column (0.6 × 40 *cm*) of Amberlite CG-20 for chromatography. Subsequent procedures were the same as described in the previous paper.[9]

Moreover, the determination of hexosamine was also performed by another way. Referring to ROSAN's report[10], the authors devised a conveniently improved procedure of hexosamine estimation, using a short column for basic amino acids in automated amino acid analysis and were able to separate amino acids and hexosamines successfully.

The dried hydrolysate mentioned above was dissolved and diluted to be 50 *ml.* with citrate buffer (0.15 *N* as Na ion, *pH* 5.70). One *ml.* of this solution was applied on a short Aminex A-5 column of Yanagimoto LC-5s amino acid analyzer. Conditions employed were as follows: column; 0.9 × 7.5 *cm*, column temperature; 52°*C* constant, flow rate; buffer 100 *ml./hr*, ninhydrin 50 *ml./hr*. A known amount of authentic glucosamine and galactosamine were independently eluted.

Amino acid analysis of the quail ovalbumin —— Five *ml.* of 12 *N* HCl was added to 5 *ml.* of 1 % ovalbumin aqueous solution in the pyrex tube. After the removal of air by the suction followed by the introduction of nitrogen gas, the tube was sealed. The ovalbumin separated by the crystallization repeated five times with salt and the ovalbumin refined by the salting-out followed by the CM-cellulose chromatography and Sephadex G-100 gel-filtration, were used respectively. The hydrolysis was carried out for 24 and 40 *hr* at 110°*C*. The filtrate was concentrated and diluted to be 100 *ml.* with citrate buffer (0.2 *N* as Na ion, *pH* 2.2). An aliquot of the solution was used for the amino acid analysis using the analyzer, as was described preceedingly.

Measurement of heat-coagulating point of the ovalbumin —— Five *ml.* of ovalbumin aqueous solution (approximate 2 %) was poured into the pyrex tube (1.7 × 11 *cm*) with reflux as shown in Fig. 1. After the sample solution was exactly kept at various programmed temperatures for 10 min., it was cooled to the room temperature and

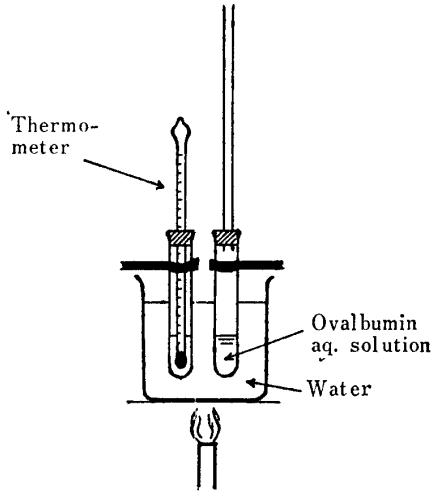

Fig. 1. Equipment for the measurement of the heat-coagulating point of ovalbumin.

centrifuged, and then the protein concentration of the supernatant was measured. From the plotting of the concentration against various temperatures, the heat-coagulating point was estimated. Through the whole experiments, basing on the equation of the calibration curve for quail ovalbumin, the protein concentration or the amount was determined from the absorption value at 280 *nm*.

RESULTS AND DISCUSSION

CM-cellulose chromatography of the quail ovalbumin separated by the salting-out procedure was represented in Fig. 2. The diagram indicates that the ovalbumin was not refined only by the salting-out, owing to the inclusion of small amount of alien proteins which were inferred to be ovomucoid and conalbumin. The fractions corresponding to a major albumin were collected and sieved on Sephadex G-100 column for refining. The elution diagram was shown in Fig. 3. The diagram reveals only one sharp peak concerning the protein and the sugar, respectively.

