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The Physical and Chemical Properties of an Immunologically Cross-Reacting Protein from Avian Egg Whites*

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ABSTRACT: The physical and chemical properties of an immunologically cross-reacting macroglobulin in avian egg whites were studied. This protein has comparatively high antigenicity in rabbits and cows and shows extensive immunological cross-reactivity when the proteins prepared from the egg whites of different avian species are tested against antibodies to the chicken protein. The chicken protein was a large protein and had a molecular weight of approximately 0.8×10^6 g mole^{-1} by sedimentation-velocity and light scattering measurements and had an isoelectric pH of approximately 4.5.

In an effort to understand the relatively extensive cross-reactivity, studies were made of the properties of the protein from four distantly related avian species: chicken, duck, penguin, and tinamou. All four proteins had very similar physical and chemical properties.

The avian egg white proteins have provided an extensive and unique system for studying the comparative biochemistry of proteins (Feeney *et al.*, 1960a; Clark *et al.*, 1963; Feeney, 1964). Comparison of structures and functions have shown widely varying properties. These proteins have also been used in fundamental studies of immunochemical phenomena by many investigators. Landsteiner *et al.* (1938) in earlier classical studies found cross-reactions among several ovalbumins. Deutsch (1953) and Wetter *et al.* (1952, 1953) found that components seen in moving boundary electrophoresis cross-reacted with varying degrees in the turkey, pheasant, duck, and chicken. Extensive studies of cross-reacting proteins from several species were recently reported from this laboratory (Miller and Feeney, 1964). In general, it was shown that the more closely related two species are, the greater was the similarity of the corresponding proteins; however, it was found that the various proteins differed in their degree of similarity. The conalbumins gave the most extensive cross-reaction of the major proteins but it was shown that antiserum to a minor component from chicken egg white cross-reacted strongly with the homologous protein from all other egg whites tested, except where this protein did not exist. This minor constituent described by Lush (1961) and later confirmed in our laboratory (Feeney *et al.*, 1963) has been previously called component 18 or C-18.[1] This protein was the only component from egg whites found to have this wide spectrum of cross-reactivity.

The present investigation has been made in order to describe more fully the physical and chemical properties of this purified constituent from chicken egg white and to attempt to understand the reasons for the extensive cross-reactivity between species. The approach to understanding the cross-reactivity has been through a comparative study of the purified component from four distantly related avian species: the chicken, tinamou, duck, and penguin.

Materials and Methods

Procurement of Eggs. The eggs of the chicken (*Gallus gallus*), Japanese quail (*Coturnix coturnix Japonica*), and turkey (*Meleagres gallopavo*) were obtained from the Poultry Department of the University of California at Davis. The eggs of the duck (*Anas platyrhynchos*) were obtained locally. Tinamou eggs (*Eudromia elegans*) were obtained from the San Diego Zoo. The eggs of the penguin (*Pygoscelis adeliae*) were obtained directly from a natural rookery in Antarctica, and those of the kiwi (*Apteryx mantelli*) from a game sanctuary in New Zealand. All eggs were refrigerated within 24 hr after

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[1] The authors propose that component 18 (C-18) be named ovomacroglobulin.

being laid and the blending of the egg whites and the storage of the frozen whites were done as previously described (Feeney *et al.*, 1963).

Antisera. Antigens were freshly prepared solutions of the purified proteins in 1% NaCl or the individual egg whites diluted with an equal volume of 1% NaCl. Freund's adjuvant was employed. The antisera were prepared by Antibodies, Inc., Davis, Calif.

Physical Methods. Microimmunoelectrophoresis was done according to the method of Scheidegger (1955) except longer microscope slides were used. Starch gel electrophoresis was done routinely at pH 8.6 with the discontinuous buffer as described by Poulik (1957) as adapted in our laboratory (Clark *et al.*, 1963; Feeney *et al.*, 1963). The electrophoretic mobilities and the isoelectric pH were determined by moving boundary electrophoresis employing a Perkin-Elmer electrophoresis apparatus Model 238. The diffusion constant was determined by immunodiffusion as described by Allison and Humphrey (1959) and by free diffusion in a Tiselius cell of the electrophoresis apparatus. The $s_{20,w}$ values were calculated from sedimentation velocity experiments in the Beckman Model E analytical ultracentrifuge. Light scattering experiments were performed in the Brice-Phoenix light scattering photometer, series 2000. The partial specific volume was calculated as outlined by Linderstrøm-Lang (1962).

