VIRUS ANTIHEMAGGLUTININ ACTIVITIES OF AVIAN EGG COMPONENTS

by

T. F. SUGIHARA, L. R. MacDONNELL*, C. A. KNIGHT** AND R. E. FEENEY***

Western Utilization Research Branch,† Albany, Calif. and
University of California, Berkeley, Calif. (U.S.A.)

The white of avian egg contains several unique and biochemically active proteins, as follows: conalbumin (iron-binding), lysozyme (bacteriolytic), ovomucoid (trypsin inhibiting), and avidin (biotin binding)[1-4]. A fifth egg-white protein, ovomucin, has unique gelatinous properties and contributes to gel strength in thick white[5]. More recently the virus antihemagglutinin (AH)†† activity of egg-white has been found to be closely associated with ovomucin[4,6,7,8]. This active substance has characteristics in common with the mucoids and other mucoproteins, and it resembles the receptor substances in the erythrocyte membrane[8]. Studies at this Laboratory have confirmed the relationship of the AH activity of egg-white to its ovomucin[9]. However, only part of the ovomucin fraction contains the activity[6,7,9] and the activity has been found to be stable to severe chemical and physical treatments that usually denature or inactivate proteins[9].

We are now reporting phases that are more characteristically biological: (1) the assay method for AH activity, (2) the activities of different structures of the chicken egg, (3) the activities of the albumens of several other avian species, and (4) inactivation of AH activity by incubation with several different viruses.

MATERIALS AND METHODS

Swine-influenza virus

Embryonated 10-day-old eggs were inoculated in the allantoic sac with egg-adapted swine-influenza virus (Shope's strain 15), and the allantoic fluids were harvested after 48 hours at 35° C. The pooled fluids were purified by Sharples centrifugation, diluted with saline to give 2 mg of protein per ml, and stabilized with 0.05% formaldehyde. The virus solution was stored at 4° C. To prepare the virus solution for use, it was diluted with buffered saline to give approximately 8 hemagglutinating units per ml. This solution was then heated at 53° C for 30 minutes to inactivate the virus and thus to convert it to an "indicator virus".

Influenza virus A

Puerto Rico strain, PR-8, was prepared as described above for swine influenza virus.

* Present address: Brentwood Egg Company, Emeryville, California.
** The Virus Laboratory, University of California, Berkeley.
*** Present address: Department of Biochemistry and Nutrition, University of Nebraska, Lincoln.
† Agricultural Research Service, United States Department of Agriculture.
†† This term does not have any immunological significance.

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Red blood cells

Chicken blood was obtained by cardiocentesis. The blood was immediately mixed with 0.5% potassium citrate to prevent coagulation. The red cells were separated by centrifugation and washed three times with buffered saline (0.81% sodium chloride and 0.005 *M* phosphate at pH 7.3). The packed red cells were then diluted with buffered saline to give a 1.5% suspension. The preparation was stored at 4° C until use (0–10 days).

Egg-white

A "standard" solution of egg-white consisted of a 20% dispersion in buffered saline preserved with 0.02% Merthiolate (Lilly), according to LANNI *et al.*[11]. The "standard" solution was stored at 4° C for as long as three months without any apparent change in AH activity.

Preparation of samples for assay

To obtain homogeneous preparations of egg-white all samples were carefully blended. In preparing single egg samples, a small electrical blender was used and with larger quantities of pooled samples, a conventional blender was employed. Aeration of the samples during blending was prevented by covering the surface of the liquid with a suitable pyrex Erlenmeyer flask. Such preparations kept for 3 to 4 weeks at 4° C without any change in AH activity. When longer storage periods were necessary, the samples were lyophilized and stored at −20° C. The lyophilized samples showed no loss of AH activity when carefully rehydrated and dispersed by homogenizing. Erratic and low activities were obtained without these precautions.

In preparing chalazae samples numerous (24–72) eggs were broken and the chalazae carefully dissected from the contents. The chalazae were placed into buffered saline and stored for several hours. They were then picked out singly and cleaned of extraneous thick white by teasing with the aid of tweezers and a scalpel. The cleaned chalazae were pooled in a small amount of buffered saline. Thioglycol was then added to give a concentration of 200 p.p.m. and the mixture homogenized by means of a ground-glass hand-homogenizer. The resultant solution was stored at 4° C until assayed. Nitrogen determinations (Micro-Kjeldahl method) were made and calculations of AH activity were done on a nitrogen basis. Yolk and shell membrane samples were prepared in a similar manner with the exception of the cleaning process. To remove extraneous material from the membranes, they were washed repeatedly in buffered saline. The "bloom" sample was obtained by washing the outer surface of very clean eggs with 1 *N* sodium hydroxide.

