The Dependence of Immunological Cross-Reactivity upon Sequence Resemblance among Lysozymes

I. MICRO-COMPLEMENT FIXATION STUDIES*

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SUMMARY

Rabbit antiserum were prepared against seven different lysozymes purified from the egg whites of chicken, bobwhite quail, turkey, Japanese quail, ring-necked pheasant, and duck. Cross-reactivity tests by means of quantitative micro-complement fixation were conducted among all possible pairs of these lysozymes as well as with three other lysozymes. The amino acid sequences are known for five of the 10 proteins we examined. A correlation was observed between degree of immunological cross-reactivity and degree of amino acid sequence similarity, with the limitation that proteins differing from each other by 40% or more in sequence exhibited no cross-reactivity in micro-complement fixation tests. This relationship between sequence resemblance and immunological resemblance is compatible not only with the fact that amino acid replacements have generally occurred on the outer surface of the lysozyme molecule during bird evolution but also with current ideas about the size and number of antigenic determinants on proteins and with the idea that the conformation of the polypeptide backbone is strongly conserved during evolution.

Cross-reactivity measurements are affected by several parameters that were investigated, including the length of the immunization program, variability among rabbits, and the degree to which the results of reciprocal tests agree. Antiserum specificity broadened with time and reached a plateau after several months of immunization. Considerable variability among rabbits necessitated pooling several sera in inverse proportion to their degree of reaction with the homologous antigen in order to obtain a more representative measurement. The results of reciprocal tests advise measuring cross-reactivity in both directions to better evaluate the degree of antigenic difference between two lysozymes.

Experimental Procedure

Eggs

Freshly laid eggs were obtained from the following sources: bobwhite quail (Colinus virginianus), Le Jeune’s Quail Farm, Sulphur, Louisiana; turkey (Meleagris gallopavo), Armour Starr, Turlock, California; Japanese quail (Coturnix coturnix japonica), Drs. H. Abplanalp and U. Abbott, University of California, Davis, California; ring-necked pheasant (Phasianus colchicus), State Game Farm, Vacaville, California; Peking duck (Anas platyrhynchos), Reichardt’s Duck Farm, Petaluma, California; and chachalaca (Ortalis vetula), Frank Wed, Realitos, Texas. The egg whites were separated from the yolks and stored frozen at -10°C.

Lysozymes

Three times crystallized chicken lysozyme was obtained from Pentex (EZ1962); chachalaca lysozyme had been prepared in our laboratory by Dr. N. Arnheim according to the purification procedure described (4). Duck II and Duck III lysozymes (14) were a gift from Dr. P. Jollès, while human lysozyme purified from the urine of patients having monocytic and monomyelocytic leukemia (15) was kindly supplied by Dr. E. F. Osserman.

Geneticists, taxonomists, evolutionary biologists, and those who study protein heterogeneity need a quick and easy method for determining the degree of sequence resemblance between homologous proteins. It has been proposed (1-4) that quantitative immunological techniques of protein comparison may fulfill this need. This proposal is based mainly on the fact that proteins which differ by only one or a few amino acid substitutions usually differ only slightly in immunological properties, while large sequence differences invariably result in large immunological differences (reviewed in References 4 and 5). To provide a more rigorous evaluation of the postulated correlation between cross-reactivity and sequence resemblance, it is important to conduct a quantitative immunological study of a homologous series of proteins of known amino acid sequence. The most thorough test of this sort has been that recently carried out with a homologous series of weak immunogens, the cytochromes c (6). Now that a number of amino acid sequences are available for the strongly immunogenic lysozymes of warm blooded animals (chicken (7, 8), Japanese quail (9), Duck II (10), turkey (11), and human (12)), we report the results of a test of the sequence-immunology correlation with these proteins. A preliminary account of this work has appeared (13).

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† Predoctoral Fellow of the National Science Foundation.
Other Chemicals

Sephadex G-50 fine (No. 5550), Sephadex G-25 fine (No. 2155), and carboxymethyl Sephadex (C-25, No. 7762) were obtained from Pharmacia. Carboxymethylcellulose (Cellex-CM, Nos. 3908, 6738, and 6964) and Bio-Rex 70 (200 to 400 mesh, Nos. 3889 and 7593) were purchased from Bio-Rad, Richmond, California. Starch for gel electrophoresis was obtained from Connaught Laboratories, Ionagar No. 2 for immunoelectrophoresis from Oxo Ltd., London, and Amido black for protein staining of starch gels and immunoelectrophoresis slides from K and K Laboratories, Brooklyn, New York.

Antisera

Antisera to each of seven purified lysozymes were produced as follows. Lysozyme (2 mg) was emulsified with Freund’s complete adjuvant supplemented by an additional 3 mg per ml of lyophilized, phenol-killed Calmette-Guérin bacillus (supplied by the Department of Bacteriology and Immunology, University of California, Berkeley, California) and injected intradermally into three areas on the back of each male New Zealand white rabbit. Four rabbits, identifiable by number and immunogen as follows, were immunized with each lysozyme: 416 to 419, chicken; 420 to 423, bobwhite quail; 424 to 427, turkey; 428 to 431, Japanese quail; 432 to 435, Duck A; 436 to 439, Duck B; and 440 to 443, ring-necked pheasant. (Duck A and B lysozymes are described in Reference 13.) Intravenous boosts were administered at intervals over the next 7 months (10 months for rabbits immunized with chicken lysozyme). Each boost consisted of 1 mg of lysozyme in isotonic buffer (the buffer used in micro-complement fixation and described previously (3)) given every other day for a total of three injections. Postboost bleedings were taken one week after the last of three intravenous injections; preboost bleedings were taken at various other times during the immunization schedule, which is summarized in Fig. 1. Antisera were stored at −10°C; those used for micro-complement fixation were heat-treated for 20 min at 60°C to inactivate the rabbit complement present and then stored at −10°C. Antiserum pools were made by combining the four individual sera directed toward a given antigen from a particular bleeding in inverse proportions to their micro-complement fixation titers against the immunogen (2, 17).

