Quantitative Immunological Comparison of Bird Lysozymes*

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SUMMARY

Rabbit antisera were prepared against crystalline chicken lysozyme and characterized by immunodiffusion, immunoelectrophoresis, inhibition of enzyme activity, and quantitative micro-complement fixation.

Lysozyme was purified by Sephadex G-75 chromatography from the egg whites of 17 species of birds in the order Galliformes and was tested for reactivity with anti-chicken lysozyme. The chicken and its closest relative, the jungle fowl, have lysozymes that appear to be indistinguishable from each other. The lysozymes of all the other species tested (e.g., partridges, quails, and pheasants) could be distinguished from the chicken enzyme by quantitative micro-complement fixation. Immunodiffusion failed to detect most of these differences in antigenic structure.

A particularly close relationship was observed between the lysozymes of the chicken, partridges, and American quails. This suggests that these lysozymes may show very small sequence differences. This finding may also have taxonomic significance.

The comparison of the structure of homologous proteins from different species can contribute towards an understanding of the relationship between protein structure and function as well as the nature of evolutionary processes and taxonomic relationships. Data on the amino acid sequence of cytochrome c (1), hemoglobin (2, 3), and insulin (4) have been analyzed from these points of view. By comparison, little information is available concerning the structural variations of lysozyme (muramidase, EC 3.2.1.17) among species (5–12). The only known lysozyme sequences are those of the T4 phage and the chicken (7, 13, 14). Since the three-dimensional structure of chicken lysozyme is known (15), information on structural variation among species is especially valuable. In the present article the lysozymes of 16 species of birds are compared with that of the chicken. Immunological methods were employed, with particular emphasis on the quantitative micro-complement fixation method (16). This method has been shown to be sensitive to small differences in amino acid sequence (17) and capable of providing an approximate measure of the degree of structural relatedness between homologous proteins (18, 19).

EXPERIMENTAL PROCEDURE

Materials

Eggs—Table I indicates the species and source of supply of all the egg whites utilized in this investigation. Most eggs were refrigerated within 24 hours after laying. The egg whites were separated from the yolks and stored frozen at −8 °C.

Proteins—Commercially prepared, crystalline chicken egg white lysozyme was obtained from Robins Chemical Corporation (Division of Armour, control No. B18309), Worthington (crystallized twice, LY644A), and Pentex (crystallized three times, EZ1662). Ovalbumin (crystallized five times, No. 5614) and conalbumin (No. 7168) were purchased from Nutritional Biochemicals. Crystalline ovalbumin, further purified by chromatography on carboxymethyl cellulose, was supplied by D. Wachter of this laboratory.

Antisera—Antibodies to chicken lysozyme were obtained by injecting commercially prepared lysozyme into male New Zealand white rabbits. Initially, 2 mg of any one of the three commercial lysozyme preparations were injected into each rabbit intradermally on the back. The lysozyme was suspended in Freund's complete adjuvant (Difco) together with an additional 4 mg per ml of lyophilized, phenol-killed BCG (supplied by the Department of Bacteriology and Immunology). One month after the initial immunization, each rabbit was given an intravenous injection of lysozyme (2 mg per ml in saline). Sera were collected 11 days later. Two months later each rabbit was given an intravenous injection (2 mg of lysozyme per ml of saline) every other day for a total of three injections. Seven days after the final injection, sera were collected. Each antiserum (A) is identified by 2 digits; for example, A24 indicates the antiserum obtained from rabbit 2 after four intravenous injections. Rabbit 2 and 3 were immunized with Worthington lysozyme, 4 and 5 with Pentex lysozyme, and 6 and 7 with the Armour preparation.

Methods

Lysozyme assay—Lysozyme was assayed by the lytic activity against Micrococcus lysodeikticus (20). The assay mixture con-
Under our conditions, Armour crystallized lysozyme has a specific activity of 100 units per mg. We have defined 1 unit of lysozyme activity as that amount which causes a change of 1% transmittance per min at 540 mμ. Under our conditions, Armour crystallized lysozyme has a specific activity of 600 units per mg.

