

Amino Acid Sequence Studies on Bobwhite Quail Egg White Lysozyme*

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SUMMARY

To test the immunological prediction that there should be two amino acid sequence differences between the lysozymes of the bobwhite quail and the chicken, the sequences of these two lysozymes have now been compared. Lysozyme purified from bobwhite quail egg white was reduced, carboxymethylated, and digested with trypsin. The resulting 18 peptides were separated and their compositions determined. Four of them differed in composition from the corresponding chicken egg white lysozyme peptides. The compositional differences could be accounted for by four amino acid substitutions. The positions at which the substitutions relative to chicken lysozyme occur were found by analysis and sequential degradation of these four tryptic peptides to be serine for threonine₄₀, valine for isoleucine₅₅, lysine for arginine₆₈, and threonine for serine₉₁. It is remarkable that three of the four interchanges appear from the three-dimensional model of chicken lysozyme to have occurred at buried positions. The remaining interchange, lysine for arginine₆₈, has occurred at an exposed position and in a region shown by others to bear an antigenic determinant. The implications of the bobwhite quail sequence information with respect to the sequence-immunology correlation, lysozyme evolution, and the phylogeny of phasianoid birds are discussed.

Immunological cross-reactivity studies (1, 2) revealed that bobwhite quail egg white lysozyme was unexpectedly similar to chicken lysozyme. Bobwhite quail lysozyme was more similar to chicken lysozyme than was pheasant lysozyme, even though pheasants are taxonomically closer (3) to the chicken than is the bobwhite quail. Subsequent chemical studies (2) revealed that, on the basis of over-all amino acid composition and peptide map comparisons, chicken and bobwhite quail lysozymes differed by a minimum of two amino acid interchanges, whereas chicken and pheasant lysozymes differed by a minimum of seven interchanges. The chicken and quail tryptic maps (2)

were identical except for one spot, which was arginine-negative in the quail but arginine-positive in the chicken.

Since that time availability of the sequences of five different lysozymes from warm-blooded animals—chicken (4, 5), Japanese quail (6), Duck II (7), turkey (8), and human (9, 9a)—made possible a more rigorous evaluation of the proposed correlation (1, 2, 10, 11) between amino acid sequence resemblance and immunological cross-reactivity with a homologous series of lysozymes. Such a study was conducted (12, 13) by quantitative immunological techniques, and a good correlation was found. This correlation predicted two differences between chicken and bobwhite quail lysozymes. A literature survey of many different proteins (13) revealed that a similar correlation between immunological cross-reactivity and amino acid sequence resemblance in tests requiring a multivalent antigen seems to hold for many other proteins as well.

We undertook determination of the actual sequence of bobwhite quail lysozyme to find out if the original immunological predictions and supporting chemical studies were correct, or whether significantly more substitutions, not suggested by either type of earlier work, existed. The quail sequence would be of special interest, since the chicken-bobwhite quail cross-reaction is the strongest observed between the lysozymes of two different species (1, 2, 12, 13). Antibody-antigen interactions are principally a surface phenomenon, and x-ray data (14, 15) on the spatial location of all amino acid side chains in chicken lysozyme are available. Thus location of the amino acid substitutions in very closely related homologous lysozymes could also be expected to provide information on the antigenic determinants in the lysozyme molecule. This approach to the location of antigenic determinants has been used in studies involving cytochromes *c* from several species (16, 17).

EXPERIMENTAL PROCEDURE

Eggs

Freshly laid bobwhite quail (*Colinus virginianus*) eggs were obtained from the Motley Quail Farm, Alvord, Texas, and from Le Jeune's Quail Farm, Sulphur, Louisiana. The egg whites were separated from the yolks and stored frozen at -10° .

Lysozymes and Trypsin

Three times crystallized chicken lysozyme was obtained from Pentex (EZ1962) and used without further purification. Bobwhite quail lysozyme was purified and characterized as pre-

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viously described (2, 12). TPCK¹ trypsin was a gift of Dr. T.-Y. Lin, Department of Biochemistry, University of California, Berkeley, or was obtained from Worthington.

