Biochemical studies on chick embryo during incubation*

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Abstract The change of sialic acid content and trypsin inhibitory activity of chicken egg white during incubation were studied. The sialic acid concentration of egg white slightly increased during incubation, indicating the absorption of sialo-proteins of egg white into embryo occurred later than that of others. In early period of incubation, trypsin inhibitory activity rapidly and extensively decreased and after this period, trypsin inhibitory activity was kept at approximately constant level.

Neuraminidase (sialidase) of chorioallantoic membrane (CAM) was able to release the endogenous sialic acid and showed the strong activity toward exogenously added ovomucoid and ovomucin. When neuraminidase was mixed with CAM in a buffer, sialic acid at the membrane surface was removed. Added neuraminidase remained bound at the surface of CAM despite of sufficient washing and shows strong activity on sialo-proteins.

The experiments for CAM-sialo-protein binding showed that desialylated proteins (desialylated ovomucoid and ovomucin) bound to native CAM in greater amount than to desialylated CAM. Further, it was found that native sialo-proteins (native ovomucoid and ovomucin) bound to desialylated CAM rather than native CAM. ^{125}I-labelled sialo-proteins were prepared and used in these binding experiments.

Introduction

Chick embryo incubated for 21 days under a suitable condition develops into chick. During incubation chorioallantoic membrane appears and develops around embryo and egg white proteins are gradually absorbed through the membrane. The absorption mechanism, however, is still uncertain, though it has been considered to be closely related with the chorioallantois.

Sialic acid are widely distributed in animal tissues, being components of milk oligosaccharides, serum glycoproteins, gangliosides and epithelial mucins. The biological roles of sialic acid are to a large degree imperfectly understood.

The presence of neuraminidase (sialidase) in the chorioallantois of the chick embryo was

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shown by Ada and Lind[1]. They purified this enzyme and determined the activity on three substrates; sialyl-lactose, fetuin and ovine submaxillary gland mucin[2,3]. In general, the substrates of neuraminidase are sialyl-lactose, fetuin and gangliosides. Chick egg proteins, another type of sialo-protein, have not been used yet as neuraminidase substrates. This paper deals with the determination of neuraminidase activity on two chick egg white proteins, ovomucoid and ovomucin.

Morell et al[4]. have been found that the removal of the terminal sialic acid residues from many glycoproteins results in the rapid and specific uptake of these proteins by rat liver tissue within ten minutes after their injection. In the case of ceruloplasmin, it has been shown that the asialo-molecule is degraded within the lysosomes upon entering the liver[5]. The signal residue for the uptake is the galactose moiety exposed on sialic acid-free ceruloplasmin[6].

Recent results by Pricer and Ashwell[7] and by Van Lenten and Ashwell[8] characterize the properties of the in vitro binding of asialo-glycoproteins by isolated rat liver plasma membranes. Plasma membranes of liver were found to be the major locus of binding for circulating glycoproteins. The binding process involves a dual role of sialic acid in which its presence on the glycoprotein is incompatible with binding. Enzymatic restoration of sialic acid residues onto partially desialylated membranes is accompanied by increased binding activity and is suggestive of the presence of an intrinsic membrane-associated sialyltransferase. The sialyltransferases comprise a family of enzymes that catalyze the transfer of sialic acid from CMP-sialic acid to appropriate acceptores[9]. In general, individual sialyltransferases are distinguished from each other on the basis of their specificities for particular acceptor molecules.

This paper deals with the relationship between the chorioallantoic membrane and chick egg white sialo-protein and also the identification of the chorioallantoic membrane as the site of glycoprotein binding.

Materials and Methods

1. Materials.

Fertile eggs were obtained from Takada Furanjo (Kyoto) and Yamamoto Shukeijo Kyoto; the species were White Leghon and Hybro. Neuraminidase, purified from Bacterium 3831-1, was kindly supplied by Dr. T. Sugimori, Kyoto Research Laboratories, Marukin Shoyu Co., Ltd (Kyoto). Trypsin (2 X cryst., from bovine pancreas) was from Sigma Chemical Co. and α-N-benzoyl-D, L-arginine-p-nitroanilide (BApNA) was from the Institute for Protein Research, Osaka University (Osaka). Na^{125} I was the generous gift of Dr. T. Mori, the Research Institute for Food Science, Kyoto University (Kyoto). All other chemicals were reagent grade.