For the determination of specific activities of purified lysozymes, the temperature was maintained at 25 ± 0.1° C. A semi-quantitative assay for lysozyme activity in the eluate from chromatographic columns was carried out by adding aliquots from each fraction collected to test tubes containing the standard assay mixture. The change in the percentage of transmittance was recorded after 1 hour of incubation at 23° with a Zeiss spectrophotometer. Routine assays were made at room temperature (23 ± 1°). For the determination of specific activities of purified lysozymes, the temperature was maintained at 25 ± 0.1° C.

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Protein was determined by measuring the absorbance at 280 mμ with a Zeiss spectrophotometer. The extinction coefficient (E1%280) for chicken lysozyme at pH 5.4 is 26.3 (21).

Immunological Methods—Immunodiffusion tests were conducted at room temperature by means of Ouchterlony’s double diffusion technique. The gel medium was a modification of that described by Fujio et al. (8). A 1% solution of Ionagar No. 2 (Oxo Ltd., London) was prepared in a 0.02 m sodium phosphate buffer (pH 7.1) containing 0.01% merthiolate and 0.14 m NaCl. Antigen and antiserum wells, each 3 mm in diameter, were placed 3 mm apart. In most cases, 10 μl of solution containing 250 μg of lysozyme per ml were added to each antigen well. Each antiserum well received 10 μl of undiluted antiserum.

Immunoelectrophoresis was performed in agar gels on glass slides (1 × 3 inch) (22). The gels contained 1% Ionagar No. 2, NaCl (0.14 m), sodium phosphate (0.0064 m), and citric acid (0.0008 m), the final pH being 6.9. Electrophoresis was carried out at room temperature with a voltage gradient of 3 volts per cm for 6 hours. Antiserum was then added. Twenty-four hours later the slides were examined for the presence of precipitin arcs, before and after staining with Amido black.

Micro-complement fixation experiments were performed according to the technique of Wasserman and Levine (16). Seven-milliliter reaction volumes were used. Hemolysin and guinea pig complement were obtained from Baltimore Biological Laboratory or Hyland Laboratories, Los Angeles. Sheep red cells were supplied by the Bennett Ranch, Woodland, California. All reagents were diluted in a buffer containing 0.14 m NaCl, 0.01 m Tris-hydrochloride, 5 × 10⁻⁴ m MgSO₄, and 1.5 × 10⁻⁴ m CaCl₂ at a final pH of 7.45. The reaction time at 5° was standardized at 18 hours.

Ultracentrifugation—Sedimentation velocity analyses were made with a Spinco model E analytical ultracentrifuge. Lysozyme solutions of 0.8% were centrifuged at 59,780 rpm in a buffer containing 0.02 m sodium acetate, pH 5.3, and 0.15 m KCl at 21°.

Sephadex Chromatography—Lysozyme was purified from the egg whites of 17 species by gel filtration. Columns of Sephadex G-75 (Lot T07118) 40 or 50 cm in length and 1.5 cm in diameter were equilibrated with 0.14 m NaCl. In most cases, 2 ml of a 1:10 dilution of egg white were applied to the column. Flow rates in any one experiment were constant and were between 0.95 and 1.5 ml per min. All fractionations were carried out at 23°.

Amberlite Chromatography—Chromatography of crystalline lysozyme was carried out according to the method of Tallan and Stein (23). Bio-Rex 70 (200 to 400 mesh) resin was employed. A 0.2 m sodium phosphate buffer, pH 7.18, was used as an eluant. Chromatography was carried out at 23° on a column, 18 × 2.5 cm, with a flow rate of 1.5 ml per min.

Starch Gel Electrophoresis—Horizontal starch gel electrophoresis was carried out in a phosphate-citrate buffer, pH 7.0 (34), for 18 hours with a voltage gradient of 6 volts per cm at 4°. The position of the protein was determined by staining with Amido black.

