

Studies on the Atopic Allergen in Hen's Egg

II. Further Characterization of the Skin-Reactive Fraction in Egg-White; Immuno-Electrophoretic Studies

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Introduction

Hen's egg is one of the foods to which, not infrequently, positive immediate skin reactions are observed. In a preceding paper [2] we reported the preliminary results of our studies undertaken to characterize the component(s) in egg responsible for the wheal and erythema phenomena occurring in some atopic individuals upon intracutaneous injection of minute amounts of egg-white. In boiled eggs the skin-reactive potency was found associated with the heat stable ovomucoid fraction; the yolk showed residual activity only. Fractionation studies indicated that the active component in fresh egg white resides largely in the ovomucoid fraction as well [2]. The protein fractions containing the major egg-white components ovalbumin and conalbumin and the ovoglobulins proved to have much lower skin-reactivities (on a dry weight basis) in individuals with positive skin responses to egg-white. The lysozyme fraction had an activity comparable to that of fresh egg-white [2]. It was considered, however, that the latter substance caused non-specific irritant skin responses (presumably by virtue of the carbohydrase activity).

In order to evaluate these findings further, fractionation and identification experiments were performed. The isolation and purification experiments were followed by means of agar- and starch gel electrophoresis and with the aid of immuno-electrophoresis with antisera to egg-white and to individual egg-white proteins. These experiments are described in this paper.

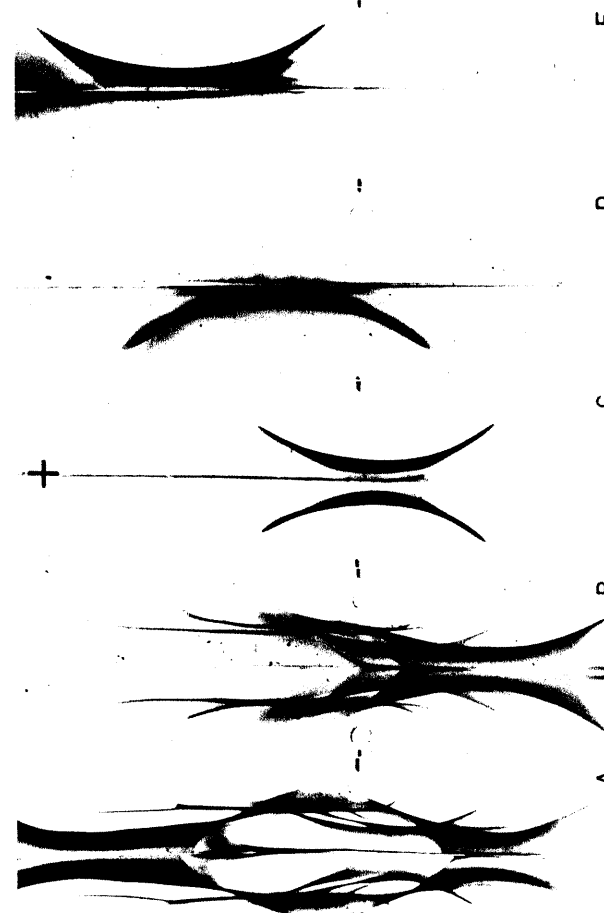


Fig. 1. Immuno-electrophoresis of egg-white components. A: Egg-white against antiserum to fresh egg-white. B: Egg-white against anti-egg-white serum. C: Egg-white against antiserum to purified ovomucoid. D: Antiserum to ovomucoid isolated by method of FREDERICQ and DEUTSCH [9]. E: Antiserum to crystalline ovalbumin. Left hand side: ovomucoid. Right hand side: ovalbumin.

Methods

Analytical Methods

Methods for the determination of the total hexose content, of the hexosamine and sialic acid content have been described before [2].

Column chromatography was performed at 4° as outlined earlier [2].

Methods used for agar gel and starch gel electrophoresis have also been given earlier [2]. Immuno-electrophoresis was performed on microscope slides according to SCHLEIDEGGER [22] with standard LKB outfit.

Antisera to egg-white proteins were raised in adult albino rabbits. Each animal received several subcutaneous injections in the foot pads consisting of totally 1 ml fresh egg-white (adjusted to pH 7) mixed with 1 ml Freund's complete adjuvant (Difco Laboratories). After 4 weeks the animals were given an intraperitoneal booster dose of 0.5 ml lyophilized egg-white (0.5% in saline, pH 7). Seven days later the rabbits were bled and the sera were stored at -20°. In a similar way antisera were raised to crude ovomucoid (isolated by the method of FREDERICQ and DEUTSCH [9]), to crystalline ovalbumin and to purified ovininhibitor (isolated after the method of TOMIMATSU [23]). The animals were immunized with 0.5 ml 5% protein solutions in saline mixed with 0.5 ml Freund's adjuvant, followed by a booster of 2.5 mg protein in 0.5 ml saline. In the case of ovomucoid (weak immunogen) this procedure was repeated twice.