2. Incubation and preparation of egg white.

(1) Incubation of fertile eggs.

Temperature (38±1°C), humidity (65±5%) and ventilation should be carefully controlled for successful hatching. After 3 day incubation, the fertile eggs must be turned over several times a day to expose every portion of the egg evenly to the surroundings. At the incubation in this laboratory the fertile eggs were allowed to stand with the air cell upside. The temperature was set at 37.8°C and water in a flask was placed in the incubator to keep suitable humidity.

(2) Preparation of egg white samples.

To investigate the change of egg white proteins during incubation, egg white of White Leghon was taken and pooled every day. At the first stage of incubation, a hole of about 5 mm diameter was made at the bottom of shell to collect the egg white. At the developing stage, the shell was gently broken and the egg white was collected. Care should be taken to avoid contamination with chalazae and blood.

3. The chorioallantoic membrane from chick embryo.

(1) Preparation of the chorioallantois.

The chorioallantoic membrane (CAM) from 14 days-old chick embyros were rinsed with citrate saline (0.15 M NaCl and 0.08 M sodium citrate solution) by removing blood and egg white. This preparation was stored at freezing stage.

(2) Preparation of desialylated CAM.

Hydrolytic removal of sialic acid from the CAM was performed by exposure to 0.1 N H_2SO_4 for 60 min at 80°C. Enzymatic desialylation of the CAM was based on the following method. The reaction mixture contained 0.02 ml of neuraminidase solution, 2.0 ml of 0.2 M acetate buffer, pH 4.3 and 200 mg of CAM. Incubation was conducted for 120 min at 37°C[10]. Desialylated CAM was separated by centrifuging the reaction mixture and was washed with the same buffer. The removal sialic acid was estimated by the thiobarbituric acid reaction[11].

(3) Assay of neuraminidase activity in CAM.

Neuraminidase activity in CAM on the endogenous substrate and on the exogenously added substrate were determined. The reaction mixture consisted of 2.0 ml of 0.2 M acetate buffer, pH 4.3, 0.5 ml of 0.01 M $CaCl_2$ and 200 mg of CAM with and without 60 mg of ovomucoid.

4 Preparation of sialo-proteins from egg white.

Ovomucoid was prepared from egg white by the method of Kanamori and Kawabata[12] and further purified by DEAE cellulose chromatography[13]. Soluble ovomucin was isolated from egg white by using the procedure of Smith[14]. Desialylation of the both sialo-proteins obtained from egg white was performed by acid hydrolysis and by the action of neuraminidase. The methods are the same as used for CAM.

5 Radioiodination of ovomucin.

Ovomucin was labelled with ^{125}I essentially as described by Greenwood et al.[15] Reaction was carried out in a plastic vial which contained sodium (^{125}I) iodide (113 μCi), 0.045 ml. The radioactivity was counted by using a auto gamma scintillation counter, Packard Auto-Gamma Scintillation Spectrometer 5220.

Into the vial was added 0.025 ml of 0.5 M phosphate buffer, pH 7.5. Immediately thereafter ovomucin (25 μg / 0.05 ml of water) and fresh chloramin-T (100 μg / 0.005 ml of 0.05 M phosphate buffer, pH 7.5) were added in this turn. After each addition, the contents of vial were briefly mixed. Immediately after mixing the chloramin-T, sodium metabisulfite was added [0.1 ml of a solution (2.4 mg/ml) in 0.05 M phosphate buffer, pH 7.5]. The residual iodide was diluted with carrier KI [0.2 ml of a solution (10 mg/ml) in 0.05 M phosphate

buffer, pH 7.5]. The reaction mixture was transferred onto a Sephadex G-50 column, followed by a single wash of the vial with another aliquout of KI solution of 0.2 ml.

Gel equilibration and elution were carried out with 0.05 M phosphate buffer, pH 7.5. Before the application of the radioactive reaction mixture, crystalline bovine serum albumin (20 mg in 1 ml of the same buffer) was passed through the Sephadex column to avoid nonspecific adsorption of ovomucin, followed by a 20 ml wash with the same buffer. The radioactive protein peak was collected into a plastic vial.

