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Immunochemical Studies on Duck (Anas Platyrhynchos) Ovotransferrin-Isolation, Isoelectric Fractionation and Antigen–Antibody Reaction (38194)

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Transferrin is an iron binding globular glycoprotein present in various vertebrate fluids (1). Avian egg white constitutes a principal source of this protein (also called ovotransferrin or conalbumin) and the protein derived from hen (HOT) egg white, has been extensively studied. All known transferrins bind ionically 2 atoms of Fe^{3+} per protein molecule and iron transferrin complexes exhibit similar absorption spectra. Transferrins show extreme heterogeneity upon starch gel electrophoresis (SGE)[1] and this has been the basis of much genetic and biochemical studies (2). In the case of hen (*Gallus domesticus*) ovotransferrin[2] the SGE heterogeneity has been shown to depend on any one of the following reasons: (1) The number of sialic acid residues bound per protein molecule (3); (2) The presence of either 1 or 2 Fe^{3+} atoms per protein molecule (4); and (3) The structural difference arising from the sequential or conformational aspects (5, 6). There is considerable evidence in the literature suggesting conformation difference between iron free and iron saturated HOT based on physico-chemical (7–10) and immunochemical studies (11). There is, however, no immunochemical study on the SGE variants of transferrin saturated with iron although there are reports suggesting similarity of these components in terms of their aminoacid composition and peptide maps (5, 6).

An earlier immunoelectrophoretic study reported significant cross reactions of ovotransferrins of eight avian species with either hen or the cassowary (Casuarius auruensis) ovotransferrin antisera wherein the duck represented an intermediate group showing weak interaction (12). The present report deals with the preparation and isoelectric fractionation of ovotransferrin derived from duck (Anas platyrhynchos) egg wherein a similar structural situation as that of HOT is shown to obtain, as well as the immunoreaction between rabbit anti-DOT antiserum and the electrophoretic components of DOT.

Experimental Procedure. Isolation of DOT. Isolation of DOT was done by a modification of the procedure described in literature (13). Duck eggs were obtained from local supermarkets. Egg white (30–36 ml/egg) was carefully separated from yolk and pooled. Iron-nitrilotriacetate complex (14) was added to pooled egg white (1 ml 100 m*M* Fe^{3+}-NTA/100 ml egg white) and thoroughly mixed. An equal vol of saturated ammonium sulfate solution was dripped into egg white under constant stirring. The mixture was left overnight in a refrigerator and then centrifuged at 1500 rpm for 40 min at 5°. Supernatant solution was adjusted to pH 4.7 with 0.5 *N* H_2SO_4 and the precipitate was filtered. Filtrate was adjusted to pH 7.0 with 0.5 *N* NaOH and solid crystalline ammonium sulfate was

[1] The abbreviations used are: SGE, starch gel electrophoresis; HOT, hen ovotransferrin; DOT, duck ovotransferrin, Fe^{3+}-NTA, ferric nitrilotriacetate complex; PB, phosphate buffer, PBS, phosphate buffer saline.

[2] Hen ovotransferrin used in this study was a gift from Dr. J. Williams.

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added to 100% saturation. The mixture was left in a refrigerator overnight and then centrifuged as before. The resulting precipitate was dissolved in the least amount of distilled water and dialyzed in cold against water followed by 2 changes of 5 m*M* PB, pH 7.5. Any precipitate formed was removed by centrifugation and the clear protein solution was further purified by ion exchange chromatography.

