The Trypsin and Chymotrypsin Inhibitors from Avian Egg Whites*

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Studies of the comparative properties of proteins and enzymes from closely related species are receiving increasing attention as an approach to understanding the relationships between molecular structures and functions (1). Outstanding examples of these are the studies on the hemoglobins by Pauling et al. (2) and the studies on ribonucleases by Anfinsen et al. (3). The purpose of this approach is to utilize the similarities or differences provided by nature in the proteins or enzymes from closely related species as a tool. By so doing, the investigator may be able to differentiate between those parts of the molecule which are important for the biological, chemical, or physical characteristics of the molecule and those parts which are less important. Such an approach is similar in purpose to the use of chemical and physical means to modify proteins for study.

The eggs of different avian species offer an excellent area for comparative biochemical research. This is true not only because of the wide variety of species but also because a number of very similar eggs may be obtained from the same bird. Furthermore, considerable study has already been made of the biologically active proteins of chicken egg white (4, 5).

One of the more interesting constituents of egg white is ovomucoid. Chicken ovomucoid has been identified as an inhibitor of trypsin (6). Although it has not been crystallized, it is easily isolated in apparently essentially pure form and the assay of biological activity (trypsin inhibition) is readily conducted (7). Ovomucoid inhibits trypsin by the formation of an equimolar complex, and this complex is relatively stable for the periods which are necessary to conduct many biochemical experiments (6, 8). In addition to the ovomucoid in chicken egg white, Matsushima et al. (9) recently reported another inhibitor of a proteolytic enzyme which they named the ovoinhibitor. The ovoinhibitor was reported to inhibit microbial proteolytic enzymes prepared from Bacillus subtilis var. biotechnicus and an unidentified Aspergillus.

Indications as to the quantities of the ovomucoids, or materials resembling ovomucoids, in the whites of the eggs of different avian species have been given by the electrophoretic studies of Bain and Deutsch (10), and of Sibley and Johns-...
solution with buffers of stepwise changes in pH values predetermined by separate experiments using stepwise and gradient elution procedures.

Analyzes—Total nitrogen was determined by micro-Kjeldahl analysis. Estimations of protein in fractions were routinely obtained by measuring the optical densities of the eluate at 280 nm with a Beckman model DU spectrophotometer.

Trypsophan was determined chemically (17) and both tryptophane and trypsophan were determined spectrophotometrically (18). Sialic acid content of the proteins was determined by the method of Warren (19) and by use of Ehrlich reagent as given by Werner and Odin (20). The sialic acid used for a analysis. Estimations of protein in fractions were routinely obtained by measuring the optical densities of the eluate at 280 nm with a Beckman model DU spectrophotometer.

Protestan (ol-chymotrypsin) was an activated, three times crystallized chymotrypsinogen preparation containing 15.0 % N. prepared on the basis of this nitrogen content and were made up daily in 0.004 M acetic acid and 0.02 M CaCl₂. The chymotrypsin (α-chymotrypsin) was an activated, three times crystallized chymotrypsinogen preparation containing 15.0 % N. The enzyme was used as received and made up daily in 0.004 M acetic acid and 0.02 M CaCl₂.

Trypsin substrate, TAMe, and the chymotrypsin substrate, BTE, were purchased from Mann Research Laboratories, Osaka, Japan, through the Biddle Spyder Corporation, 20 Vesty Street, New York.

The trypsin substrate, TAMe, and the chymotrypsin substrate, BTE, were purchased from Mann Research Laboratories, Inc. Part of the BTE used in this study was synthesized in this laboratory essentially according to the procedure of Fox (22) which he presented for the synthesis of benzoyl-L-diodotyrosine ethyl ester.

Trypsin Inhibitor Assays—Trypsin inhibitor assays were done with the use of a Beckman model DU spectrophotometer connected to a Bristol's Dyanamaster recording potentiometer through a Beckman energy recording adapter, model 5800, as has been described (7) with slight modifications as given below. The reaction mixture in the cuvette of the spectrophotometer consisted of 0.3 ml of a solution containing 333 μg of trypsin per ml in 0.004 M acetic acid and 0.02 M CaCl₂, plus 0.0 to 0.7 ml of solution containing 150 μg per ml of inhibitor in 0.006 M Tris buffer, pH 8.2, and 0.7 to 0.0 ml of Tris buffer to bring the volume to 1.0 ml. Two milliliters of the substrate-buffer-indicator solution (0.02 M TAMe, 0.015 M Tris-HCl buffer, and 0.015% m-nitrophenol, respectively, with a final pH value of 8.2) were added to the cuvette just before starting the recorder. The speed of the chart was 4 inches per minute. Calculation of activities is as previously given (7).