6 Procedure for binding of sialo-proteins to CAM.

(1) Procedure for binding of ovomucoid to CAM.

The reaction mixture in a tube contained 200 mg of CAM (14 days-old), 2.0 ml of 0.1 M Tris-HCl buffer, pH 8.0, containing 0.01 M $CaCl_2$ and 30-100 μg of ovomucoid (native or desialylated ovomucoid). At the end of incubation the reaction mixture was centrifuged for 5 min at 2000 rpm and the pellet (CAM) was rinsed with the same buffer. The supernatant was used for the determination of trypsin inhibitory activity. The pellet was again rinsed with the same buffer. After the second washing and centrifugation of the pellet, the trypsin inhibitory activity of the supernatant was again measured. The rinsed CAM was mixed with 2 ml of the same buffer and 40 μg of trypsin in a tube and incubated at 37°C for 10 min. After centrifugation at 2000 rpm for 5 min, the residual trypsin activity of the supernatant was determined. From the results, the amount of bound ovomucoid to CAM was calculated. Trypsin and trypsin inhibitory activity were measured using BApNA as the substrate by the method described in the previous paper[16].

(2) Procedure for binding of ^{125}I-labelled proteins to CAM.

The reaction mixture contained 100 mg of CAM (14 days-old) and 0.1 ml of ^{125}I-labelled ovomucin (or ovomucoid) (20,000 counts per sec). Further, this reaction mixture was brought up to 2.0 ml with 0.1 M Tris-HCl buffer, pH 8.0, containing 0.01 M $CaCl_2$. After incubation at 37°C for 60 min, the reaction mixture was centrifuged (2000 rpm, 5 min) and the radioactivity of the supernatant was counted. The CAM was washed with the same buffer twice and the radioactivity of the washing was also counted. The amount of bound protein was calculated from the counts of the CAM by using an auto gamma scintillation counter.

Results

1 Change of sialic acid content of chick egg white during incubation.

The sialic acid concentration did not increase as sharply as had been expected to conpensate the drastic decrease of egg white resulting in the sharp decrease of total sialic acid contents particularly in the early period of incubation. However, the rate of the decrease slowed down in the later period, indicating relatively slower absorption of sialo-proteins, ovomucoid and ovomucin during this period. The results are shown in Fig. 1.

2 The change of trypsin inhibitory activity of chick egg white during incubation.

The trypsin inhibitory activity of egg white extremely decreased in the early period of incubation while it increased after the 5th day of incubation. Then, trypsin inhibitory activity

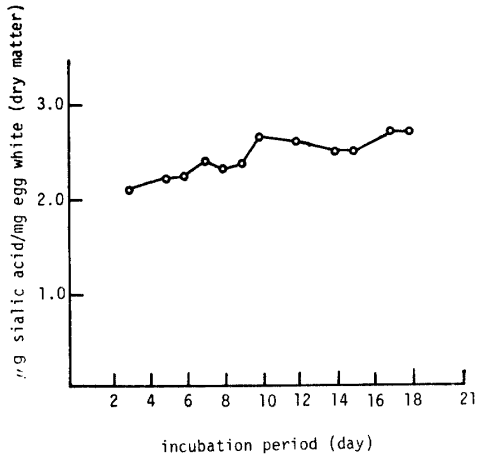

Fig. 1. Change of sialic acid content of chick egg white during incubation. Sialic acid was determined by thiobarbituric acid reaction.

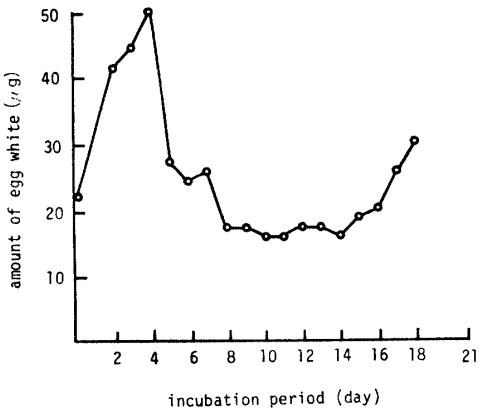

Fig. 2. Change of trypsin inhibitory activity of chick egg white during incubation. The amount of egg white which inhibits 1.0 μg of trypsin is indicated.

was maintained at approximately constant level untill large portion of egg white had been absorbed. The early period corresponds to the time in which egg york rather than egg white is being absorbed. Thereafter, egg white begins to be absorbed at faster rate. Figure 2 shows these results above described.