RESULTS

Purity of Immunizing Antigens—Sedimentation velocity ultracentrifugation of all three commercial lysozyme preparations gave single symmetrical peaks having an average s₂₀,₆ of 1.82, which is in agreement with the reported behavior of chicken lysozyme under the experimental conditions that were employed (21).

The three commercial preparations of chicken lysozyme were subjected to starch gel electrophoresis. All showed minor, anodally migrating, protein impurities in addition to the cathodally migrating chicken lysozyme. A comparison with the electrophoretic separation of whole chicken egg white revealed that the Armour lysozyme preparation was contaminated with egg white albumins, globulins, and conalbumin. When similar amounts of the Pentex and Worthington preparations (approximately 1 mg) were applied to the gel, the globulin fraction was the only contaminant observed.

Amberlite chromatography of 90 mg of Armour lysozyme revealed one major protein peak. Two minor peaks, one eluting at the effluent volume characteristic for ovalbumin and another eluting just before the major protein peak were also observed. Together, the minor peaks accounted for 4% of the total absorbance at 280 mμ (Fig. 1).
Electrophoretic and chromatographic behavior therefore indicate the presence of minor impurities in the commercial preparations of chicken egg white lysozyme that had been utilized as immunizing antigens.

**Homologous Immunological Reaction**—Six antisera were tested for the presence of antibodies directed against chicken lysozyme. Immunodiffusion revealed that every antiserum gave rise to a single precipitin line when tested against Armour crystalline lysozyme that had been further purified by Amberlite chromatography.

One of the antisera, A54, was tested and found to inhibit the enzymatic activity of chicken lysozyme. The antiserum did not inhibit human salivary lysozyme. It is known that antiserum directed against human lysozyme does not inhibit the enzymatic activity of chicken lysozyme (6).

Each antiserum was tested against chicken egg white by immunoelectrophoresis. In every case, a strong precipitin arc was observed at a cathodal position, which was determined by parallel experiments with crystalline enzyme to be characteristic for lysozyme. Antisera A24, A31, and A54 also produced a second precipitin arc with egg white. Since an arc of identical mobility was produced upon immunoelectrophoresis of crystalline ovalbumin, these antisera must contain antibodies to ovalbumin as well as to lysozyme. The remaining three antisera (prepared against Armour lysozyme) were shown by analogous methods to contain antibodies to both ovalbumin and conalbumin as well as to lysozyme.

Micro-complement fixation tests were conducted with antisera A24 and A54. In both cases it was demonstrated that the degree of complement fixation is independent of the purity of lysozyme. As shown in Fig. 2, the height and shape of the complement fixation curves and the lysozyme concentration required for peak fixation are identical whether egg white or chromatographically purified crystalline lysozyme is used. Hence, the antibodies to ovalbumin in antisera A24 and A54 do not interfere with our complement fixation analysis of lysozyme. This conclusion was confirmed by directly measuring the concentration of antibodies against ovalbumin in these antisera. Micro-complement fixation experiments conducted with chromatographically purified crystalline lysozyme demonstrated that the concentration of antibodies against ovalbumin was more than 10 times lower than that against lysozyme in both A24 and A54. The sharp dependence of complement fixation on antibody concentration (see Reference 19 and below) insured that our micro-complement fixation analysis of lysozyme is free from any interference by antibodies against other egg white proteins.