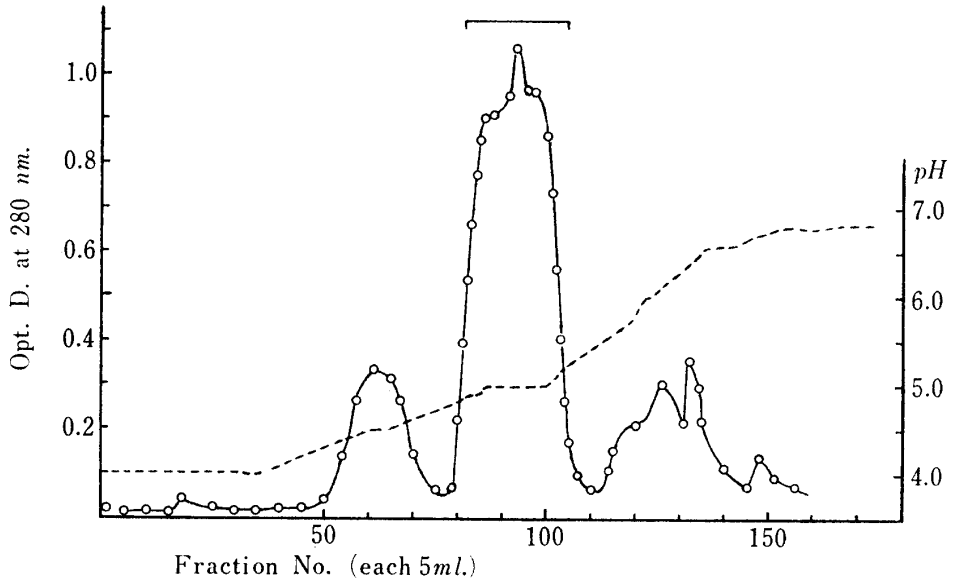

Fig. 2. CM-cellulose column chromatography of the quail ovalbumin separated by the salting-out
(Ovalbumin : crystallized five times with sodium sulfate)
—○—○— Protein, - - - - - *pH*, Eluting buffer; 0.025 *M* acetic A.-Na-acetate

From the protein and sugar amount in the eluates at the peak and the near position, the neutral sugar content of ovalbumin was ascertained to be 2.30 %. Sugar content of the ovalbumin separated only by the salting-out procedure was fixed to be 2.51 %, which was slightly higher than the value of the refined ovalbumin.

In the gas chromatography preliminarily carried out to investigate the trimethylsilylated reaction mixture, it was inferred that the sample contained no amount of sugars such as glucose and galactose except mannose. Subsequently, trimethylsilyl (TMS) derivative of mannose, TMS derivative of the hydrolysate of the quail ovalbumin, into which glucose was introduced as an internal standard, prior to hydrolysis, and TMS derivative

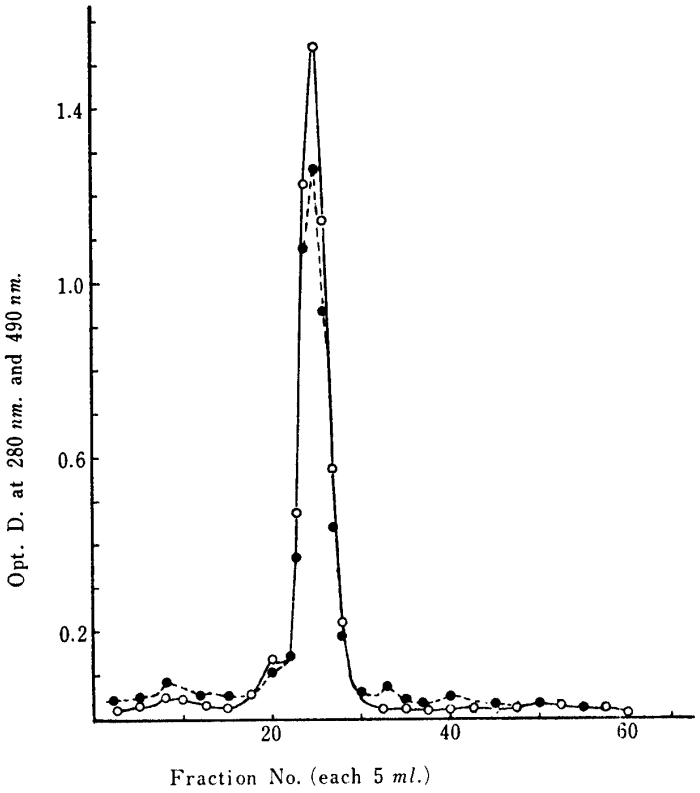

Fig. 3. Sephadex gel-filtration of the quail ovalbumin separated by the salting-out followed by the CM-cellulose column chromatography
Sephadex ; G-100 column (2.2 × 52 *cm.*) was used.
—○—○— Protein, —●—●— Sugar

of the ovalbumin hydrolysate were separately run for the analysis with gas-liquid chromatograph under the same condition. The gas chromatograms were as shown in Fig. 4. TMS derivatives of mannose and quail ovalbumin hydrolysate revealed three peaks, resulting from the isomerization, and the retension times of these peaks were equal one another. RICHEY et al[11]. reported also the resolution of TMS derivative of mannose into three peaks. Therefore, neutral sugar of the quail ovalbumin was identified as mannose and the content was ascertained to be amounting to 2.39 %, calculating from glucose amount added on the sample as an internal standard. Mannose contents estimated by both of the phenol-sulfuric acid method and the gas-liquid chromatography was agreeing mutually.