Trypsin and chymotrypsin inhibitor assays were performed spectrophotometrically according to methods described by RICK [19, 20] with the synthetic substrates N- α -benzoyl-arginine-ethyl ester (BAEE, obtained from Nutritional Biochemicals Corporation) and L-tyrosine-ethyl ester (TEE, purchased by Koch-Light England) respectively.

Enzyme activities were obtained from the slopes of the extinction-time plots. The ratio of the slope with inhibitor to that without inhibitor present, times 100, gives the percentage of residual activity. Enzyme and inhibitor substance were incubated prior to assay for 10 min at 20° in the standard buffer solution used for enzyme assay. Measures were made of the residual activity of several different ratios of enzyme to inhibitor concentrations. Extrapolation of the linear portion of the residual activity curve to 100% inhibition gives the moles of enzyme bound per mole of inhibitor. Inhibitor concentrations were estimated from the weights of dry protein used. For trypsin and chymotrypsin optical factors of 0.585 and 0.500 respectively were applied to the measured optical density at 280 nm of the freshly prepared stock solutions (in 0.001 M HCl) to obtain concentrations in mg/ml [19, 20].

Trypsin assays were done at 20° and pH 8 (Tris-HCl buffer 0.05 M, 0.02 M CaCl₂) with 3 ml substrate (0.001 M BAEE) solution to which 0.2 ml aliquots of trypsin-inhibitor mixtures were added, containing about 10 μ g trypsin (specific activity 35.4 μ Mol BAEE/min mg) and 5-50 μ g inhibitor material. The digestion was determined spectrophotometrically by measurement of the extinction change at 254 nm with time.

Chymotrypsin assays were performed at 20° and pH 7 (Tris-HCl buffer 0.05 M, 0.02 M CaCl₂) with 3 ml 0.001 M L-tyrosyl-ethyl ester-HCl to which at time zero 0.2 ml aliquots of chymotrypsin-inhibitor mixtures were added containing 30 μ g chymotrypsin (specific activity 8.3 μ Mol TEE/min mg) and 5-50 μ g inhibitor material. The digestion was determined by measurement of the change in extinction at 234 nm/min (against 0.001 M tyrosine as a blank).

Trypsin inhibitor assays also were performed with denatured casein (Hammersten quality, obtained from Nutritional Biochemicals Corporation) as substrate, according to the method described by RICK [20]. Assays with chymotrypsin-inhibitor mixtures and casein as substrate gave inconsistent results [19].

Skin Reactivity Assays

The skin reactive potency of the protein preparations was determined in 13 atopic patients with a positive intracutaneous immediate reaction to egg-white [2].

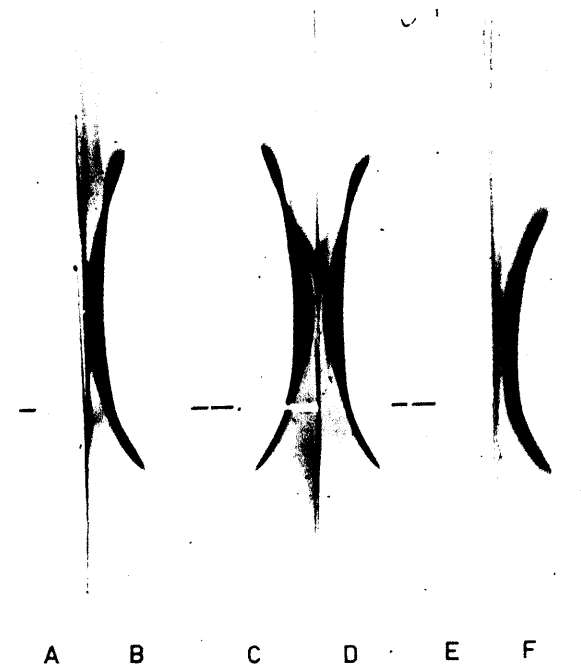


Fig. 2. Immuno-electrophoresis with antiserum to ovomucoid. A: Lysozyme. B: Ovomuroid (VE 20) isolated after LINEWEAVER and MURRAY [13]. C: Ovomuroid (VE 32) isolated after KETTERER [12]. D: Ovomuroid purified by means of column chromatography on P-cellulose. E: Ovalbumin. F: Ovomuroid from boiled eggs (VE 6).

The clinical data of the atopic patients tested for skin-reactivity with various egg-fractions have been given in a preceding paper [2]. All these persons had atopic dermatitis, some of them (4 of 13) also asthma and/or rhinitis. The majority of the atopic patients showed urticaria, dermatitis and angioedema after eating (minute amounts) of egg.

In addition to egg, the patients also had positive skin reactions to inhalant allergens and some other foods.

The skin tests were performed as outlined previously [1]; the skin-reactivity being expressed in units/mg dry weight of material.

Isolation and Purification of Egg-white Components

As starting material fresh egg-white was used, obtained from 24 h old eggs.