Antisera A24, A54, A64, and A74 directed toward chicken lysozyme have been described (4), as has the antiserum to whole botwhite quail egg white (16).

Lysozyme Assays

Lysozyme activity was assayed by recording the change in percentage of transmittance at 540 nm in a Zeiss spectrophotometer at 23°C during the course of lysis of Micrococcus luteus (formerly named Micrococcus lysozymicus) (18).

Protein

Protein was measured by the absorbance at 280 nm. Lyophilized protein was weighed.

Immunological Methods

Immunoelectrophoresis, Ouchterlony double diffusion, and micro-complement fixation were carried out as described (3). A total of 88 antisera was used in the micro-complement fixation experiments reported here. The degree of antigenic difference between pairs of lysozymes is expressed as the index of dissimilarity or as the immunological distance. The index of dissimilarity is the experimentally determined factor by which the antiserum concentration must be raised in order for a heterologous lysozyme (a lysozyme other than the one used for evoking the antibodies being used) to produce a complement fixation curve the peak of which is equal in height to that produced by the homologous lysozyme (the lysozyme used for immunization) (3). (We note here the strictly immunological definition of homologous. Elsewhere in this work it is used in the genetic sense to refer to proteins which are similar enough in sequence so that one can say that the genes coding for their sequences stemmed from the same ancestral gene.) The immunological distance is defined as 100 times the log of the index of dissimilarity (19), and it is this latter derived quantity which appears to be proportional to the actual amount of change which has occurred since two proteins diverged from a common ancestor (19).

Ultracentrifugation

Sedimentation velocity studies were carried out with a Spinco model E analytical ultracentrifuge. Lysozyme solutions of 0.5 to 0.8% protein were centrifuged at 67,770 rpm in a buffer (20) of 0.02 M sodium acetate, pH 5.3, and 0.15 M KCl at 23°C.

Amino Acid Composition

Lysozyme samples were hydrolyzed under vacuum in 6 N HCl at 105-110°C for 24, 42, or 72 hours. The hydrolysates were evaporated to dryness, dissolved in 0.2 M citrate buffer at pH 2.2, and analyzed with either a Beckman 121 or 120B automanalyzer as previously described (4, 21). Values for serine and threonine were extrapolated to zero time of hydrolysis (22), while those for valine and isoleucine were taken from the 72-hour hydrolysate (23). To calculate the number of residues of each amino acid, it was assumed that each lysozyme has 129 amino acids, as has been done previously (4, 16). All those bird lysozymes sequenced, i.e. chicken (7, 8), Japanese quail (9), Duck II (10), and turkey (11), have this length. Additionally, glycine was allowed to equal previously published values for each species as a baseline for the other amino acids, i.e. glycine equals 12.00 for chicken (7, 8), 12.00 for botwhite quail (4), 13.00 for turkey (11), 11.00 for Japanese quail (9), 12.00 for Duck A, B, C (16), and 14.00 for ring-necked pheasant (4). Values close to integers were then obtained for the other amino acids. Tryptophan and half-cystine and the amides asparagine and glutamine were not determined. Furthermore, proline was not determined because it was not possible to obtain a reliable value on a lysozyme sample in which the half-cystines were neither carboxymethylated nor otherwise modified. However, many of these values are available from sequence data and other work, and reference is made to them where appropriate.
Horizontal starch gel electrophoresis at pH 5.3, 7.0, and 11.9 was performed at 4° as has been described (4), but starch was used at a concentration of 11%. Electrophoresis at pH 5.3 under modified conditions (higher voltage, cold air cooling of the gel) was also carried out. After electrophoresis, protein was located by staining with Amido black as described (16).

**Purification of Lysozyme**

The purification procedures utilized were based upon published methods (4, 14, 24). The general procedure is outlined below, with any modifications for individual lysozymes subsequently indicated.