3 Neuraminidase activity of chorioallantoic membrane (CAM).

(1) Sialic acid content of CAM.

Sialic acid content of CAM was shown in Table 1. The average value was about 60 μg per 200 mg of CAM. It was about 0.03% of CAM weight.

(2) Neuraminidase activity of CAM.

Figure 3 shows how much sialic acid was released by neuraminidase treatment of CAM. The amount of released sialic acid reached a plateau after 3 hours incubation. CAM was treated with sufficient amount of neuraminidase, and only 10% of the total sialic acid were released. This result indicates the surface sialic acid is located further inside the membrane not to be exposed to the enzyme.

It has been reported that CAM is able to release sialic acid from the sialo-protein or sialo-lipid substrates[2]. In this experiment, ovomucoid which is a sialo-protein of egg white was mixed as substrate with CAM in a suitable buffer. The release of sialic acid from ovomucoid is shown in Fig. 4. Neuraminidase activity of CAM on ovomucoid was appreciable strong. Also, this

Table. 1. Sialic acid content of chorioallantoic membrane

age	μg of sialic acid*/200 mg of CAM**
12 days-old	54.9
15 days-old	68.2
16 days-old	54.8
17 days-old	64.4

* Sialic acid was removed by acid hydrolysis.
** chorioallantoic membrane.

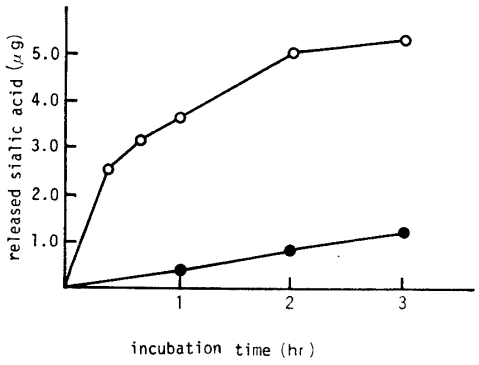

Fig. 3. Neuraminidase treatment of chorioallantoic membrane. The reaction was carried out wiht (○) or without (●) neuraminidase.

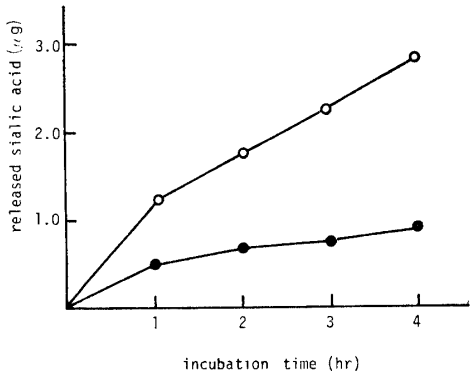

Fig. 4. Neuraminidase activity of chorioallantoic membrane. The reaction was performed with (○) or without (●) ovomcoid.

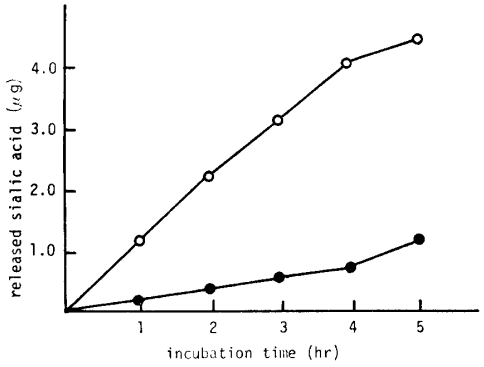

Fig. 5. Neuraminidase activity of desialylated chorioallantoic membrane. The reaction was carried out with ovomucoid (30mg). $CaCl_2$ was not added in the reaction mixture. (●), native chorioallantoic membrane (CA M) ; (○), desialylatedCAM.