Chemical Methods. With the exception of tryptophan which was estimated spectrophotometrically, amino acids were determined after acid hydrolysis by column chromatography on ion exchange resins (Technicon Amino Acid Analyzer) (Moore *et al.*, 1958). The other chemical analyses were determined by the methods indicated: nitrogen by micro-Kjeldahl analysis; hexose (Dubois *et al.*, 1950); sialic acid with thiobarbituric acid (Warren, 1959) as used in this laboratory (Feeney *et al.*, 1960b); hexosamine (Boas, 1953); and sulfhydryl using 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman, 1959; Fernandez-Diez *et al.*, 1964).

Results

Identification of C-18. C-18 could be provisionally identified by its position in starch gel electrophoretic patterns. The other egg white proteins[2] were available in purified or crystallized form and the known positions of these in starch gel and in immunoelectrophoresis aided in differentiating and identifying C-18. A further and most helpful aid in identifying C-18 was the use of the egg whites of a strain of Japanese quail which did not contain C-18. Figure 1 shows a starch gel pattern of several avian egg whites. It can be seen that the whites of these particular strains of quail, turkey, and the kiwi lack C-18. The duck and the penguin appear to have elevated levels of this protein as compared to chicken egg white.

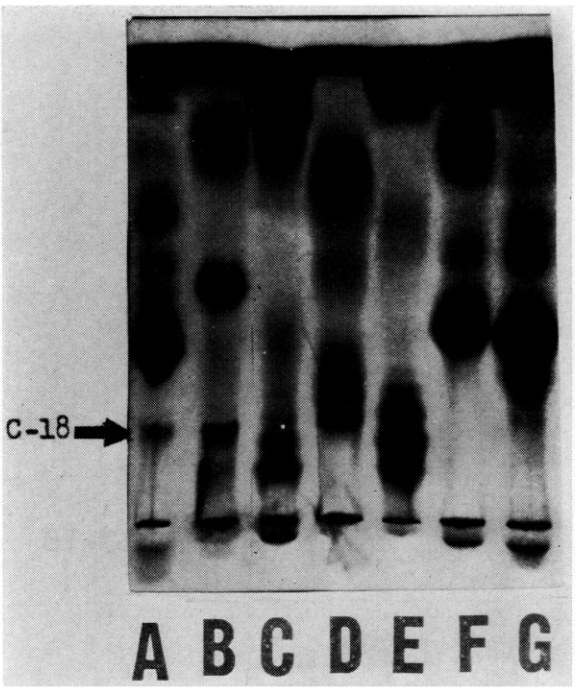

FIGURE 1: Starch gel electrophoretic patterns of various avian egg whites showing the presence and absence of component 18. (A) chicken; (B) duck; (C) kiwi; (D) penguin; (E) tinamou; (F) turkey; (G) quail. The buffer was the discontinuous buffer of Poulik (1957) as employed in this laboratory (Feeney *et al.*, 1963). The pH was 8.6; the gel buffer contained 0.076 M Tris, 0.005 M citric acid, and 0.02 M urea; the electrode buffer contained 0.3 M boric acid and 0.06 M NaOH.

Purification of C-18. Chart I is a flow sheet for the purification of C-18 and Figure 2 shows the starch gel patterns of the major fractions obtained during purification. Pattern A of Figure 2 is untreated egg white. The egg white was adjusted to pH 4.5 with 0.5 N H_2SO_4 and centrifuged to remove most of the ovomucin. The resulting supernatant was adjusted to pH 6.7 with 1.0 N NH_4OH and made to 35% saturation with solid $(NH_4)_2SO_4$. The solution was left for 24 hr and again centrifuged. The precipitate which resulted from this $(NH_4)_2SO_4$ treatment is shown in pattern B. It can be seen that this fraction is highly enriched in C-18 and the A_1A_2 genetic globulins (Feeney *et al.*, 1963). Pattern C is the supernatant from this salting out. When B was adsorbed on CM-cellulose at pH 5.3 in 0.05 M acetate buffer, the proteins which did not adsorb are shown in pattern D. It has been possible to purify all of C-18 from fraction B by this procedure. When fraction D was subjected to gel filtration on G-200 Sephadex, C-18 was eluted as a single leading peak as shown in Figure 3. The larger second peak contained ovalbumin, ovomucoid, and other unidentified globulins. Figure 4 shows a starch gel pattern of purified C-18 from chicken

[2] The chicken egg white proteins available in crystalline form were ovalbumin, ovotransferrin (conalbumin), and muramidase (lysozyme). Those available in highly purified form were ovomucoid, A_1A_2 genetic globulins, the ovoflavoprotein and ovoinhibitor (Feeney, 1964).