**General Procedure**—Pooled egg white (500 ml to 1000 ml, depending on the level of lysozyme activity present) was diluted 1:5 in ammonium acetate buffer (pH 9.0, 0.1 M acetate), homogenized for 1 to 2 min in a Waring Blender, and filtered through tissue paper (KimWipes). About 30 to 45 g (depending on the total volume and total lysozyme present) of carboxymethyl cellulose equilibrated in the same buffer were added, and the slurry was stirred at 4° overnight. The remainder of the purification was carried out at room temperature. The resin, with the lysozyme bound to it, was allowed to settle, and the supernatant, containing the vast majority of the egg white proteins, was decanted. The carboxymethylcellulose was poured onto a Buchner funnel lined with eight layers of KimWipes and thoroughly washed with the ammonium acetate buffer. The lysozyme was then eluted with 0.4 M ammonium carbonate (pH 9.2) and lyophilized. It was next subjected to gel chromatography on a Sephadex G-50 column, 4.5 x 53 cm, equilibrated with the ammonium acetate buffer. The material in the largest peak (in some cases the only peak), which contained all of the lysozyme activity, was lyophilized. The residue, dissolved in 0.05 M ammonium carbonate, was then put onto a C-25 carboxymethyl Sephadex column, 2.4 x 14.5 cm, equilibrated with the same buffer. A linear gradient produced by utilizing 250 ml each of 0.05 M and 0.4 M ammonium carbonate eluted the lysozyme (with the exception of Duck C), which was then lyophilized. The residue was dissolved in 1% acetic acid and desalted by passage over a Sephadex G-25 column, 4.5 x 30 cm, equilibrated with the 1% acetic acid. The lysozyme was then lyophilized. Several hundred milligrams of each lysozyme were generally obtained. Significant losses of lysozyme during purification were accepted to obtain the maximum possible purity so vital for any immunization program.

**Gel filtration and sedimentation velocity behavior of bird lysozymes**

<table>
<thead>
<tr>
<th>Lysozyme</th>
<th>$V_v/V_s$</th>
<th>$s_{20,w}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chicken</td>
<td>1.58</td>
<td>1.92</td>
</tr>
<tr>
<td>Bobwhite quail</td>
<td>1.65</td>
<td>1.91</td>
</tr>
<tr>
<td>Turkey</td>
<td>1.70</td>
<td>1.82</td>
</tr>
<tr>
<td>Japanese quail</td>
<td>1.54</td>
<td>1.85</td>
</tr>
<tr>
<td>Duck</td>
<td>1.56</td>
<td>1.93</td>
</tr>
<tr>
<td>Duck C</td>
<td>1.56</td>
<td>1.89</td>
</tr>
<tr>
<td>Ring-necked pheasant</td>
<td>1.58</td>
<td>1.88</td>
</tr>
</tbody>
</table>

$V_v$ is the elution volume and $V_s$ is the void volume on a column of Sephadex G-50.

**Chicken**—Three times crystallized Pentex lysozyme was further purified (for immunization purposes) by the procedure above, with elimination of the carboxymethylcellulose batch step.

**Bobwhite Quail**—After the final lyophilization described above, part of the residue was further purified on a Bio-Rex 70 column, 2.5 x 44 cm, equilibrated with 0.2 M sodium phosphate at pH 7.18. A single lysozyme peak was eluted by 1.60 column volumes of this buffer; a switch to 0.8 M sodium phosphate, pH 7.10, did not elute any more lysozyme. The material in the peak tubes was then placed in cellulose casing, dialyzed extensively against distilled water at 4°, and lyophilized. The immunological work was conducted with that portion of the bobwhite quail lysozyme which was carried through this extra step.

**Turkey and Japanese Quail**—No modifications of the general procedure were made.

**Duck A, B, C**—The purification procedures utilized and the low yields obtained have been described in detail (16).

**Ring-necked Pheasant**—After lyophilization following elution from the carboxymethyl Sephadex column, the residue was applied to a Bio-Rex 70 column like that used for the bobwhite quail enzyme and eluted in the same manner. The multiple peaks previously reported on Bio-Rex 70 for pheasant lysozyme (4) were not detected; a single peak emerged after 1.75 column volumes of 0.2 M sodium phosphate had passed over the resin. The lysozyme was then dialyzed and lyophilized as described above.

**Results**

**General Properties of Lysozymes**

**Criteria of Purity**—Each of those lysozymes (i.e. all except chicken, turkey, Japanese quail, and Duck C prepared from two individual eggs (16)) tested on an Amberlite (Bio-Rex 70) column emerged as a single symmetrical peak with constant specific activity across the peak. Each lysozyme moved as a single protein zone upon starch gel electrophoresis at pH 5.3, 7.0, and 11.9. In sedimentation velocity studies each lysozyme showed a single, symmetrical peak. Immunoelectrophoresis with an antiserum against whole bobwhite quail egg white produced only one precipitin arc with each lysozyme. All rabbits immunized with these seven lysozymes produced antisera which, when tested against whole, homologous egg white, displayed only one precipitin arc. This situation held even after 6 months of immunization, at which time antibodies to minor contaminants would be most likely to appear.

**Specific Activity**—In their lysis of M. luteus under the conditions used, these bird lysozymes exhibited very similar specific activities, within ±25%, as has been noted (4, 9, 16, 25, 26).

**Molecular Size**—During the purification of lysozyme from pooled egg white, a single active peak was obtained on Sephadex G-50 for each lysozyme at the same point at which Pentex chicken lysozyme emerged. Sedimentation velocity studies gave $s_{20,w}$ values close to the 1.92 obtained for chicken lysozyme under these conditions. Table 1 summarizes these two measures of molecular size and shape. These results are consistent with previously reported sedimentation velocity (4, 16) and Sephadex G-50 (16) data for some of the enzymes listed above.