Ion exchange chromatography. The dialyzed protein solution was passed through a column (2 × 40 cm) of DEAE sephadex A-50 (Pharmacia) previously washed and equilibrated with 5 m*M* PB, pH 7.5. Adsorbed ovotransferrin was washed free of impurities by passing initial buffer (250 ml) through the column. Elution of the protein was started with a linear gradient consisting of initial buffer and 25 m*M* PB, pH 7.5 (200 ml) and completed with the latter buffer. Eluted protein was dialyzed against 2 changes of distilled water followed by 2 changes of 5 m*M* PB, pH 5.6, in the cold. The protein solution was centrifuged to remove any precipitate and then applied on a column (2 × 40 cm) of CM sephadex C-50 (Pharmacia) prepared with 5 m*M* PB, pH 5.6. Adsorbed protein was washed with initial buffer (200 ml) and with a linear gradient consisting of initial buffer and 20 m*M* PB, pH 7.2 (300 ml). Elution of protein was accomplished with a linear gradient consisting of 20 m*M* PB, pH 7.2 and 50 m*M* PB, pH 7.2 (200 ml). Eluate was thoroughly dialyzed against distilled water, protein was saturated with Fe^{3+}-NTA and then dialyzed against 2 changes of 2 m*M* ammonium hydroxide and freeze dried (Virtis). The purity of ovotransferrin was tested by SGE.

Starch gel electrophoresis. The method as described by Smithies (15) was followed employing a discontinuous buffer system (16). Voltage was maintained at 7V/cm and the duration of electrophoresis was for 120–150 min at room temperature. Protein sample (50 μl of 2% solution) was applied on filter paper (Whatman #3) rectangles and inserted into the hydrolyzed starch gel slab (Connaught Medical Research Laboratories, 12.5%). After electrophoresis, starch gel was sliced and stained with amidoblack 10B. Relative electrophoretic mobility is expressed as the ratio of distance moved by protein component to that of the buffer front.

Isoelectric focusing. The method described by Wenn and Williams (5) was followed for isoelectric focusing of DOT. Isoelectric focusing column (110 ml, LKB) and ampholine carrier ampholyte, pH 5–7 (Batch 14, LKB) were used. Current, not exceeding 1 W, was passed for 68 hr. Voltage was maintained at 500 during the final 24 hr. Purified iron saturated DOT (40 mg) was distributed in the middle of the sucrose gradient. After electrofocusing, fractions (0.65 ml/tube) were collected by draining the column (1 ml/min). Absorbence of fractions at 280 mμ (unicam S.P. 500 spectrophotometer) using micro cuvettes (0.5 ml; 1 cm light path) and pH (Unicam, pye pH meter model 79) were measured. Peak fractions were pooled and thoroughly dialyzed against water to remove sucrose and carrier ampholytes and tested for their purity by SGE.

Antiserum. A male albino rabbit (3 kg body wt) was injected with a preparation of purified iron saturated DOT (20 mg/ml 150 m*M* NaCl) emulsified with an equal volume of complete Freund's adjuvant (Baltimore Biologicals). Three injections (approximately 0.5 ml) were given by distribution into foot pads and flanks (intramuscular), 1 week apart. A test bleeding through marginal ear vein was done at 6 weeks. An intramuscular booster (1 ml) was given at week 7 and 10 days later the animal was bled (30 ml) through the ear vein. Serum was separated by centrifugation of clotted blood. Complement components were inactivated by incubating the serum for 30 min at 56°. Serum was preserved in cold with the addition of thimerosal (Eli Lilly, 1:10,000). Studies reported in this paper were done with this antiserum sample. After resting the rabbit for 4 weeks, a second booster (1 ml, im) injection was given and the animal exsanguinated 10 days later by drawing blood by cardiac puncture. Serum was processed as before.

Absorption of antiserum was carried out

by graded addition of 250 μg of antigen to 1 ml antiserum until no further precipitation occurred. Completion of precipitation was further checked by immunodiffusion. Ouchterlony double diffusion method was followed as described in (17), using microscope slides (26 × 76 mm) coated (1 mm thick) with 1% agar (Bacto agar, Nobel,