Ovalbumin was isolated according to KEKOWICK [11] and recrystallized 4 times from water at pH 4.7 and 20° with the aid of Na₂SO₄ (16 g/100 ml protein solution). From 20 eggs

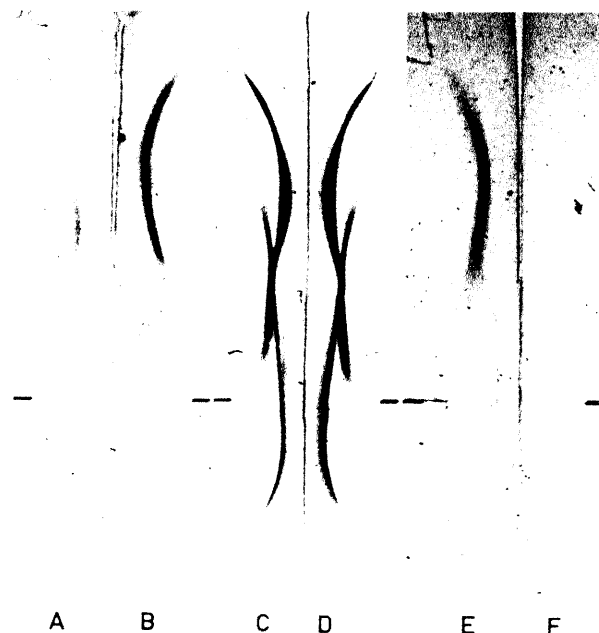


Fig. 3. Immuno-electrophoresis with antiserum to egg-white. A: Ovomuroid from boiled eggs (VE 6). B: Ovomuroid from fresh egg-white (VE 9). C: Ovomuroid (VE 20) isolated after LINEWEAVER and MURRAY [13]. D: Ovomuroid (VE 39) isolated after KETTERER [12]. E: Ovomuroid (VE 63) isolated after RHODES [18]. F: Ovomuroid (VE 54b) purified by means of column chromatography on P-cellulose. (As may be observed, antisera to fresh egg-white did not contain antibodies to ovomuroid.)

about 25 g dialyzed and lyophilized (electrophoretic homogeneous) protein material was obtained.

Lysozyme was obtained from Mann Research Laboratories and was purified by means of column chromatography on carboxy-methyl-cellulose (CM-cellulose or Cellex-CM, capacity 0.8 mEq/g, obtained from California Corporation for Biochemical Research) similarly to the procedure employed for the fractionation of egg-white [2, 16]. The major peak encompassing lysozyme was eluted at pH 9–10; various minor peaks were always obtained at lower pH values.

Ovoinhibitor was isolated by means of two different methods:

a) After the procedure of TOMIMATSU [5, 23] in which a 5% solution of crude ovomuroid (prepared according to LINEWEAVER and MURRAY [13]) was titrated to pH 6 and made 50% saturated with respect to $(\text{NH}_4)_2\text{SO}_4$. The precipitate that formed was collected by centrifugation, dissolved in water and dialyzed against 0.25 M NaCl overnight; the bag

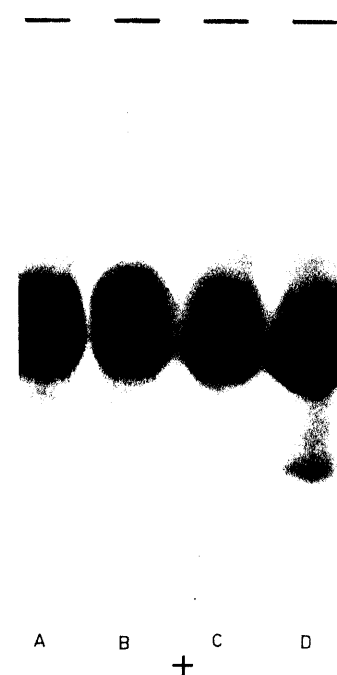


Fig. 4. Starch gel electrophoretic pictures of VE 54 fractions, obtained by means of column chromatography on P-cellulose of VE 54 (isolated after FREDERICQ and DEUTSCH [9]). A: VE 54A (eluted at pH 3.5). B: VE 54B (eluted at pH 4.4). C: VE 54C (eluted at pH 4.6). D: VE 54D (eluted at pH 5.0). Electrophoresis performed in 0.01 M 2-mercapto-ethanol medium. Stain: Amido Black.

content was centrifuged and the supernatant made 35% saturated with $(\text{NH}_4)_2\text{SO}_4$. The precipitate was collected by centrifugation, dissolved in water, dialyzed and lyophilized.

From 5 g crude ovomuroid about 100 mg ovoinhibitor was obtained ($E_{1\text{cm}}^{1\%}$ 280 nm = 6.2, galactose content 2.25%).

b) After the procedure of MATSUSHIMA [14], modified to some extent. To fresh egg-white (pH 6), an equal volume of 0.25 M NaCl solution was added (ovomucin was removed by centrifugation) and the solution saturated to 50% with $(\text{NH}_4)_2\text{SO}_4$; ovoinhibitor will precipitate during this treatment. The precipitation step was repeated, after which the protein solution was dialyzed extensively against distilled water. Then solid trichloroacetic acid was added to 0.15 M (pH 3.5), after 10 min the heavy precipitate was collected