Chymotrypsin Inhibitor Assays—Chymotrypsin assays were performed essentially by the same method as the trypsin inhibitor assays described above. The reaction mixture in the cuvette of the spectrophotometer consisted of 0.3 ml of a solution of chymotrypsin (333 μg per ml) in 0.004 M acetic acid and 0.02 M CaCl₂ plus 0.0 to 0.7 ml of a solution containing 150 μg of inhibitor per ml in 0.006 M Tris buffer at pH 8.2, and 0.7 to 0.0 ml of Tris buffer to bring the volume to 1.0 ml. To this were added 2 ml of substrate-buffer-indicator solution (0.008 M BTE, 30% methanol, 0.0075 M Tris-HCl buffer, and 0.01% m-nitrophenol with a final pH value of 8.2) and the recorder started. The speed of the chart was 2 inches per minute. Activities were calculated in the same manner as previously described for trypsin inhibitor activity (7).

Physical Analyses—Diffusion experiments were carried out in a portable electrophoretic apparatus (American Instrument Company, Inc.) at 20-22°. Diffusion constants were routinely corrected to 20° and partial specific volumes were determined according to Bull (25). These determinations were made at 30°. Sedimentation analyses were performed in a Spinco model E ultracentrifuge. Paper electrophoretic analyses were performed with the use of horizontal strip apparatus employing a constant current for 16 to 20 hours.

RESULTS

Fractionation and Purification of Ovomucoid—Initial fractionation of the egg whites of all species studies were performed on CM-cellulose. Columns of this exchange agent, consisting of 4 g of CM-cellulose per g of egg-white protein to be fractionated, were equilibrated with 0.1 M acetic acid titrated to pH 4.3 with NH₄OH. Quantities of 4 to 10 g of exchange agent were used as columns with a diameter of 2 cm and lengths appropriate to the amounts of CM-cellulose employed. Larger quantities of exchange agent (15 to 100 g) were used as short, compact columns on Buchner funnels of the appropriate size. In both cases, dialyzed egg white (0.1 M acetic acid-NH₄OH, pH 4.3) was run through the exchange agent. Elution was performed with the same buffer. With all the whites investigated, primarily only ovomucoid was unadsorbed and passed directly through the exchange column, whereas all of the other proteins were adsorbed. These ovomucoid fractions were then refractionated between pH 3.5 and 4.3 to separate different types of ovomucoids and to remove impurities. DEAE-cellulose was employed to remove small traces of apoprotein from the ovomucoid when present. The ovomucoid fractions equilibrated at pH 4.3 (0.1 M acetic acid-NH₄OH) were run through a short column of DEAE-cellulose (approximately 10 g of exchange agent per g of apoprotein). Under these conditions, the majority of the ovomucoid passed directly through the exchange agent unretained. The adsorbed apoprotein and ovomucoid containing a relatively high percentage of sialic acid were then eluted at pH 3.5 with 0.1 M acetic acid-NH₄OH in 1 M NaCl. When this mixture was refractionated on CM-cellulose starting at pH 3.5, ovomucoid passed directly through the exchanger while the apoprotein was adsorbed.

The purified ovomucoids were subjected to paper electrophoretic analysis employing 0.1 M sodium acetate-acetic acid, pH 4.5, as the buffer and also at pH 6.9 employing 0.1 M potassium phosphate buffer for 16 hours at 8 milliamperes. In all cases, the presence of contaminating proteins was not evident.
When the figures are multiplied by 0.9, the values obtained are approximately equivalent to the mg of trypsin inhibited per mg of inhibitor. The relative trypsin inhibitory activity of the ovomucoid isolated from chicken egg white was determined by the procedure recently reported by Matsushima (9). The principal advantage was that much less exchanger was needed to isolate a gram of this fraction since primarily only the flavoprotein-apoprotein and the high sialic acid ovomucoid are retained on the DEAE-cellulose in the initial step while the other proteins of egg white are unadsorbed. When CM-cellulose was employed for direct isolation, only the high sialic acid ovomucoid was unadsorbed by this exchanger and all the other egg-white proteins had to be retained.

The fourth of the fractions possessing trypsin inhibitor activity was eluted between pH 4.9 to 5.5. In contrast to the ovomucoid fractions which were obtained in a nearly pure state in a single step, the ovoinhibitor contained three other proteins, none of which were any of the well characterized egg-white proteins. Refractionation on both CM-cellulose and DEAE-cellulose did not result in a completely homogeneous protein. As a result, trichloroacetic acid and acetone were employed as outlined by Matsushima (9) to obtain a protein which was homogeneous as indicated by paper electrophoresis in several buffers at different pH values.

