

Multiple Lysozymes of Duck Egg White*

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ELLEN M. PRAGER† AND ALLAN C. WILSON

From the Department of Biochemistry, University of California, Berkeley, California 94720

SUMMARY

Lysozyme was purified from a pooled sample of Peking duck egg whites by ion exchange chromatography and gel filtration. Three different lysozymes were found, and each was shown to be pure by several criteria. The three enzymes were like chicken lysozyme in molecular size and specific activity, although they could be distinguished from it, as well as from one another, by electrophoretic and immunological criteria. The three duck enzymes were shown to be very similar to one another on the basis of amino acid composition (in particular, all lacked histidine) and immunological tests.

Lysozyme was then partially purified from 44 individual duck egg whites and subjected to starch gel electrophoresis at pH 5.3, at which the three different lysozymes are readily distinguishable from each other. Each individual egg white contained either any one of the three lysozymes alone or a combination of any two of them. No single egg white contained all three duck lysozymes. The frequency of the six different phenotypes, as well as the marked similarity of the three enzymes to one another, leads us to suggest the existence of three alleles at one locus in the duck population which we examined.

Although a single type of lysozyme occurs in chicken egg white, multiple lysozymes have been reported to be present in duck egg white (1-3). Since lysozyme is being used for evolutionary studies (4, 5), it is important to elucidate the basis of this multiplicity in order to determine whether one is examining the product of only one lysozyme locus or whether, in the duck, there are multiple lysozyme loci, only one of which may be homologous with the lysozyme locus expressed in the chicken and other birds examined (4, 5).

Previous investigations have reported the presence of two (3) or three (1) different lysozymes in pooled egg white from the duck.¹ We have isolated three histidine-free duck lysozymes from pooled egg white of the Peking duck, one of the domesti-

cated breeds of the mallard *Anas platyrhynchos*. Two of them may match those already reported. We could not find in the Peking duck the histidine-containing duck lysozyme mentioned by Jollès, Spotorno, and Jollès (1).

Seeking to determine the basis for the observed multiple lysozymes in the duck, we investigated the lysozyme present in each of 44 individual duck eggs. The finding of no more than two lysozymes in any one egg and phenotypic frequencies consistent with the model of three alleles at one locus lends support to a three allele-one locus model, as opposed to such models as multiple loci or posttranslational protein modification.

EXPERIMENTAL PROCEDURE

Eggs

Peking duck (*A. platyrhynchos*) eggs, freshly laid, were bought from Reichardt's Duck Farm, Petaluma, California. Bobwhite quail (*Colinus virginianus*) eggs, used in eliciting antibodies to whole egg white, were from LeJeune's Quail Farm, Sulphur, Louisiana. The egg whites were separated from the yolks and stored frozen at -10°.

Lysozymes

Three times crystallized chicken lysozyme was obtained from Pentex (EZ1962), and human lysozyme purified from the urine of patients with monocytic and monomyelocytic leukemia (10) was kindly supplied by Dr. E. F. Osserman.

Other Chemicals

Sephadex G-50 fine (No. 5556) and carboxymethyl Sephadex (C-25, No. 7762) were obtained from Pharmacia. Carboxymethyl cellulose (Cellex-CM, No. 6964) and Bio-Rex 70 (200 to 400 mesh, No. 3889) were purchased from BioRad. Starch for gel electrophoresis was obtained from Connaught Laboratories, Ionagar No. 2 for immunoelectrophoresis and Ouchterlony double diffusion from Oxo Ltd., London, England, and Amido black, used for protein staining of starch gels and immunoelectrophoresis slides, from K and K Laboratories, Brooklyn, New York.

Antisera

The four antisera to chicken lysozyme, designated A24, A54, A64, and A74, have been described (4). Antiserum to whole bobwhite quail (closely related to the chicken (4, 5)) egg white was produced by immunizing, over a 15-week period, a male New Zealand white rabbit intradermally with 10 mg of egg white suspended in Freund's complete and then Freund's incomplete adjuvant (Difco) and subsequently intravenously with 1.5 mg of egg white suspended in isotris buffer (the buffer used in micro-

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† Predoctoral Fellow of the National Science Foundation.

¹ While these investigators make no mention of the particular species and breed of duck used in their studies, we presume that they worked with a domesticated *Anas platyrhynchos* (6-9).

complement fixation and described previously (4)). Antisera to each of the two duck lysozymes found in greatest amount (A and B) were produced as follows. The lysozyme (2 mg) was suspended in Freund's complete adjuvant supplemented by an additional 3 mg per ml of lyophilized, phenol-killed BCG (supplied by the Department of Bacteriology and Immunology) and injected intradermally into three spots on the back of each male New Zealand white rabbit. Four rabbits (Nos. 432 to 435) were injected with lysozyme A and four rabbits (Nos. 436 to 439) with lysozyme B. Sera (first bleeding) were collected 24 days later. Thirty-eight days after the primary immunization, intravenous boosts of 1 mg of lysozyme in isotris buffer were given every other day for a total of three injections. One week after the last injection, antisera (second bleeding) were collected. Antisera were stored at -10° ; those used for micro-complement fixation were heat-treated at 60° for 20 min to inactivate the rabbit complement present and then stored at -10° .