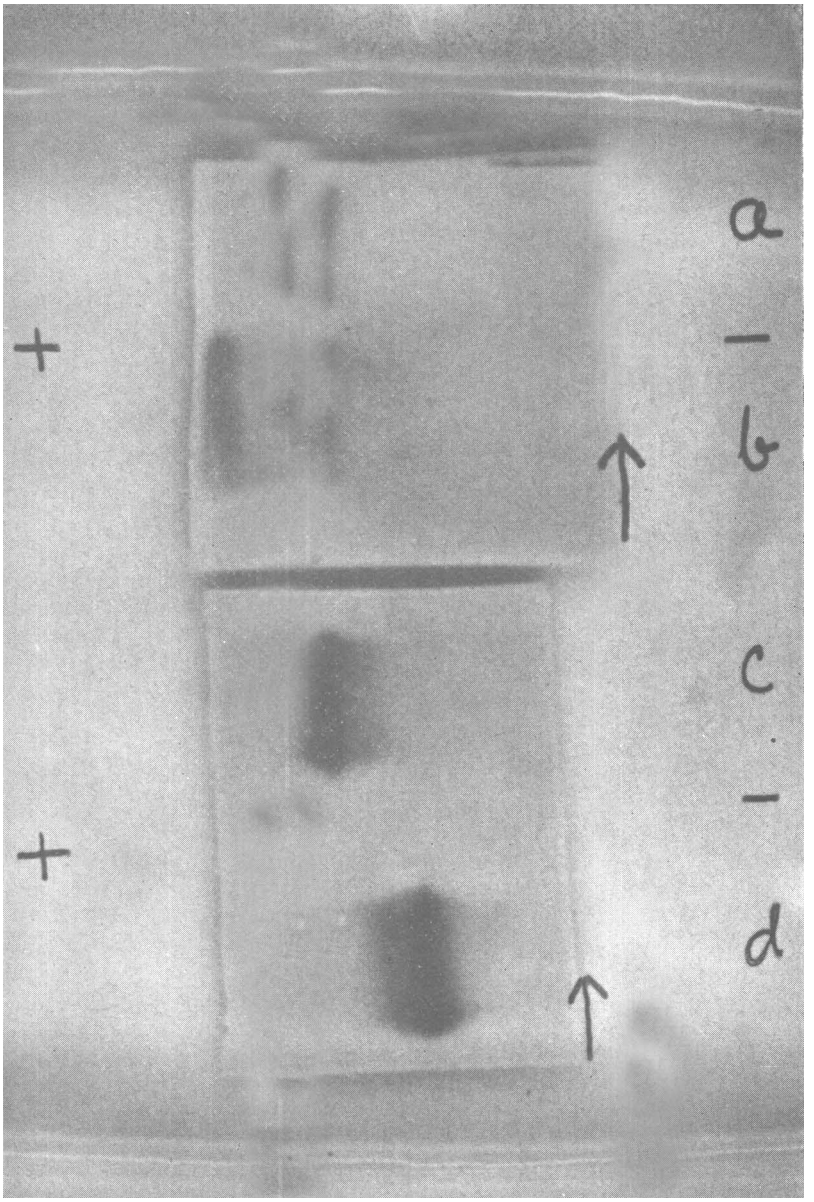

FIG. 1. Starch gel electrophoresis patterns of duck ovotransferrin (DOT) and hen ovotransferrin (HOT). (A) purified DOT; (B) DOT before purification by ion exchange chromatography; (C) purified DOT; (D) purified HOT. Conditions of electrophoresis: A and B, 7V/cm, 150 min; C. and D, 7V/cm, 120 min. Note both DOT and HOT have a minor fast moving and a major slow moving component. Arrow denotes the point of application of sample; +, anode and −, cathode.

Difco) in PBS (50 m*M* PB, pH 7.2 containing 150 m*M* NaCl). Standard procedure described in (17) was followed for passive hemagglutination and hemolysis studies employing the tanning and antigen coating method of Stavitsky (18). A 2 mg/ml concentration of antigen was found optimal for coating tanned sheep erythrocytes. Guinea pig serum served as the source of complement in hemolysis studies.

Quantitative immunoprecipitation was done according to standard methods. To 1 ml of PBS containing 50–500 μg of antigen in 5 ml conical centrifuge tubes, in duplicates, was added 1 ml diluted (1:10 in PBS V/V) antiserum. Contents were mixed, incubated at 37° for 60 min, then at 5° for 5 days and centrifuged in the cold. The precipitates were carefully washed in ice cold PBS and finally dissolved in 3 ml of 0.1 *N* NaOH. Protein content of these samples was estimated using an auto analyzer (Technicon) following the manufacturer's recommended procedure based on the method of Lowry *et al.* (19) using bovine crystalline serum albumin (Sigma) as standard.