These values are more realiable than the value (4.32 %) described in the previous report[1], since the latter was calculated from the calibration curve for galactose. Moreover, mannose content of the chicken ovalbumin was fixed to be amounting to 1.79 %, which agrees with the minimum mannose content (1.77 %) obtained by FRANÇOIS et al.[11] with the radio isotope dilution method.

Hexosamine of the quail ovalbumin was identified as glucosamine with the Amberlite CG-120 column chromatography as shown in Fig. 5, and identified also from the chromatogram on a short column in the automated amino acid analysis as shown in Fig. 6.

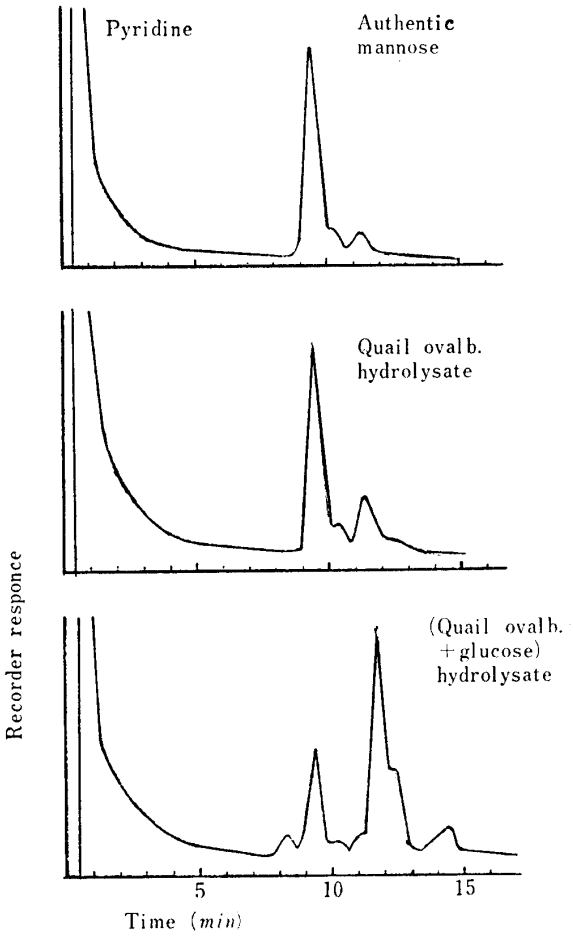

Fig. 4. Gas chromatograms of TMS-derivatives of the known sugars and the quail-ovalbumin hydrolysate

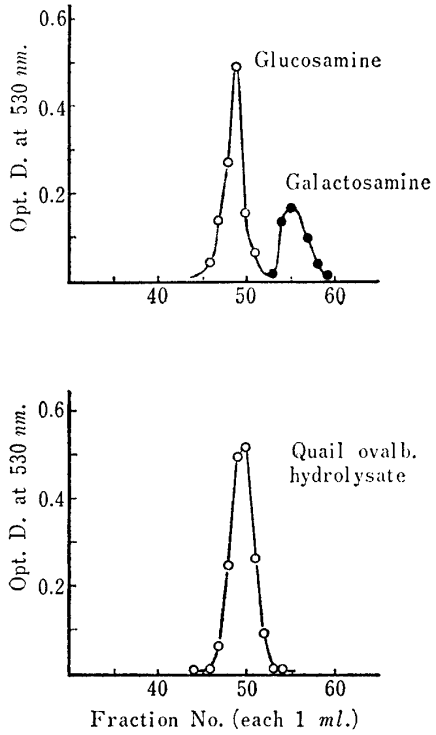

Fig. 5. Chromatography of hexosamine on the Amberlite CG-120 column
Column ; 0.6×40 *cm.* Solvent; 0.3 *N HCl.*

The content was ascertained to be amounting to 0.6 % according to the Elson-Morgan method modified by BOAS[8], and amounting to 0.75 % according to the conveniently improved procedure with amino acid analyzer.