![Fig. 2. Reactivity of antiserum A24 with chicken egg white (○) and chromatographically purified crystalline chicken lysozyme (□), as determined by the micro-complement fixation technique. The reaction mixtures contained 1 ml of a 1:11,000 dilution of antiserum.](image)

![Fig. 3. Sephadex G-75 chromatography of chicken egg white. Two milliliters of a 1:9 dilution of egg white were applied to a column, 52 × 1.5 cm. Absorbance at 280 nm (○), lysozyme activity (△). See "Methods" for further details.](image)
**Immunological Cross-reactions** In order to measure the reactivity of these antisera with the lysozymes of other species, lysozyme was first partially purified from the egg whites of the species listed in Table I. Since avian lysozymes are small molecules (mol wt ~14,500), compared with other egg white proteins (8-10, 25), chromatography on Sephadex G-75 was employed for this purpose. Fig. 3 shows the result of an experiment in which chicken egg white was subjected to this procedure. One major protein peak was eluted beginning at the void volume of the column. This peak was devoid of lysozyme activity. At an effluent volume of 60 ml, a second peak was observed which contained 94% of the initial lysozyme activity. The specific activity of the lysozyme in the peak tube was 615. Chromatographically purified, crystalline lysozyme has a specific activity of 660 (see "Methods" for definition of specific activity). When lysozyme purified from chicken egg white by Sephadex G-75 chromatography was tested against antisera A24 and A54 by immunoelectrophoresis, a single precipitin arc was observed at a position characteristic for lysozyme. This shows that Sephadex chromatography effectively separated ovalbumin from lysozyme.

![Fig. 4. Sephadex G-75 chromatography of egg white from the Japanese quail. Two milliliters of a 1:9 dilution of egg white were applied to a column, 53 X 1.5 cm. Absorbance at 280 nm (○), lysozyme activity (△). See "Methods" for further details.](image1)

![Fig. 5. Reactivity of antiserum A54 with lysozymes purified by Sephadex chromatography from the egg whites of chicken (○), turkey (□), and ring-necked pheasant (△). The reaction mixtures contained 1 ml of a 1:8800 dilution of antiserum.](image2)

When other egg whites were subjected to Sephadex G-75 chromatography, similar elution profiles resulted. As an example, Fig. 4 shows the results of an experiment with egg white from the Japanese quail.

The degree to which lysozyme from each species was purified by the Sephadex procedure was estimated by comparing the specific activity of the peak lysozyme tube with that of chromatographically purified crystalline chicken lysozyme (i.e. 660). Almost all the preparations had specific activities greater than 500 and were thus more than 75% pure according to this criterion.1

Our first cross-reaction experiments were done with turkey and ring-necked pheasant lysozymes. Fig. 5 shows the results of an experiment with antiserum A54 used at a concentration of 1:8800. At this antiserum concentration the homologous reaction with chicken lysozyme gave the greatest degree of complement fixation, measured at the peak of the curve. The reaction with turkey lysozyme was weaker, while essentially no reaction was detected with ring-necked pheasant lysozyme.

In another experiment with turkey lysozyme, the antiserum concentration was raised by a factor of 1.16. At this concentration of antiserum, turkey lysozyme fixed as much complement as the chicken enzyme had at the original antiserum concentration. In a reaction with ring-necked pheasant lysozyme, the peak height could be made to equal that of chicken lysozyme if the antiserum concentration was raised by a factor of 1.70.

Experiments with both chicken and ring-necked pheasant lysozyme have shown that the height of the complement fixation curve is dependent on antiserum concentration.

1 The lysozyme preparations that had lower specific activities may have resulted from either one of two phenomena. It is possible, first of all, that some species have an unusual abundance of low molecular weight material which exhibits absorbance at 280 nm and is eluted with lysozyme. On the other hand, the turnover number of lysozyme in some of the species may differ from that of the chicken. Although crystallized turkey lysozyme has been shown to have the same specific activity as crystallized chicken lysozyme (25), no other close relatives of the chicken have been examined with respect to the turnover number of lysozyme.
TABLE II
Cross-reactions of lysozymes from various species with anti-chicken lysozyme

