Studies on the Emu Egg White Proteins

(I) Electrophoresis, CM-cellulose chromatography and gradient extraction with salt on the whole white

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Introduction

The emu (*Dromiceius novae hollandiae*), a flight-less bird, has been classified in a group called the ratite as the general name, together with the ostrich, cassowary, rhea, etc. Among all the birds living on the earth at present, this species is the bird of big type ranking next to the ostrich, and it is regarded as the most primitive one. Though they were inhabiting previously all over the area of Australia, the number of them has been considerably reduced, due to the reckless huntings to get their meat and eggs for foodstuffs of human beings. They lay 9–13 large eggs in the pit built by digging in the land, the incubation period of 70–80 days being required[1]. In the earlier days, Bain and Deutsch[2] performed electrophoretic comparative studies on the egg whites of different species birds belonging to various species of Galliformes, Anseriformes and Columbiformes, but they have not reported on the ratite group. Since those days, a great many papers have been published on the avian egg whites, particularly on the chicken egg white, but the papers on the ratite group are very scarce, while only Feeney and coworkers' papers are to be available.

Feeney et al. studied on the avian egg whites from the standpoint of the comparative biochemical aspect and reported that the emu egg whites had extremely small quantity of lysozyme content[3], and that the activity of lysozyme resembled that of the chicken egg white[4], while ovomucoid content was large, with antiproteolytic action strength[5] and types[4][5] different from those of the chicken egg white. And the electrophoretic behavior of conalbumin of the ratite group is different from that of Galliformes[6]. In taxonomy the Japanese quail is regarded belonging to the same family of the *Phasianidae* together with the chicken, and it is reported simultaneously by the author in another paper that the structure of the egg white constituent protein is more less different from that of the latter[7].

As the emu is widely different phylogenetically from the birds of *Phasianidae*, its egg is of dark green, it is 12 folds as large as that of the chicken and general properties of its egg white are also different from that of chicken, the relative proportion of the constituent proteins in the egg white and both its chemical composition and structure are supposed to be different from those of chicken. Therefore, the author's present studies were intended to know the characteristics of them step by step.

When the experiment was performed on the general chemical properties, electrophoresis, CM-cellulose column chromatography and gradient extraction with ammonium sulfate, of the egg white the interesting and significant facts were observed.

Materials and method

Materials—Fresh emu eggs within 20 hours after being laid were obtained through the good offices of the Kamoike Zoo, Kagoshima City. After procurement of the eggs, their weights and sizes were immediately measured. Then they were broken and the egg white was separated as completely as possible from the yolk with the aid of injector. Then the weights of the white, yolk and shell were measured respectively. The chalaza of egg white was removed with a pair of tweezers and it was blended with a homogenizer at a fixed speed. The homogenized egg white was made the sample to know the general chemical properties and electrophoretic behavior. The remaining egg white homogenate was ready after being freeze-dried to be used for analysis at need.

Examination of general chemical properties—The specific gravity of the egg white was measured with the Ostwald picnometer in the water at the constant temperature of 30°*C*. The nitrogen content determination was done by the semi micro Kjeldahl method. The electric resistance was measured with the Kohlrausch bridge in the water at the constant temperature of 15°*C*. And the specific conductance was calculated from the value of electric resistance measured beforehand. The total sugar content was obtained by the phenol-sulfuric acid method described by Dubois et al.[8] Its color intensity was measured at 490 mμ with the Shimazu-Bausch Lomb spectronic 20 spectrophotometer.

Moving boundary electrophoresis—Electrophoretic analysis was performed with the Tiselius apparatus (Hitachi Ltd., HTD - 1 type) under the analysis conditions of 1.5% protein concentration, 4–5 *mA* constant current and the constant temperature of 15°*C*. And the buffers applied are : 0.05 *M* carbonate *pH* 10.0, μ 0.10, 0.05 *M* phosphate *pH* 7.80, μ 0.144, *pH* 6.81, μ 0.10, and 0.1 *M* acetate *pH* 4.40, 4.00, μ 0.10.

Chromatographic separation on CM-cellulose—The separation of the whole egg white on CM-cellulose was performed by the method of Rhodes et al.[9] The summary of the experimental method is as follows :

The double times dilution of homogenized egg white was dialyzed against 0.025 *M* sodium acetate buffer, *pH* 4.0 for 2–3 days at 5°*C*, and after removing precipitates via centrifuge, the protein concentration was adjusted to 1.0 %. The protein was adsorbed while pouring slowly 20 *ml.* of the solution mentioned above on the CM-cellulose column (1.8 × 20 *cm*) which was bufferized beforehand with 0.025 *M* acetate buffer *pH* 4.0. After that, 30 *ml.* of the same buffer was slowly dropped on it. The buffers used for gradient elution due to the continuous *pH* raise were of the following series :