Lysozyme Assays

Lysis of *Micrococcus luteus* (formerly named *Micrococcus lysodeikticus*) (11) was measured by recording the change in percentage of transmittance at 540 m μ in a Zeiss spectrophotometer at 23° .

Protein

The absorbance at 280 m μ was used to measure protein. Lyophilized protein was weighed.

Immunological Methods

Immunoelectrophoresis, Ouchterlony double diffusion, and micro-complement fixation were performed as described previously (4). The degree of antigenic difference between pairs of lysozymes is expressed as the index of dissimilarity. This index is defined as the factor by which the antiserum concentration must be raised in order for a heterologous lysozyme to produce a complement fixation curve the peak of which is equal in height to that produced by the homologous lysozyme (4).

Ultracentrifugation

Sedimentation velocity studies were carried out with a Spinco model E analytical ultracentrifuge. Lysozyme solutions of 0.6 to 0.7% protein were centrifuged at 67,770 rpm in a buffer (12) of 0.02 M sodium acetate, pH 5.3, and 0.15 M KCl at 23° .

Amino Acid Composition

Lysozyme samples were hydrolyzed under vacuum in 6 N HCl at $105-110^{\circ}$ for 24, 42, or 72 hours. The hydrolysates were evaporated to dryness and subsequently dissolved in 0.2 M citrate buffer at pH 2.2. Amino acid analyses were then carried out with either a Beckman 121 or a Beckman 120B autoanalyzer as previously described (5, 13). Values for serine and threonine were extrapolated to zero time of hydrolysis (14), whereas those for valine and isoleucine were taken from the 72-hour hydrolysate (3, 14). To calculate the number of residues of each amino acid, it was assumed that each lysozyme has 129 amino acids, as has been the case for all those bird lysozymes which have been sequenced, i.e. chicken (15, 16), Japanese quail (17), Duck II (18), and turkey (19). This assumption has also been made for other bird lysozymes, such as those of bobwhite quail and ring-necked pheasant (5). In addition, glycine was allowed to equal 12.00

as a base-line for the other amino acids, in line with glycine values given for duck lysozymes previously described (1, 3). Values close to integers were then obtained for the other amino acids. Tryptophan and half-cystine, as well as the amides asparagine and glutamine, were not analyzed for. Furthermore, proline was also not determined because it was impossible to obtain a reliable value on a lysozyme sample in which half-cystine was neither carboxymethylated nor otherwise modified.

Starch Gel Electrophoresis

Horizontal starch gel electrophoresis at pH 5.3, 7.0, and 11.9, was performed at 4° as has been described (5). However, starch was used at a concentration of 11%. The three duck lysozymes were most clearly distinguishable at pH 5.3. In order to separate them cleanly, a voltage gradient of 10 volts per cm for 18 hours was used. This high voltage and the concomitant high current necessitated cooling the gel by directing a stream of cold air from an Oster hair dryer over the gel during the run. Under these conditions the current was 50 ma or less per gel.

Following electrophoresis, protein was located by staining with 0.6% Amido black dissolved in a solution containing methanol-water-acetic acid in the ratio 45:45:10. Excess Amido black was removed by thorough rinsing of the gel in the same solvent.

Densitometry

Photographs of the Amido black-stained starch gels were made with Polaroid type 46-L film. The lysozyme bands of those eggs containing two lysozymes were then scanned on a Joyce-Loebl double beam recording microdensitometer using a narrow slit. The amount of protein represented by each peak was determined by cutting out the peak from the densitometric tracing and weighing the paper.

Purification of Lysozyme

The purification procedure utilized was based upon published methods (1, 5, 20).

Pooled Preparation—Pooled Peking duck egg white (1 liter) was diluted 1:5 in ammonium acetate buffer (pH 9.0; 0.1 M acetate), homogenized for 1 to 2 sec in a Waring Blendor, and filtered through tissue paper (KimWipes). About 42 g of carboxymethyl cellulose equilibrated in the same buffer were added, and the slurry was stirred at 4° overnight. The rest 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 carboxymethyl cellulose was then poured onto a Buchner funnel lined with eight layers of KimWipes and thoroughly washed with the ammonium acetate buffer. The lysozyme was eluted with 0.4 M ammonium carbonate (pH 9.2) and lyophilized. It was then subjected to gel chromatography on a Sephadex G-50 column, 4.5×53 cm, equilibrated with the ammonium acetate buffer. As Fig. 1 shows, one large protein peak emerged, as well as two small peaks. The material in the large peak, which included all of the lysozyme activity, was lyophilized. The residue, dissolved in 0.05 M ammonium carbonate, was then put onto a carboxymethyl Sephadex column, 2.4×14.5 cm, equilibrated with the same buffer. A linear gradient produced by utilizing 255 ml each of 0.05 M and 0.4 M ammonium carbonate eluted two lysozymes, designated A and B (Fig. 2). Then 0.8 M ammonium carbonate was passed over the