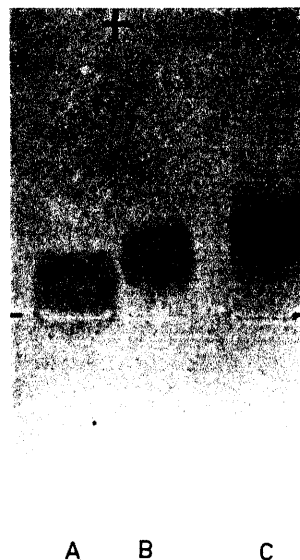


Fig. 5. Agar gel electrophoresis of VE 54 fractions obtained by means of column chromatography on DEAE-cellulose. A: Ovomucoid eluted with 0.02 M phosphate buffer pH 6.5 + 0.04 M NaCl. B: Ibidem + 0.1 M NaCl. C: Ibidem + 0.3 M NaCl. Stain: Periodate fuchsin Schiff (PAS).

by centrifugation. More impurities were removed by subsequent addition of acetone (40%) after which ovomucoid was precipitated at 60% acetone.

From 1,000 ml egg-white about 400 mg ovomucoid was obtained ($E_{1\text{ cm}}^{1\%}$ 280 nm = 6.9, galactose content 2.55%).

Both preparations were purified further by column chromatography on phosphate-cellulose (Cellex-P, capacity 0.98 mEq/g, obtained from California Corporation for Biochemical Research). For this purpose, 200 mg of ovomucoid was dissolved in pH 4 ammonium-acetate buffer and applied to a column (bedvolume 40 ml) packed with the resin. The phosphate-cellulose was treated with 0.1 M, pH 4 and pH 4.6 ammonium-acetate buffers and subsequently ovomucoid was eluted with 100 ml 0.1 M, pH 4.6 buffer, containing 1 M NaCl.

The preparations obtained by these procedures were found to be potent inhibitors both of trypsin and chymotrypsin: 10 μ g of ovomucoid were found to inhibit 9.6–10.8 μ g chymotrypsin (or on a molar ovomucoid/chymotrypsin ratio of 1:2), 10 μ g inhibits 8.5–9.0 μ g trypsin (a molar ovomucoid/trypsin ratio of 1:1.7–1.8).

Apoprotein-flavoprotein (binds riboflavin) was prepared according to RHODES [17] by means of chromatography on DEAE-cellulose. (Cellex-D, capacity 0.8 mEq/g, obtained from

California Corporation for Biochemical Research). Ovomucoid-free and lyophilized egg-white (10 g = 100 ml egg-white) was dialyzed overnight against ammonium-acetate buffer (pH 4.3, 0.1 M) and applied to a column (bedvolume 300 ml) containing DEAE-cellulose. The resin was washed with pH 4.3, 0.1 M ammonium-acetate buffer, the eluate contains the majority of the egg-white proteins (only proteins with a relatively low iso-electric point – such as flavoprotein – will be adsorbed by the resin) the flavoprotein fraction was eluted with pH 3.5 ammonium-acetate buffer containing 0.5 M NaCl and purified further by means of chromatography on CM-cellulose as outlined in detail by RHODES [17, 18]. From 10 g egg-white about 50 mg flavoprotein was obtained ($E_{1\text{ cm}}^{1\%}$ 280 nm = 6.5–7.0, trypsin inhibitor activity below 10%, electrophoretic mobility on agar gel and pH 8.6 between that of ovalbumin and ovomucoid).

Crude ovomucoid preparations were isolated from fresh egg-white by 4 different techniques (the original isolation methods were all modified to some extent).

1. After LINEWEAVER and MURRAY [13], involving the use of trichloroacetic acid and acetone. Egg-white was adjusted to pH 3.5 by the slow addition of 1 vol trichloroacetic acid-acetone solution (1 vol of aqueous 0.5 M TCA plus 2 vol of acetone). The bulk of the egg-white proteins precipitate during this treatment; the supernatant fluid contained the ovomucoid; it can be separated from the solution by the addition of 2 $\frac{1}{2}$ vol of cold acetone. The acetone precipitation step was repeated once, after which the ovomucoid solution was dialyzed (insoluble matter removed by centrifugation) and lyophilized (fractions VE 20 and 61). From 100 ml egg-white about 750 mg crude ovomucoid may be obtained. These preparations were found to be far from homogeneous and usually contained, besides ovomucoid, lysozyme and ovomucoid (denatured), ovalbumin and some other unidentified egg-white constituents, detectable with the aid of immuno-electrophoresis.

2. After the method of FREDERICQ and DEUTSCH [9], in which a combination of sodium-trichloroacetate precipitation and ethanol fractionation is used. Fresh egg-white was adjusted to pH 3.5 with 1 N H_2SO_4 , 1 vol of 10% sodium-trichloroacetate pH 3.0 was added and the final mixture was allowed to stand overnight at 4°C, after which the precipitate was removed by centrifugation. To the supernatant, adjusted to pH 6.0 with 0.5 N NaOH and cooled to 4°, 2 vol of precooled ethanol (–20°) were added; after 4 h the precipitate was collected by centrifugation, dissolved in water and the solution obtained was dialyzed extensively against 0.02 M NaCl (some sediment was discarded). Subsequently the solution was adjusted to pH 3.2 and 1 vol precooled 96% ethanol was added. After 6 h the mixture was centrifuged, the precipitate dissolved in a minimum amount of water, dialyzed and lyophilized (fractions VE 32 and VE 54). Yield 300–560 mg ovomucoid for each 100 ml of fresh egg-white. In this preparation small amounts of (denatured) ovalbumin and 2 minor protein components were detectable.