Firstly, 0.025 *M* acetate buffer *pH* 4.0, 500 *ml.* (A)—0.025 *M* CH_3COONa 500 *ml.* (B), secondly, 0.025 *M* phosphate buffer *pH* 5.30, 500 *ml.* (A)—0.025 *M* Na_2HPO_4 500 *ml.* (B), thirdly, 0.05 *M* carbonate buffer *pH* 9.00, 200 *ml.* (A)—0.05 *M* Na_2CO_3 200 *ml.* (B), fourthly, 0.05 *M* Na_2CO_3 100 *ml.*, finally, 0.05 *M* NaOH 100 *ml.*

The *pH* rising apparatus was used as described in the other paper[7]. The eluate was collected with the Tōyoo Kagaku Siphon fraction collector in which the fraction size

was adjusted to 5 *ml.* The flow rate was fixed at 1.0–1.2 *ml.* per minute. The *pH* of the eluate was measured at each fraction tube. Then the optical density at 280 *mμ* was measured with the Hitachi 101 spectrophotometer, from which value the protein content was calculated. The purified ovalbumin of chicken eggs was used as the standard of the protein content.

Separation by gradient extraction with ammonium sulfate—The full saturation was achieved by adding excess ammonium sulfate to the dilution (3.6 %) 4 *ml.* of the homogenized egg white. The precipitated proteins and the supernatant were poured slowly on the celite layer of the extracting apparatus shown in Fig. 1. The extracting apparatus employed was that which Soejima[10] recommended. The extraction of precipitated proteins was performed by decreasing continuously the concentration of ammonium sulfate from its full saturation to approximately zero. The eluate was collected with the siphon fraction collector with the capacity of 5 *ml.* The flow rate was fixed at 0.6–0.7 *ml.* per minute. With a syringe inserted into the introducing gum tube leading to the extracting part at every given time, a very small quantity of ammonium sulfate solution was taken, and the refractive index was measured at 19°*C* with the Abbe's refractometer. Separately, the standard line of the refractive index at 19°*C* of the ammonium sulfate with various concentrations was established. Basing on the line, the concentrations of ammonium sulfate were determined from those really measured values. The protein and sugar contents in each fraction tube were measured as stated before.

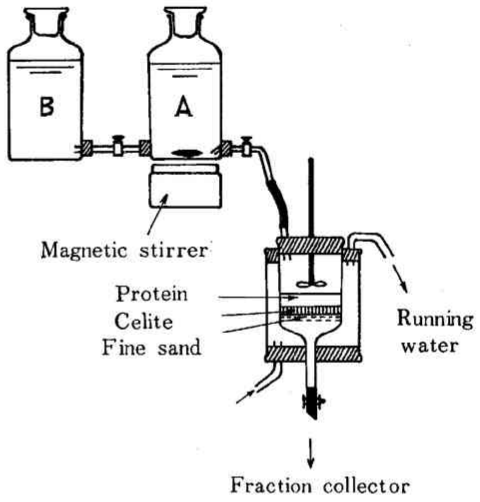

Fig. 1. Apparatus for gradient extraction with ammonium sulfate
A: saturated $(NH_4)_2SO_4$
B: water

Preparation of protein not precipitated by full saturation of ammonium sulfate—After the homogenized egg white was brought to its full saturation with the addition of ammonium sulfate crystals, it was left as it was, for one day, and then it was centrifuged for ten minutes at 13,000 *r.p.m.* Its supernatant was dialyzed against the water. Subsequently, the supernatant thus treated was dialyzed against 10 % polyethylene glycol (Av. M.W. 7,500) solution, and the protein content and the sugar content of the solution were determined.

Results and discussion

General chemical properties—The general chemical properties of the emu egg white are as shown in Table I. The egg supplied for the experiment was as shown in Fig. 2 with the size : the longer axis : 14.3 *cm*, the shorter axis : 9.5 *cm*, the thickness of the egg shell : about 1 mm.

Feeney et al.[3] stated that the ratio of an egg white to a yolk of 25 different species

birds resembled with one another. But the ratio of the emu egg white to its yolk was about 1.0, which was nearly half of that of chicken and quail eggs. Compared with the egg white of birds belonging to the *Phasianidae*, it was observed that the specific gravity of the emu egg white was smaller and the nitrogen content in dry matter (12 %) was remarkably low, while on the other hand, the sugar-protein ratio (S-P ratio) was rather large, amounting to 8.10×10^{-2}, in which the free sugar content was also included. Then the ratio of the combined sugar to the protein was estimated after removing the free sugar by dialysis. The carbonate buffer 0.05 *M*, *pH* 9.80 which would prevent protein precipitates was used for the dialysis. The ratio of the combined sugar to the protein was revealed very high, amounting to 7.53×10^{-2}. This fact suggests that the ovomucoid content is to be found much in the emu egg white or that the specific protein containing much combined sugar, which is consistent with the fact of its low nitrogen content. The *pH* value of the egg white is a little higher than that of the chicken egg white. And the specific conductance of the former is the same with that of the chicken, while it was revealed larger than that of the latter, when both of them were adjusted to the same concentration.