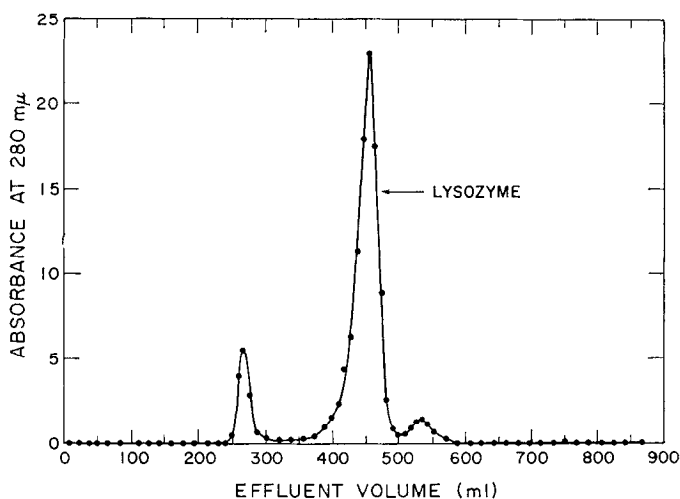


FIG. 1. Gel filtration of partially purified duck lysozyme on a column of Sephadex G-50.

column, eluting the lysozyme designated C (Fig. 2). A further increase in buffer concentration to 1.0 M ammonium carbonate failed to elute any more lysozyme. After lyophilization, each individual lysozyme was then further purified on a BioRex 70 column, 57×1.5 cm, equilibrated with 0.2 M sodium phosphate at pH 7.60. The column volume was 100 ml. After several column volumes of buffer had been used, a switch was made to 0.8 M sodium phosphate at pH 7.55. The peak of lysozyme A emerged after 1.82 column volumes of 0.2 M buffer had passed over it; the peak of lysozyme B emerged after 4.61 column volumes of 0.2 M buffer had passed over it. Although 5 column volumes of 0.2 M buffer failed to elute lysozyme C, 0.91 column volume of the 0.8 M phosphate succeeded. Each lysozyme was then placed in cellulose casing, dialyzed extensively against distilled water at 4°, and lyophilized. The yield of lysozyme was approximately 70 mg each of A and B and 3 mg of C. The low yield could be anticipated, since duck egg white has a low lysozyme content, in contrast to that of gallinaceous birds (21).

Individual Egg Whites—Thirty milliliters of each individual egg white were diluted 1:5 in the ammonium acetate buffer, homogenized, filtered, and stirred overnight at 4° with 2 g of carboxymethyl cellulose. The supernatant was decanted, the resin washed, and the lysozyme eluted and lyophilized as above. The residue was then dissolved in 1 ml of distilled water, centrifuged at $26,000 \times g$ for 15 min, and stored frozen. The lysozyme was, at this point, sufficiently pure (60 to 70%) and sufficiently concentrated (10 mg per ml) to be run on starch gels and detected easily by the Amido black stain. On each gel a pooled sample purified to this same point was run as a marker, enabling one to classify the lysozyme phenotype of each individual egg white. The major nonlysozyme contaminant had a mobility at pH 5.3 which was markedly different from the lysozymes, as shown below. The low molecular weight contaminating material (see Fig. 1, Sephadex G-50 column) did travel in the lysozyme region at this pH. However, it was soon washed out when the Amido black-stained gel was rinsed with the methanol-water-acetic acid solvent, and thus it could not interfere with the determinations of the type(s) and amount of lysozyme present in each egg.

Duck C Lysozyme from Two Individual Eggs—In order to obtain additional Duck C lysozyme for ultracentrifugation and