3. After the method of KETTERER [12], originally developed for the isolation of ovoglycoprotein (a protein without trypsin and chymotrypsin inhibitor activity, but which has many chemical characteristics in common with ovomucoid). Sodium chloride was added to egg-white to 0.15 M and the pH was adjusted at 6.2 with acetic acid. Ethanol was then added to give a concentration of 20% (v/v), the precipitate that formed was discarded. The supernatant was then adjusted to pH 4.6 and more ethanol added to 30% (v/v), a copious precipitate settled which was also rejected. Finally the supernatant was made 70% with respect to ethanol and the precipitate that formed was collected, dissolved in water at pH 7, dialyzed and lyophilized (VE 39). Yield: 500 mg/100 ml of egg-white. Electrophoresis showed the presence of at least 5 other components.

4. After the method of RHODES [18] employing column chromatography on CM-cellulose at pH 4.3. To this end 10 g (= 100 ml) egg-white was dissolved in ammonium-acetate buffer (0.1 M, pH 4.3), dialyzed overnight against the same buffer and applied to a column (bedvolume 300 ml) packed with CM-cellulose (equilibrated with the starting

buffer); elution was performed with this buffer. Primarily only ovomucoid was unabsorbed. The material, not absorbed by the exchange agent, was pooled, dialyzed and lyophilized. In this way about 500 mg crude ovomucoid (VE 63) was obtained containing also some other egg-white components.

Purification of Ovomucoid

Crude ovomucoid, obtained after the method of FREDERICQ and DEUTSCH (ovomucoid preparation 2), was purified further by means of column chromatography on phosphate-cellulose. One gram of ovomucoid was dissolved in 20 ml 0.01 M, pH 3.5 sodium-acetate buffer and dialyzed overnight against 2 l of the same buffer and applied to a column (bedvolume 300 ml) packed with P-cellulose (cellulose-P, capacity 0.98 mEq/g); the resin was equilibrated at pH 3.5 (0.01 M acetate buffer). The column was eluted with 250 ml of the following buffers: 0.1 M pH 3.5; 0.1 M pH 4.0; 0.1 M pH 4.4; 0.1 M pH 4.6 and 0.1 M pH 5.0 sodium-acetate-acetic acid solutions. As a rule 3 peaks were obtained viz: one at pH 4.4 (fraction VE 54 B, 600–650 mg), a peak at pH 4.6 (fraction VE 54 C, 150–200 mg) and one at pH 5.0 (fraction D, 25–50 mg); the total weight recovery was about 85%. Sometimes a small amount of material could be eluted at pH 3.5 (fraction A, 10 mg). In fraction A small amounts of flavoprotein could be detected; fraction B contained the bulk of the ovomucoid material; electrophoretic studies showed in C the presence of ovomucoid and a minor protein component; in D in addition to small amounts of ovomucoid ovalbumin two other minor protein components were found to be present.

Results and Discussion

a) Identification Experiments

The biological activities and protein composition of the isolated ovomucoid preparations have been collected in table I. The skin-reactivities of the various ovomucoid samples, obtained from hen's egg by quite different methods, are all of the same order of magnitude, ranging from 200,000 to 360,000 units/mg protein. The average reactivity of the eleven preparations, tested for skin-reactivity in atopic individuals with strong wheal and erythema reactions to egg-white, was found to be $267,000 \pm 60,000$ (or a standard deviation of 22.5%). The standard deviation is remarkably low considering the inaccuracy of the skin assays and taking into account that the preparations had been isolated by quite different methods. As may be observed from table I, there exists no clear-cut correlation between skin-reactivity and trypsin inhibitor activity. There are 3 ovomucoid samples with a low inhibitor activity (VE 6 and VE 63) indicating that in these cases marked protein denaturation may have occurred (the low activities of these preparations cannot be attributed to significantly lower ovomucoid contents). The average trypsin inhibitor activity for the latter 3 preparations (see table I) was found to be 0.43 ± 0.05 , whereas the

other ovomucoid samples had an average inhibitor activity of 0.71 ± 0.05 . In contrast to the significantly low trypsin inhibitor activities, the ovomucoid preparations VE 6 and VE 63 showed no marked reduced skin-reactivities. These data could suggest that skin-reactivity is not associated with trypsin inhibitor activity or in other words that separate sites or properties of the molecule are responsible for the observed activities.