Table I. General chemical properties of the emu egg white compared with those of the Galliformes birds

	Emu	Chicken (White leghorn)	Quail (Japanese quail)
Weight of entire egg (*g.*)	736	52.4—67.2 (10)	9.5—11.4 (20)
White/Yolk (*w/w*)	1.0	1.9—2.3 (10) AV. 2.1	1.5—2.1 (20) AV. 1.8
White/Egg ×100 (%)	44.0	58.4—62.5 (10)	53.2—59.6 (20)
Solids in egg white (%)	10.6	10.5—11.5 (5) AV. 11.1	10.7—12.0 (10) AV. 11.4
Water in egg white (%)	89.6	89.5—88.5 (5) AV. 88.9	89.3—88.0 (10) AV. 88.6
Specific gravity at 30°*C*	1.0296	1.039—1.052 (5)	1.032—1.036 (5)
Nitrogen in dry matter (%)	12.2	13.8—14.1 (5) AV. 14.0	17.3—17.8 (5) AV. 17.5
Sugar-protein ratio	8.10×10^{-2}	7.18×10^{-2}	8.03×10^{-2}
Sugar-protein ratio after dialysis against carbonate*, *pH* 9.80	7.53×10^{-2}	3.71×10^{-2}	4.60×10^{-2}
pH	8.51	7.90—8.30 (20)	8.40—8.78 (50) 8.60—8.70 (in general)
Electric resistance, 15°*C*	161 *ohm*	161—154 *ohm*	150—144 *ohm*
Specific conductance at 15°*C*	6.23×10^{-3}*mho*	$6.23—6.51\times10^{-3}$ *mho*	$6.68—6.96\times10^{-3}$ *mho*
Specific conductance in *C*=4.9 % at 15°*C*	3.45×10^{-3}*mho*	3.25×10^{-3} *mho*	3.54×10^{-3} *mho*

Numbers in parentheses are the eggs used for measuring.
AV.: Average value, * *M*/20 carbonate buffer

Electrophoretic analysis — The electrophoretic patterns and mobilities of the emu egg white in ascending side are as shown in Fig. 3 and in Table II. The emu egg white was separated into four components with the carbonate buffer, *pH* 10.0, while the chicken and quail egg whites into three components with the same buffer. The mobility of the quantitatively largest component is considerably larger than that of the chicken egg white. Judging from the results, which will be described later on, of CM-cellulose chromatog-