Table I shows some chemical and biological properties of the chicken ovomucoid prepared by trichloroacetic acid-acetone precipitation, of the three ovomucoid fractions isolated, and of the ovoinhibitor. As can be seen, the ovomucoid fractions and the trichloroacetic acid-acetone prepared protein were all similar to each other except for differences in content of sialic acid. In contrast to the ovomucoids, the ovoinhibitor was eluted above pH 5 and had a higher tyrosine content, little or no sialic acid, and most significantly, was found to inhibit both trypsin and chymotrypsin.

**Inhibitor from Duck Egg White**—The ovomucoids isolated from both Peking duck egg white and from Khaki Campbell duck white were studied in detail. The ovoinhibitor present in chicken egg white appeared to be absent or present in very low concentration in duck white.

In Table II are given some physical properties of Peking duck ovomucoid compared to chicken ovomucoid. The mean sedimentation values, diffusion constants, and apparent specific volumes are similar. The calculated molecular weights of 27,000 and 28,000 for chicken and duck ovomucoid, respectively, are close to the values reported for chicken ovomucoid (7). Concentration of the proteins in the above sedimentation and diffusion analyses was 1%. The values given were not corrected to infinite dilution.

Chemical and biological properties of the duck ovomucoids are given in Table III. Comparison of the chemical properties with those of chicken ovomucoid as shown in Table I indicates a similarity in elution pH from CM-cellulose, in nitro-

### Table I

**Properties of chicken ovomucoids and proteinase inhibitor**

<table>
<thead>
<tr>
<th>Property</th>
<th>Ovomucoid prepared by</th>
<th>Elution from CM-cellulose</th>
<th>Ovoinhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trichloroacetic acid-acetone method</td>
<td>pH</td>
<td>pH</td>
</tr>
<tr>
<td>pH of peak elution</td>
<td>3.5</td>
<td>4.1</td>
<td>4.4</td>
</tr>
<tr>
<td>N, %</td>
<td>10.8</td>
<td>10.8</td>
<td>11.8</td>
</tr>
<tr>
<td>Tyrosine, % (spectrophotometric)</td>
<td>3.8</td>
<td>2.8</td>
<td>3.5</td>
</tr>
<tr>
<td>Tryptophan, % (spectrophotometric)</td>
<td>0.5</td>
<td>0.5</td>
<td>0.6</td>
</tr>
<tr>
<td>Total trypsin inhibitor activity</td>
<td>&lt;0.3</td>
<td>&lt;0.3</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>Relative chymotrypsin inhibitor activity</td>
<td>1.0</td>
<td>0.9</td>
<td>1.1</td>
</tr>
<tr>
<td>Relative chymotrypsin inhibitor activity</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

* Fraction passed through CM-cellulose unadsorbed at this pH value.

### Table II

**Physical properties of chicken and duck ovomucoid**

<table>
<thead>
<tr>
<th>Property</th>
<th>Ovomucoid</th>
<th>Ovomucoid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Poultry</td>
<td>Duck</td>
</tr>
<tr>
<td>Sedimentation constant, $s_{20, w}$</td>
<td>2.49 S°</td>
<td>2.50 S°</td>
</tr>
<tr>
<td>Diffusion constant (20°), cm²</td>
<td>$7.7 \times 10^{-7}$</td>
<td>$8.1 \times 10^{-7}$</td>
</tr>
<tr>
<td>Apparent partial specific volume, ml g⁻¹</td>
<td>0.71</td>
<td>0.73</td>
</tr>
<tr>
<td>Molecular weight, g mole⁻¹</td>
<td>$27 \times 10^3$</td>
<td>$28 \times 10^3$</td>
</tr>
</tbody>
</table>

- The chicken ovomucoid was a composite fraction eluted from pH 3.5 to 4.4. The duck (Peking) ovomucoid was unadsorbed on CM-cellulose at pH 4.1.
- Determinations were made on 1% solutions of the respective proteins; values were not corrected to infinite dilution.
- Averages of two determinations.