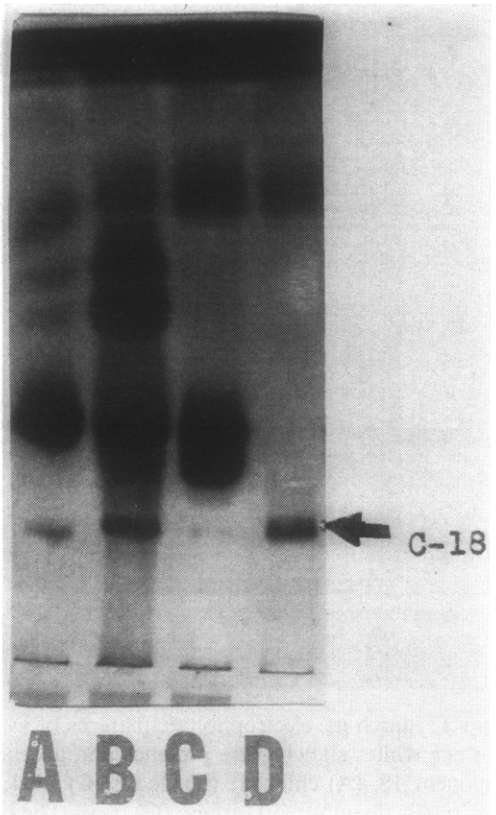

FIGURE 2: Starch gel electrophoretic patterns of fractions of component 18 at various stages of purification. (A) chicken egg white; (B) precipitate resulting from 35% saturated ammonium sulfate; (C) supernatant from same treatment; (D) eluate from crude chromatography on CM-cellulose at pH 5.2. The buffer system was as in Figure 1.

egg white. In the borate buffer usually employed as the electrode buffer the protein gave a major band with a very faint leading component. When barbital buffer was used as the electrode buffer, this leading faint band was not observed.

The C-18 fraction from Sephadex G-200 sometimes contained 3 to 5% of a contaminating material as determined ultracentrifugally and by starch gel electrophoresis. This contaminating material appeared to have a lower molecular weight and a more negative charge than C-18. It could be removed by chromatography on CM-cellulose. The exchanger, which had been equilibrated with 0.05 M sodium acetate buffer at pH 4.9, was put into a column and the fraction of C-18 was applied. The unadsorbed material under these conditions was the contaminant. A gradient was then applied which consisted of 0.05 M acetate at pH 4.9 changing to 0.1 M acetate buffer at pH 6.0. The major peak (C-18) was eluted at pH 5.2–5.3 and at a buffer concentration of 0.066 M. This fraction isolated in this manner contained neither the faster moving band seen in starch gel analysis nor the slower sedimenting peak seen in the analytical ultracentrifuge.

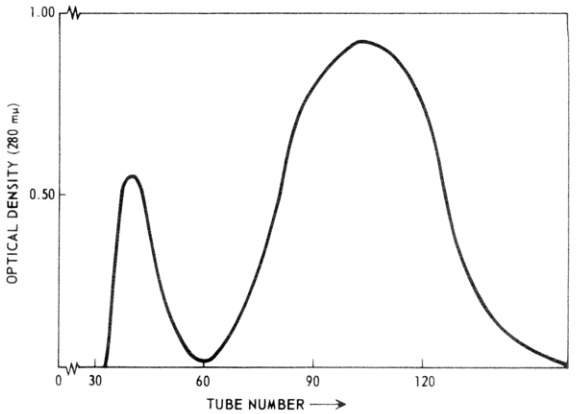

FIGURE 3: A typical gel filtration pattern of crude component 18 on G-200 Sephadex. Fraction employed was dialyzed and lyophilized eluate from CM-cellulose chromatography.