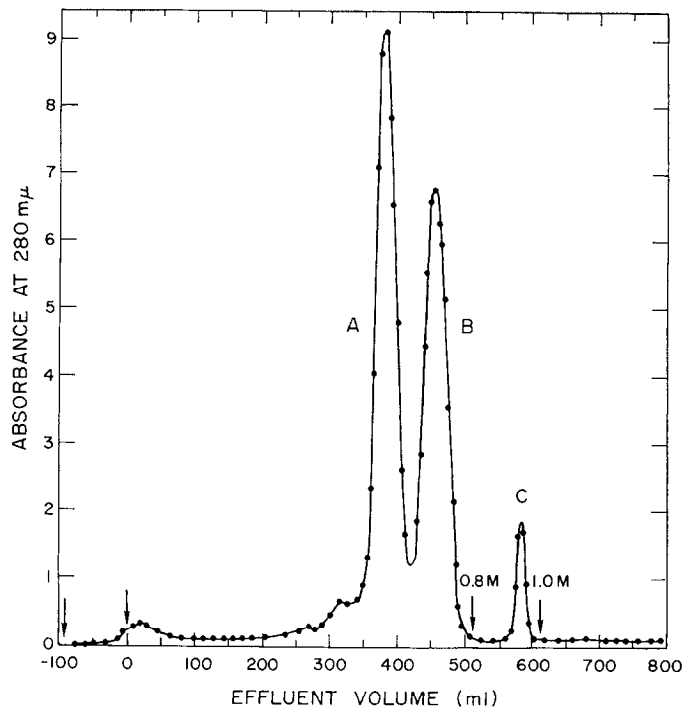


FIG. 2. Carboxymethyl Sephadex chromatography of duck lysozyme. The first and second unlabeled arrows indicate, respectively, the points at which the sample was put onto the column and at which the gradient was started.

amino acid composition studies, the partially purified lysozyme from two egg whites shown by starch gel to have only the C type lysozyme was further purified. It was passed over a Sephadex G-50 column, 1.5×60 cm, equilibrated with the ammonium acetate buffer described above, and the material in the large peak was then pooled and lyophilized. Duck C prepared in this manner satisfied all but two of the purity criteria described below: it was not tested on the Amberlite column, nor was it injected into rabbits.

RESULTS

Criteria of Purity of Lysozymes—Each of the duck lysozymes tested emerged as a single symmetrical protein peak from a column of Amberlite (BioRex 70). The specific activity was constant across the peak. In addition, each lysozyme moved as a single protein zone upon starch gel electrophoresis at pH 5.3, 7.0, and 11.9. The previously described difficulties of trailing and concentration dependence in the pH 7.0 system (5) were also encountered here. A single, symmetrical protein peak was exhibited by each lysozyme in sedimentation velocity studies. Immunoelectrophoresis, with an antiserum against whole bobwhite quail egg white, produced only one precipitin arc with each duck lysozyme.²

An additional immunological criterion of purity could be applied to lysozymes A and B. Rabbits immunized with A or B produced antisera which, when tested by immunoelectrophoresis against duck egg white, revealed only one precipitin arc.

Specific Activity—In their capacity to lyse *M. luteus* under the conditions used, the three duck lysozymes did not differ significantly from one another or from the chicken enzyme.

² Lysozyme C, however, partially precipitated in the agar before the antiserum was added.

TABLE I
Amino acid composition of duck lysozymes

Amino acid	Duck A				Duck B			Duck C			
	Time of hydrolysis			Integral value	Time of hydrolysis		Integral value	Time of hydrolysis			Integral value
	24 hrs	42 hrs	72 hrs		42 hrs	72 hrs		24 hrs	42 hrs	72 hrs	
	residues/molecule				residues/molecule			residues/molecule			
Lysine.....	6.57	6.08	5.96	6	6.00	6.10	6	6.68	6.50	5.60	6
Histidine.....	0	0	0	0	0	0	0	0	0	0	0
Arginine.....	13.38	12.67	12.70	13	13.60	13.74	14	15.9	15.0	14.1	15
Aspartic acid.....	18.75	19.00	18.92	19	18.60	18.59	19	18.95	18.50	18.70	19
Threonine.....	6.76	6.68	6.15	7 ^a	6.45	5.66	7 ^{a, b}	6.82	6.55	6.54	7 ^a
Serine.....	9.65	9.46	8.26	11 ^a	8.41	6.37	11 ^{a, b}	8.21	8.37	7.16	9 ^a
Glutamic acid.....	5.03	5.01	5.02	5	5.08	5.04	5	5.08	5.16	4.97	5
Glycine.....	12.00	12.00	12.00	12	12.00	12.00	12	12.00	12.00	12.00	12
Alanine.....	11.00	10.81	11.08	11	11.00	10.98	11	11.28	10.62	11.5	11
Valine.....	6.81	6.70	6.93	7 ^c	6.72	7.05	7 ^c	6.55	6.59	7.00	7 ^c
Methionine.....	2.20	0.94	1.70	2	1.62	1.77	2	2.34	1.50	1.66	2
Isoleucine.....	5.47	5.65	5.60	6 ^c	5.63	5.54	6 ^c	5.26	5.32	5.65	6 ^c
Leucine.....	7.90	8.04	8.01	8	8.16	7.98	8	7.95	7.73	7.86	8
Tyrosine.....	5.05	3.91	4.61	5	4.60	4.65	5	5.05	4.97	5.00	5
Phenylalanine.....	1.06	1.12	1.05	1	1.06	1.00	1	1.16	1.06	1.14	1

^a Extrapolated to zero time of hydrolysis.

^b The unusually steep decline in values between 42 and 72 hours of hydrolysis leads us to question the extrapolated values. Threonine would still be 7 even with a much shallower slope, whereas serine would give an integral value of 10.