Agar- and starch gel electrophoresis and immuno-electrophoresis showed that all ovomucoid samples contained other protein constituents. Figures 2 and 3 showed some immuno-electrophoretic pictures of ovomucoid samples with antisera raised in rabbits against crude ovomucoid and egg-white proteins respectively. Figure 2 revealed in some ovomucoid preparations the presence of ovalbumin and in VE 20 also a postalbumin – probably avidine – could be detected [21]. With an antiserum to egg-white in two preparations (VE 20 and VE 39) at least 6 other protein antigens are detectable. The strong line at the anodic side is ovalbumin, the weak line with the same mobility as ovalbumin is probably seralbumin (hen's serum albumin) identified recently by RODRIGUEZ-BURGOS [21], the α_1 -component is flavo-protein-apoprotein, α_2 has the same mobility as the α_2 -1,75 antigen of RODRIGUEZ-BURGOS [21], the β_1 component is probably lysozyme, the β_2 antigen has an electrophoretic mobility comparable to that of ovomucoid. Ovomucoid obtained from boiled eggs (VE 6) and that purified by means of column chromatography on CM-cellulose (VE 9) also contained (traces of) ovalbumin. Fraction VE 63 proved to contain ovalbumin and component α_2 -1,75 [21].

A point of special note is that antisera raised in rabbits to fresh egg-white did not give precipitation arcs with purified ovomucoid. This may become evident from figure 3 which shows the immuno-electrophoretic pictures of purified ovomucoid with an anti-egg-white serum. Although with the antisera used, at least 15 separate egg-white protein antigens were detectable, no precipitating antibodies to ovomucoid proved to be present.

This is quite remarkable because of the relatively high content of ovomucoid (10–12%) in egg-white, while 12 of the 15 egg-white constituents capable of initiating antibody production in rabbits are only present in minute amounts ($\leq 1\%$). These findings indicate that ovomucoid has weak immunogenic properties. RODRIGUEZ-BURGOS and OTEIZA [21] obtained similar results. They immunized rabbits

subcutaneously with 1 ml egg-white (plus 1 ml incomplete Freund's adjuvant) at weekly intervals during 7 weeks. After 20 days the animals received another series of injections for a period of 5 weeks. With the antisera thus obtained 22 antigenic components were detectable. The immuno-electrophoretic pictures revealed, however, only a very weak line with ovomucoid [21].

Of the isolation methods employed, column chromatography on carboxymethylcellulose, using stepwise elution with buffers of increasing pH (fraction VE 9) and the technique of FREDERICQ and DEUTSCH [9] – fractions VE 32 and VE 54 – yielded the most pure preparations. The latter fractions were therefore used for further fractionation and identification studies. To this end, fraction VE 54 was subjected to column chromatography on CM-cellulose. Elution was performed with 0.1 M ammonium-acetate buffer of pH 4.0, 4.4, 4.6 and 5.0. At pH 4.4 a protein fraction was obtained with a skin-

Table I. Skin-reactivities and composition of crude ovomucoid preparations

Fraction number	Obtained from/ isolated after	Isolation technique employed
VE 6 (6a, 6b)	Boiled eggs after BLEUMINK [2]	(NH ₄) ₂ SO ₄ precipitation, column chromatography on P-cellulose
VE 9 and 9a	fresh egg-white after BLEUMINK [2]	column chromatography on CM-cellulose
VE 20 and 61	after LINEWEAVER and MURRAY [13]	precipitation with TCA-acetone mixture at pH 3.5, acetone precipitation
VE 32 and 54	after FREDERICQ and DEUTSCH [9]	Na-TCA precipitation at pH 3.5, ethanol fractionation
VE 39	after KETTERER [12]	ethanol fractionation
VE 63	after RHODES [18]	column chromatography on CM-cellulose at pH 4.3

¹ As identified by starch- and agar gel electrophoresis and by means of immune-electrophoresis with rabbit antisera to purified ovalbumin, crude ovomucoid (VE 32), ovomucoid and fresh egg-white (compare fig. 1, 2 and 3).

² Average skin-reactivity of 4–7 patients with an egg-hypersensitivity [2]. Several preparations were tested 2 or 3 times.

reactivity of 360,000 units/mg. With starch gel electrophoresis (in 0.01 M 2-mercaptoethanol, compare fig. 4) and immuno-electrophoresis with antisera to egg-white and crude ovomucoid (compare fig. 2 and 3) the homogeneity of this fraction was established. In the system ovomucoid-anti-ovomucoid a single broad precipitation line was found, with an anti-egg-white serum, no precipitating antibodies to egg-white constituents could be detected. Starch gel electrophoresis revealed one band only.

Some proteins usually present as minor constituents in the crude ovomucoid sample also were tested for skin-reactivity in atopic individual with strong positive skin responses to egg-white. The skin-reactivities and some chemical data of the flavoprotein-apoprotein (binds riboflavin), ovomucoid, ovalbumin and lysozyme have been collected in table II. The skin-reactivities of these proteins proved at least 15 times lower than the biological potency of purified ovomucoid.