Resins

Sephadex G-10 (No. 4252), Sephadex G-15 (No. 8507), and Sephadex G-25 Fine were obtained from Pharmacia. Bio-Rad AG50W-X4 (200 to 400 mesh, No. 9446) (Dowex 50-X4) and the mixed bed resin Bio-Rad AG501-XS(D) (No. 9479) were purchased from Bio-Rad, Richmond, California, as was the 2-aminoethylaminomethyl-polystyrene-1% divinylbenzene supporting resin used in the solid phase degradation.

Chemicals

Urea was obtained from Fisher or Baker or B & A and was either recrystallized from 95% methanol-5% water or deionized with a Bio-Rad AG501-XS(D) mixed bed resin. *p*-Chloromercuribenzoate was a gift from J. Haber. Iodoacetic acid, from Eastman, was recrystallized from *n*-heptane or ethyl acetate. Pyridine, from Mallinckrodt or Matheson, Coleman, and Bell, was redistilled from phthalic anhydride, b.p. 114–115°, for all uses except paper chromatography and electrophoresis. Ninhydrin, dimethylformamide (Sequanal grade), and dansyl chloride (No. 8299-3) were purchased from Pierce. Trifluoroacetic acid, butyl chloride (1-chlorobutane), and hydriodic acid (50%) from Eastman were used without further purification, whereas triethylamine from Eastman was distilled from phthalic anhydride, b.p. 89–90°, after treatment with sodium hydroxide to remove any acid present and then sodium sulfate to remove water. Dioxane, from Matheson, Coleman, and Bell, was passed over alumina to remove peroxides and aldehydes. The water-soluble carbodiimide EDC was obtained from Cyclo Chemical. The coupling reagent TATG was synthesized as described (18, 19), based on the method of Jensen and Pedersen (20). Acetone, absolute methanol, and hydriodantoin were obtained from Mallinckrodt, B & A, and Sigma, respectively, whereas *N*-ethylmorpholine and *t*-boc azide were from Aldrich Chemical Co.

Sulphydryl Group Determination

Analysis of the sulphydryl groups in the native enzyme was made with *p*-chloromercuribenzoate in urea as described (21). However, we used 10 M rather than 7.0 or 8.8 M urea.

Carboxymethylation of Lysozyme

Reduction and carboxymethylation of lysozyme were carried out as described (2), based on the method of Crestfield, Moore, and Stein (22) but with 10 M in place of 8 M urea. The pH during the carboxymethylation, which was allowed to run for 30 min, was generally maintained at 8.3 to 8.4 by the addition of 1 N NaOH. Excess mercaptoethanol was added prior to dialysis. Two cycles of reduction and carboxymethylation were often required to effect complete carboxymethylation under these conditions.

¹ The abbreviations used are: TPCK, L-1-tosylamino-2-phenylethyl chloromethyl ketone; EDC, 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide·HCl; TATG, thioacetylthioglycolic acid; *t*-boc, *tert*-butoxycarbonyl; RCM, reduced, carboxymethylated; Cys(Cm), carboxymethylcysteine dansyl, 5-dimethylaminonaphthalene-1-sulfonyl.

Tryptic Hydrolysis

Tryptic hydrolysis of RCM chicken and bobwhite quail lysozyme was carried out at 37° for 9 to 12 hours. The proteolytic enzyme was added either in one portion (3%, w/w, TPCK trypsin to lysozyme) or sequentially (2% at zero time, 1% at 4 hours, ½% at 8 hours). The pH was maintained at 8.0 (pH meter reading) by manual addition of dilute NH₄OH or at times by use of a Radiometer pH-stat. Digests used for peptide mapping and Sephadex G-25 chromatography were lyophilized. Those used for ion exchange chromatography on Dowex 50-X4 were acidified to pH 2.8 with formic acid and stored refrigerated or frozen until use.