As shown in Table I, during the hydrolysis performed, no significant destruction was observed. Table II represents mannose and glucosamine contents of the quail ovalbumin compared with those of the chicken and duck ovalbumins. JOHANSEN, MARSHALL and NEUBERGER (1958, 1960)[13,14] reported 2 % of mannose and 1.2 % of glucosamine contained in the chicken ovalbumin (Mol. No., 5 : 3 per one mol. of ovalbumin). After that, determining the mannose in ovalbumin by the radioisotope dilution method (1962)[12], they showed the content to be amounting to 1.77 %. BRAGG and HOUGH (1961, 1966)[15,16] stated 2.75 % of mannose and 1.34 % of glucosamine (Mol. No., 6.9 : 3.4 *per* one mol. of ovalbumin). LEE and MONTGOMERY (1961)[17,18] proposed 2.39 % of mannose and 1.25 % of glucosamine in hen ovalbumin, correcting a loss of sugar through the hydrolysis and pointed to the presence of 6 moles of mannose and 3 moles of glucosamine per 45000g of the ovalbumin. After several years, MAKINO and YAMASHINA (1966)[19] proposed 5 moles of mannose and 3 moles of glucosamine per one mol. of ovalbumin and they inferred the whole structure of carbohydrate moiety linked to the asparagine residue in hen ovalbumin.

As shown in Table II, mannose content of the quail ovalbumin was amounting to 2.30

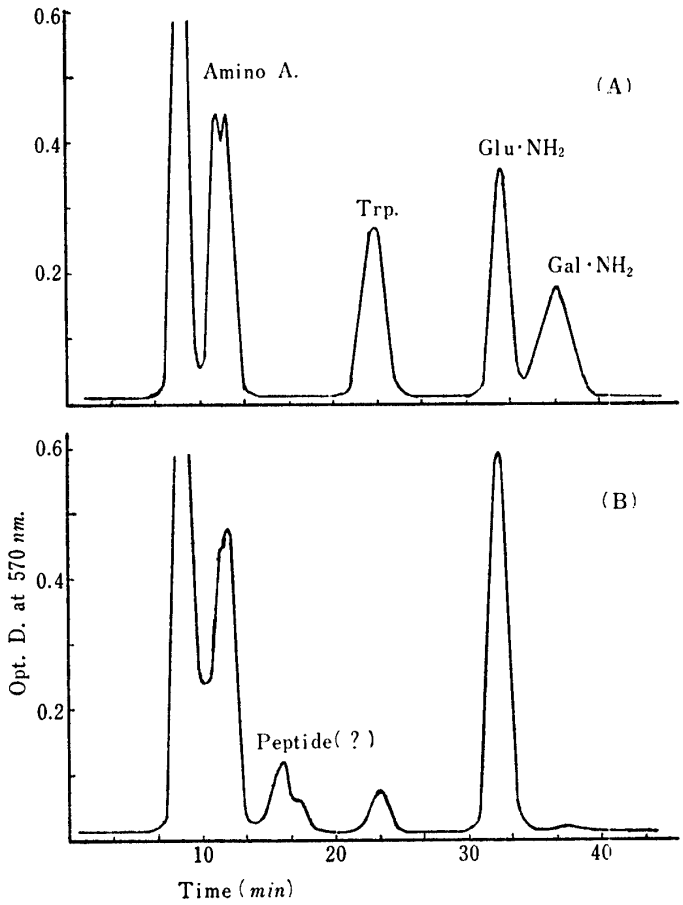

Fig. 6. Chromatograms of hexosamine on a short column in the automated amino acid analysis
Column ; Aminex A-5 (0.9×7.5 *cm*)
(A) Chromatogram of authentic hexosamines mixed with authentic amino acid mixtures.
(B) Chromatogram of the quail ovalbumin hydrolysate
Glu·NH_2 ; glucosamine, Gal·NH_2 ; galactosamine, Trp ; tryptophan.

Table I. Variation of the glucosamine contents of the quail ovalbumin during the hydrolysis

Time of hydrolysis (*hr*)	6	8	10	15
Glucosamine content (%)	0.76	0.73	0.75	0.75

Hydrolysis temperature ; 100°C (in a sealed tube)
Identification and determination of glucosamine were done by the chromatography employing a short column in the automated amino acid analysis.