CHART I: Preparation of Component 18.[a]

Blended Egg White (A)

adjusted to pH 4.5 with 0.5 N H_2SO_4

Supernatant adjusted to pH 6.7 with 1 N NH_4OH. Solid $(NH_4)_2SO$ to give 35% saturation

Precipitate (discard)

Precipitate (B) enriched in C-18 A_1, A_2 genetic globulins

Supernatant (C) major portion of ovalbumin, conalbumin

dialyzed *vs.* water, lyophilized, adsorbed on CM-cellulose (0.05 M sodium acetate, pH 5.3)

5.3 eluate (D) C-18, ovalbumin, ovomucoid

CM-cellulose Conalbumin Globulins Lysozyme

dialyze, lyophilize

G-200 Sephadex (0.05 M sodium phosphate, pH 6.5) Peak I = component 18 Peak II = ovalbumin, ovomucoid, and globulins

[a] Letters in parentheses refer to fractions cited in text and to starch gel electropherograms of Figure 2.

C-18 could also be partially fractionated by centrifuging in a preparative ultracentrifuge. In general, the precipitate which resulted from 35% saturated ammonium sulfate was dissolved, dialyzed, and centrifuged

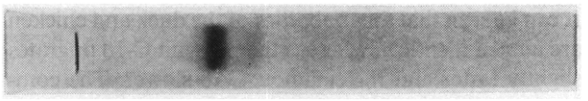

FIGURE 4: A starch gel electrophoretic pattern of purified component 18 from chicken egg white. The buffer system was as in Figure 1.

for 16 hr at 80,000*g*. In one procedure, the pellet was dissolved in pH 9.0 glycine–sodium glycinate buffer and applied to a G-200 Sephadex column. This fraction contained 50–60% C-18. The material from the Sephadex filtration always gave a smear rather than a sharp band on starch gel electrophoresis and lysozyme was always a contaminant; therefore this procedure was not used as an early step in routine isolations.

Physical Properties. Moving boundary electrophoresis was done at five different pH values at 0.1 ionic strength for all buffers. Acetate buffers were used at pH values of 3.5, 4.5, and 4.9. Cacodylate buffers were used at pH 6.0, phosphate at pH 7.8, and glycine at pH 9.0. The protein was most soluble in the pH 9.0 glycine buffer. The plot of mobility *vs.* pH is shown in Figure 5. The isoelectric point is 4.5–4.7. From starch gel analysis comparing the other known egg white proteins, the isoelectric point could be predicted to be near 7.0.[3] No migration was observed in acrylamide gel electrophoresis as performed in this laboratory (Clark *et al.*, 1963). The gel filtration properties of the starch and the acrylamide apparently affected the mobility markedly.

The diffusion constant was determined by free diffusion at a protein concentration of 0.5% in $\Gamma/2 = 0.1$ glycine–sodium glycinate buffer at pH 9.0 and 25°. The value corrected to 20° was 1.98×10^{-7} cm^2 sec^{-1}. By immunodiffusion the measured diffusion constant was 1.95×10^{-7} cm^2 sec^{-1}. The quantitative precipitin test showed that at optimum proportions 0.375 mg/ml of C-18 reacted with the maximum amount of rabbit antiserum to C-18. No calculations were made for the molar ratios of antibody to antigens because specific antibody was not isolated from the purified γ-globulin fractions. The samples of antigen and antibody which gave the optimum precipitate were used in the immunodiffusion experiment for the diffusion constant (Allison and Humphrey, 1959). The angle formed by the line of precipitation and the antigen trough was 36.0° and this value was substituted in the original equation, $\tan \theta = (D_{Ag}/D_{Ab})^{1/2}$.

Light scattering experiments on chicken C-18 showed that concentrations of 0.05–0.15% gave a molecular weight of approximately 900,000 g by both the dissymmetry method (Doty and Steiner, 1950) and by the method of Zimm (1948). Chicken C-18 was examined in the analytical ultracentrifuge in several different buffers and at several concentrations. In Figure 6 are ultracentrifugal patterns showing C-18 in glycine buffer after 4, 27, and 43 min of sedimentation. By this method of analysis, the protein appeared homogeneous. The values of $s_{20,w}$ employing glycine buffer at pH 9.0 were 13.8, 14.1, and 14.8 for protein concentrations of 1.0, 0.4, and 0.08%, respectively. The plot of values was linear and the $s_{20,w}$ value extrapolated to zero concentration was 15.1.