Table I. (continued)

Identified components (besides ovomucoid) ¹	Skin-reactivity (units/mg) ²	Trypsin inhibitor activity ³
traces of ovalbumin and two other minor components	300,000 240,000 200,000	0.49 n.d. 0.40
ovalbumin	240,000 360,000	0.70 0.80
lysozyme, apoprotein, ovalbumin, ovomucoid, two unidentified components, avidine	200,000 200,000	0.65 0.70
ovalbumin, two minor components	360,000 280,000	0.77 0.70
ovalbumin, lysozyme, ovomucoid, apoprotein, unidentified component	320,000	0.67
(den.) ovalbumin, apoprotein, unidentified component	240,000	0.39
Average	267,000	0.63
Standard deviation	± 60,000	± 0.14

³ Mg of trypsin, inhibited by 1 mg of inhibitor substance, BAEE as substrate (for conditions compare method section). One mg of VE 9a was found to inhibit 0.80 mg trypsin, that means a molar inhibitor/trypsin ratio of 1:1.07 (mol weight of trypsin 23,500 and of ovomucoid 31,500).

Table II. Skin-reactivities of purified egg-white compounds

Protein component	Prepared after	Skin-reactivity (units/mg) ¹	Isoelectric point	Mol weight	Biological activity
Ovomucoid	FREDERICQ and DEUTSCH [9] followed by column chromatography on P-cellulose	360,000	4.1-4.4	31,500	inhibits trypsin
Flavo-apoprotein (crude)	RHODES [17]	10,000	3.9	35,000	binds riboflavin
Ovoinhibitor	TOMIMATSU [23] and MATSU-SHIMA [14]	24,000		47,500	inhibits trypsin and chymotrypsin
Ovalbumin (Recrystallized)	KEKWICK [11]	10,000	4.6	45,000	
Lysozyme	obtained from Mann Research Laboratories purified by chromatography on CM-cellulose [16]	2,000	10.7	15,000	carbohydrase (N-acetylmuramide glucanohydrolase EC 3.2.1. 17).

¹ Average skin-reactivity of patients with a positive skin response to egg-white [2].

Table III. Analytical data of ovomucoid¹

Hexose content (expressed as galactose)	7.4 ± 0.5 (n=6)
N-acetyl glucosamine content	14.9 ± 0.9% (n=6)
N-acetyl neuraminic acid content	0.59 ± 0.07% (n=12)
E ₁ ¹ % 280 nm = 4.1	
Molecular weight 31,500 [6]	
Trypsin inhibitor activity: 1 mol ovomucoid inhibits 1 mol of trypsin	

¹ Compare references 2, 3, 4, 6, 7 and 8.

For ovoinhibitor (a trypsin and chymotrypsin inhibitor) an activity of 24,000 units/mg protein was found, for the other preparations even lower activities were observed. It can be excluded therefore, that these proteins account for the biological activity of the particular active ovomucoid fractions.

The present findings together with the results of the fractionation experiments described earlier [2] strongly imply that ovomucoid may be considered as the specific skin-reactive protein in egg-white responsible for the wheal and erythema reactions in 'egg-sensitive' atopic individuals.

b) Some Properties of Ovomucoid

In table III some analytical data of ovomucoid have been collected. As stated above ovomucoid inhibits the proteolytic activity of trypsin. For the molar ovomucoid/trypsin ratio values of 0.99-1.18 were found, indicating that a one-to-one complex between trypsin and ovomucoid is being formed.

A remarkable point is the charge polydispersity of ovomucoid [7, 13]. This may become apparent from the electrophoretic and immuno-electrophoretic pictures shown in figures 5 and 6. The protein may be separated into several subfractions differing only in electrophoretic mobility. Chromatography of ovomucoid on DEAE-cellulose according to Feeney [7], using stepwise elution with 0.02 M, pH 6.5 phosphate buffer, containing increasing amounts of NaCl (0, 0.04, 0.1, 0.3 and 0.5 M) yielded 3 homogeneous ovomucoid fractions eluted at 0.04, 0.1 and 0.3 M NaCl in relative amounts of 58, 38 and 4%. At 0.0 and 0.5 M NaCl also small amounts of ovomucoid may be eluted; in these fractions as a rule some other proteins components were detectable. Immuno-electrophoretic pictures of the 3 homogeneous ovomucoid samples showed distinct lines with increasing electrophoretic mobility at higher molarity of the elution solution.

Between the 3 fractions no significant differences were observed in sialic acid, hexose or hexosamine content and in trypsin inhibitor activity. The charge poly-dispersity is reduced by reduction of S-S linkages with 2-mercapto-ethanol and subsequent alkylation of thiol-groups with iodo-acetamide; by these treatments skin-reactivity markedly decreased.

Finally, a point of special notice is the finding that ovomucoid; although showing strong reactivity in skin tests in some atopic patients, possesses only weak immunogenic activity (compare III, a). Only injection of high amounts of ovomucoid, followed by repeated booster injections gave rise to perceptible antibody production.