**Amino Acid Composition**—The amino acid compositions of the lysozymes we purified agreed with published values. Table II summarizes the most complete amino acid compositions avail-
**Table II**

Amino acid compositions

| Amino acid | Lysozymes |  |  |  |  |  |  |  |
|------------|-----------|---|---|---|---|---|---|
|            | Chicken   | Bobwhite quail | Turkey | Japanese quail | Duck A | Duck B | Duck C | Ring-necked pheasant | Duck II | Human |
| Lysine     | 6         | 7            | 7      | 7             | 6     | 6     | 6     | 8                | 6       | 5     |
| Histidine  | 1         | 1            | 2      | 2             | 0     | 0     | 0     | 2                | 0       | 1     |
| Arginine   | 11        | 10           | 10     | 10            | 13    | 15    | 15    | 9                | 13      | 14    |
| Aspartic acid | 21     | 21           | 20     | 20            | 19    | 19    | 19    | 20               | 19      | 18    |
| Threonine  | 7         | 7            | 7      | 7             | 7     | 7     | 7     | 7                | 7       | 5     |
| Serine     | 10        | 10           | 10     | 10            | 11    | 10-11 | 9     | 10               | 11      | 11    |
| Glutamic acid | 5      | 3            | 5      | 5             | 5     | 5     | 5     | 5                | 5       | 9     |
| Proline    | 2         | 2            | 2      | 2             | N.D.  | N.D.  | N.D.  | 2                | 2       | 2     |
| Glycine    | 12        | 13           | 13     | 12            | 12    | 12    | 12    | 12               | 12      | 11    |
| Alanine    | 12        | 12           | 12     | 12            | 11    | 12    | 11    | 11               | 11      | 14    |
| Half-cystine| 8         | 8            | 8      | 8             | N.D.  | N.D.  | N.D.  | 8                | 8       | 8     |
| Valine     | 6         | 7            | 5      | 7             | 7     | 7     | 7     | 7                | 7       | 8     |
| Methionine | 2         | 2            | 2      | 2             | 2     | 2     | 2     | 3                | 2       | 2     |
| Isoleucine | 6         | 5            | 6      | 6             | 6     | 6     | 6     | 6                | 6       | 5     |
| Leucine    | 8         | 8            | 9      | 8             | 8     | 8     | 8     | 8                | 8       | 8     |
| Tyrosine   | 3         | 3            | 4      | 4             | 5     | 5     | 5     | 4                | 5       | 6     |
| Phenylalanine | 3     | 3            | 2      | 2             | 1     | 1     | 1     | 2                | 1       | 2     |
| Tryptophan | 6         | 6            | 6      | N.D.          | N.D.  | N.D.  | N.D.  | 6               | 6       | 5     |

* From the sequence (7, 8).
* Reference 4.
* From the sequence (11).
* From the sequence (9).
* Reference 16.
* From the sequence (10).
* From the sequence (22).
* Although the proteins do contain asparagine and glutamine, they are included in the totals for aspartic acid and glutamic acid, since amino acid compositions are experimentally determined on hydrolysates.
* N.D., not determined.
* Computed indirectly in Reference 4.
* Consistent with over-all composition and extinction coefficient reported in Reference 4.

**Table III**

Comparison of number of compositional and number of sequence differences among lysozymes

The number of sequence differences between any two lysozymes is given in the upper right-hand part of the matrix, while the number of differences in amino acid composition between any two lysozymes is given in the lower left-hand part.

<table>
<thead>
<tr>
<th>Species compared</th>
<th>Chicken</th>
<th>Japanese quail</th>
<th>Turkey</th>
<th>Duck II</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chicken</td>
<td>6</td>
<td>7</td>
<td>22</td>
<td>53</td>
<td></td>
</tr>
<tr>
<td>Japanese quail</td>
<td></td>
<td>8</td>
<td>10</td>
<td>25</td>
<td>55</td>
</tr>
<tr>
<td>Turkey</td>
<td></td>
<td></td>
<td>12</td>
<td>8</td>
<td>22</td>
</tr>
<tr>
<td>Duck II</td>
<td></td>
<td></td>
<td>12</td>
<td>12</td>
<td>54</td>
</tr>
<tr>
<td>Human</td>
<td></td>
<td></td>
<td>28</td>
<td>26</td>
<td>32</td>
</tr>
</tbody>
</table>

Some authors (27, 28) have proposed that the degree of compositional difference can be used to estimate the degree of sequence resemblance among proteins. Table III compares the number of compositional and sequence differences for those lysozymes of known sequence. Duck II lysozyme (10) has the same composition as Duck A and, as shown below, by electrophoretic and, more importantly, immunological criteria, seems very similar to Duck A. Duck A is therefore likely to have about the same number of sequence differences from the other species listed as does Duck II. Fig. 2 presents these data in graphical form. Clearly, the number of compositional differences can be a rough guide to the number of sequence differences over a rather broad range. However, this method is rather insensitive, even for sequence differences of around 20% or less.1 For example, 12 compositional differences are the reflection of anywhere from 7 to 25 sequence differences. It is in this lower

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1 Amino acid composition data can of course set a lower limit on the number of sequence differences which must exist, since these must be equal in number to at least half the number of compositional differences.
amino acid sequence differences for lysozymes of known sequence.