The stability of C-18 to various treatments was observed. Heating a 1% solution of C-18 in pH 9.0 glycine buffer for 30 min at 60° caused starch gel patterns to become very diffuse. Employing starch gel analysis as

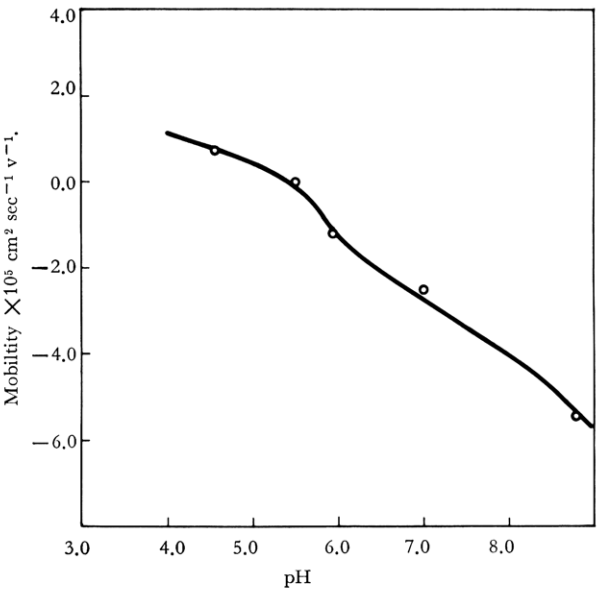

FIGURE 5: Electrophoretic mobility of chicken component 18 as a function of pH. Ionic strengths of all buffers were 0.1.

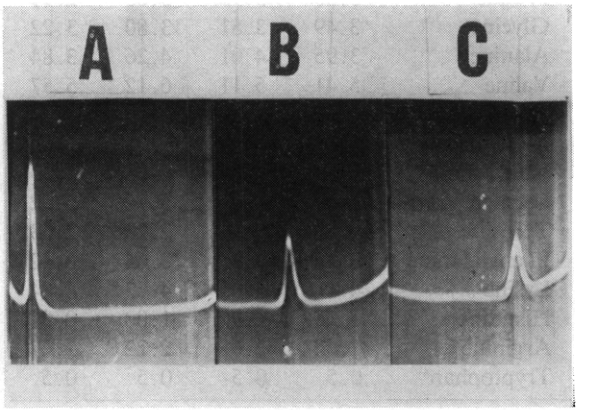

FIGURE 6: Ultracentrifuge patterns of chicken component 18. Sedimentation times: (A), 4 min; (B), 27 min; and (C), 43 min. Bar angle, 65°; protein concentration, 1% in $\Gamma/2 = 0.1$ glycine–sodium glycinate buffer at pH 9.0.

[3] This statement is based on observations of the relative mobilities of C-18 and proteins whose isoelectric pH values are known. Comparisons were made with the chicken proteins, ovalbumin, conalbumin, and lysozyme, and bovine β-lactoglobulin as relative standards in starch gel electrophoresis.

the criterion of stability, 8 M urea and 1.0% sodium dodecyl sulfate denatured this protein. When C-18 was treated with trypsin in a ratio of 10:1 in pH 8.1 Tris buffer for 24 hr at 37°, the starch gel pattern was not affected. Exhaustive dialysis of a solution of C-18 caused the protein to change to a turbid suspension (pH 5.8). When the pH was raised to 6.5 or higher with 0.002 M NH_4OH the suspension again became a clear solution. In some experiments with some preparations there was evidence for degradation or dissociation in acidic solutions (*e.g.*, pH 4.0).

Chemical Composition. The amino acid analyses of different preparations of C-18 accounted for 84–87% of the molecule as amino acids (Table I). The hexose content was determined to be 3.8% with mannose as a standard and 3.5% with glucose as a standard. The hexosamine content was measured to be 5.2%. These values account for 93–96% of the molecule. Other chemical and physical values are shown in Table II.