Peptide Maps and Peptide Stains

Up to 3 mg of a lysozyme tryptic digest were mapped on paper essentially as described (2). Chromatography was done with the butanol-pyridine-acetic acid-water (15:10:3:12) system described (2), but electrophoresis was carried out for 80 min at 2300 volts at pH 3.5 with a pyridine-acetic acid-water (1:10:189) system. The maps were stained with the cadmium-ninhydrin reagent (23), except that immediate color development was brought about by heating the papers at 90–110°. To detect arginine the maps were stained by the phenanthrenequinone technique (24) or very occasionally the Sakaguchi procedure (25). Tryptophan was located with the Ehrlich stain (26). Chlorination (26) was occasionally used for weakly ninhydrin-positive spots. To obtain peptides for elution, sequential narrow strips of paper were cut and stained until the edge of a peptide spot was stained. Then the remainder of the spot was cut out. Peptides were eluted with 3 ml of 0.01 N NH₄OH; after evaporation the residue was hydrolyzed in 0.4 ml of 6 N HCl for 24 hours at 110°.

Low Voltage Electrophoresis

Electrophoresis at pH 2.0 was carried out for 3 hours at 300 volts with 2% formic acid-8% acetic acid in water. Bands were located with the cadmium-ninhydrin stain.

Quantitative Ninhydrin Determination

Quantitative ninhydrin analysis following alkaline hydrolysis was performed with some modifications of described procedures (27–29). To 0.3 ml of column effluent 0.5 ml of 4 N NaOH was added. The solutions were autoclaved for 30 min, cooled, and then neutralized with 0.5 ml of 46% acetic acid. Ninhydrin solution (27), 0.5 ml, was added and the tubes then covered and incubated in boiling water for 20 min. The tubes were cooled, 3 ml of 50% ethanol were added, and the absorbance at 570 nm was read.

Amino Acid Composition

Peptides were hydrolyzed under vacuum in 6 N HCl at 105–110° for 24 hours. Thiazolinones (see below under "Solid Phase Degradation") were hydrolyzed, without vacuum, in 5.7 N HCl at 130° for 2 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 autoanalyzer as previously described (2, 30). Frequently, however, a single column was used, in which case the third buffer, needed to elute the basic amino acids, was 1.6 N in sodium (*i.e.*, 1.4 N NaCl and the remainder sodium citrate) at pH 6.60 to 6.95. For analysis of

whole tryptic peptides, lysine or arginine was set equal to 1.00 to obtain the ratios of the other amino acids. Tryptophan was determined qualitatively with the Ehrlich stain as indicated above, or, in the case of tryptic peptide T-9, quantitatively by assuming an $E_{280}^{1\text{M}} = 5 \times 10^3$ (Reference 31).

Sephadex G-25 Chromatography

A tryptic digest (200 to 250 mg) of RCM-lysozyme in 2% acetic acid was added to a Sephadex G-25 column, 110×2.5 cm, equilibrated with the same solvent. Fractions of 3.6 ml were collected at a flow rate of 19 ml per hour, and the column was monitored by reading the absorbance at 280 nm. The contents of peak tubes were pooled and lyophilized and then analyzed by chromatography, high voltage electrophoresis, and amino acid composition.

Dowex 50-X4 Chromatography

A tryptic digest (160 to 200) mg of RCM-lysozyme adjusted to pH 2.8 with formic acid was put onto a Dowex 50-X4 jacketed column, 140×2 cm, heated to 35°. The eight-chambered pyridine-acetate gradient system described by Canfield (32) was used, with 500 ml of buffer in each chamber. Ten-minute fractions were collected, at a flow rate of 60 to 72 ml per hour. The column was operated only until the original buffer supply (32) was used up, since by that time the peptides of interest to us had emerged. Adding more Chamber 8 buffer, raising the column temperature to 50°, and ultimately using ammonium acetate, as done by Canfield (32) in order to elute all of the tryptic peptides, were therefore unnecessary. The column was monitored by quantitative ninhydrin analysis after alkaline hydrolysis and also by applying a few microliters of effluent to paper and staining with cadmium-ninhydrin and phenanthrenequinone. The material in tubes from peaks and, at times, shoulders of peaks was pooled and lyophilized and then analyzed by chromatography, high voltage electrophoresis, and amino acid composition.