Results. Starting with 860 ml egg white from 24 eggs, the amount of DOT obtained in a typical experiment was 1.94 g, which amounts to about 2% total solids of egg white. A typical starch gel electrophoretic pattern of purified DOT along with a sample of HOT for comparison, is shown in Fig. 1. Both transferrins have a minor component with relatively faster anodic electrophoretic mobility at pH 8.6 than the major component. Under the described experimental conditions (current −7V/cm; time −150 min), the relative mobility of minor DOT component is 0.70 (hen 0.617) and major DOT component is 0.51 (hen 0.425).

The 2 electrophoretic components were isolated by subjecting iron saturated DOT to isoelectric focusing and sucrose gradient column (Fig. 2) as described in the experimental part. The pH value for the peak tube of the minor component is 5.52 ± 0.02 (hen 5.62 ± 0.02) and the major component is 5.70 ± 0.02 (hen 5.78 ± 0.02). These pH values represent the isoelectric

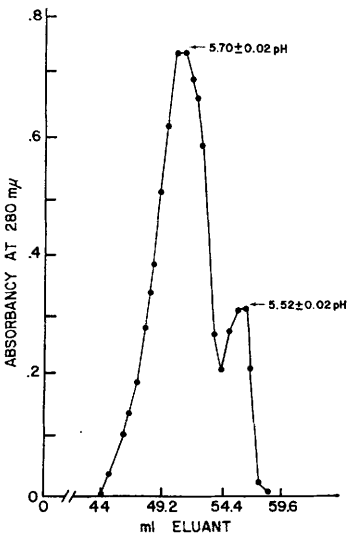

FIG. 2. Isoelectric fractionation of iron saturated duck ovotransferrin (DOT). Isoelectric point of the peak tube with major fraction is 5.70 ± 0.02 pH unit. Isoelectric point of the peak tube with minor fraction is 5.52 ± 0.02 pH unit.

points of the components. The 2 components differ in their isoelectric points by 0.18 pH unit.

Results of a typical experiment on the precipitation of DOT, DOT components and HOT by an anti-DOT antiserum, employing Ouchterlony double diffusion method, is shown in Fig. 3. There is a precipitin line of identity with DOT and DOT components whereas HOT shows a reaction of partial identity as indicated by the formation of a spur. Attempts to obtain antiserum specific for each component of DOT proved unsuccessful. In a typical experiment, no further precipitation of antibody was observed after the graded addition and incubation of a total of 250 μg of the DOT major component to 1 ml of anti-DOT antiserum whereas to obtain similar results, more than 2000 μg of the DOT minor component was required.

Results on the ability of the anti-DOT antiserum to agglutinate and lyse tanned sheep erythrocytes coated with DOT, DOT components and HOT are presented in Table I. DOT and DOT major component have comparable activities whereas the ac-