^c Determined from the 72-hour hydrolysis.

TABLE II
Amino acid composition of histidine-free duck lysozymes and of chicken lysozyme

Amino acid	Duck lysozymes							Chicken lysozyme ^b
	Prager and Wilson, this work			Jollès <i>et al.</i>		Imanishi <i>et al.</i> ^a		
	Duck A	Duck B	Duck C	Duck II ^c	Duck III ^d	DL-1 ^e	DL-2 ^e	
Lysine	6	6	6	6	6	5	5	6
Histidine	0	0	0	0	0	0	0	1
Arginine	13	14	15	13	13-14 ^f	14	14	11
Aspartic acid	19	19	19	19	18-19	19	19	21
Threonine	7	7	7	7	7	7	7	7
Serine	11	10-11	9	11	10-11	11	10	10
Glutamic acid	5	5	5	5	5	5	5	5
Proline	N.D. ^g	N.D.	N.D.	2	2	2	2	2
Glycine	12	12	12	12	12	12	12	12
Alanine	11	11	11	11	11	11	11	12
Half-cystine	N.D.	N.D.	N.D.	8	8	8	8	8
Valine	7	7	7	7	7	7	7	6
Methionine	2	2	2	2	2	2	2	2
Isoleucine	6	6	6	6	6	6	6	6
Leucine	8	8	8	8	8	8	8	8
Tyrosine	5	5	5	5	5	5	5	3
Phenylalanine	1	1	1	1	1	1	1	3
Tryptophan	N.D.	N.D.	N.D.	6	6	6	6	6

^a Reference 3.

^b References 15 and 16.

^c From the sequence (18).

^d Reference 22.

^e DL, duck lysozyme.

^f Duck III is specifically said (22) to have more arginine than Duck II, at a time when the arginine value for Duck II was given as 12 to 13.

^g N.D., not determined.

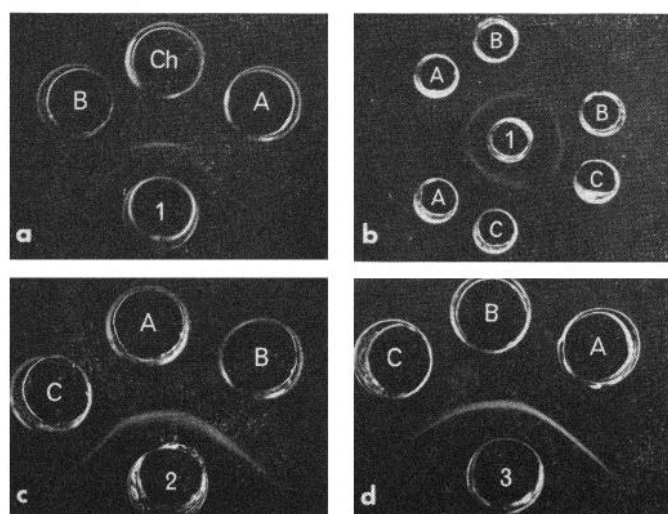


FIG. 3. Photographs of Ouchterlony double diffusion patterns obtained by reacting antichicken and antiduck lysozyme sera with chicken and duck lysozymes. 1, antichicken A24; 2, antiduck A, No. 432, second bleeding; 3, antiduck B, No. 436, second bleeding; Ch, chicken lysozyme; and A, B, and C, Duck A, Duck B, and Duck C lysozyme, respectively.

Molecular Size—When the purification of lysozyme from pooled egg white was carried out, a single active peak was obtained on Sephadex G-50, at the same point at which Pentex chicken lysozyme emerged when passed over that column. Sedimentation velocity studies gave $s_{20,w}$ values of 1.93 for Duck A, 1.89 for Duck B, and 1.91 for Duck C, in agreement with a value of 1.92 obtained for the chicken enzyme under the same conditions. Thus, duck lysozymes A, B, and C probably do not differ from each other or from chicken lysozyme in molecular size.

Amino Acid Composition—Table I shows the amino acid composition of the three duck lysozymes. The three enzymes appear to be identical in composition except that Duck B has one more arginine than Duck A and that Duck C has one more arginine than Duck B and two fewer serines than Ducks A and B (although the serine value for Duck B is open to question and is possibly equal to 10 rather than 11). Table II summarizes the amino acid composition (integral values of residues per molecule) of our three duck lysozymes, of the histidine-free duck lysozymes investigated by other workers, and of chicken lysozyme. Our Duck A agrees in composition with Duck II, and our Duck B seems to fit the compositional values given for Duck III. Our values (and also those for Duck II and Duck III) differ from those given for DL-1 and DL-2 (3) in the case of lysine and of arginine; in the latter instance, Imanishi *et al.* (3) found no variation in arginine value between their enzymes, in marked contrast to our findings and to the findings for Duck II compared with Duck III (22). The chicken enzyme differs considerably from the duck enzymes in its content of histidine, arginine, aspartic acid, serine, alanine, valine, tyrosine, and phenylalanine; its sequence differs from that of Duck II in 22 positions (18).