Table II. Mannose and glucosamine content of the quail ovalbumin compaired with those of the chicken and the duck ovalbumins

	Quail ovalb.	Chicken ovalb.	Duck ovalb.
Mannose (%)	2.30 (a) 2.39 (b)	1.79 (a)	4.00 (a)
Glucosamine (%)	0.60 (c) 0.75 (d)	1.20 (c)	2.03 (c)

(a) Obtained from the phenol-sulfuric method
(b) Obtained from the gas chromatography of TMS-derivative
(c) Obtained from the Elson-Morgan method
(d) Obtained from the devised method in automated amino A. analysis (by authors).

or 2.39 %, glucosamine content, 0.60 or 0.75 %. The former is noticed to be slightly larger than in the chicken ovalbumin, while the latter, smaller. Comparing with the sugar content of the duck ovalbumin, both sugar contents of the quail ovalbumin were quite small, respectively. These contents point the presence of 6 or 7 moles of mannose and 2 moles of glucosamine in one mol. of quail ovalbumin, differing from the mannose and glucosamine ratios in the chicken ovalbumin. The molecular weight of quail ovalbumin was estimated to be 45000, being equal to that of chicken ovalbumin as mentioned later. Therefore, authors are going to propose the difference of the structure between the carbohydrate moieties of both albumins. One mol. of duck albumin was ascertained to be containing 12 moles of mannose and 6 moles of glucosamine.

Estimation of the molecular weight of the quail ovalbumin by Sephadex gel-filtration —— The elution curves obtained for several authentic proteins on a 2.64 × 64 *cm.* Sephadex G-100 column were as shown in Fig. 7. From the plotting of relative elution volumes of authentic proteins, V/V_0, against the logarithm of the molecular weights of the proteins, a linear relationship between the two was confirmed as shown in Fig. 8. After the relative elution volumes of the quail and the duck ovalbumins were measured employing the same column, those molecular weights were estimated to be 45000 and 47000, respectively. The molecular weight of the quail ovalbumin was found to be equal to that of the chicken ovalbumin, and smaller than that of the duck ovalbumin.

The calibration curve for quail ovalbumin —— The relation between the absorbance and the concentration of the quail ovalbumin compared with those of the chicken and the duck ovalbumins is as shown in Fig. 9. The equation of calibration curve for quail ovalbumin could be represented as $y=5.60\times10^{-3}x$, and the equations for chicken ovalbumin and duck ovalbumin, $y=6.41\times10^{-3}x$ and $y=7.80\times10^{-3}x$, where y is optical density at 280 *nm* and x, *mg* %. These equations suggest that the amount of aromatic amino acids is smaller in the quail ovalbumin than in the chicken and duck ovalbumins.

Amino acid composition of the quail ovalbumin —— The amino acid composition of the quail ovalbumin calculated from the diagram recorded (recorder response) in the automated analysis was as shown in Table III. Those of the crude quail ovalbumin crystallized five times with sodium sulfate and the purified chicken ovalbumin were presented together in that table. Hydrolysis was carried out for 24 and 40 *hr* respectively, however the resultant analytical data showed no noticeable differences between the two periods.

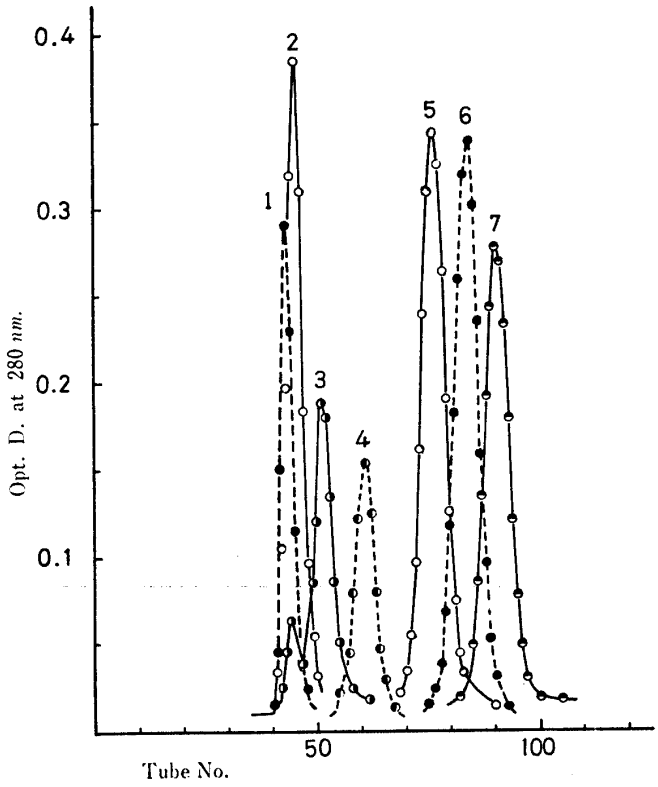

Fig. 7-a. Elution curves of several authentic proteins from a 2.64×64 *cm.* column of Sephadex G-100.