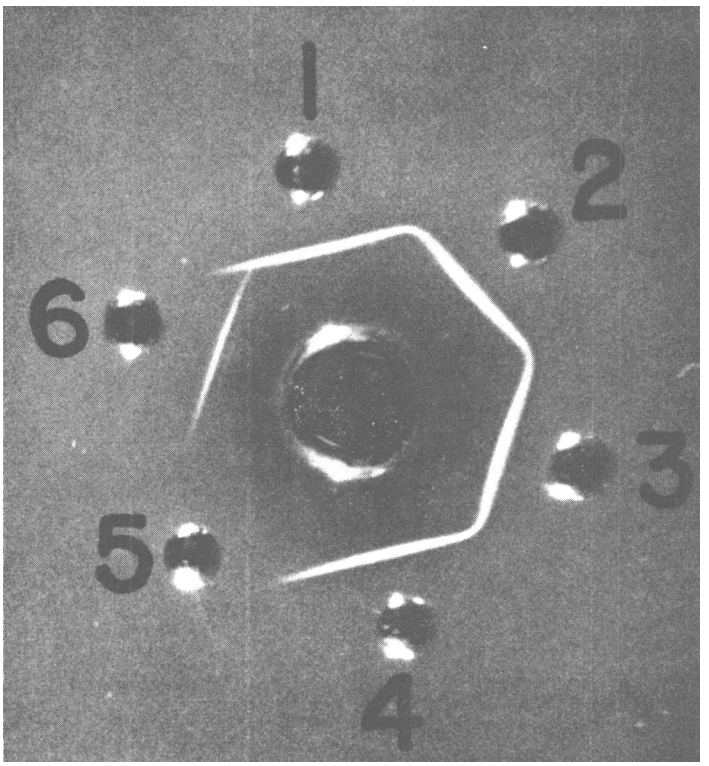

FIG. 3. Ouchterlony double diffusion analysis of duck ovotransferrin (DOT), DOT components and hen ovotransferrin (HOT) with rabbit anti-DOT antiserum. Wells 1 and 4 contain DOT; wells 2 and 3, DOT isoelectric fraction major and minor respectively; well 5, saline and well 6, HOT. Central well contains anti-DOT antiserum. Note reaction of identity with DOT and DOT components and partial identity with HOT.

tivity of DOT minor component is 25–50% lower than that of the major component. HOT treated erythrocytes also show agglutination and hemolytic activity although at a very low level.

Results on the quantitative immunoprecipitation of rabbit anti-DOT antiserum with DOT, DOT components and HOT are presented in Fig. 4. There is much similarity between the precipitin curve of DOT and DOT major component. DOT minor component, however, shows some similarity with the major component in the antibody excess region of the precipitin curve although somewhat increased amounts of the antigen is required to give comparable amounts of immune precipitate. In the regions of maximum antibody precipitate and antigen excess this difference is further magnified. HOT gives a positive but weak interaction with anti-DOT antiserum.

Discussion. Ovotransferrins of duck and hen egg whites have been demonstrated in this study to have similar electrophoretic heterogeneity on starch gel. The duck components show comparatively higher mobility than the hen components. DOT components can be separated like the analogous HOT components by isoelectric focusing on sucrose gradient columns and the difference in isoelectric points between the 2 components, in either case, is 0.18 pH unit. However, the isoelectric points of DOT components are less than the corresponding HOT components by 0.08–0.1 pH unit. This fact, together with the published report (3) on the lower mol wt of DOT might account for the increased anodic electrophoretic mobility on starch gel of DOT components observed in the present study.

TABLE I. Hemagglutination and Hemolysis Activities of Antiduck Ovotransferrin (DOT) Antiserum Against DOT, DOT Components and Hen Ovotransferrin.

Antigen coated on "tanned" sheep erythrocytes	Activity[a] of anti-DOT antiserum														
	Hemagglutination Antiserum dilution × 100									Hemolysis Antiserum dilution × 100					
	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	1:4096	1:5	1:10	1:20	1:40	1:80	1:160	1:320
Duck ovotransferrin (DOT)	4+	4+	4+	4+	4+	4+	2+	+	—	4+	4+	4+	3+	2+	—	—
DOT major component (isoelectric focusing)	4+	4+	4+	4+	4+	4+	3+	2+	—	4+	4+	4+	4+	3+	+	—
DOT minor component (isoelectric focusing)	4+	4+	4+	4+	2+	+	—	—	—	4+	4+	4+	2+	+	—	—
Hen ovotransferrin (HOT)	4+	2+	+	—	—	—	—	—	—	+	—	—	—	—	—	—

[a] Hemagglutination and hemolytic activities are graded as: 4+, complete; 3+ to 2+, partial; +, trace and —, none.