Turkey ...................... 114 83
Chachalaca. ................. 117 -77
Japanese quail ............... 111 71
Duck C .................... 113 176
Ring-necked pheasant. ...... 119 -37
Duck B ................... 104 106
Duck I1 .................... 96 51
Bobwhite quail .............. 100 65
Duck A ..................... 96 51
Duck II ..................... 96 51

Japanese quail.. . 111 122 71 -4
Turkey. . . 114 122 83 -4

plus 9 at pH 5.3 from its amino acid sequence.

predicted by adding up the individual charges.

amino acid sequence, as opposed to the relative charge, is tabulated since the direction of migration is actually opposite to that

DuckIII............. 96 89 51 -5
Chicken. . . . . 100 100 100 -3
Human.............. 103 111 97 -4

immunological approach is most useful.

range of sequence differences, as we shall see below, where an immunological approach is most useful.

Electrophoretic Mobility—Table IV summarizes the electrophoretic mobilities at pH 5.3 and pH 11.9 for all the lysozymes included in this work relative to chicken lysozyme. Lysozymes having very different origins and sequences, as, e.g. chicken and human, may nevertheless have almost identical mobilities. Fur-

thermore, charge is a conservative property for this enzyme; at
pH 11.9, when nearly all other proteins move toward the anode, ten of these lysozymes still move toward the cathode and the other two move only slightly toward the anode; at pH 5.3, the difference in mobility between the fastest and slowest of these diverse lysozymes is only 23%.

Table V compares the relative mobilities and charges at pH 5.3 and pH 11.9 for those five lysozymes of known sequence. The charges are computed from the compositions as determined from the sequences. At pH 5.3 we assumed that 10 of 11 carboxyls in the chicken are ionized (29) and that, similarly, all carboxyls in the other lysozymes except glutamic acid 35 at the active site are ionized. We assumed, furthermore, that all amino-terminal, histidyl, lysyl, and arginyl residues would be charged at pH 5.3. We see that the order of difference, if not the exact magnitude, is the same whether one compares relative mobilities or relative charges at this pH.

At pH 11.9 we assumed that all arginyl and tyrosyl residues and all carboxyl groups are charged and that all other potentially chargeable groups are uncharged. Calculated, rather than relative, charges are tabulated for this pH because migration is not a simple function directly related to the sum of the charges; migration is actually toward the cathode (albeit a very short distance), rather than toward the anode as predicted from the amino acid compositions. However, electrical endosmosis at high pH in a low ionic strength buffer would be expected to cause significant migration in a cathodal direction (30). Thus, at this pH also, the order of difference is the same whether one compares charges or relative mobilities.

Considering that the individual pK values for all the chargeable groups on the molecules are not accurately known and that the microenvironment of each residue (e.g. whether it be internal or external in the molecule) can influence its ionization, the agreement obtained both at pH 5.3 and pH 11.9 is quite good.

**Immunological Studies of Lysozymes**

**Antiserum Production**—Each of the seven lysozymes tested was strongly immunogenic in rabbits. Every rabbit injected responded and the response, as measured either by micro-complement fixation or immunodiffusion tests, was evident even before administration of the first booster injection. By contrast, most cytochromes c tested (6) do not elicit production of any precipitating or complement-fixing antibodies and, in the case of those few cytochromes c that do, only some of the rabbits injected respond, and these animals produce only low titer antiserums even after repeated boosting (6).

Fig. 3 shows the variation in concentration of complement-fixing antibodies during the course of immunization of rabbits with chicken lysozyme. The titer of an antiserum is defined as that factor by which the antiserum is diluted in order to give 75% complement fixation with the homologous antigen at the peak of the micro-complement-fixation curve. The titers rose slowly after the primary injection and quickly after an intravenous boost and then gradually declined as expected. Continued boosting subsequent to the second intravenous boost did not increase the titers above the maximum values reached immediately after the second boost. Rabbit variability, both at any given time and from one boost to the next, is evident.

The titers of pools formed from the antiserum elicited by each of our seven antigens after 6 months of immunization (sixth bleeding) used in our cross-reactivity survey varied over a 2-
fold range, i.e. from about 7,000 to about 13,000. Within a set of four rabbits immunized with the same lysozyme, titers generally varied 4-fold. We infer that these lysozymes did not differ significantly from one another in their ability to stimulate antibody production.

Variation in Specificity with Time—Fig. 4 shows the variation in index of dissimilarity between chicken and six heterologous lysozymes for the pools of antichicken sera. The indices gradually declined to a constant value during the course of immunization. The index for the bobwhite quail lysozyme, the most closely related enzyme to that of the chicken by this and previous tests (4), reached a plateau by the second bleeding (7 weeks), and those for the turkey and ring-necked pheasant leveled off by the third bleeding (12 weeks). Reaction with the weakly cross-reacting chachalaca lysozyme reached a plateau by the fourth bleeding (15 weeks), while the indices for Duck A and Duck B did not do so until the sixth bleeding (26 weeks).

Fig. 5 shows a similar phenomenon for the pooled anti-Duck A sera. The index of dissimilarity for the closely related Duck B was constant throughout the immunization program, but the index for the chicken did not plateau until 6 months had elapsed.

A similar broadening of antiserum specificity with time, until a plateau is reached, has been noted for alkaline phosphatase (31) and ovalbumin, while for serum albumin the specificity is constant essentially throughout an immunization program. A possible explanation for the broadening of specificity is that more avid antibodies are produced with time. Thus, those determinants in the heterologous antigen which do not resemble homologous determinants sufficiently to react with early sera could react with later, more avid sera.