Antihemagglutinin assay

The AH activity assay, using 4 HU units, was conducted according to the techniques of MILLER AND STANLEY[10,12,13].

Inhibitor inactivation studies

Ovomucin was prepared by first removing the bulk of the lysozyme from egg-white by direct crystallization, precipitating the crude ovomucin by acidification and further purifying the crude material by salt fractionation[4].

For the inactivations, 0.25% solutions of ovomucin were made in pH 7.3 phosphate buffered saline. The solution was dispensed in 2.0 ml aliquots into 16 × 150 mm test tubes. The active virus preparations were diluted with saline to give an activity of approximately 1000 H.U. (hemagglutinating units). The virus solutions were pipetted (0.1 ml or 100 H.U.) into the test tubes containing the inhibitor and mixed by shaking. The tubes were then incubated at 37° C for three hours. A duplicate set of tubes were run using the same procedure but with heat-inactivated (100° C for 5 minutes) virus preparations. After incubation all tubes were assayed against swine-influenza virus to determine the remaining AH activity.

Glassware cleaning technique

All glassware used in the AH assay was acid-washed and then rinsed 15 times with tap water, twice with distilled water, and once with a final rinse of a very dilute solution of ammonium hydroxide. The glassware was dried in a hot air oven.

RESULTS

Assay reproducibility and variations in activities between different samples

Extensive studies were made on: (a) reproducibility of assays on individual egg-white samples, (b) comparisons of activities of different egg-whites, and (c) use of a reference "standard" of egg-white and means for its preparation and preservation. Large

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differences in AH activities of egg-whites had been obtained and it was necessary to consider whether such differences were real or caused by inaccuracies of the biological method, variations in sample preparation, *etc.* Accordingly, experiments were designed in which a number of different whites were assayed singly over a relatively long period of time in the same assays with a repeatedly assayed, preserved, "standard", pooled sample of several whites. An eggwhite "standard" was prepared according to LANNI *et al.*[11] and assayed 22 different times over a period of 3 months. Each of these 22 assays included a different egg-white from a single egg. Thus, single assays were obtained on 22 different egg-whites and the one pooled sample was assayed 22 times. Distribution of the activities found are plotted in Fig. 1.

It is evident that identical distributions were obtained for both the 22 different egg-whites and the pooled sample. Ranges in activities were 0.33 to $1.6 \cdot 10^7$ AH units per g of N for the 22 different egg-whites and 0.30 to $1.6 \cdot 10^7$ AH units for the pooled sample with means of 0.97 and $0.91 \cdot 10^7$ and standard errors of 0.071 and 0.061, respectively. These mean values agreed very closely with the mean value ($1.1 \cdot 10^7$) obtained 2 years previously in which 15 assays were performed on 6 different pooled samples of white over a 9-month period. The data of Fig. 1 also illustrate that variations found in activities between the whites of different eggs may be explained by variations in the assay and not in the inhibitor contents; *i.e.* in this study no detectable differences in the AH activities of the whites of different eggs were found. This was further confirmed in assays of egg-whites from widely different sources.

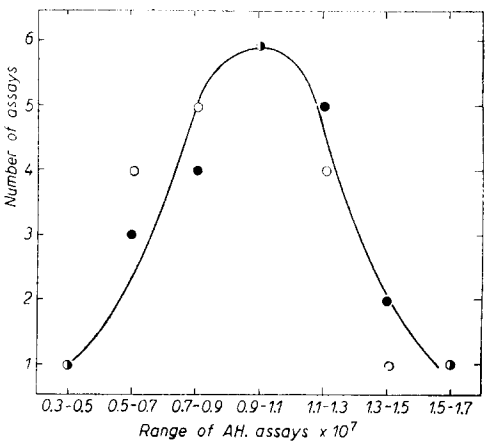

Fig. 1. Distribution of AH assays. O st'd. egg-white, 22 assays, mean 0.91; st'd. err. 0.061. ● 22 different egg-whites, mean 0.97; st'd. err. 0.071.