1 ; Blue dextran (200×10⁴)
2 ; r-Globulin (16×10⁴)
3 ; Serum albumin (6.7×10⁴)
4 ; Hen ovalbumin (4.5×10⁴)
5 ; Chymotrypsinogen A (2.3 ×10⁴)
6 ; Myoglobin (1.78×10⁴)
7 ; Cytochrome C (1.24×10⁴)

Figures in parentheses are the values of molecular weight. Solvent ; 0.05 *M* carbonate buffer, *pH* 10.0. Fraction size ; 3 *ml.*

Amino acid contents in the table are all the values when hydrolyzed for 40 *hr.* Comparison of the amino acid composition of the quail ovalbumin purified with that of the chicken's one represents that threonine and leucine are more in the quail than in the chicken, while lysine, glutamic acid, cystine, methionine and tyrosine in the former are less than in the latter. Total aromatic amino acid content is smaller in the quail than in the chicken. This relation was agreeing with the preceeding description concerning the calibration curves. Compared with the purified quail ovalbumin, in the crude ovalbumin, lysine, aspartic acid, cystine and tyrosine are found to be more, while glutamic acid, alanine, isoleucine, leucine and phenylalanine, less.

Compared with the amino acid composition of duck ovalbumin reported previously[19], in quail ovalbumin, histidine, arginine, aspartic acid, alanine, isoleucine and leucine was observed to be more, while threonine, serine, glutamic acid, proline, methionine, tyrosine and phenylalanine, less in quantity. In taxonomy the duck belongs to the Anseriformes, differing from the quail and the chicken belonging to the Galliformes. The quail and chicken ovalbumins were both ascertained to be containing more histidine, arginine,

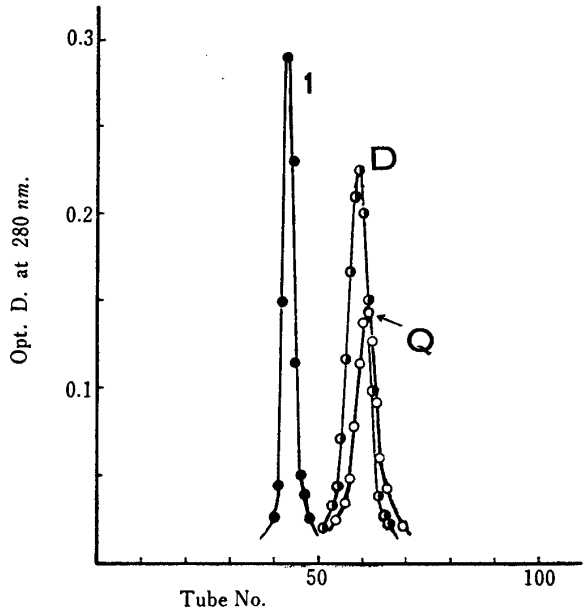

Fig. 7-b. Elution curves of the quail and the duck ovalbumins from a 2.64 × 64 *cm.*column of Sephadex G-100.
1 ; Blue dextran, D ; Duck ovalbumin, Q ; Quail ovalbumin
Solvent ; 0.05 *M* carbonate buffer, *pH* 10.0 Fraction size ; 3 *ml.*

aspartic acid, alanine and isoleucine, while less threonine, serine, glutamic acid, proline, methionine, tyrosine and phenylalanine than those contained in the duck ovalbumin. These facts may be attributed to the difference of the taxonomic situations.

Heat-coagulating point of ovalbumin —— From the plotting of the concentration (%) of the supernatant against the programmed temperatures, at which ovalbumin aqueous solution was heated, the curve as shown in Fig. 10 was obtained. Authors propose that the critical point marked with arrow on the curve is to be the heat-coagulating point. Therefore, the heat-coagulating point of quail ovalbumin was ascertained to be 64°*C*, being equal to that of chicken ovalbumin. These facts suggest the similarity of the conformation between the two albumins. No visible denaturation of the quail ovalbumin after storage under the freezing condition described in the previous report[1] may be attributed to a small amount of other protein contained accompanying in that protein.

SUMMARY

1) The purification of the quail ovalbumin from the egg white homogenate could be carried out by the crystallization with sodium sulfate followed by the CM-cellulose column chromatography and Sephadex gel-filtration.