### Inhibitors from Chicken Whites

When chicken egg white was fractionated on CM-cellulose starting at pH 3.5, four fractions were obtained which possessed trypsin inhibitor activity. These included three ovomucoid fractions and, the fourth, the ovoinhibitor recently reported by Matsushima (9). The principal difference detected among the ovomucoid fractions was their content of sialic acid as seen in Table I. This variation in sialic acid content was reflected in their respective pH values of elution from CM-cellulose. As initially eluted, the fraction unadsorbed at pH 3.5 comprised approximately 1% of the total ovomucoid and each of the other two fractions comprised approximately 50% of the remainder. The exact amount of sialic acid in each fraction varied slightly with each preparation and hence further work would be needed to prove homogeneity of each fraction. Somewhat larger quantities of the high sialic acid ovomucoid fraction (that unadsorbed on CM-cellulose at pH 3.5) were prepared essentially by the procedure recently reported for isolation of the apoprotein of the egg-white flavoprotein (26). This fraction of ovomucoid was reported there as an unidentified acidic protein. This procedure, which employed DEAE-cellulose, had several advantages. The main advantage was that much less exchanger was needed to isolate a gram of this fraction since primarily only the flavoprotein-apoprotein and the high sialic acid ovomucoid are retained on the DEAE-cellulose in the initial step while the other proteins of egg white are unadsorbed. When CM-cellulose was employed for direct isolation, only the high sialic acid ovomucoid was unadsorbed by this exchanger and all the other egg-white proteins had to be retained.
chymotrypsin and trypsin in-gene, and in low tryptophan content. However, the duck ovomucoids contained approximately twice the tyrosine content of chicken ovomucoids and very little or no sialic acid compared to variable amounts in the chicken ovomucoids.

Duck ovomucoid differed extensively from chicken ovomucoid in its inhibitory activity (Fig. 1). Curves A and C represent the curves obtained for chymotrypsin and trypsin in inhibition by chicken ovomucoid. Extrapolation to the abscissa represented the curves obtained for chymotrypsin and trypsin in the presence of increasing amounts of chicken or duck ovomucoid. In this instance, a 1:1 molar complex was indicated. As previously reported (7), 100 μg of trypsin were inhibited by approximately an equal weight of chicken ovomucoid, indicating a 1:1 molar complex.

In this study, the results represented by Curves B and D were obtained for duck ovomucoid. Curve B indicated that 100 μg of chymotrypsin were inhibited by an equal weight of duck ovomucoid, again indicating a 1:1 molar complex. On the other hand, Curve D showed that 100 μg of trypsin were inhibited by 50 μg of duck ovomucoid. In this instance, a 2:1 molar complex of trypsin and duck ovomucoid was indicated. Thus, it appeared, in contrast to chicken ovomucoid which inhibited only trypsin as a 1:1 molar complex, duck ovomucoid would inhibit chymotrypsin as a 1:1 molar complex and would also inhibit trypsin by forming a 2:1 molar complex.

**Inhibitors from the Egg Wholes of Different Avian Species**—The results given in the previous section concerning duck ovomucoid stimulated extension of this comparative biochemistry study to include the ovomucoids of 9 other avian species. Chemical characteristics and biological activities of these ovomucoids are presented in Table III. In general, the conditions for adsorption and elution of these ovomucoids from CM-cellulose or DEAE-cellulose were approximately the same as for chicken or duck ovomucoids. In addition, all of the ovomucoids had similar nitrogen contents and very low tryptophan contents. Some variations were noted in the tyrosine content and in the sialic acid content of the various ovomucoids. In addition, the fractions of ovomucoid from the egg white of certain species also varied in sialic acid content. For example, the three fractions of turkey ovomucoid varied in sialic acid content similarly to chicken ovomucoid, although the content was significantly higher.