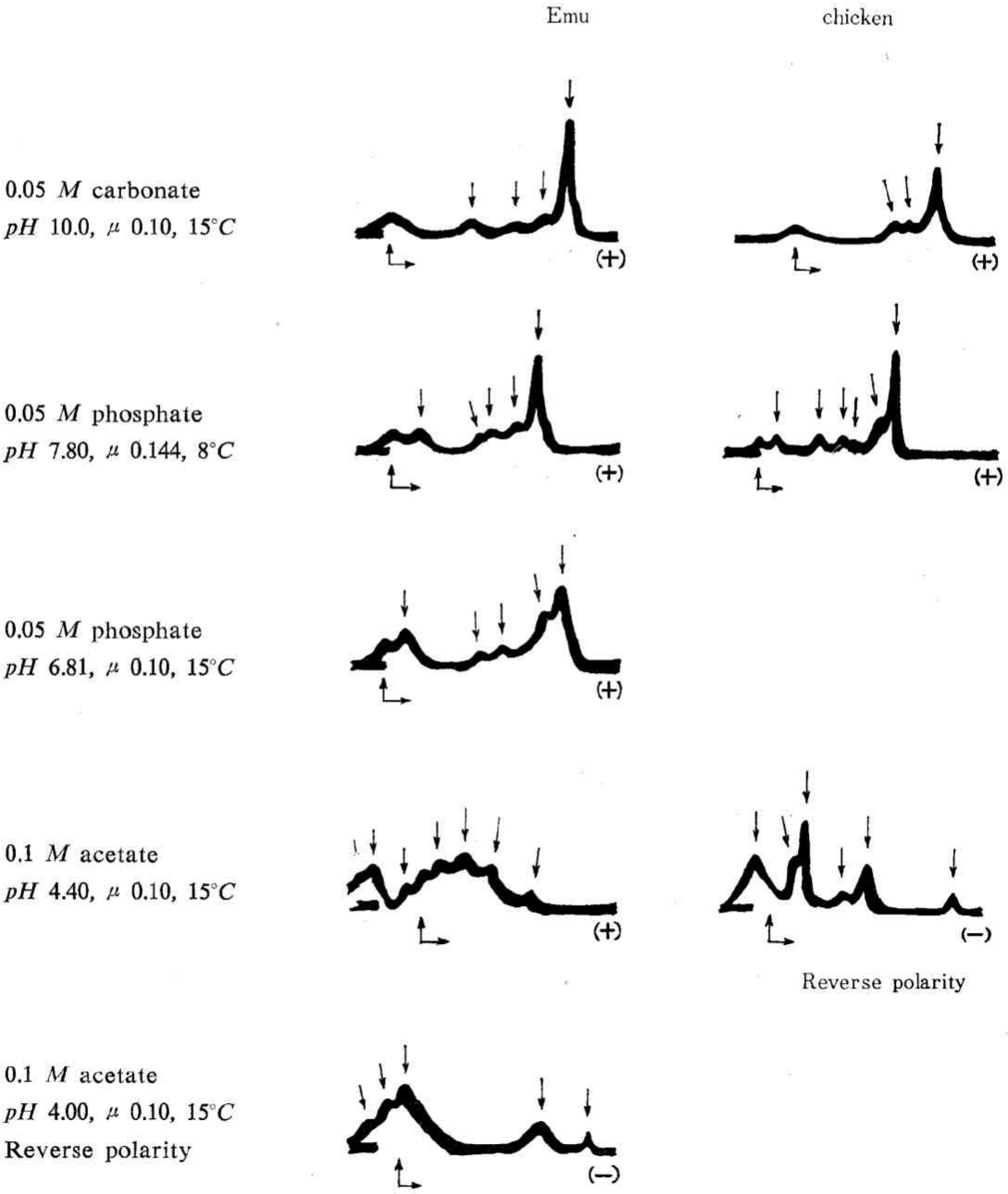

Fig. 3. Electrophoretic diagrams of emu egg white proteins compared with chicken egg white proteins in the ascending side
These diagrams show the traced photographs.

raphy and gradient extraction, this component is considered to be the mixture of ovomucoid and ovalbumin. Sugano[11] observed eight components separated from the chicken egg white under the conditions of *pH* 7.80, μ 0.144 and 2°*C*, with Tiselius apparatus of his own making, while at the author's experiment, the number of obviously confirmed components was five. In the case where the same buffer was used, the emu egg white revealed five components. When these five components were correspondingly compared with each component of the chicken egg white respectively, the mobilities of each of the former was found large. Longsworth et al.[12] observed, under the condition of *pH* 4.45, μ 0.10, six components : ovomucoid, ovalbumin, G_3-globulin, G_2-globulin, conalbumin and lysozyme (G_1). The author too observed six components of the chicken egg white under the conditions of *pH* 4.40, μ 0.10, at 15°*C*. Forsythe and Foster[13] state that at least one anionic protein will be observed even under the condition of *pH* 3.9 when electrophoresis is applied to the chicken egg white.

Table II. Electrophoretic mobilities of the egg white proteins at different *pH* values in the ascending side

Buffer / Species	0.05 *M* carbonate *pH* 10.0, μ 0.10, 15°*C*	0.05 *M* phosphate *pH* 7.80, μ 0.144, 8°*C*	0.05 *M* phosphate *pH* 6.81, μ 0.10, 15°*C*
Emu	6.5, 9.8, 12.4, 14.1	1.7, 5.1, 5.7, 7.3, 8.5	1.1, 6.2, 7.9, 10.5, 11.1
Chicken	8.5, 9.2, 11.6	0.8, 3.6, 4.8, 5.5, 7.1, 7.7	

Buffer / Species	0.1 *M* acetate *pH* 4.40, μ 0.10, 15°*C*	0.1 *M* acetate *pH* 4.0, μ 0.10, 15°*C*
Emu	+4.2, +3.3, +0.8, 0.8, 2.5, 3.6, 6.0	Reverse polarity 5.0, 2.0, 0, +25.4, +33.3
Chicken	Reverse polarity 0.7, +1.8, +2.2, +5.2, +6.2, +12.4	

Units of mobilities are $\cdot 10^{-5}$ *cm² per volt second.*

At the electrophoresis applied to the emu egg white with *pH* 4.40, seven components, which were greater in number than those of the chicken egg white, were observed, but its separation patterns were revealed entirely different from those of the latter. And as shown in Fig. 3, five components among the six components of the chicken egg white separated with *pH* 4.40 were found positively charged, while on the other hand four components among the seven components separated with the buffer of the same *pH* that was applied to the emu egg white were observed negatively charged. Moreover, even with *pH* 4.00 buffer, at the least two negatively charged components were observed. In other words, it was demonstrated that four components with the isoelectric point below 4.40 and two components with the isoelectric point below 4.0 were existing. It is a matter of great interest that such phenomena are not to be observed in the *Phasianidae* egg white.