<table>
<thead>
<tr>
<th>Species</th>
<th>Antiserum A24</th>
<th>Antiserum A54</th>
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</thead>
<tbody>
<tr>
<td>Fowl</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Domestic chicken</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Burmese red jungle fowl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Partridges</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sharp's francolin</td>
<td>1.02</td>
<td>1.07</td>
</tr>
<tr>
<td>Chukar</td>
<td>1.13</td>
<td>1.10</td>
</tr>
<tr>
<td>American quails</td>
<td>1.04</td>
<td>1.01</td>
</tr>
<tr>
<td>Bobwhite quail</td>
<td>1.08</td>
<td>1.00</td>
</tr>
<tr>
<td>California quail</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other gallinaceous birds</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Domestic guinea fowl</td>
<td>1.33</td>
<td>1.45</td>
</tr>
<tr>
<td>Ruffed grouse</td>
<td>1.35</td>
<td>1.32</td>
</tr>
<tr>
<td>Blue-eared pheasant</td>
<td>1.37</td>
<td>1.60</td>
</tr>
<tr>
<td>Lady Amherst’s pheasant</td>
<td>1.44</td>
<td>1.60</td>
</tr>
<tr>
<td>Blue peafowl</td>
<td>1.52</td>
<td>1.31</td>
</tr>
<tr>
<td>Golden pheasant</td>
<td>1.63</td>
<td>1.48</td>
</tr>
<tr>
<td>Domestic turkey</td>
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</tr>
<tr>
<td>Swinhoe pheasant</td>
<td>1.81</td>
<td>1.87</td>
</tr>
<tr>
<td>Japanese quail</td>
<td>1.86</td>
<td>1.81</td>
</tr>
<tr>
<td>Reeves’s pheasant</td>
<td>1.88</td>
<td>1.70</td>
</tr>
<tr>
<td>Ring-necked pheasant</td>
<td>2.20</td>
<td>1.70</td>
</tr>
</tbody>
</table>

* Average for at least two experiments.

* Experiments with a third antiserum confirm that these lysozymes are immunologically distinct from chicken lysozyme, the index of dissimilarity for all four species being between 1.1 and 1.2.

* Experiments with three other antisera gave the following values for turkey lysozyme: 1.33, 1.37, and 1.63.

The peak is linearly related to the log of the antiserum concentration (Fig. 6). The slopes of these two lines are identical, allowing us to use as a measure of the cross-reactivity of any lysozyme the factor by which the antiserum concentration must be raised in order that a peak height equal to that given by chicken lysozyme is obtained. This factor is called the index of dissimilarity (18, 19). This procedure for measuring cross-reactions has been used before in investigations of species differences in other proteins (18, 19).

Table II presents the results of cross-reaction experiments with lysozymes from 16 species. Two antisera were used, A24 and A54. In all cases the peak of the complement fixation curve occurred in the reaction tube containing 0.03 enzyme units of lysozyme (about 0.05 μg). With the exception of the red jungle fowl, all the species have a lysozyme that can be distinguished from that of the chicken by at least one of the antisera. These species fall into two categories. The first includes the partridges and American quails. These species have a lysozyme that is barely distinguishable from that of the chicken. Experiments with another antiserum confirm this conclusion. The remaining species have a lysozyme that is more easily distinguishable from that of the chicken by micro-complement fixation.

Immunodiffusion proved to be a far less discriminating technique. The lysozymes from 15 of the 16 species gave lines of identity with chicken lysozyme in experiments with antisera A24 and A54. Ring-necked pheasant lysozyme, however, was exceptional in that a weak spur was formed with chicken lysozyme when tested with antiserum A24 (Fig. 7a). With antiserum A54, ring-necked pheasant and chicken lysozyme gave lines of identity (Fig. 7b).

**DISCUSSION**

All of the species tested, with the exception of the chicken's closest relative, the red jungle fowl, have an egg white lysozyme which differs in structure from that of the chicken as judged by the micro-complement fixation technique. As judged by immunodiffusion, however, all the species except the ring-necked pheasant have a lysozyme which is identical with that of the chicken. The relative insensitivity of immunodiffusion to small differences in protein structure has been shown before in studies on hemoglobin (17), serum albumin (19), and growth hormone (26).