The importance of a reference standard is also shown by the data of Fig. 1. The mean value of the standard sample, $0.91 \cdot 10^7$ AH units, was taken as the reference figure and the activities of individual egg-whites in each assay were corrected proportionally to the activity obtained for the reference standard in each particular assay. When this was done, the AH activities of the 22 different samples ranged from 0.7 to $1.3 \cdot 10^7$ and the standard error was reduced to approximately half (0.033).

Although it was possible to obtain satisfactory figures with lyophilized preparations, the liquid samples of egg-white preserved according to LANNI *et al.*[11] were considerably simpler to employ and more reliable. They were therefore preferred as standard samples.

Antihemagglutinin activities of the various structural components of the egg

The only known chemical and biochemical differences of any consequence between thick and thin egg-whites are their contents of ovomucin (as determined by chemical precipitation techniques) and virus antihemagglutinin activity[4]. There is approximately 4 times as much of both of these substances in the thick as in the thin white.

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Fig. 2 contains average values found in the 6 major structural components of the egg. Both the yolk membrane and the chalazae were much higher in AH activity than the egg-white fraction, while the other components were very low. Similar data for the yolk membrane and chalazae were obtained with 4 separate preparations in 4 different experiments. The conalbumin and lysozyme contents of the chalazae were determined. Conalbumin values similar to those of egg-white were found: 10–21% (as based on a nitrogen content of egg-white of 15.8%). However, lysozyme was found in amounts of 2 to 3 times greater than in egg-white, 7 to 10%.

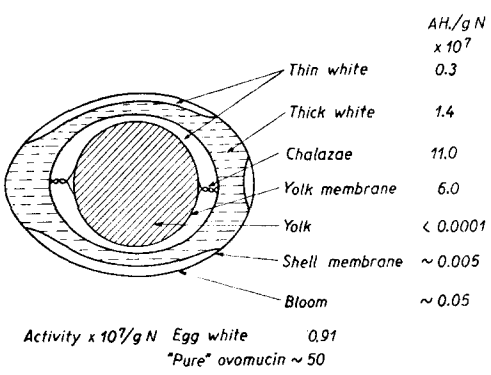

Fig. 2. AH activities of the various components of the egg.

Of particular interest were the assays on whites of eggs from hens recovering from infectious coryza. Very thin whites and low total solids in the whites were found in these eggs but the AH activities were normal on a dry-weight and nitrogen basis.

Antihemagglutinin activities of egg albumens of different avian species

Determinations of AH activities in pooled whites (from 6 to 12 eggs) gave an average value for turkey egg-white similar to that of chicken egg-white, namely $0.9 \cdot 10^7$ AH units per g of nitrogen, while a lower value, $0.6 \cdot 10^7$, was found with duck egg-white (values based on 10 to 20 assays). This lower value for duck egg-white is in line with values found for the other egg-white proteins. Significant differences occur between egg-white proteins of chicken, turkey, and duck[2]. The lysozyme content of turkey egg-white is half that of chicken egg-white, while the lysozyme and conalbumin contents of duck egg-white is about one-fourth that of chicken egg-white.

Inactivation of antihemagglutinin activity by several virus preparations

It has been previously reported that active viruses when mixed with an inhibitor reduce or destroy the AH titer by an enzyme-like action[7,14,15]. Our studies confirm the inactivation. Table I shows the effect of several different virus preparations on the AH activity of the ovomucin inhibitor. In 5 of the 6 tests the titer dropped to approximately 1/7 to 1/5 that of the original after 3 hours of incubation at 37° C.

DISCUSSION

The results of this study support the conclusion that the virus antihemagglutinin activity of egg-white resides in a normal chemical component, which exists in rather similar amounts in the dry matter of eggs laid by different normal hens. In this respect it is similar to the major structural egg-white proteins, ovalbumin, conalbumin, ovomucoid, and lysozyme[2,4].