CM-cellulose column chromatography — In the fractionation of the chicken egg white

which was carried out by Rhodes et al.[9] the all proteins were eluted near their isoelectric points. From the further studies on twenty-five different species belonging to six orders, they described that the major proteins of all the whites were eluted from *CM*-cellulose within approximately 0.2 to 0.4 *pH* unit of the *pH* values at which the corresponding proteins from chicken egg whites are eluted. The separation pattern from the emu egg white shown in Fig. 4 is remarkably different from that which was separated from the chicken and quail egg whites[7]. The separation pattern of the emu egg white is not so good as those of the latter, but the number of the substances which are to be regarded as a component is 11, excelling the number of those from the chicken and quail egg whites. Those components were marked A, B, C ····· K in order of their elution. The relative ratio and *pH* of eluate at peak are as shown in Table III.

Although, in the present state where both the isolation of the major proteins composing the emu egg white and the measurement of their isoelectric point have not been done, each separated component shown in the figure can not be correctly identified, it is not impossible for us to estimate the separated component from the order of elution and *pH* of eluate at peak.

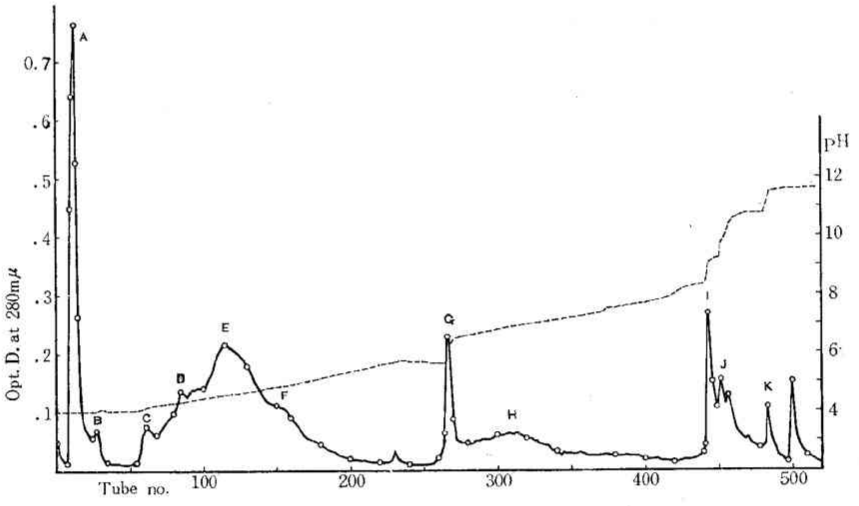

Fig. 4. Separation of the emu egg white proteins by CM-cellulose chromatography
—o—o— Protein, ----- pH

Judging from the result of electrophoretic analysis, Component A can be inferred to be the mixture of the protein of approximately 4.0 isoelectric point with the protein showing an anionic behavior at *pH* 4.0, probably the anionic protein (I.E.P.$<$4.0). The eluate *pH* of a small amount of Component B is also 4.0, while it is supposed to be composed of anionic protein which is a different kind of protein from the protein contained in Component A. This component is the component which has surely appeared in several experiments by chromatography and has never been found in the chicken egg white.

It is to be inferred that Component C and D are respectively ovomucoid, E ovalbumin, F globulin, G conalbumin and K lysozyme. The point different from the case of the chicken and quail egg whites is that each of ovomucoid and ovalbumin is continuously eluted as the separation of one from the other is not so good. The separation pattern

resembling to that of the emu egg white is also noticeable in the case of the pigeon egg white[14]. The relative proportions of each component among them to one another are as shown in Table III. When compared with the component separated from the chicken egg white, in the case of the emu egg white proteins, much of those separated are anionic protein, ovomucoid, globulin, or globulin like protein, while the components corresponding to ovalbumin and lysozyme are small in quantity. The result indicating that the ovomucoid content in the case of the emu egg white is excelling that of the chicken egg white is agreeing with the description by Rhodes et al.[5], while the result indicating that the lysozyme content is less than that of the latter is also agreeing with the report by Feeney et al[3].