Partial Acid Hydrolysis

One micromole of bobwhite quail tryptic peptides T-7 and T-8 was hydrolyzed under vacuum at 105–110° for 28 hours in 0.03 N HCl-40% glacial acetic acid to cleave almost exclusively before and after aspartic acid and asparagine, as previously described (33, 34).

Sephadex G-10 and G-15 Chromatography

Columns (51×1.5 cm) were packed with Sephadex G-10 and G-15 equilibrated with 5% acetic acid. Twelve-minute fractions at a flow rate of 12 ml per hour were collected. Effluent fractions were monitored like those from the Dowex 50 column, except that the phenanthrenequinone stain was not used. The material in tubes from peaks or shoulders of peaks was pooled and lyophilized and then analyzed by low voltage electrophoresis and amino acid composition. One micromole of the partial acid hydrolysates of T-7 and T-8 was run, respectively, on G-10 and G-15.

Solid Phase Peptide Degradation

The procedure developed by Mross and Doolittle for sequential degradation of peptides attached to resin was performed essentially as described (18, 19), with some modifications, and is

summarized briefly here. In essence, peptides are attached to a derivatized polystyrene supporting resin and degraded in stepwise fashion with TATG. The main advantages of this method over conventional sequencing techniques are (a) after hydrolysis of the cleaved thiazolinone, nearly all residues are recovered as the free amino acids, allowing quantitative identification with an automatic amino acid analyzer; and (b) the peptide being degraded is not lost into either aqueous or organic solvents because it remains bound to the resin throughout.

In the work reported here, peptides were used without blocking the side chain carboxyls, since these do provide additional points of attachment to the resin and since one can differentiate glutamic acid *versus* glutamine and aspartic acid *versus* asparagine even without blocking these carboxyls; if the amide is present, the yield is essentially equal to those of the preceding and succeeding steps, whereas if the acid is present, the yield is much less because much of the thiazolinone amino acid (see below) remains bound to the resin by the β - or γ -carboxyl and is not cleaved off by trifluoroacetic acid (see below).

The peptide amino groups are blocked reversibly with 10% *t*-boc azide in 50% aqueous dimethylformamide and 10% triethylamine or *N*-ethylmorpholine at 40–50° for 12 to 24 hours. After evaporation of the solvents by lyophilization, dry resin (2-aminoethylaminomethyl-polystyrene-1% divinylbenzene) is added to the blocked peptide, and 50% ethanol is added to facilitate distribution of the peptide on the resin surface; the ethanol is then evaporated. Water-soluble carbodiimide (0.1 M) in 50% aqueous dioxane at pH 5 is then added to couple the peptide to the resin. After 4 to 6 hours at room temperature, the resin is washed with 50% dioxane and then absolute methanol and dried. The *t*-boc group is removed by reaction with 50% trifluoroacetic acid in butyl chloride at 40° for 20 min; the resin is washed with butyl chloride and then methanol and dried. An aliquot of the resin is hydrolyzed in 50% concentrated HCl in propionic acid at 120° for 4 hours to determine the quantity of peptide coupled.