2) The molecular weight of the quail ovalbumin was ascertained to be equal to that of the chicken ovalbumin and smaller than that of the duck ovalbumin by Sephadex G-100 gel-filtration.

3) Neutral sugar of the quail ovalbumin was fixed to be mannose, the content amount-

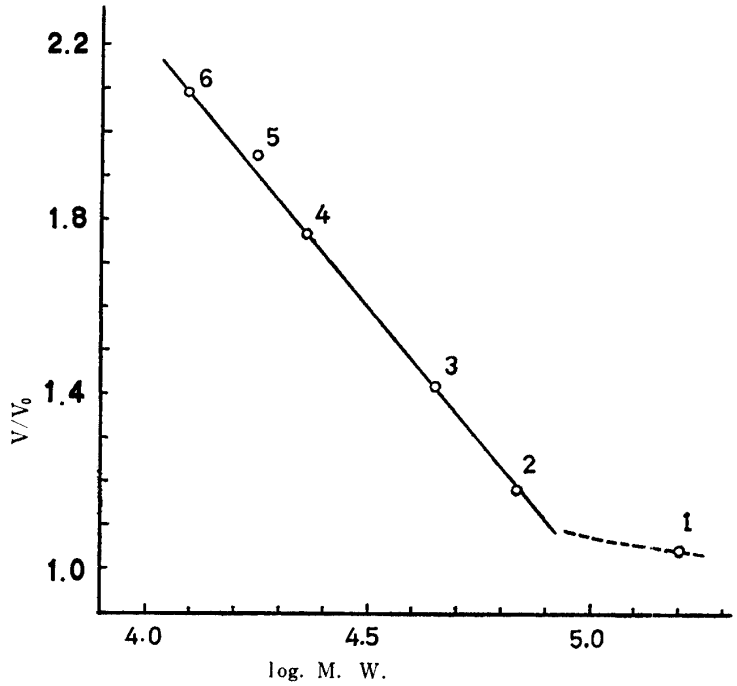

Fig. 8. Relationship between elution volume and molecular weight of several proteins when Sephadex G-100 column was used

1 ; r-Globulin (16 $\times 10^4$)
2 ; Serum albumin (6.7$\times 10^4$)
3 ; Hen ovalbumin (4.5$\times 10^4$)
4 ; Chymotrypsinogen A (2.3$\times 10^4$)
5 ; Myoglobin (1.78$\times 10^4$)
6 ; Cytochrome C (1.24$\times 10^4$)

Figures in parentheses are the values of molecular weight.
V ; elution volume of each protein Vo ; elution volume of blue dextran.

ing to 2.30 % by the phenol-sulfuric acid method and 2.39 % by the gas-liquid chromatography. These values were slightly larger than the mannose content of the chicken ovalbumin.

4) Hexosamine of the quail ovalbumin was identified as glucosamine, the content amounting to 0.60 % by the Elson-Morgan method modified by Boas, and 0.75 % according to the convenient procedure with amino acid analyzer devised by authors. These values were smaller than the glucosamine content of the chicken ovalbumin. These sugar contents point the presence of six or seven moles of mannose and two moles of glucosamine in one mol. quail ovalbumin.

5) Compared with the chicken ovalbumin, in the quail ovalbumin, threonine and leucine were ascertained to be more, while lysine, glutamic acid, cystine, methionine and tyrosine, less in quantity.

6) Heat-coagulating point of the quail ovalbumin was found to be 64°C, being equal to that of the chicken ovalbumin.