In view of these results, it is wise to verify that antiserum specificity has reached a plateau for the antigen one is studying before undertaking an extensive survey of cross-reactivity. Our survey with antibody pools to all seven immunogens was done with sera from the sixth bleeding (over 6 months of immunization) because these were the earliest, postboost sera for which the index of dissimilarity was constant. It is undesirable to prolong an immunization program longer than necessary and desirable to work with high titer sera in order to measure cross-reactions readily and to work at a point in time after which the specificity is no longer changing significantly.

Individual Rabbit Variability and the Case for Pooling Sera—Fig. 6 shows the variation in the index of dissimilarity between chicken and Duck A lysozymes with time for two individual antichicken rabbits. While No. 418 reached a plateau after only 7 weeks, No. 417 did not do so until 6 months had elapsed. The magnitude and pattern of change in specificity also varied widely between these two rabbits.

At the time of the sixth bleeding the four rabbits immunized with chicken lysozyme gave indices of dissimilarity for Duck A ranging from 3 to 5. A more striking case of rabbit variability involves antisera A24, A54, A64, and A74 (3) to chicken lysozyme which were pooled to give an index of 6.67 for Duck A (16); the individual sera gave indices of 4.6 (A54), 5.9 (A64), 7.1 (A24), and 13.3 (A74). (Since these sera were the result of a shorter,
less intense immunization program than used in this present work, it is not surprising that the indices are higher, indicating greater antisem specificity.) Furthermore, one cannot assume that an antisem will give relatively high or relatively low indices with all heterologous antigens on the basis of its index with one heterologous antigen. A74, which gives 13.3 with Duck A, gives 1.4 with Japanese quail, while A54, which gives 4.6 with Duck A, gives 1.8 with Japanese quail.

Pools were made by combining two of these four sera at a time in inverse proportions to their micro-complement fixation titer. When the indices of Duck A were measured, those given by the pools were intermediate between those of the individual components. Furthermore, when two individual components were combined in varying proportions, the indices of dissimilarity varied in a parallel manner, i.e. the greater the proportion of the pool composed of the serum giving the higher index, the higher the index of the pool. This last observation provides a justification for the practice of combining sera in inverse proportions to their micro-complement fixation titer. Using inverse proportions implies that one obtains equal numbers of complement-fixing antibodies from each rabbit (17). If one simply used equal volumes without regard for titer, one could create a pool having, e.g. a 70% contribution in number of antibodies from one rabbit (should be produced an exceptionally high titer serum) and a 10% contribution from each of three other rabbits. Measurements made with such a pool would yield an index strongly biased in favor of that given by the one serum alone and would in fact hardly be superior to using only one serum. We wish to emphasize that the use of equal volumes of sera without regard to their immunological properties (whether these be, for instance, precipitin, agglutination, or micro-complement fixation titer) is a completely arbitrary practice, since the serum protein concentration can vary considerably due to the animal's health and recent fluid consumption, the amount of anticoagulant used (if any), and the amount of hemolysis which occurs.

We note that, although extensive pooling of sera on the basis of titer has been done in the albumin system (2, 17, 19, 32-34), the relatively small amount of rabbit variability did not allow for a good test of this practice. The great variation obtained among the antilysozyme sera emphasized the need to form pools in this manner. It should be noted that Dietrich (35), with the use of inhibition techniques, did observe considerable variation in the specificity of sera produced in response to immunization with mouse serum albumin.
Reciprocity—In an ideal system, the difference between two proteins, X and Y, should be the same regardless of the direction in which the comparison is made, i.e., it should be the same as determined by antibodies to X (considering Y the heterologous antigen) and antibodies to Y (considering X the heterologous antigen) (2). Table VI shows the immunological distances between the homologous and each of nine heterologous lysozymes obtained with the seven serum pools used in this work. The table is arranged so that the reciprocal comparison, where available (i.e., for all lysozymes other than Duck C, chachalacas, and human, to which antisera were not produced), is in the adjacent column. The average values in those cases where both measurements could be made are also tabulated.

The results of reciprocal tests agree only approximately in the lysozyme system. Fig. 7 presents in graphical form the results of the 21 pairs of reciprocal tests. The smaller value of each pair is arbitrarily plotted on the horizontal axis. A line through the data points deviates from the line of perfect reciprocity; the former has a slope of about 1.28, while the latter, by definition, has a slope of 1.00. A further evaluation of the deviation from reciprocity can be made by calculating $100 \times \frac{(\text{anti-X versus Y}) - (\text{anti-Y versus X})}{(\text{anti-X versus Y}) + (\text{anti-Y versus X})}$, with all values in immunological distance units. The average for our 21 lysozyme pairs is 33%. By comparison, in the mammalian albumin system, in which reciprocity is excellent, such a calculation gives a value of 6.5% (Reference 34). To obtain an accurate estimate of the degree of antigenic difference between two lysozymes, it is therefore clearly desirable to produce antiserum to each of them and measure cross-reaction in both directions.