Table III. Relative proportions and *pH* values for elution of each component of the emu egg white separated by CM-cellulose column chromatography

Component	A	B	C	D	E	F	G	H	I	J	K	Others	Total
Protein (%)	11.0	1.3	2.2	10.8	23.5	11.5	4.9	10.8	4.4	7.1	2.0	10.5	100
pH of eluate at peak	4.0	4.0	4.13	4.39	4.60	4.85	6.10	6.90	9.0	9.80	10.93		

Presumption A,B : Proteins being anionic at *pH* 4.0 (may be ovomucoid)
C,D : Ovomucoid fraction
E : Ovalbumin 〃
F : Ovoglobulin 〃
G : Conalbumin 〃
K : Lysozyme 〃

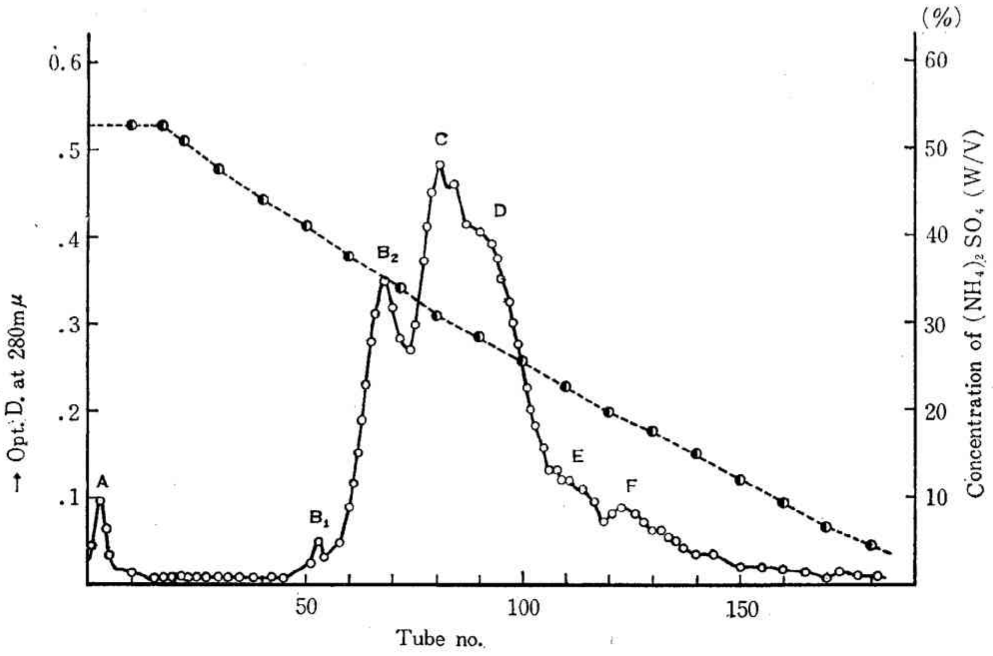

Fig. 5. Separation of the emu egg white proteins by gradient extraction with salt
—○—○— Protein, ---◐--◐--- Concentration of ammonium sulfate

Gradient extraction with ammonium sulfate— The separation of the protein and sugar of the emu egg white by the gradient extraction with ammonium sulfate is as shown in Fig. 5 and 6. The separation pattern is greatly different from the case of the chicken and quail egg whites. The component extracted by full saturation of ammonium sulfate was marked A, and each component extracted was marked in the order of B_1, B_2, C, D, E, and F respectively. The protein and sugar contents of each component are as shown in Table IV. In general, as the classification of proteins is based on solubility, judging from that standpoint it is to be inferred that each of the Component A, B_1, B_2 is ovomucoid, C ovalbumin and conalbumin, D, E, F are each globulins or globulin like protein, respectively.

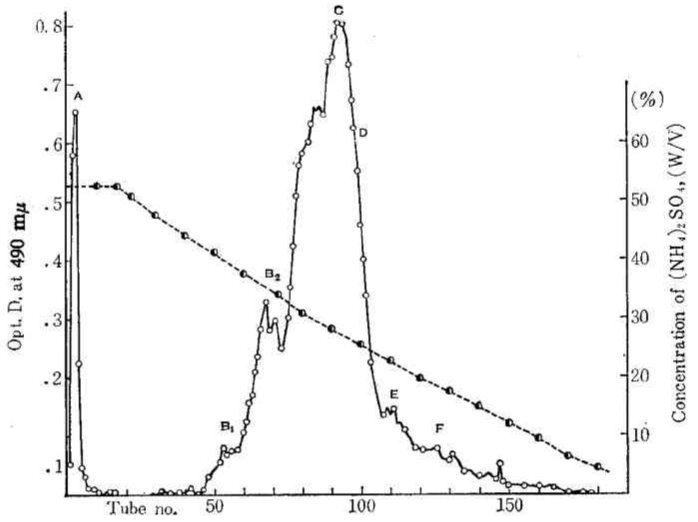

Fig. 6. Separation of emu egg white sugars by gradient extraction with salt
—○—○— Sugar, ---◐--◐--- Concentration of ammonium sulfate