Extensive differences were observed in the biological prop-

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**Table III**

<table>
<thead>
<tr>
<th>Species</th>
<th>pH of elution from CM-cellulose</th>
<th>N</th>
<th>Tyrosine</th>
<th>Tryptophan</th>
<th>Relative trypsin inhibit activity</th>
<th>Relative chymotrypsin inhibit activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peking duck</td>
<td>4.1</td>
<td>12.7</td>
<td>5.7</td>
<td>0.8</td>
<td>&lt;0.3</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Khaki Campbell duck</td>
<td>4.1</td>
<td>12.6</td>
<td>5.1</td>
<td>0.5</td>
<td>&lt;0.3</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Turkey</td>
<td>3.5</td>
<td>12.4</td>
<td>2.9</td>
<td>0.6</td>
<td>&lt;0.3</td>
<td>4.8</td>
</tr>
<tr>
<td>Turkey</td>
<td>3.5-4.1</td>
<td>11.1</td>
<td>2.8</td>
<td>0.6</td>
<td>&lt;0.3</td>
<td>2.8</td>
</tr>
<tr>
<td>Turkey</td>
<td>4.1-4.5</td>
<td>11.7</td>
<td>3.4</td>
<td>0.4</td>
<td>&lt;0.3</td>
<td>1.9</td>
</tr>
<tr>
<td>Guinea</td>
<td>4.3</td>
<td>12.6</td>
<td>2.9</td>
<td>0.7</td>
<td>&lt;0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Goose</td>
<td>DEAE</td>
<td></td>
<td>4.7</td>
<td>0.7</td>
<td>&lt;0.3</td>
<td>1.7</td>
</tr>
<tr>
<td>Goose</td>
<td>4.1</td>
<td>11.6</td>
<td>4.8</td>
<td>0.6</td>
<td>&lt;0.3</td>
<td>1.7</td>
</tr>
<tr>
<td>Emu</td>
<td>DEAE</td>
<td></td>
<td>12.9</td>
<td>6.3</td>
<td>&lt;0.3</td>
<td>10.6</td>
</tr>
<tr>
<td>Emu</td>
<td>4.1</td>
<td>10.8</td>
<td>4.4</td>
<td>0.6</td>
<td>0.1</td>
<td>3.6</td>
</tr>
<tr>
<td>Golden pheasant</td>
<td>4.3</td>
<td>13.2</td>
<td>4.0</td>
<td>0.6</td>
<td>&lt;0.3</td>
<td>0.6</td>
</tr>
<tr>
<td>Cassowary</td>
<td>4.1</td>
<td></td>
<td>4.2</td>
<td>0.5</td>
<td>&lt;0.3</td>
<td>5.4</td>
</tr>
<tr>
<td>Red jungle fowl</td>
<td>4.3</td>
<td></td>
<td>3.5</td>
<td>0.7</td>
<td>&lt;0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>California valley quail</td>
<td>4.3</td>
<td></td>
<td>4.9</td>
<td>0.7</td>
<td>&lt;0.3</td>
<td>1.4</td>
</tr>
<tr>
<td>Painted quail</td>
<td>4.3</td>
<td></td>
<td>4.0</td>
<td>0.6</td>
<td>&lt;0.3</td>
<td>0.4</td>
</tr>
</tbody>
</table>

- a Chicken ovomucoid prepared by the method of Lineweaver and Murray (6), corrected to 13.3% N, was used as the standard. Figures expressed on a dry weight basis.
- b Duck ovomucoid (Peking) was used as the standard. Figures expressed on a dry weight basis.
- c Fraction passed through CM-cellulose unadsorbed at this pH value.

**Fig. 1.** Activity of 100 μg of trypsin or chymotrypsin in the presence of increasing amounts of chicken or duck ovomucoid. Conditions for assay are given under methods.
The complex consists of two trypsins, one chymotrypsin, and one duck ovomucoid. Extension of the investigation to the chymotrypsin, respectively. Results of one such experiment were presented in Fig. 2. Curves A and B represent percentage activity of 100 μg of chymotrypsin in the presence of increasing amounts of duck ovomucoid. Curve A was obtained in the presence of 400 μg of trypsin. Similarly, Curves C and D represent percentage activity of 100 μg of trypsin. Curve D was obtained in the presence of 400 μg of chymotrypsin. These results indicate: (a) The sites of trypsin inhibition are distinct from the site of chymotrypsin inhibition, (b) both enzymes may be inhibited simultaneously, and (c) the molecular complex consists of two trypsins, one chymotrypsin, and one duck ovomucoid. Extension of the investigation to the other ovomucoids indicated that those of turkey, guinea, emu, cassowary, red jungle fowl, painted quail, and chicken. Next, the only ovomucoid thus far found which inhibits primarily chymotrypsin was that from golden pheasant. Third, the ovomucoids which inhibit approximately equal molar amounts of trypsin and chymotrypsin were those from turkey and guinea, and the fourth class, the ovomucoids which inhibit twice as much trypsin as chymotrypsin, were those from duck, emu, and California valley quail. Sedimentation constants obtained for the turkey, guinea, and golden pheasant ovomucoids indicate that the turkey and guinea ovomucoids form equal molar complexes with trypsin or chymotrypsin and that the golden pheasant ovomucoid forms an equal molar complex with chymotrypsin. "Multiheded" Inhibitors—As a result of finding that some of the ovomucoids would inhibit either trypsin or chymotrypsin, it was necessary to determine, if possible, whether the same inhibitor sites were involved or whether the site of trypsin inhibition was distinct from the site of chymotrypsin inhibition. One approach available was to determine the inhibition of one enzyme in the presence of high concentrations of the other enzyme. Such an approach was possible through the use of the specific synthetic substrates, TAMe and BTE for trypsin and chymotrypsin, respectively. Results of one such experiment are presented in Fig. 2. Curves A and B represent percentage activity of 100 μg of chymotrypsin in the presence of increasing amounts of duck ovomucoid. Curve A was obtained in the presence of 400 μg of trypsin. Similarly, Curves C and D represent percentage activity of 100 μg of trypsin. Curve D was obtained in the presence of 400 μg of chymotrypsin. These results indicate: (a) The sites of trypsin inhibition are distinct from the site of chymotrypsin inhibition, (b) both enzymes may be inhibited simultaneously, and (c) the molecular complex consists of two trypsins, one chymotrypsin, and one duck ovomucoid. Extension of the investigation to the other ovomucoids indicated that those of turkey, guinea, emu, and California valley quail each have several distinct inhibitory sites. Inhibition of both trypsin and chymotrypsin occurs simultaneously, and excess of one enzyme does not effect inhibition of the other enzyme.