Wetter, Cohn, and Deutsch (11) reported that differences between chicken lysozyme and turkey, guinea fowl, and pheasant lysozymes can be detected by quantitative precipitation with an

![Figure 7](http://www.jbc.org/)

**FIG. 7.** Photographs of immunodiffusion patterns obtained by reacting anti-chicken lysozyme sera (A) with Sephadex preparations of chicken (C) and ring-necked pheasant (P) lysozyme. The antisera used were a, A24 and b, A54. See "Methods" for further details.

**TABLE III**

Conventional zoological classification of species whose lysozymes were investigated

<table>
<thead>
<tr>
<th>A. Family Phasianidae</th>
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<tbody>
<tr>
<td>Pheasants</td>
</tr>
<tr>
<td>Domestic chicken</td>
</tr>
<tr>
<td>Burmese red jungle fowl</td>
</tr>
<tr>
<td>Ring-necked pheasant</td>
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<tr>
<td>Reeves's pheasant</td>
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<tr>
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<td>Sharp's francolin</td>
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<td>Chukar</td>
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<table>
<thead>
<tr>
<th>B. Other Families</th>
</tr>
</thead>
<tbody>
<tr>
<td>Domestic guinea fowl</td>
</tr>
<tr>
<td>Ruffed grouse</td>
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<tr>
<td>Domestic turkey</td>
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</tbody>
</table>
tisera to chicken lysozyme. Other workers, however, state that chicken lysozyme and turkey lysozyme “can be regarded as having almost identical behavior in the quantitative precipitin test and in gel diffusion experiments” even though these lysozymes differ in amino acid composition (10). All of our antisera clearly distinguish turkey from chicken lysozyme in the micro-complement fixation test.

The fact that the immunological reactivity of partridge and American quail lysozymes is nearly equal to that of the chicken suggests that these enzymes differ only slightly from each other in chemical structure. Hemoglobins S and C, which differ from hemoglobin A by a single amino acid residue, are distinguishable from A1 by means of micro-complement fixation tests conducted with antisera prepared against hemoglobin A1 (17). The index of dissimilarity was observed to be 1.3 in each case (18). As a smaller index of dissimilarity (1.1) was observed in the comparison of partridge and quail lysozymes with chicken lysozyme, it is possible that these enzymes differ by not more than 1 amino acid residue from chicken lysozyme.

It is of interest that over 100 additional species of partridges and American quails exist (27). Such a large number of lysozymes, possibly differing slightly from each other in amino acid sequence, would be of value in relating protein structure to catalytic and immunological activity.

Our cross-reaction data may also have taxonomic implications. All the species studied here are considered by zoologists to be closely related to the chicken. Many of them have been reported to be capable of hybridizing with the chicken (28). According to classical zoological criteria, they belong to four related families in the order Galliformes (27, 29–35). Further details regarding the zoological classification of these species are given in Table III. It will be observed, for example, that the chicken and jungle fowl are grouped among the pheasants. Our data on lysozyme suggest instead a close relationship among the chicken, jungle fowl, partridges, and American quails.3

Clearly, one of these alternatives is incorrect. Either the chicken is more closely related to pheasants, as suggested by conventional zoological criteria, or it is more closely related to partridges, as our lysozyme experiments indicate. Unpublished experiments which we have performed with the lactic dehydrogenase of these species agree with the relationships suggested by the lysozyme data. Further studies on ovalbumin and serum albumin are in progress. Information concerning several proteins will aid in the evaluation of the taxonomic significance of data on protein structure and may determine whether the conventional taxonomic scheme is in need of revision.

Micro-complement fixation may receive increasing use by biochemists interested in protein structure. The technique is exceedingly economical of materials. Cross-reactivity experiments are conveniently carried out with millimicrogram quantities of protein antigens and microliter quantities of antiserum. Information concerning several proteins as well as proteins from different species.

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REFERENCES

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