Component A is protein with relative content of 3.6 % and it does not precipitate even by full saturation of ammonium sulfate. As free sugar is mixed in this fraction, the fluctuation of S-P ratio is great so that the value itself is meaningless. Therefore the solution of protein which did not contain free sugar, free amino acid and other low molecular substances was exclusively prepared separately. The S-P ratio of the protein preparations above was 43.33×10^{-2} as shown in Table V. Glycoprotein, 43 % of which is sugar content has not been obtained in the egg white. The study on this component is now under way in the author's laboratory. Component B_1 and B_2 are equally glycoprotein extracted by the salt with higher concentration than ovalbumin, which is generally called ovomucoid and its relative content is much more than that of the chicken or quail. The relative content of the albumins is much less than that of the *Phasianidae* ovalbumin, while on the contrary the S-P ratio is very high, coming to 6.24×10^{-2}, which phenomenon is considered as one of the causes indicating bad separation of ovomucoid and ovalbumin in CM-cellulose chromatogram of the emu egg white. The content of three components of the globulins is less than that of the chicken egg white, while their S-P ratios are of considerably large values, coming to 9.38, 5.11 and 2.47 $\times 10^{-2}$. In consideration of solubility, the protein belonging to the ovalbumin or the

globulin which contains such a large quantity of sugar has not yet been reported.

Table IV. Protein and sugar contents of each component of the emu egg white separated by gradient extraction with ammonium sulfate

Component	A	B_1	B_2	C	D	E	F	Others
Concn. of salt (*w/v*, %)	52.7	42.7 | 38.5	38.0 | 33.7	33.0 | 29.0	28.8 | 23.8	23.6 | 20.1	20.0 | 4.5	
Protein (*mg.*)	3.59	2.93	28.16	45.66	44.03	10.22	16.83	1.7
Protein index	2.34	1.92	18.41	29.85	28.79	6.68	11.00	1.11
Sugar (*mg.*)	0.61	0.20	1.26	2.85	4.13	0.52	0.42	
S-P ratio ($\cdot 10^{-2}$)	16.89	6.68	4.49	6.24	9.38	5.11	2.47	

Table V. Sugar protein ratio of the component not precipitated by full saturation with ammonium sulfate

Species	Emu	Chicken	Japanese quail
S-P ratio	43.3×10^{-2}	23.0×10^{-2}	37.2×10^{-2}

Fredericq and Deutsch[15] prepared ovomucoid under the mild condition from the chicken egg white, and they stated that it could be observed as a single component by the application of electrophoresis in the buffer μ 0.1. Longsworth et al.[12] suggested the electrophoretic heterogeneity of ovomucoid. Bier et al.[16] reported that ovomucoid will be separated into three major components and into two minor components, with different isoelectric point respectively. Rhodes et al.[5] divided the ovomucoids from 11 different avian species into four classes on the basis of their inhibitory activities, and gave the term "multiheaded" inhibitor implied by Wu and Laskowski[17] to the emu ovomucoid.

At present heterogeneity of avian ovomucoid is generally accepted. Judging from the experimental results of electrophoresis, CM-cellulose chromatography and gradient extraction with the salt, it would be apprehended that the heterogeneity degree of the emu ovomucoid is higher than the *Phasianidae* birds ovomucoid. In respect of the biological function, much importance has been particularly attached to the study of ovomucoid. In consideration of the facts, however, that the sugar content of Japanese quail ovalbumin is more than that of the chicken ovalbumin[7], the sugar content of the emu ovalbumin is much more than that of Japanese quail ovalbumin and that the sugar content of the chicken ovalbumin decreases in quantity along with the proceeding of the incubation stage[18], the necessity of the comparative biochemical studies on ovalbumin is widely to be asserted. And moreover, it is a matter of great interest from the standpoint of phylogenetics that the S-P ratio of each component of the emu is higher than the S-P ratio

of each corresponding component of the *Phasianidae* egg white.

Summary

1) The author examined the general chemical properties of the emu egg white, and studied on the constituent proteins by electrophoresis, chromatography on CM-cellulose column and gradient extraction with ammonium sulfate. He compared the results thus obtained with the analytical results of his studies on the chicken egg white and discussed them.

2) The specific gravity and the nitrogen content (12.2 %) of the emu egg white are each smaller than each corresponding value of the chicken egg white. However, *pH* and the specific conductance are rather high. Particularly the ratio of the combined sugar with the protein is remarkably high, amounting to 7.53×10^{-2}.

3) The egg white proteins were separated electrophoretically into four components on the alkaline side and into five components near the neutral. It was also separated into seven components by the application of the buffer *pH* 4.40, μ 0.10, four components of which were ascertained to be negatively charged. And even by the application of *pH* 4.0, at least two components carrying negative charge were observed.

4) The emu egg white proteins were observed to have been separated into 11 components by chromatography on CM-cellulose column, though the appearance of separation was not so good as that of the proteins of the *Phasianidae* bird egg white. The separation pattern of ovomucoid and ovalbumin from the egg white was especially bad. When the emu egg white proteins were compared with the chicken egg white proteins, in the former there were more the protein being anionic at *pH* 4.0, ovomucoid, and globulin like protein, than in the latter, while ovalbumin and lysozyme like protein appeared less in quantity.