Prior to thioacetylation, the resin is neutralized by washing with 50% aqueous pyridine. TATG (0.3 M) in aqueous 60% pyridine-13% triethylamine adjusted to pH 9.4 to 9.6 with trifluoroacetic acid is added and the resin incubated at 40° for 1 hour. To remove excess reagents, the resin is washed first with 50% pyridine and then with methanol and then dried. The resin is subsequently incubated with 50% trifluoroacetic acid in butyl chloride for 15 min at 40° to cleave off the NH_2 -terminal residue as the thiazolinone amino acid, which is extracted by washing with butyl chloride. The resin is subsequently washed with methanol and dried and is then ready for the next degradative cycle. The thiazolinone is mixed with about 0.2 ml of 5.7 N HCl and the organic phase evaporated with nitrogen; the thiazolinone is hydrolyzed and the free amino acid analyzed, as indicated above ("Amino Acid Composition"). Each cycle involves the steps described in this paragraph. The amino acids tryptophan, cystine, cysteine, carboxymethylcysteine, threonine, and serine are not recovered, but serine gives a pattern of derivatives (carboxymethylcysteine, the mixed disulfide of cysteine and thioglycolic acid, cysteine, and cystine) which generally allows it to be recognized.

Toward the end of the work described in this communication, we found that an alternate method of hydrolysis of the thiazolinone allowed serine to be identified in good yield as several

	T-1 ← T-2 → T-3 → T-4 ←	
		10
Chicken	Lys--Val--Phe--Gly--Arg--Cys(Cm)--Glu--Leu--Ala--Ala--Ala--Met--Lys--Arg--His--Gly--Leu--	
Bobwhite quail	Lys-(Val--Phe--Gly)-Arg-(Cys(Cm)--Glu--Leu--Ala--Ala--Ala--Met)-Lys-(Arg--His--Gly--Leu--	
	T-5 → T-6 →	
	20 30	
Chicken	-Asp--Asn--Tyr--Arg--Gly--Tyr--Ser--Leu--Gly--Asn--Try--Val--Cys(Cm)--Ala--Ala--Lys--Phe--	
Bobwhite quail	-Asp--Asn--Tyr)-Arg-(Gly--Tyr--Ser--Leu--Gly--Asn--Try--Val--Cys(Cm)--Ala--Ala)-Lys--Phe--	
	T-7 → T-8 →	
	40 50	
Chicken	-Glu--Ser--Asn--Phe--Asn--Thr--Gln--Ala--Thr--Asn--Arg--Asn--Thr--Asp--Gly--Ser--Thr--Asp--	
Bobwhite quail	-Glu--Ser--Asn--Phe--Asn- Ser -Gln--Ala--Thr--Asn--Arg-(Asn--Thr--Asp--Gly--Ser--Thr--Asp)	
	T-9 → T-10 →	
	60	
Chicken	-Tyr--Gly--Ile--Leu--Gln--Ile--Asn--Ser--Arg--Try--Try--Cys(Cm)--Asn--Asp--Gly--Arg--Thr--	
Bobwhite quail	-Tyr--Gly- Val -Leu-(Gln--Ile--Asn)-Ser--Arg-(Try--Try--Cys(Cm)--Asn--Asp--Gly)- Lys -(Thr--	
	T-11 → T-12 →	
	70 80	
Chicken	-Pro--Gly--Ser--Arg--Asn--Leu--Cys(Cm)--Asn--Ile--Pro--Cys(Cm)--Ser--Ala--Leu--Leu--Ser--	
Bobwhite quail	-Pro--Gly--Ser)-Arg--Asn--Leu-(Cys(Cm))-Asn--Ile--Pro-(Cys(Cm))-Ser--Ala--Leu--Leu--Ser--	
	T-13 → T-14 → T-15 →	
	90 100	
Chicken	-Ser--Asp--Ile--Thr--Ala--Ser--Val--Asn--Cys(Cm)--Ala--Lys--Lys--Ile--Val--Ser--Asp--Gly--	
Bobwhite quail	-Ser--Asp--Ile-(Thr)-Ala- Thr -Val--Asn-(Cys(Cm))-Ala--Lys-(Lys--Ile--Val--Ser--Asp--Gly--	
	T-16 → T-17 → T-18 →	
	110 120	
Chicken	-Asp--Gly--Met--Asn--Ala--Try--Val--Ala--Try--Arg--Asn--Arg--Cys(Cm)--Lys--Gly--Thr--Asp--	
Bobwhite quail	-Asp--Gly--Met--Asn--Ala--Try--Val--Ala--Try)-Arg--Asn--Arg--Cys(Cm)--Lys-(Gly--Thr--Asp--	
	T-19 → T-20 →	
	130 140	
Chicken	-Val--Gln--Ala--Try--Ile--Arg--Gly--Cys(Cm)--Arg--Leu--	
Bobwhite quail	-Val--Gln--Ala--Try--Ile)-Arg-(Gly--Cys(Cm))-Arg--Leu--	