TABLE I: Amino Acid Analysis of Component 18 in Residues per 10,000 g.[a]

Amino Acid	Chicken	Duck	Penguin	Tinamou
Aspartic acid	6.50	5.85	7.65	6.41
Threonine	4.55	4.21	4.42	3.62
Serine	5.05	5.21	5.53	4.83
Glutamic acid	7.82	8.11	9.05	7.21
Proline	3.65	3.75	3.65	2.91
Glycine	3.49	3.81	3.80	3.22
Alanine	3.95	4.01	4.26	3.84
Valine	5.41	5.11	6.12	5.57
Cystine	1.15	1.47	0.60	1.24
Methionine	1.43	1.61	1.05	1.44
Isoleucine	4.42	3.52	4.02	3.73
Leucine	6.25	5.61	5.76	5.52
Tyrosine	2.68	2.68	2.20	2.53
Phenylalanine	3.45	3.34	3.64	3.42
Lysine	4.11	4.05	4.63	4.04
Histidine	1.25	1.92	1.00	0.81
Arginine	2.60	2.61	2.22	2.47
Tryptophan[b]	0.5	0.5	0.5	0.5

[a] All values, except those for tryptophan, are averages of duplicate determinations by ion exchange chromatography on all four proteins after 22-hr hydrolysis and also of duplicate determinations of the chicken and tinamou protein after 72-hr hydrolysis. [b] Tryptophan values were estimated from ultraviolet absorption spectra and are only approximate.

Comparative Properties of C-18 of the Chicken, Duck, Penguin, and Tinamou. This protein has been isolated from the egg white of the duck, penguin, and tinamou as well as from the chicken. The purified proteins from these species are shown in starch gel patterns in Figure 7. It can be seen that the mobilities of the duck and chicken are almost identical, whereas the penguin C-18 migrates slightly faster and the tinamou C-18 slower. The comparative amino acid analyses are shown in Table II.

TABLE II: Properties of Component 18.

Chemical Composition (%)	
Nitrogen	14.0
Hexose	3.5
Hexosamine	5.2
Sulfhydryl	<0.03
Sialic Acid	<0.03
Physical Constants	
Isoelectric pH (acetate, $\Gamma/2 = 0.1$)	4.5–4.7
Sedimentation Constant	15.1
Apparent Partial Specific Volume	0.745
Diffusion Constant ($cm^2\ sec^{-1}$)	
Free (Tiselius Cell)	1.98×10^{-7}
Agar (Immunodiffusion)	1.95×10^{-7}
Molecular Weight ($g\ mole^{-1}$)	
Sedimentation–Diffusion	~760,000
Light Scattering	~900,000

Although the amino acid contents are quite similar, the duck and chicken proteins give only lines of partial identity in immunodiffusion tests (Ouchterlony, 1949). Using antichicken C-18 serum, the duck and penguin proteins show lines of complete identity as observed in Figure 8A. The duck, penguin, and tinamou are lacking determinants which are present on the chicken C-18, as shown when antichicken C-18 is the antiserum. Similar cross-reactivities were obtained when bovine antiserum to chicken egg white was employed.

In Figure 8B antiserum to chicken C-18 was allowed to diffuse against whole egg whites from various species. It can be seen that the genetic strain of quail, 5448, as well as the turkey lack C-18 in their egg whites, while the other strain of Japanese quail and the chicken contain C-18. By immunoelectrophoretic analysis employing antisera to chicken C-18, C-18 was not found in the blood serum or in the egg yolk of the chicken.

Discussion

The main objectives of this study were to determine the basis for the apparent extensive immunological cross-reactivity and the apparent high antigenicity of C-18. Both its cross-reactivity and antigenicity can be attributed at least in part to its relatively high molecular weight. The explanation of the high antigenicity as a result of the relatively high molecular weight is, however, at best only indirect. A certain minimum molecular size is required for good antigenicity and larger proteins have a larger number of effective antigenic determinants than smaller proteins (Singer, 1964), but the require-

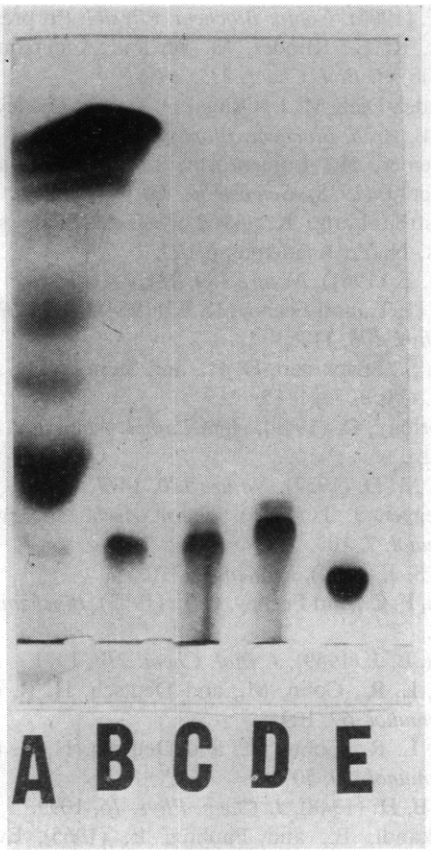

FIGURE 7: Starch gel electrophoretic patterns of purified component 18 from various species. (A) chicken egg white (control); (B) chicken; (C) duck; (D) penguin; (E) tinamou. The buffer system was as in Figure 1.