Immunological Cross-Reactivity—Chicken lysozyme produced spurs over all three duck lysozymes when tested by the Ouchterlony double diffusion technique with antisera directed toward chicken lysozyme (Fig. 3a, for example), indicating partial identity but significant structural differences between the duck lysozymes and that of chicken. However, these antisera could

TABLE III

Cross-reactions of duck, chicken, and human lysozymes with antisera to duck and chicken lysozymes

Lysozyme	Index of dissimilarity		
	Antiduck A ^a	Antiduck B ^b	Antichicken ^c
Duck A.....	1.00	1.30	6.67
Duck B.....	1.08	1.00	7.00
Duck C.....	1.50	1.51	6.83
Chicken.....	7.8 ^d	7.0	1.00
Human.....	>100 ^d	>100	>100 ^e

^a Antiserum 432, second bleeding.

^b Antiserum 436, second bleeding.

^c Antisera A24, A54, A64, and A74 pooled in reciprocal proportions to their micro-complement fixation titers (24, 26).

^d Tested with antiserum 434, second bleeding, since the anti-complementarity of 432, second bleeding, did not allow it to be used at the concentrations needed to test for cross-reactivity with chicken and human lysozyme.

^e Antisera A24, A54, and A74 tested individually.

not distinguish one duck enzyme from the other by this technique (Fig. 3b). Fujio *et al.* (23) showed that the one duck lysozyme which they studied, with the use of an antiserum to chicken lysozyme, gave a reaction of partial identity with the chicken enzyme.

In micro-complement fixation tests, our four antichicken sera did show differences among the duck enzymes relative to chicken lysozyme. A pool of the four sera gave indices of dissimilarity (4, 24) of 6.67 for Duck A, 7.00 for Duck B, and 6.83 for Duck C. Indices of this magnitude indicate structural relatedness yet considerable structural differences between chicken lysozyme and the duck enzymes, in agreement with the Ouchterlony double diffusion results. Similarly, Maron *et al.* (25) have observed, in experiments with antichicken lysozyme, that Duck II and Duck III are related to chicken lysozyme, although not very closely, and that these two duck lysozymes are distinguishable from each other by means of an immunological competition technique.

Antisera produced in response to Duck A and Duck B gave lines of identity among the three duck lysozymes in the Ouchterlony test (Fig. 3, c and d), indicating close structural similarities among them. Micro-complement fixation with antisera elicited by duck lysozymes A and B gave small indices of dissimilarity among the duck enzymes, in comparison to the indices given by antichicken sera tested against duck lysozyme and antiduck sera tested against chicken lysozyme. Nevertheless, real differences are readily detected among the three duck lysozymes, as shown in Table III, which summarizes the micro-complement fixation results, and in Fig. 4. The leukemic human lysozyme that we tested, which differs greatly in amino acid sequence from bird lysozymes (27), gave no detectable complement fixation with either antichicken or antiduck lysozyme sera. Others have also observed that antichicken lysozyme sera react weakly (25), or not at all,³ with human lysozymes.

Electrophoretic Mobility—At pH 11.9, Duck C moved most rapidly toward the cathode and Duck A most slowly, with Duck B having an intermediate mobility. Chicken lysozyme moved

³ N. Arnheim, J. Sobel, and R. E. Canfield, personal communication.

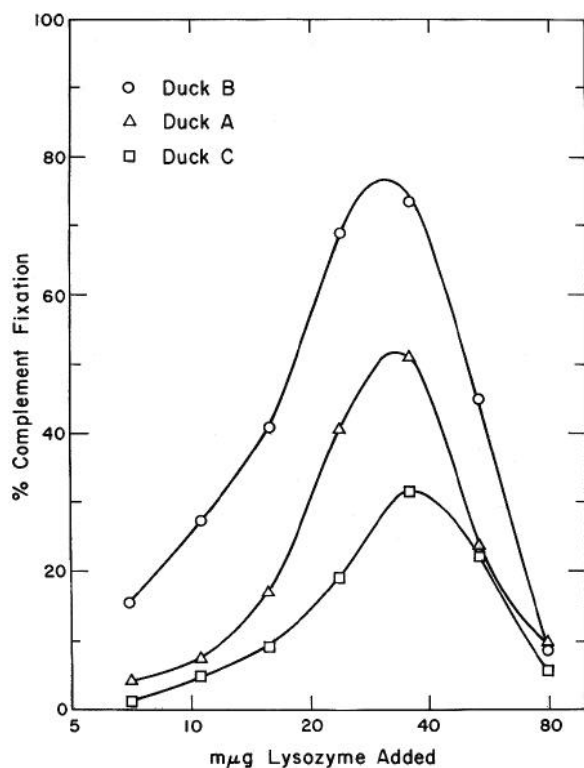


FIG. 4. Micro-complement fixation with antiserum prepared against Duck B lysozyme. The reaction mixtures contained 1 ml of 1:10,000 dilution of antiserum 436, second bleeding.