5) Seven components were identified by the gradient extraction of emu egg white proteins with ammonium sulfate. The sugar content of the protein which was not precipitated by full saturation of ammonium sulfate was 43.3 %, which was acknowledged to be very high. The quantity of glycoprotein extracted with ammonium sulfate of concentration higher than that of the extraction of ovalbumin was very large. The albumins appeared in a small quantity but with very high sugar-protein ratios. The relative content of the globulins was large and its sugar-protein ratios were considerably high, too.

6) It was inferred from the results obtained in the present experiments performed by the author that ovomucoid of the emu egg white has high heterogeneity degree.

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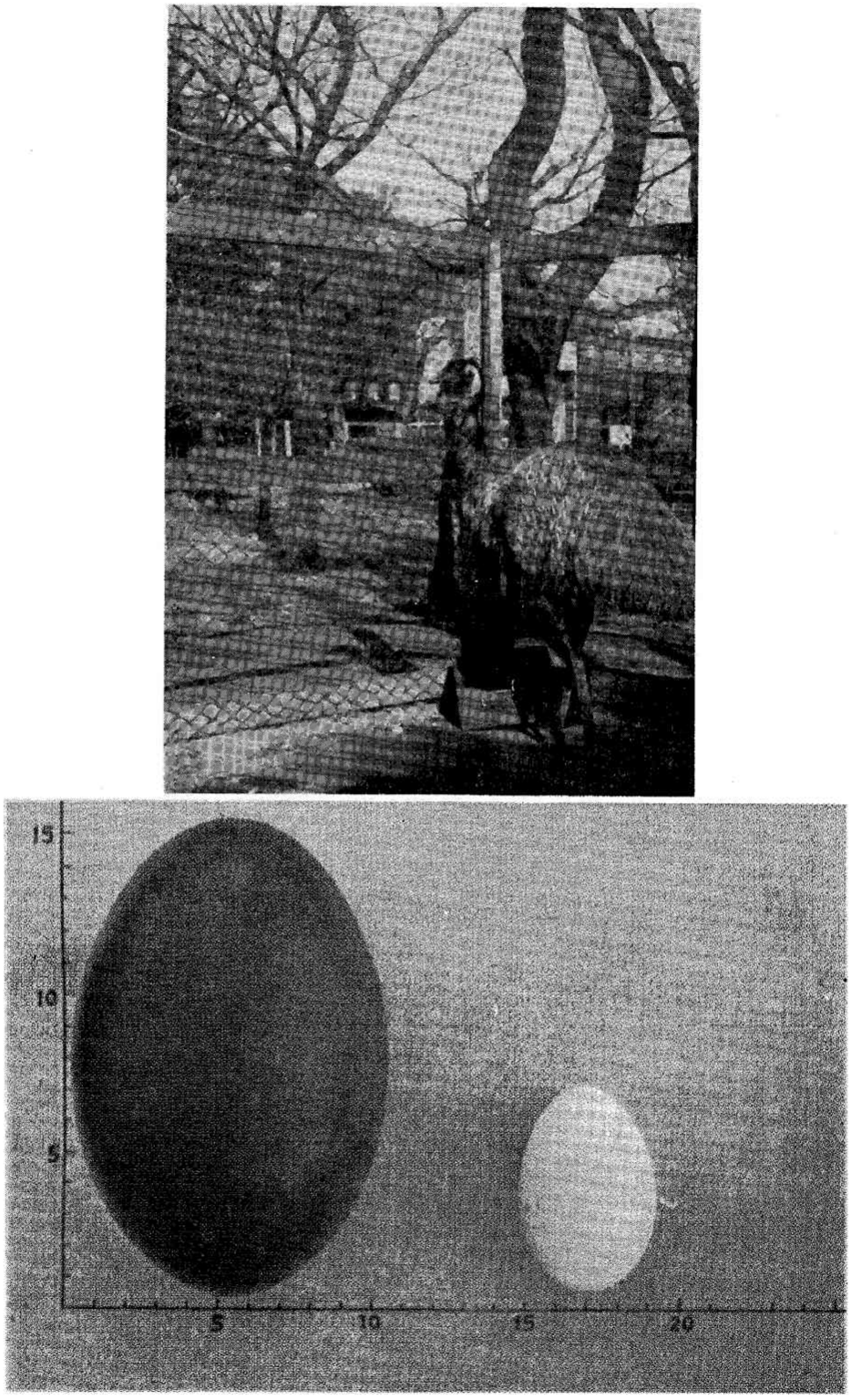

Fig. 2. Emu (*Dromiceius novae hollandiae*) and its egg compared with chicken (white leghorn) egg
A graduation in the photograph shows one centimetre.