Further evidence for the simultaneous inhibition of trypsin and chymotrypsin by duck ovomucoid was obtained by the use of a mixed substrate of TAMe and BTE. The activity of the enzyme-inhibitor complex of two trypsins, one chymotrypsin, and one duck ovomucoid molecules was much lower than an equivalent amount of either enzyme on the mixed substrate.

Physical Studies of Enzyme-Inhibitor Mixtures—Further evidence for the enzyme-ovomucoid complexes was obtained through the use of paper electrophoresis. For these analyses, 0.1 M/2 potassium phosphate buffer at pH 6.9 was employed and the analyses were run at 8 milliamperes for 16 hours. In all cases, 0.02 ml of the enzymes, ovomucoids, or enzyme-ovomucoid mixtures prepared in the buffer was placed on the paper strips. The concentration of ovomucoid was 1% in all cases. The enzyme-ovomucoid mixtures given below contained additional amounts of enzymes on a weight basis. A single spot for the chicken ovomucoid-trypsin (1:1) mixture was obtained. This was easily distinguishable from the spot for trypsin or chicken ovomucoid alone and moved to an intermediate position. Analyses of the chicken ovomucoid-chymotrypsin (1:1) mixture did not show a new spot but showed only the presence of two spots corresponding to those for chymotrypsin and chicken ovomucoid. Both the duck ovomucoid-trypsin (1:2) mixture and the duck ovomucoid-chymotrypsin (1:1) mixture gave single spots which were distinguishable from one another and the individual components of the mixtures. In the case of the duck ovomucoid-trypsin (1:2) mixture, however, a trace of a second component was observed which might correspond to the 1:1 duck ovomucoid-trypsin complex. The duck ovomucoid-trypsin-chymotrypsin (1:2:1) mixture gave a single spot which was indistinguishable from the duck ovomucoid-trypsin (1:2) mixture or to chymotrypsin alone. Finally, the duck ovomucoid-trypsin-chymotrypsin (1:1:1) mixture gave two spots. These corresponded to the duck ovomucoid-chymotrypsin (1:1) mixture and the duck ovomucoid-trypsin-chymotrypsin (1:2:1) mixture.

The golden pheasant ovomucoid-chymotrypsin (1:1) mixture gave one spot distinguishable from the spots of the chymotrypsin or golden pheasant ovomucoid alone as expected from the biological data. In contrast, the golden pheasant ovomucoid-trypsin (1:1) mixture gave two spots corresponding to trypsin and golden pheasant ovomucoid, respectively. These observations are the reverse of those obtained with chicken ovomucoid as would be expected from the enzymatic studies.

Further evidence for the enzyme-inhibitor complexes was furnished by ultracentrifugal analyses. Sedimentation patterns for duck, chicken, and golden pheasant ovomucoids and for several enzyme-ovomucoid mixtures are presented in Fig. 3. In all instances, the final protein concentration was 1%. The buffer employed for Pattern A and B was 0.1 M sodium acetate-acetic acid in 0.1 M KCl at pH 4.5. For the remaining analyses described, the buffer was 0.1 M Tris-HCl in 0.1 M NaCl at pH 8.5 except for Pattern G which was obtained in 0.01 M Tris-HCl in 0.01 M NaCl at pH 8.5. The speed of the rotor was 52,640 r.p.m. Only a single ultracentrifugal analysis was made in most cases. The sedimentation constants for the golden pheasant (Pattern F, 1.8 S) and for the turkey and guinea...
ovomucoids, 2.0 S and 2.1 S, respectively (not shown in Fig.
3), are somewhat lower than for duck or chicken ovomucoids
(Patterns A and B, respectively). This does not necessarily
indicate lower molecular weights in the former cases since cal-
culations of molecular weights depend also on other physical
measurements. Only single components were noted in each
case, but more detailed studies would be necessary to prove
homogeneity by this criterion.