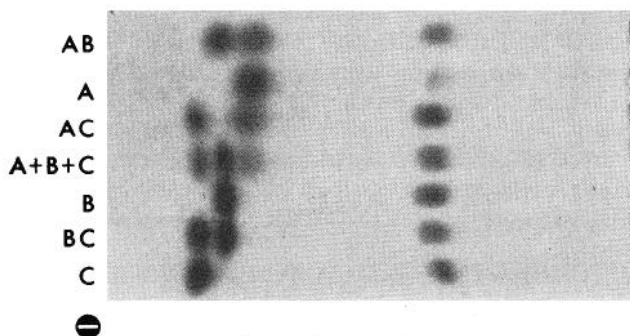


FIG. 5. Starch gel electrophoresis at pH 5.3 of the six possible duck egg white lysozyme phenotypes and of an artificial mixture of the three duck lysozymes (centermost slot). The prominent less basic band is the major nonlysozyme contaminant.

with the same mobility as Duck B. At pH 5.3, the same pattern held, with Duck C moving most rapidly and Duck A most slowly toward the cathode; the calculation of relative mobility of the three enzymes at this pH was 100 for Duck A, 108 for Duck B, and 116 for Duck C. In this system, chicken had a mobility intermediate between that of Duck A and Duck B. This behavior on starch is consistent with the behavior of the three duck enzymes on the carboxymethyl Sephadex and BioRex 70 columns, with Duck C being most basic and binding most strongly and Duck A being least basic and binding most weakly.

The electrophoretic mobility results are also consistent with the amino acid composition results. Assuming that the number of asparagine and glutamine residues is equal and that an equal number of carboxyl groups are exposed (and ionized at pH 5.3,

TABLE IV

Individual variation in lysozymes of duck egg white

Type of lysozyme	No. of eggs*
A	5
B	10
C	2
A and B	19
A and C	3
B and C	5
Total	44

* One egg per individual duck.

TABLE V

Theoretical and observed lysozyme phenotype frequencies in individual duck egg whites

For calculation of the theoretical values, we assumed a randomly mating population, three alleles at one locus, and no selective pressure. In this situation, the Hardy-Weinberg law predicts an $(\alpha + \beta + \gamma)^2 = \alpha^2 + \beta^2 + \gamma^2 + 2\alpha\beta + 2\alpha\gamma + 2\beta\gamma$ distribution, with α being the allele frequency of *a*, β of *b*, and γ of *c* in the population. α^2 , for example, represents the frequency of the *aa* genotype (A phenotype), whereas $2\alpha\beta$, for instance, represents the frequency of the *ab* genotype (AB phenotype).

Phenotype	Presumed genotype	No. of eggs		Frequency	
		Theoretical	Observed	Theoretical	Observed
A	<i>aa</i>	5.82	5	0.132	0.114
B	<i>bb</i>	11.0	10	0.250	0.227
C	<i>cc</i>	0.815	2	0.019	0.046
AB	<i>ab</i>	16.0	19	0.364	0.432
AC	<i>ac</i>	4.35	3	0.099	0.068
BC	<i>bc</i>	5.98	5	0.136	0.114

as well as at pH 11.9) in the 3 molecules, Duck B, by virtue of its 14 arginines, can be expected to have 1 unit of charge in excess of Duck A (which has 13 arginines), and Duck C, by virtue of its 15 arginines, can be expected to have 1 unit of charge above that of Duck B and 2 units above that of Duck A. This situation would prevail at both pH 11.9 and pH 5.3.

Survey of Lysozymes Present in Individual Duck Eggs—The partially purified lysozymes from 44 individual duck eggs were subjected to starch gel electrophoresis at pH 5.3. An individual egg white contained either a single type of lysozyme or a combination of any two of the three. No single egg contained all three lysozymes. Fig. 5 shows the six possible phenotypes found as well as an artificially made mixture of the three lysozymes. Table IV shows the frequency of each phenotype.

Densitometry performed on photographs of the Amido black-stained starch gels showed that when two lysozymes occurred together in one egg they were present in very nearly equal amounts. When A and B were present, we found 55% A and 45% B; when A and C were present, we found 58% A and 42% C; when B and C were present, we found 58% B and 42% C. The slight deviation from equality is not surprising because any streaking or trailing on our starch gels would raise the measured amount of the more anodal, slower moving component.