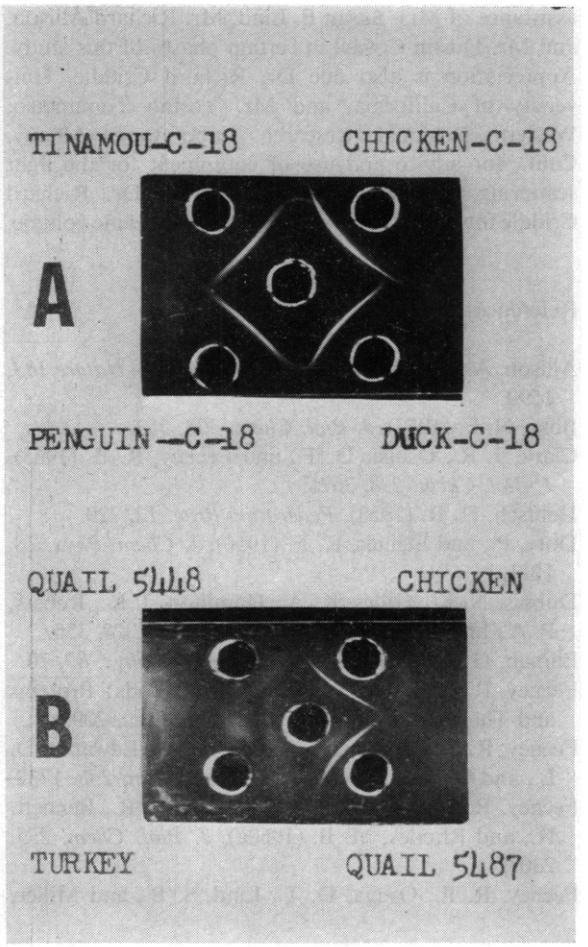

FIGURE 8: Immunodiffusion patterns of (A) purified component 18 from four species against anti-chicken component 18; and (B) various whole egg whites against anti-chicken component 18. Rabbit antiserum to chicken C-18 was in the center cells.

ments for high antigenicity are not understood. The wide spectrum of cross-reactivity of C-18 can probably be more easily explained on the basis of closely similar structures as evidenced by similarities in molecular weights, electrophoretic migrations in starch gel, and in the contents of most of the amino acids. Although differences were found in the contents of amino acids and these differences would be magnified in terms of the numbers of residues per mole of protein because of its large size, the large size of the molecule might also supply numerous antigenically similar sites and the differences in amino acids might only affect antigenically unimportant parts of the molecule. In contrast, other homologous egg white proteins show larger differences in chemical and physical properties (Clark *et al.*, 1963; Fernandez-Diez *et al.*, 1964; Stevens and Feeney, 1963; Feeney *et al.*, 1966; H. T. Miller, D. T. Osuga, and R. E. Feeney, 1965, unpublished data) and very large differences in immunological cross-reactivity (Miller and Feeney, 1964).

No function or biochemical activity has yet been found or suggested for C-18. The absence from egg yolk and from the whites of at least certain strains of the Japanese quail, turkey, and the kiwi show that it cannot have an important role in embryological development. Its absence from blood serum indicates that when it is present it is probably synthesized in the oviduct along with the other egg white proteins. Since the egg white proteins are frequently homologous with proteins present in other fluids, such as the lysozymes and transferrins, C-18 may also have a functionally important homolog elsewhere in higher vertebrates. The value of a study of its possible enzymic activities or other biochemical functions is obvious. A knowledge of a function of C-18 would also aid considerably in understanding the apparent evolutionary stability of its structure. Of closely related importance is a consideration of why the other egg white proteins may evidence much less structural conservation. Invariance in homologs is apparently now being considered to be restricted to minor portions of the protein molecule (Zuckerkandl and Pauling, 1965).

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