Examination of the patterns obtained for the enzyme-ovo-
mucoid mixtures indicates single peaks in all cases where com-
plexes were expected from the biological and electrophoretic
studies described above. This is apparent from Pattern C and
G, duck ovomucoid, trypsin, and chymotrypsin in a 1:2:1 mix-
ture; Pattern D, duck ovomucoid and trypsin in a 1:2 mix-
ture; and Pattern E, chicken ovomucoid and trypsin in a 1:1
mixture. In addition, the calculated sedimentation constants
increase in magnitude in the order which would be expected.
The enzymes involved here have reported sedimentation con-
stants which are similar to the ovomucoids (27). Another anal-
ysis not shown in Fig. 3 gave a calculated sedimentation con-
stant for a duck ovomucoid-chymotrypsin mixture of 3.5 S and
a single peak. This is in contrast to the results with the mix-
ture of chicken ovomucoid and chymotrypsin where no com-
plex was expected (Pattern H). No increase in sedimentation
constant was obtained over chicken ovomucoid alone, and an
unsymmetrical peak was obtained.

Inhibitor Content of Various Avian Egg Whites—In Table IV
are given the percentage inhibitory activity against trypsin and

![Fig. 3. Ultracentrifugal patterns for certain ovomucoids and
enzyme-ovomucoid mixtures. See text for details. Patterns are
as follows: A, duck ovomucoid; B, chicken ovomucoid; C and G,
duck ovomucoid-trypsin chymotrypsin (1:2:1); D, duck ovomuc-
coid-trypsin (1:2); E, chicken ovomucoid-trypsin (1:1); F, golden
peahen ovomucoid; and H, chicken ovomucoid-chymotrypsin
(1:1). Minutes indicated are the lengths of time run at 52,640
r.p.m. and figures on the patterns are the calculated sedimentation
constants.](http://www.jbc.org/)

per centage inhibitory activity against chymotrypsin of various
avian egg whites employing chicken ovomucoid and duck ovo-
mucoid as the respective standards. The percentage activities
obtained in this study do not agree with those obtained in earlier
studies on some of these whites (12, 14). These disagreements
might be explained in that different substrates were used in
these studies. Table IV also gives the ratios of inhibitory ac-
tivities against trypsin and chymotrypsin for the different whites.
In general, these ratios agree with the ratios obtained for the
respective isolated ovomucoids (see Table III). The ovomo-
coid contents of the whites (calculated on the basis of the
biological activity of the isolated ovomucoids) indicated chicken
white to contain the least amount and cassowary white to
contain the highest amount. The amount of ovoinhibitor of
only the chicken and turkey were considered in these calcula-
tions since the amounts present in the other whites have not
been established.

Additional Observations—A bacterial proteolytic enzyme mar-
keted as Nagarse was found to be inhibited by duck ovomucoid
as well as by the ovoinhibitor which had been previously re-
ported (9). This enzyme has very slow activity on TAME, com-
pared to equal amounts of chymotrypsin and apparently no
activity on TAME. A study of the effect of Nagarse proteinase
on the inhibition of trypsin or chymotrypsin by duck ovo-
mucoid gave the following results: (a) Inhibition of trypsin in
the presence of 4 times the amount of Nagarse (on a weight
basis) was unaffected, (b) inhibition of chymotrypsin in the
presence of 4 times as much Nagarse (on a weight basis) was
reduced approximately 40%, and (c) equal amounts of Nagarse
(on a weight basis) with chymotrypsin caused only an approxi-
mate 20% reduction in inhibition of chymotrypsin. Evidence
supporting these data was obtained by paper electrophoresis
with the methods described above for the other enzyme-inhi-
bitor complexes.

<table>
<thead>
<tr>
<th>Species</th>
<th>Relative trypsin inhibitor activitya</th>
<th>Relative chymotrypsin inhibitor activityb</th>
<th>Approximate ratio of trypsin inhibitor to chymotrypsin inhibitor activity</th>
<th>Approximate ovomucoid contentsc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chicken</td>
<td>16</td>
<td>22</td>
<td>3.3</td>
<td>18</td>
</tr>
<tr>
<td>Peking duck</td>
<td>22</td>
<td>20</td>
<td>1.1</td>
<td>11</td>
</tr>
<tr>
<td>Khaki Campbell duck</td>
<td>19</td>
<td>18</td>
<td>1.0</td>
<td>22</td>
</tr>
<tr>
<td>Turkey</td>
<td>20</td>
<td>15</td>
<td>1.3</td>
<td>22</td>
</tr>
<tr>
<td>Guinea</td>
<td>21</td>
<td>15</td>
<td>1.4</td>
<td>22</td>
</tr>
<tr>
<td>Goose</td>
<td>24</td>
<td>12</td>
<td>2.0</td>
<td>22</td>
</tr>
<tr>
<td>Golden pheasant white</td>
<td>47</td>
<td>22</td>
<td>2.1</td>
<td>22</td>
</tr>
<tr>
<td>Cassowary white</td>
<td>30</td>
<td>3</td>
<td>10</td>
<td>22</td>
</tr>
<tr>
<td>Rhea</td>
<td>12</td>
<td>10</td>
<td>1.0</td>
<td>11</td>
</tr>
</tbody>
</table>

a Chicken ovomucoid prepared by the method of Lineweaver and Murray (6), corrected to 13.3% N, was used as the standard. Figures calculated on a dry weight basis.