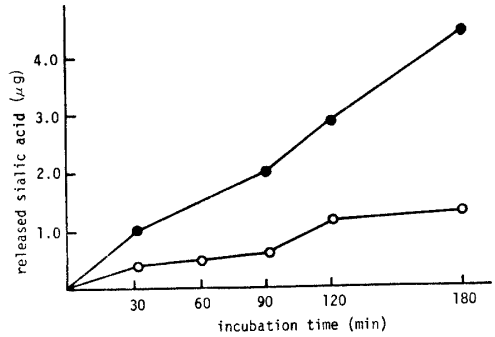

Fig. 6. Neuraminidase activity of chorioallantoic membrane. The reaction mixture consisted of 2.0ml of 0.2 M acetate buffer, pH 4.3, 1.0ml ovomucin solution (epuivalent to 135 μg as sialic acid) and 200 mg of native chorioallantoic membrane (CAM) (○) or 100 mg of desialylated CAM (●).

result indicated that neuraminidase of CAM reacted to endogenous substrates. When ovomucoid was mixed with desialylated CAM, neuraminidase activity was found stronger in desialylated CAM than in native CAM (Fig. 5). It was considered that neuraminidase of CAM was activated by desialylation or exogenously added neuraminidase which tightly bound to CAM, in spite of sufficient washing, reacted to ovomucoid, as reported by McQuiddy and Lilien[17]. They proved that exogenously added neuraminidase bound at the cell surface or tissue prepared from chick embryo.

Figure 6 shows the results of the action of CAM on soluble ovomucin which is another sialo-protein of chick egg white. Neuraminidase of desialylated CAM showed stronger activity on ovomucin than that of native CAM.

4 Binding of ovomucoid and ovomucin to CAM.

The binding of sialo-proteins to liver plasma membrane has been investigated by many workers[18]. They made many discussions about the phenomenon that desialylation of sialo-proteins resulted in the rapid uptake of desialylated protein by liver plsama membrane. This section describes the results of the studies on the CAM binding of ovomucin and ovomucoid which

are the sialo-proteins of egg white.

(1) Binding of ovomucoid to CAM.

Egg white ovomucoid shows the trypsin inhibitory acitivity. This trypsin inhibitor was isolated and the experiment for CAM-ovomucoid binding was carried out with the guidance of this trypsin inhibitory activity. Twenty three and a half μg of pure ovoumucoid was found to inhibit completely 20 μg of trypsin. The amount of bound ovomucoid was calculated from the decrease of trypsin inhibitory activity in the solution. Table 2 shows that the amount of desialylated ovomucoid bound to CAM was much more than that of native ovomucoid. More than 96% of the total sialic acid was released by enzymatic desialylation, but trypsin inhibitory activity of this ovomucoid did not decrease.

Time course of CAM-ovomucoid binding reaction is shown in Fig. 7. Desialylated ovomucoid was bound to CAM in greater amount than native one as shown in Table 2. The amount of bound ovomucoid increased extremely untill 60 min and at 90 min of incubation about the half amount of added ovomucoid (desialylated) bound to CAM. The bound amount decreased at 120 min of incubation probably because the once bound ovomucoid was released from CAM.

Ashwell reported that the enzymatic desialylation of plasma membrane resulted in the inhibition of binding of desialylated protein[7]. It may be expected from his report that ovomucoid may not bind to desialylated CAM. As shown in Fig. 8, however, the amount of bound

Table. 2. Binding of ovomucoid to chorioallantoic membrane

	native ovomucoid (μg)			desialylated ovomucoid (μg)**		
added	100	40	30	100	40	30
bound*	60	30	26	78	39	28

* The amount of bound ovomucoid was calculated from the decrease of trypsin inhibitory activity in the solution.