Correlation of Number of Sequence Differences with Immunological Cross-Reactivity—Table VII compares those animal lysozymes of known sequence by immunological and sequence techniques. The immunological distance values given for any two bird lysozymes are the averages of anti-X versus Y and anti-Y versus X. Here the sequence of Duck II (10) is used with the immunological data for Duck A, since these two are undoubtedly very similar; Duck II has an index of dissimilarity of 1.00 with anti-Duck A and an index of 1.36 with anti-Duck B. The index for Duck A with anti-Duck B by comparison is 1.24. Fig. 8 displays these data in graphical form. A reasonable correlation is observed. The values for human versus bird lysozymes presumably fall in a region where this correlation no longer holds, since there is no evidence of a micro-complement fixation reaction between the anti-bird sera and human lysozyme.

### DISCUSSION

#### The Sequence-Immunology Correlation

We have presented new evidence for the proposal (4) that quantitative immunological methods can be used to obtain an approximate measure of the number of sequence differences between related lysozymes. The other two methods we have examined and discussed briefly, amino acid composition and electrophoretic mobility, are less useful in determining degree of structural relatedness among lysozymes. Utilization of the immunological approach requires, as we have demonstrated, that certain precautions be taken; an immunization program of several months should be used, sera from several rabbits should be pooled in inverse proportion to their titers, and reciprocal measurements should be made.

A further variable in cross-reactivity measurements which we have not yet considered is the species in which the antiserum are produced. That is, would antisera produced in an animal other than the rabbit yield a different picture of the degree of
sequence resemblance among the lysozymes examined here? Studies using chicken and rabbit sera to human serum albumin and other human proteins (39) as well as investigations involving rabbit, guinea pig, rat, and human antibodies to human growth hormone (37) have shown that cross-reactivity measurements are fairly insensitive to the species in which the antisera are produced unless that species is taxonomically very closely related to the species from which the immunogen was obtained. The rabbit is not a very close relative of any of the species whose lysozymes we investigated. The correlation between degree of immunological cross-reactivity and degree of sequence homology of lysozymes, shown in Fig. 8, can be described by the equation $y = 5x$, where $y$ is expressed in immunological distance units and $x$ is the percentage of sequence difference. The value 5 in this equation is an approximation; we realize that it is subject to refinement as more points are obtained in the lysozyme system and may well be different for other systems.

Human lysozyme did not react with any of these sera against bird lysozyme even when the serum concentration was raised by a factor of 100 over that needed to give 75% complement fixation in the presence of the homologous antigen. Others have similarly observed that human and chicken lysozymes do not cross-react by complement fixation or precipitin tests (see, for example, Reference 38 for a review). Human lysozyme has 53 to 55 amino acid differences compared to the four bird lysozymes whose sequences are known and therefore differs by more than 40% from them. Thus, as pointed out, the difference falls in a region where the correlation just described no longer holds. Other workers (39-41) have noted that bovine $\alpha$-lactalbumin, which has about a 63% sequence difference from those bird lysozymes of known sequence (42), also fails to show immunological cross-reactivity. Goose lysozyme, which appears to have a radically different sequence (12, 24), also fails to cross-react with chicken lysozyme (43). Knowledge of the sequence of chachalaca lysozyme, which is immunologically quite distant from the seven lysozymes to which we produced antisera, would be useful for determining more precisely the percentage sequence difference above which there is no longer any apparent immunological cross-reactivity in the lysozyme system.

A similar sequence-immunology correlation has been shown for the poor antigen cytochrome $c$ (6), as depicted in Fig. 9. Cross-reactivity in that system seems to disappear at around 20 to 40% sequence difference (cytochrome $c$ has 104 residues). It should be pointed out that in those studies antisera to only three cytochromes $c$ were used, so that only a very limited test of reciprocity was possible. Furthermore, only one or two sera were prepared against each cytochrome, and the antisera variability evidenced by even these limited measurements suggests that pooling of several sera would have been advisable. Also, studies concerning changing of specificity with time were not carried out. It would be of interest to re-examine the cytochrome $c$ cross-reactions with these experimental modifications.

Although the lack of a perfect correlation between immunological distance and the degree of sequence difference could be due mainly to such factors as the variability of cross-reactivity measurements, the existence of amino acid substitutions not in antigenic determinants, or the effects of some substitutions on the conformation of the polypeptide backbone, the possibility of errors in the determination of amino acid sequences should also be considered. Indeed, when two groups of investigators have independently determined the sequence of the same protein, they have often obtained slightly different answers (see, for example, the studies on chicken lysozyme (7, 8), tobacco mosaic virus coat protein (45, 46), and bovine ribonuclease (47-51)). As most of the lysozyme and cytochrome $c$ sequences published have never been checked by a second group of investigators and some of the reported sequences are still provisional, small errors may exist in some of the sequence data we used. Such errors may contribute to the scatter of points seen in Figs. 8 and 9.