FIG. 1. Amino acid sequences of reduced, carboxymethylated chicken and bobwhite quail egg white lysozymes. The chicken sequence is from Canfield (4), whose numbering of tryptic peptides is followed (4, 32). The quail sequence is written in **bold face**

italics at those positions where the two enzymes differ. Those residues in the quail whose positions were inferred by composition and homology with the corresponding chicken peptides are given within parentheses.

products, principally alanine, and allowed threonine to be identified by the production in very good yield of α -aminobutyric acid. The modified procedure was adapted from that described (35) for hydrolysis of phenylthiohydantoins and consisted to hydrolysis in 50% hydriodic acid (without preservative and therefore containing free iodine) under vacuum at 110° for 16 hours.

Dansylation

To 10 to 15 nmoles of dry peptide, 10 μ l of 0.2 M NaHCO_3 were added and the volatile material was then evaporated. Subsequently, 10 μ l each of water and of 2.5 mg per ml of dansyl chloride in acetone were added and the reaction mixture incubated for 1 hour at 37°. After evaporation, 50 μ l of 5.7 N HCl were added and the dansyl-peptide hydrolyzed at 110° for 18 hours. The residue after hydrolysis was taken up in 5 μ l of 50% pyridine and spotted on polyamide sheets. Chromatography and identification of the dansyl-amino acids were carried out as described (36).

RESULTS

Throughout this section reference should be made to Fig. 1, in which the amino acid sequence of chicken lysozyme from Canfield (4) is given, the tryptic peptides numbered (4, 32), and the substitutions we found in the bobwhite quail enzyme (see below) indicated. The quail peptides were numbered in the same manner as those from the chicken; no ambiguity resulted in view of the marked similarity (2) between the two lysozymes. Since no free sulfhydryl groups were found in the native quail enzyme, just as none have been reported for any of the homologous lysozymes sequenced to date, the residues indicated as carboxymethylcysteine would exist in disulfide linkages in the native molecule.

Compositions of Tryptic Peptides

All the individual bobwhite quail tryptic peptides save T-9 were isolated from peptide maps and their compositions determined (T-17 was isolated in the combined form T-17 + 18 but later as T-17 alone from the Dowex column; T-1 + 2 was isolated as well as T-1 and T-2 alone). Of these, T-7, T-8, and T-11 each had two compositional differences from the analogous chicken peptides. Relative to the chicken peptides, T-7 had 1 more serine and 1 fewer threonine, T-8 had 1 more valine and 1 fewer isoleucine, and T-11 had 1 more threonine and 1 fewer serine. T-9 was isolated by Sephadex G-25 chromatography (Peak I of Fig. 2). It was significantly retarded and did not emerge until 1.25 times the total column volume of 2% acetic acid had passed over the column. It, too, was found to have two differences in composition from chicken T-9, which was retarded in the identical manner on the Sephadex column. Bobwhite quail T-9 had 1 lysine and no arginine compared to the analogous chicken peptide. Table I summarizes the compositions and net changes for tryptic peptides T-7, T-8, T-9, and T-11 in the two species. As can be seen, the interchanges in T-7 (chicken threonine to bobwhite quail serine) and T-11 (chicken serine to bobwhite quail threonine) cancel each other to give an over-all compositional difference of 4 (2, 12) despite a minimum sequence difference of 4 as indicated by the compositions of the individual tryptic peptides.