b Duck ovomucoid (Peking was used as the standard). Figures calculated on a dry weight basis.

c Estimated from assays on whites and relative activities of preparations of ovomucoids.
Casein was also used as the substrate for trypsin or chymotrypsin instead of TAME and BTE, respectively. In general, the same amounts of inhibitor were required to inhibit the enzymes as found with the synthetic substrates. Exceptions were found with guinea ovomucoid and chicken ovoinhibitor. Approximately 2 to 3 times as much of these inhibitors was required to inhibit chymotrypsin when casein was employed as a substrate as when BTE was employed. The significance of these observations has not been further investigated.

**DISCUSSION**

A variety of inhibitors of proteolytic enzymes has been studied including the chicken ovomucoid (5), chicken ovoinhibitor (9), pancreatic inhibitor (24), soybean trypsin inhibitor (24), the ascarsis inhibitor (28), and the proteolytic enzyme inhibitors of animal sera (29). Most of these, however, have been found to be either inhibitors of trypsin or to have varying activities against trypsin and chymotrypsin and activity against bacterial proteolytic enzymes.

A “multiheaded” inhibitor with simultaneous inhibitory activity against more than one proteolytic enzyme was implied by Wu and Laskowski (24) for soybean trypsin inhibitor. Even in this instance, inhibition of molar quantities of both tryptic and chymotrypsin B was not obtained. Thus, perhaps the duck ovomucoid is the first inhibitor of this type actually established. On the other hand, Green (28) has reported the isolation of an impure mixture of proteins as possibly containing the first example of a specific inhibitor of chymotrypsin. There is a question as to whether this preparation had two inhibitors present, as was suggested, or whether the inhibitor was of the multiheaded variety. Thus, the ovomucoid of golden pheasant egg white is at least one of the few protein inhibitors known to inhibit primarily chymotrypsin.

The existence of the multiheaded inhibitors appears well supported by biological, electrophoretic, and ultracentrifugul studies. However, future work perhaps should consider other criteria for the existence of these inhibitors. Diagrammatic presentations of the types of enzyme-inhibitor complexes demonstrated in this study are given in Sketch 1. Although this presentation is only diagrammatic, the equal size of the circles is indicative of the similarity in the molecular weights of the enzymes and inhibitors involved here. In addition, the lack of overlapping of the circles indicates that the site of inhibition of one enzyme is distinct from the site of inhibition of the other enzyme and that simultaneous inhibition of both enzymes may occur.

Other results indicated that both chymotrypsin and Nagarse proteinase are inhibited by duck ovomucoid but that these enzymes appeared to occupy the same site or closely adjacent sites on the inhibitor. This might be interpreted to mean that the combination site (active site?) of Nagarse proteinase is more similar to that of chymotrypsin than to that of trypsin. The comparative results with casein and BTE as substrates for chymotrypsin would indicate that the type of substrate, in certain cases, may effect the amount of inhibition caused by any particular inhibitor.

Variable amounts of sialic acid in the several fractions of chicken ovomucoid as well as in the ovomucoid fractions of other species have apparently not been reported previously. The results of a detailed investigation of the distribution of sialic acid in these fractions will appear elsewhere.

**SUMMARY**

The ovomucoids from eleven different avian species have been isolated and studied in detail. Particular attention has been focused on their respective biological properties. The ovomucoids were all somewhat similar as to the conditions for elution from carboxymethyl cellulose, low nitrogen content, and the low content or absence of tryptophan in these proteins. Variable amounts of sialic acid in the ovomucoid fractions prepared from the egg white of a given species have been found. The types of biological activity of the ovomucoids may be divided into four classes on the basis of their inhibitory activity: First, ovomucoids which inhibit primarily trypsin; second, ovomucoids which inhibit primarily chymotrypsin; third, ovomucoids which inhibit equal molar amounts of trypsin and chymotrypsin, separately or simultaneously; and fourth, ovomucoids which inhibit twice as much trypsin as chymotrypsin, separately or simultaneously. The latter two classes of inhibitors were considered to be “multiheaded” in their biological action.

The calculated ovomucoid contents of eight avian egg whites of different species are given.

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