Rationale for the Correlation

It is pertinent to ask whether a rationale exists for the observed sequence-immunology correlation. Antibody-antigen interactions involve principally the surface of the antigen, and protein evolution appears as a general rule (52) to be primarily a surface phenomenon. Although antibody-antigen reactions are known to be very sensitive to the conformation of the antigen, x-ray studies on myoglobin, hemoglobins, and cytochromes $c$ indicate that the conformation of the polypeptide backbone is strongly conserved during evolution (52, 53). Specifically, then, we are interested in what percentage of the amino acids replacements occurring in lysozyme are likely to participate in the antigen-antibody reaction. That is, are most positions in which changes are occurring fully or partly exposed positions, or are they buried? X-ray studies have provided information about the location of all amino acid side chains in chicken lysozyme (54) and, assuming similar conformations of the polypeptide backbone, about the location of the amino acid replacements among those bird lysozymes of known sequence. Table
VIII reveals that two-thirds of the replacements occur in completely exposed positions and that less than 10% of the changes are in buried locations. Furthermore, one notes that at least one-third of all external positions can accommodate an amino acid substitution. The interior of the molecule thus appears to be more conserved. Several immunochemical investigations (55-58) have shown that antibodies to a considerable part of the lysozyme molecule are elicited. These considerations clarify why immunological techniques can be expected to detect most amino acid substitutions occurring in proteins.

The disappearance of immunological cross-reactivity when sequences differ by about 30 to 40% or more (a more precise lower limit is not yet known for lysozyme) is also explicable on the basis of what is known about antigenic determinants. Generalizing from the extensive studies on the antigenic determinants of polysaccharides, nucleic acids, and synthetic polypeptides (59-61), it is expected that each antigenic determinant on a protein can comprise about five amino acids. When protein sequences reach differences of about 30%, and the exposed parts of the molecules can be expected to differ by an even larger percentage than the molecules as a whole, as we have just discussed, most determinants would have about two changes in them, assuming that the changes are randomly distributed on the surface. It is reasonable to suppose that, while one substitution in a determinant would not be expected to abolish its cross-reactivity, two substitutions might well do so. Physiological (and teleological) considerations regarding autoimmunity also dictate abolition of cross-reaction at a point well below complete abolition of sequence similarity. Identical runs of three or so amino acid residues (in primary or tertiary juxtaposition) can be expected to occur rather frequently even in very dissimilar proteins. Thus an organism would succumb to autoimmune disease due to the cross-reaction of antibodies originally formed in response to foreign proteins with its own proteins, if such cross-reactivity would occur in the presence of only slight sequence homologies.

It is important to note that the (quantitative) sequence–immunology correlation we have been discussing applies to methods (such as precipitin and complement fixation) requiring the antigen to be multivalent, i.e., having at least two determinants capable of reacting with antibody molecules. Methods necessitating only one determinant on the antigen have, for example, detected considerable cross-reactivity among various trypsin so distinct from one another that they did not cross-react by methods requiring multivalent interactions (62). Similarly, human and chicken lysozymes cross-react by such tests (38). The rationale we have adopted above for explaining why cross-reactivity between related native proteins fades out at sequence differences of 30 to 40% would lead us to expect broader cross-reactivity between unfolded proteins. Unfolding would expose regions of sequence homology wholly or partially buried in the native protein, particularly those in the more conservative interior of the molecule. Denaturation would thus make the protein multivalent. Indeed, reduced, carboxymethylated chicken and human lysozymes cross-react strongly in microcomplement fixation tests utilizing antisera to the unfolded proteins (38).

A. Miller, B. Bonavida, J. A. Stratton, and E. Sercarz, personal communication.
The sequence data and the calculated changes in Fig. 104 indicate that the rates of evolution of bird lysozymes have been relatively constant (although some acceleration seems to have occurred in the Japanese quail lineage) along the various lineages since points of divergence. This finding is in keeping with the molecular clock concept of evolution (65). The rate of evolution of lysozymes is high compared to the rate of cytochrome c evolution in birds; out of 104 residues, the chicken-turkey and chicken-duck cytochrome c sequence differences are, respectively, 0 and 3 residues (44), compared to values of 7 and 22, respectively, in the lysozyme system.

Advantages and Disadvantages of the Immunological Approach

It seems likely that, as proposed earlier (4), amino acid replacements taking place in naturally occurring, functionally active proteins are likely to produce additive immunological effects in tests requiring a multivalent antigen and that one can obtain a reasonable correlation between these effects and the number of substitutions which have actually occurred. The immunological approach has a number of important advantages over conventional sequence determination. It is quick and easy to do, requires little material, and, if one dispenses with doing reciprocal tests (which would be acceptable when an approximate answer would suffice), permits a survey of a large number of homologous proteins without purification of all the antigens. There are, of course, disadvantages to this approach; internal substitutions may not be registered, the actual location of substitutions cannot be determined, only an approximate value for the number of substitutions can be determined, and very distantly related proteins cannot be compared quantitatively.

Despite the several advantages of the immunological approach to assessing the degree of sequence similarity between related proteins and the observed dependence of immunological cross-reactivity upon sequence resemblance described in this work, we feel we have, by obtaining as good a correlation as we did even for the relatively small antigen lysozyme, established a good foundation for the practice of using immunological cross-reactivity as an approximate measure of the number of amino acid substitutions existing between two homologous proteins.

Note Added in Proof—Our assumptions that the conformation of the lysozyme polypeptide backbone is conserved during evolution and that lysozyme evolution is primarily a surface phenomenon agree with the findings of Blake and Swan (66), whose article appeared after our paper was submitted for publication.

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REFERENCES

35. DIETRICH, F. M., Immunochemistry, 5, 239 (1968).
The Dependence of Immunological Cross-Reactivity upon Sequence Resemblance among Lysozymes: I. MICRO-COMPLEMENT FIXATION STUDIES
Ellen M. Prager and Allan C. Wilson


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