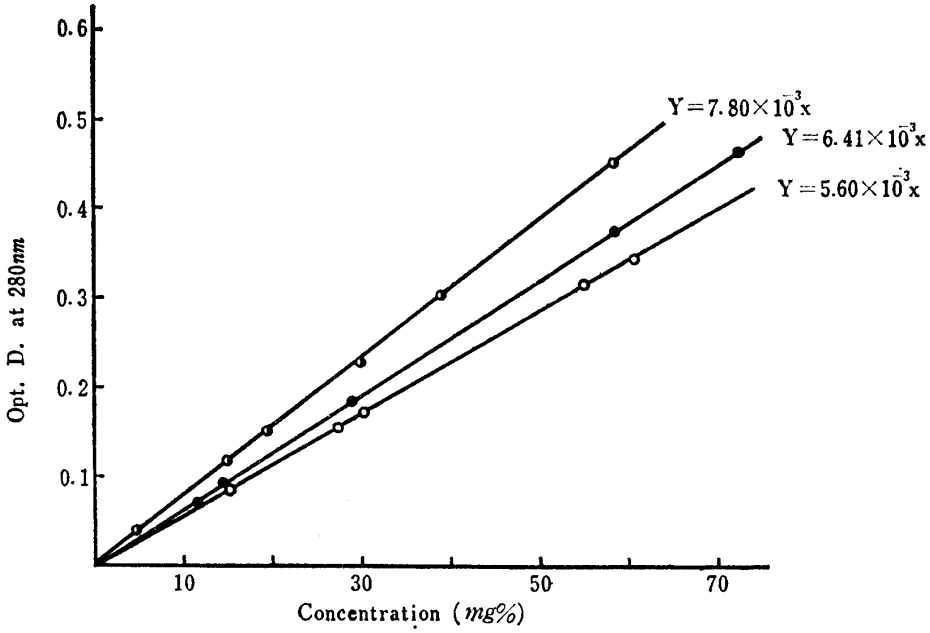

Fig. 9. Calibration curves for quail-, duck- and chicken-ovalbumins.
—○—○— Quail, —◑—◑— Duck, —●—●— Chicken

Table III Comparison between the amino acid composition of quail ovalbumin and that of chicken ovalbumin (values are grams of amino acid residues in 100 *g*. protein. Tryptophan value was calculated from the ultraviolet absorption determination.)

Amino acid	Quail ovalbumin	Crude quail ovalbumin	Chicken ovalbumin
Lysine	4. 31	6. 00	5. 30
Histidine	2. 16	2. 24	1. 94
Ammonia	2. 25	0. 70	0. 75
Arginine	4. 55	4. 04	4. 83
Aspartic A.	7. 32	8. 63	7. 49
Threonine	3. 80	3. 44	3. 09
Serine	5. 65	5. 28	5. 91
Glutamic A.	12. 50	11. 37	13. 46
Proline	2. 47	2. 43	2. 98
Glycine	2. 49	2. 77	2. 34
Alanine	5. 63	4. 45	5. 50
Cystine/2	0. 81	1. 71	1. 28
Valine	5. 74	5. 20	5. 56
Methionine	3. 78	3. 28	4. 26
Isoleucine	5. 46	4. 07	5. 77
Leucine	9. 13	7. 03	7. 48
Tyrosine	2. 89	3. 41	3. 38
Phenylalanine	6. 78	4. 92	6. 78
Tryptophan	0. 90		1. 10
Total residues	88. 62	80. 97	89. 20

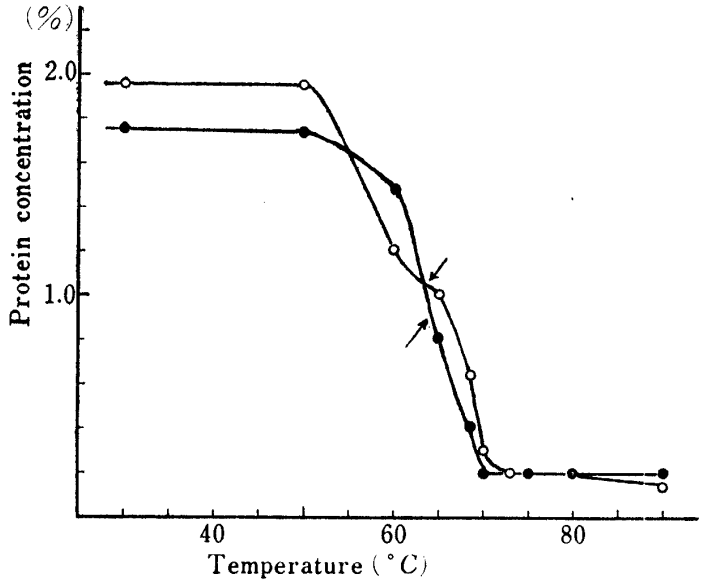

Fig. 10. Relationship between the concentration of ovalbumin aqueous solution and the temperature

Heating ; treated at the programmed temperatures for 10 min.
—○—○— Quail ovalbumin, —●—●— Chicken ovalbumin
Arrows show the heat-coagulating point.

Correction — Neutral sugar contents of the chicken, the quail and the duck ovalbumins described in the previous report[1,9] were calculated from the calibration curve for galactose respectively. On the basis of the calibration curve for mannose, those contents were corrected to the values as shown in Table II in the present paper.

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