** Ovomucoid was desialylated by the treatment of neuraminidase.

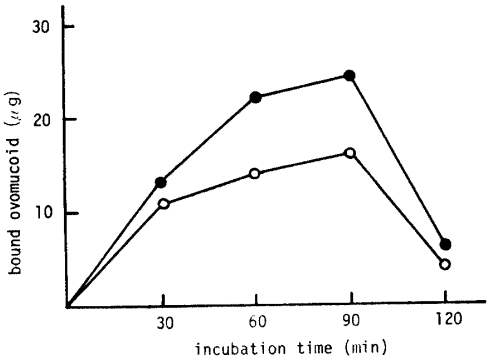

Fig. 7. Binding of ovomucoid to chorioallantoic membrane. 50 μg of ovomucoid was added in the reaction mixture. (○), native ovomucoid; (●), desialylated ovomucoid by neuraminidase. The amount of bound ovomucoid was calculated from inhibitory activity for trypsin

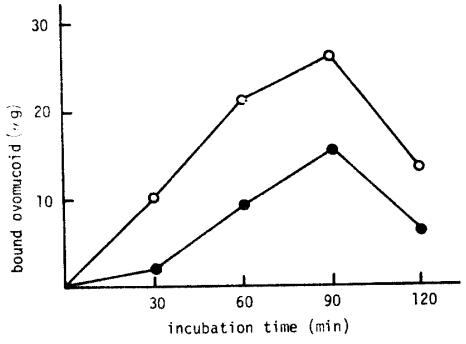

Fig. 8. Binding of ovomucoid to desialylated chorioallantoic membrane. Membrane was treated with neuraminidase. (○), native ovomucoid; (●) desialylated ovomucoid with neuraminidase.

desialylated ovomucoid to desialylated CAM was not so much, but the amount of bound native ovomucoid to desialylated CAM was quite much. The mechanism of sialo-protein binding to CAM was different from that to plasma membrane.

(2) Binding of ^{125}I-labelled sialo-proteins to CAM.

The amount of bound ovomucoid was determined with indirect but sensitive method. Then the experiment for CAM-ovomucin binding was performed by use of ^{125}I-labelled ovomucin. Ovomucin containes much more sialic acid than ovomucoid so that it is preferred as the sample for these experiments. In general, ovomucin is hardly soluble. Then soluble ovomucin was prepared accordingly to the procedure derived by Smith[14] and labelled with ^{125}I-iodide.

The result of binding of ^{125}I-labelled ovomucin to native and desialylated CAM is shown in Fig. 9. The rapid binding was observed untill 30 min. The amount of bound ovomucin to desialylated CAM was apparently much more than to native CAM.

Table 3 shows the results for binding to CAM of desialylated ovomucin and ovomucoid as well as native ones labelled with ^{125}I-iodide. These results were almost the same as shown in Table 2, Fig. 7-9 which indicated the binding of sialo-protein to CAM. The bound amount of desialylated ovomucoid to native CAM was much more than that to desialylated CAM. And the bound amount of native ovomucoid to desialylated CAM was much more than that to native CAM. Also the bound amount of native ovomucin to desialylated CAM was much more

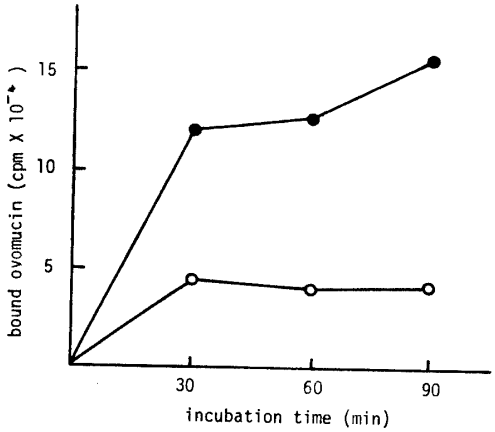

Fig. 9. Binding of ^{125}I-labelled ovomucin to chorioallantoic membrane. 0.1 ml of ovomucin (1×10^6 counts per min) was added in the reaction mixture. (○), native chorioallantoic membrane (CAM) ; (●), desialylated CAM.

Table. 3. Binding of ^{125}I-labelled sialo-protein to chorioallantoic membrane

	ovomucoid		ovomucin	
	native	desialylated	native	desialylated
added (cpm)	1,100,000	1,100,000	1,100,000	24,000
bound (cpm)*				
native CAM**	36,000	70,000	96,000	4,700
desialylated CAM	51,000	56,000	140,000	5,400

* Reaction mixture was incubated at 37°C for 60 min. The bound amount of ^{125}I-labelled protein was counted by using an auto gamma scintillation counter.