Ovomucoid is a very interesting protein. Besides trypsin inhibitor activity, this protein has also been shown to lead to anaphylactoid re-

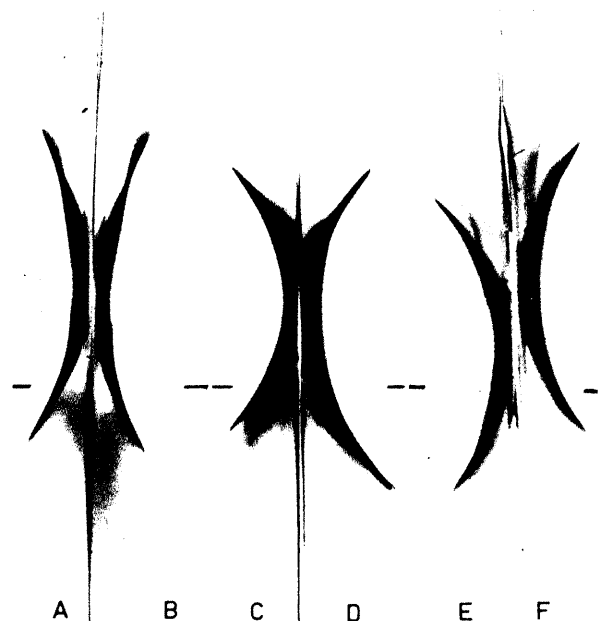


Fig. 6. Immuno-electrophoresis of ovomucoid fractions-obtained by column chromatography on DEAE-cellulose - against rabbit antiserum to ovomucoid. A+B: Ovomucoid eluted with 0.02 M phosphate buffer pH 6.5 + 0.1 M NaCl. C: Ovomucoid eluted with 0.02 M phosphate buffer + 0.01 M NaCl. D: Ovomucoid eluted with 0.02 M phosphate buffer + 0.3 M NaCl. E: Fraction eluted with phosphate buffer only. F: Fraction eluted with phosphate buffer + 0.5 M NaCl. E+F: Impure ovomucoid fractions obtained in minor amounts.

action in some strains of rats [10]. The white rat responds to a single intraperitoneal injection of ovomucoid by an acute inflammatory response, characterized by hyperaemia, gross edema of the extremities and marked pruritis [10]. Identification experiments described above indicated that this protein also may be considered to be the major skin-reactive component in egg-white. This makes ovomucoid an attractive protein for model studies on the relationship between biological activities and molecular structure or conformation of active sites. In a forthcoming paper some experimental results will be de-

scribed with regard to the influence of protein modification on the biological activity of ovomucoid.

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Summary

Ovomucoid has been isolated from egg-white by four different methods; the preparations obtained were tested for skin-reactivity in atopic individuals with a strong positive skin response to egg-white. Proteins usually present in crude ovomucoid samples were also isolated, purified and assayed for their activity in skin tests. The isolation and purification experiments were followed by means of agar- and starch gel electrophoresis (the latter in 0.01 M 2-mercapto-ethanol medium) and with the aid of immuno-electrophoresis with antisera raised in rabbits to fresh egg-white and to crude ovomucoid, ovomucoid and ovalbumin.

All the crude ovomucoid preparations isolated from fresh egg-white and boiled eggs were found to contain other minor protein constituents. Totally, eleven ovomucoid samples were assayed for skin-reactivity, the average activity was found to be 267,000 units/mg ($\sigma = 60,000$ or 22.5%). Purified and electrophoretically homogeneous ovomucoid had a skin-reactivity of 360,000 units. (This preparation elicited a one plus reaction upon intracutaneous injection of 0.003 μ g.) For ovomucoid, ovomucoid, ovomucoid and ovalbumin even much lower activities were found.

It is concluded that ovomucoid may be regarded as the major skin-reactive protein in egg-white responsible for the wheal and erythema phenomena which occur in some atopic individuals upon intracutaneous injection of hen's eggs test solutions.

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The Kinin-System of Human Plasma

I. Isolation of a Low Molecular Weight Activator of Prekallikrein¹

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Plasma kinins are one of the chemical mediators of the vascular phenomena of acute inflammation [1, 2] and there is some evidence that kinins may play some role in certain forms of anaphylaxis [3, 4]. The kinin-system can be readily activated with glass, quartz, kaolin, diatomaceous earth and many other surface active agents. Based on earlier observations of DAVIES and LOWE [5, 6], we have chosen preformed antigen-antibody precipitates as the activating agent [7, 8], since such *in vitro* activation may have *in vivo* significance. For example, kinins generated in plasma and interstitial tissues at sites where immune precipitates have formed, may, in addition to anaphylatoxin, PMN-leucocytes and platelets, be one of the mediators of the various forms of allergic inflammation and of aggregate anaphylaxis.

Guinea-pig serum activated by antigen-antibody complexes enhances vascular permeability when injected intradermally; produces hypotension when injected intravenously; contracts the rat uterus in a Schultz-Dale bath; has TAME-esterase activity; and enhances the clotting of recalcified plasma [9, 10]. Several serine esterase inhibitors suppress the vascular permeability enhancing property of activated serum. Hexadimethrine bromide, an inhibitor of Factor XII, prevents activation of serum by the immune precipitates and immune precipitates have no effect on Factor XII deficient serum. Upon incubation with fresh non-contacted serum, antigen-antibody precipitates adsorb

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