In view of the marked similarity among the three duck lyso-

zymes, the absence of an egg containing all three enzymes, and the nearly equal amounts of each lysozyme found in those egg whites containing two of them, we find it reasonable to suggest a model of one locus with three alleles, with both alleles being expressed should two occur in one individual. On that basis the allele frequency would be as follows: *a*, 0.364; *b*, 0.500; and *c*, 0.136. Here *a* represents the allele coding for lysozyme A, *b* for B, and *c* for C. This computation was made with 88 genes (from 44 diploid individuals) and assuming that the A phenotype, for example, is indicative of the *aa* genotype. We can then ask, given the allele frequencies computed, how many eggs of each phenotype would be expected if the duck population was in Hardy-Weinberg equilibrium at this locus? Table V compares the theoretical and observed results in terms of numbers of eggs and frequencies.

Agreement between the theoretical and observed values is excellent. Applying the χ^2 test, we find we can accept the hypothesis of three alleles at one locus at the significance level of $0.50 < P < 0.75$ ($n = 5$).

DISCUSSION

The principal alternatives available for explaining the presence of multiple lysozymes in Peking duck egg white are the existence of multiple lysozyme loci or the existence of multiple alleles at one lysozyme locus. Both these alternatives have been reported for other species of birds (28, 29). In the case of the black swan (*Cygnus atratus*), two nonallelic genes for egg white lysozyme appear to exist, since this species produces two lysozymes which are antigenically entirely unrelated, one rather like the chicken enzyme and the other rather like the goose enzyme (28). In contrast, Baker and Manwell (29) have implicated multiple (two) alleles at one lysozyme locus in a population of migratory quail (*Coturnix* species).

On the basis of a survey of individual duck eggs, in which we found no more than two lysozymes in any one egg, representation of each of the six possible phenotypes (any one lysozyme alone or any two together), a 1:1 ratio of the two lysozymes in every heterozygote, and a frequency distribution in accord with the Hardy-Weinberg law for three randomly assorting alleles at one locus, we propose that the lysozyme polymorphism in the duck is due to multiple alleles at one locus rather than to the presence of multiple nonallelic genes for lysozyme. If such multiple loci existed in the duck, one would expect some eggs, if not all, to have all three lysozymes, and one would not expect agreement with the Hardy-Weinberg law.

A further possibility to account for the presence of electrophoretically and immunologically different lysozymes in duck egg white is posttranslational protein modification. Examples of such modifications could include adding a phosphate or a charged carbohydrate moiety to the protein (neither of which has been reported for any lysozyme to date), or perhaps of converting asparagine or glutamine to their respective acids; an amino acid substitution would not be possible at the posttranslational stage. Our amino acid composition studies, which show Duck B to have one more arginine than Duck A, Duck C to have two more arginines than Duck A (and thus one more than Duck B), and Duck C to have two fewer serines than Duck A and one or two fewer serines than Duck B, as well as the partial sequence work comparing Duck II with Duck III (30), in which at least two amino acid differences were found, would thus argue against

posttranslational modifications as being responsible for the multiple lysozymes present in duck egg white.

In the absence of amino acid sequence studies on our three duck lysozymes, we cannot say how many amino acid substitutions occur among the three lysozymes. The immunological evidence indicates that they are few in number (*cf.* Reference 5 and Table III). However, the sequencing work (30) on Duck II versus Duck III (which may be the same as our Duck A and Duck B, respectively, on the basis of amino acid composition, relative basicities, and amounts found in a sample of pooled egg white) indicates that more than one amino acid difference most likely exists between any two of the three duck lysozymes. Although a number of reported allelic differences, such as those for human hemoglobin (31), appear to result in single amino acid differences, cases of multiple amino acid differences have been reported for the hemoglobin β chains of sheep (32, 33), cattle (34, 35), and goats (36), for rabbit hemoglobin α chains (37), and for bovine carboxypeptidase A (38, 39). The β chains of sheep hemoglobins A and B, for example, differ by at least 7 residues (33).

Thus, in view of our results in surveying individual Peking duck egg whites, we feel it is reasonable to accept a multiple allele-one locus model despite the possibility of a number of amino acid differences among the allele products. Given this model, it is possible that the histidine-containing lysozyme, Duck I, reported by Jollès *et al.* (1, 2), is the product of an allele found in some populations of ducks but not in the Peking ducks studied by us. Immunological techniques, namely immunodiffusion and micro-complement fixation, would enable one to determine whether Duck I is closely related to Duck II and Duck III, or whether it is indeed much more like chicken lysozyme, as its amino acid composition would imply. Comparative electrophoresis and micro-complement fixation should enable one to determine which of our three histidine-free lysozymes do or do not match those reported by other workers (1, 3).

Another possibility is that one or the other allele may have entered the domestic duck population through rather recent hybridization of different breeds. To shed some light on this aspect of the problem, it would be desirable to study the lysozyme(s) present in the wild species, the common northern mallard (6-9). An investigation of the lysozymes found in domestic and wild *A. platyrhynchos* populations in different areas of the world would perhaps reveal duck lysozymes we have not found in our Peking duck population and also geographic variations in allele frequencies. Genetic tests would also be valuable in obtaining further supporting evidence for the multiple allele-one locus model that we are proposing.

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