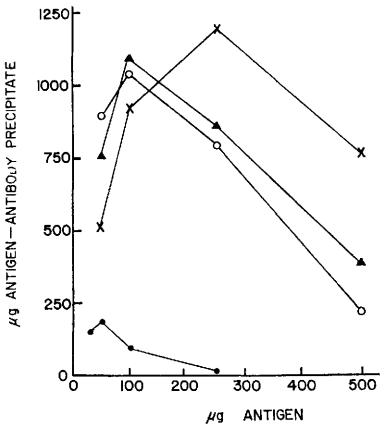

FIG. 4. Quantitative immunoprecipitation reaction of rabbit antiduck ovotransferrin (DOT) antiserum with DOT components and hen ovotransferrin. ▲—▲ precipitin curve of DOT and rabbit anti-DOT antiserum; ○—○ precipitin curve of DOT major component (isoelectric focusing) and anti-DOT antiserum; ×—× precipitin curve of DOT minor component (isoelectric focusing) and anti-DOT antiserum; ●—● precipitin curve of hen ovotransferrin and anti-DOT antiserum.

The *N*-terminal amino acid sequence of DOT (Ala-Pro-Pro-Lys-Thr) resembles HOT (Ala-Pro-Pro-Lys-Ser) and the sequence of the basic and neutral glycopeptide units with sequences ASP-Lys-Thr . . , Thr-ASP-Leu-Thr-Phe . . respectively, appears homologous with the hen glycopeptides. Furthermore, both DOT and HOT contain mannose and *N*-acetyl glucosamine as their sole carbohydrate components, although the relative amounts of these are more in DOT. DOT has a total of 8.3 residues of mannose (HOT = 4) and 8.6 residues of *N*-acetyl Glucosamine (HOT = 8) per 80,000 g protein equally distributed between the 2 carbohydrate binding sites whereas in HOT most of the carbohydrate is present at one site (5) (Personal Communication, Dr. J. Williams).

The 2 components of DOT have common antigenic sites as demonstrated by the line of identity by immunodiffusion whereas HOT shows a reaction of partial identity and therefore some difference in antigenic structure. However, quantitative hemagglutinating and hemolytic activities of anti-DOT antiserum, using tanned sheep erythrocytes coated with DOT, DOT components and HOT, revealed some differences suggesting that the major component has higher antibody combining activity than the minor component. This was also observed by the quantitative immunoprecipitation reaction of transferrin components with rabbit anti-DOT antiserum. The precipitin curve of the major component resembles DOT, the original antigen used for raising antibody. The precipitin curve of the minor component, on the other hand, shows some similarity with the major component up to the zone of equivalence but diverges from thereon. This demonstrates the relatively decreased activity of the minor component in causing inhibition of precipitation throughout the antigen excess region. While the difference in the quantitative antibody combining activity may be a reflection of the structural dissimilarity of DOT components, the possibility of contamination of the minor component with some inert material causing the observed difference in the precipitin curve cannot be ruled out. However, as mentioned in the experimental part, in order to obtain almost complete absorption of antibody, 8–10 times the amount of minor component as compared to the major component was required. HOT reacts with anti-DOT antiserum to the extent of 10%–15% of the homologous reaction. The weak reaction reported earlier (12) might be due to the fact that cross reactions of various avian ovotransferrins were not studied using antibody prepared against purified DOT.

Summary. Ovotransferrin was prepared from duck (Anas platyrhynochos) egg white by ammonium sulfate precipitation and further purified by DEAE and CM sephadex chromatography in a final yield of 2.25 mg/ml egg white, forming about 2% of total solids of egg white. Duck ovotransferrin (DOT) was shown by starch gel electrophoresis to be comprised of a minor component with fast anodic mobility at pH 8.6 and a major component with slow mobility. Isoelectric focusing on sucrose gradient column revealed isoelectric points of the minor and major components

as 5.52 ± 0.02 and 5.70 ± 0.02 pH units, respectively. These components gave identical reactions with a rabbit anti-DOT antiserum by Ouchterlony double diffusion analysis. Anti-DOT antiserum agglutinated and lysed tanned sheep erythrocytes coated with minor component 25%–50% less than the erythrocytes similarly coated with the major component. Similar difference was also observed by the quantitative immunoprecipitation reaction of transferrin components with anti-DOT antiserum.

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