The AH activity is likely fortuitous and is possibly due to the resemblance of the chemical structure of the incompletely characterized inhibitor[9] to the receptor substance in the erythrocyte membrane[8]. The data definitely oppose the concept that the AH

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TABLE I

INACTIVATION OF ANTIHEMAGGLUTININ ACTIVITY BY SEVERAL VIRUSES

*Virus**		*AH activity after incubation***	
Name	*Preparation*	*Heated**** *units*	*Unheated units*
Mumps	Methanol purified	8000	900
Mumps	Sharples purified	8000	1200
Mumps	Allantoic fluid	14000	6400
NDV, G.B. Texas	Allantoic fluid	14000	800
NDV, Hitchner	Allantoic fluid	14000	800
NDV, Wing-web	Allantoic fluid	14000	800

* These virus preparations were kindly supplied by Dr. R. B. HOULIHAN, Director, Biologica Research Department, Cutter Laboratories, Berkeley, California.

** 0.25% ovomucin solution (activity $5 \cdot 10^7$ AH units per gram of N) was employed. Assay of control solutions was 14000 units/ml, incubated at 37° C for 3 hours, heated and assayed against swine-influenza virus.

*** 100 HD (hemagglutinating dose) employed. Heated samples were treated at 100° C for 5 minutes.

activity has an immunological origin, for this would be expected to cause large differences in titer between eggs. No such differences were observed.

The relatively high AH activity of the chalazae and yolk membrane can be considered as biochemical evidence for the relationships between the chalazae and the ovomucin as postulated by CONRAD and co-workers[16,17]. These workers obtained eggs which had not developed chalazae from the upper ends of hens' oviducts and were able to produce chalazae artificially by slowly rotating the egg. They considered chalazae to consist mainly of ovomucin fibers, which are twisted out of the thick egg-white as the egg turns over while passing through the oviduct. The high figure for yolk membrane fits an extension of this theory, which postulates a structural relationship between yolk membrane, chalazae, and ovomucin. Such an idea is compatible with earlier findings of this laboratory which demonstrated that a main constituent of the total yolk membrane is solubilized by reducing chemicals as is the case with ovomucin[18]. The high lysozyme content of chalazae is similar to the high contents in preparations of ovomucin precipitated directly from egg-white[4,7]. This may be due to complex formation.

Our studies on the inactivation of AH activity by virus action confirm the findings of previous workers[8] that live viruses destroy AH activity. The mechanism of this reaction, however, remains obscure.

The desirability of a standard reference sample in the inhibitor assay was amply shown. As was evident with the lyophilized samples, sample preparation has paramount importance. Many of the variations in activities observed in this study were undoubtedly due to controllable factors, such as temperature, ages of blood cells and virus, *etc.*

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SUMMARY

1. The virus antihemagglutinin (AH) component of chicken egg-white is present in similar amounts in normal eggs.

2. The AH activity is highest in the chalazae and yolk membrane of the egg.

3. As is the case with other egg-white constituents, species differences in the AH activity exist. Turkey and chicken whites both contained approximately 50% more AH activity than egg-whites of two species of duck.

4. The inactivation of AH activity by incubation with live viruses was confirmed.

5. The use of a reference standard in the inhibitor assay is recommended.

RÉSUMÉ

1. Le constituant antihémagglutinique (AH) du virus du blanc de l'oeuf de poule est présent en quantités semblables dans les oeufs normaux.

2. L'activité AH est plus élevée dans la chalaze et dans la membrane du jaune.

3. De même que pour les autres constituants du blanc d'oeuf, des différences spécifiques existent pour l'activité AH. Les blancs de dinde et de poule ont une activité AH 50% supérieure environ à celle des blancs de deux espèces de canard.

4. L'inactivation de l'activité AH par incubation avec des virus vivants a été confirmée.

5. L'emploi d'un standard de référence pour le dosage de l'inhibiteur est recommandé.

ZUSAMMENFASSUNG

1. Die Virus-Antihaemagglutinin-(AH)-Komponente des Hühnereiweiss ist in ähnlichen Mengen in normalen Eiern vorhanden.

2. Die AH-Aktivität ist am höchsten in der Chalaze und in der Eidottermembran des Eies.

3. Im Falle anderer "Eiereiweiss"-Konstituenten bestehen spezielle Unterschiede in der AH-Aktivität. Truthahn- und Hühner-Eiereiweiss haben annähernd 50% mehr AH-Aktivität als das Eiereiweiss zweier Entenarten.

4. Die Inaktivierung der AH-Aktivität durch Inkubation mit lebenden Viri wurde bestätigt.

5. Der Gebrauch eines allgemeingültigen Standarts zum Testen der Hemmung wird empfohlen.

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