** chorioallantoic membrane.

than that to native CAM. But, the unsatisfactory result was obtained in the case of desialylated ovomucin because of its lower specific activity of ^{125}I-labelled protein.

The experiments for CAM-sialo-protein binding by the direct method as well as by indirect method resulted that native sialo-protein rather than desilylated protein bound much more to desialylated CAM.

Discussion

This paper shows the experimental results on the change of chicken egg white during incubation and discusses the possible mechanism how egg white is absorbed by embryo.

Egg white changes during incubation, and the water content decreased day after day to increase the egg white density in consequence. Egg white components, ovomucoid and ovomucin, have been known to be sialo-proteins. The physiological meaning of sialic acid, however, have not been known yet, so it is interesting to investigate the mechanism of egg white absorption by embryo in relation to sialic acid. The total amount of sialic acid of egg white decreased in proportion to absorption amount of egg white by embryo, but the concentration of sialic acid slightly increased, indicating relatively slower absorption of sialo-protein. But it has been unknown why the absorption of sialo-protein is slower.

It has been known that egg white inhibits trypsin and the activity does not change during incubation. The results of Fig. 2 shows the rapid and extreme decrease of inhibitory activity in the early period of incubation. If the absorption of ovomucoid is slower than another protein, trypsin inhibitory activity of egg white has to increase in the later period of incubation. But the clear results were not obtained in the present experiments because of the existence of another trypsin inhibitor of egg white components. It may be necessary to investigate further details of the change of egg white during incubation by the use of the technique of electrophoresis and chromatography.

CAM contains neuraminidase and sialic acid. Neuraminidase of CAM was found to release sialic acid from CAM itself, and showed strong activity toward exogenously added egg white sialo-proteins.

Desialylated egg white sialo-protein bound to CAM rapidly much more than native sialo-protein just as desialylated protein rapidly bound to plasma membrane[4]. Namely, native sialo-protein was found to bind but slightly to native CAM. It was considered from these results that ovomucoid (ovomucin) bound to CAM followed by desialylation and release, and that the released desialylated ovomucoid (ovomucin) was susceptible to bind again to CAM for the absorption by embryo.

Data in Table 2 show that sialic acid binds at the surface of CAM. Ashwell reported that desialylation of plasma membrane caused the inhibition of desialylated protein binding. They indicated that sialic acid of plasma membrane surface was necessary for binding process[7]. It is reasonable to consider that the bound amount of native sialo-protein to native CAM and that of desialylated sialo-protein to desialylated CAM to be small because of the charge repulse between sialic acid at the membrane surface and the added sialo-protein. On the binding process to CAM, the persent results showed that desialylated protein was not tend to bind to desialylated CAM. However, it was found that native sialo-protein bound to CAM from which

sialic acid was removed. This result indicates that when either CAM or protein contains sialic acid, CAM-protein binding is enhanced. This experiment demonstrates that binding mechanism of CAM-sialo-protein is different from that of plasma membrane-sialo-protein binding. The trials of solubilization of neuraminidase from CAM are now in progress.

Acknowledgement

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要旨：孵卵中の卵白のシアル酸およびトリプシン阻害活性の変化を追求した。シアル酸濃度は日と共に徐々に増加し，インヒビター活性は，最初減少したのち増加し，それ以後は変化しなかった。孵卵数日にして形成される漿尿膜（CAM）は，強いシアリダーゼ活性を有し，卵白のシアロタンパク質であるオボムチンやオボムコイドからシアル酸を遊離させた。またCAM自身もシアル酸を有し，自らもシアル酸を遊離させる。微生物由来のシアリダーゼで処理したCAMは非常に強いシアリダーゼ活性を示したが，これは加えたシアリダーゼが膜と強く結合するものと考えられる。シアロタンパク質とCAMの結合が，オボムコイドの示すトリプシン阻害活性をもって間接的に，^{125}I による標識で直接的に検討された。用いたオボムコイド，オボムチン共に脱シアル酸処理をしたCAMとよく結合し，逆に脱シアル酸処理したタンパク質は，そのままのCAMとよく結合した。