The remaining peptides had identical compositions in the two

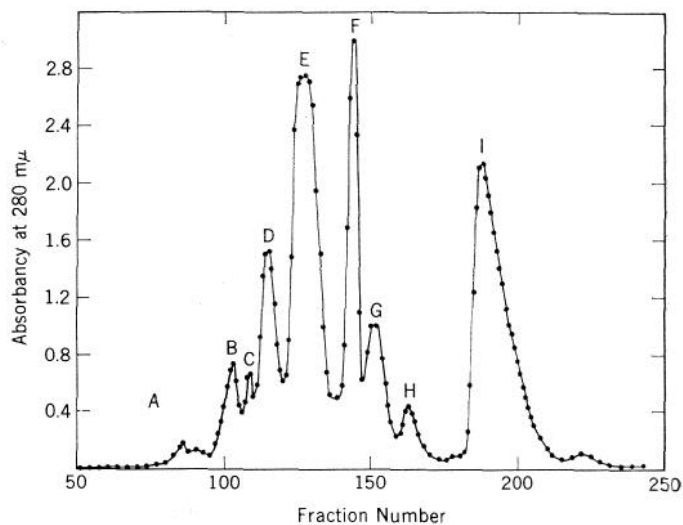


Fig. 2. Sephadex G-25 chromatography of a tryptic digest of reduced, carboxymethylated bobwhite quail lysozyme. Peak I consists of tryptic peptide T-9.

species, as Table II shows, and they were presumed to have identical sequences also. Tryptophan was detected in T-6, T-13, and T-16 by the Ehrlich stain. In view of the observations that the two lysozymes must have the same total number of tryptophans (2, 12), that T-9 in both species has two of the 6 total tryptophans, and that T-6, T-13, and T-16 in the two species match with respect to the other amino acids, it appeared unnecessary to quantitate the tryptophan in these three peptides.

It should be pointed out here that any compensating interpeptide charge exchanges—such as an aspartic acid to asparagine change in 1 peptide and an asparagine to aspartic acid change in another—are ruled out because peptides identical in composition could be isolated from identical locations on peptide maps of both species. Were there any such compensating charge interchanges, peptide positions in the electrophoretic dimension would be altered. The identity of the peptides maps further indicates that the relative number of aspartic and glutamic acids to their amides (which are of course determined as acids after hydrolysis in 6 N HCl) within each peptide must be the same in both species. The absolute sequences of the dipeptides T-14 and T-15 are also determined by composition alone in view of the specificity of trypsin.

Dowex 50-X4 chromatography (Fig. 3) was then carried out to isolate T-11, T-8, and T-7 (in order of their elution) in quantities sufficient for sequencing. Paper chromatography and electrophoresis (pH 3.5) with cadmium-ninhydrin- and phenanthrenequinone-staining as well as amino acid composition were carried out to determine their purity. T-11 and T-8 were essentially pure, whereas T-7 had a slight amount of contamination (less than 10% of the T-7 present), which was not considered sufficient to warrant further purification prior to sequencing. The tryptic peptides of chicken lysozyme when run on our Dowex column gave the same profile as shown in Fig. 3 for the bobwhite quail peptides. Bobwhite quail T-9, isolated on Sephadex, was pure by the same criteria applied to the peptides from the Dowex column; it was, however, arginine-negative in the quail. Because T-9 was so weakly positive

TABLE I

Amino acid compositions of reduced carboxymethylated bobwhite quail lysozyme tryptic peptides differing in composition from analogous reduced, carboxymethylated chicken lysozyme tryptic peptides

Lysine or arginine was set equal to 1.00 residue per molecule of peptide and the values of the other amino acids computed in relation to them. The integral values then assumed for the bobwhite quail lysozyme peptides are given in parentheses. The values for the analogous chicken lysozyme peptides are from the sequence (4, 5). For those residues for which differences exist between the two species, the integral values are underlined. The data for T-7, T-8, and T-11 are from peptides eluted from peptide maps, while the data for T-9 are from the peptide isolated by Sephadex G-25 chromatography. BWQ, bobwhite quail; Ch, chicken.

Amino acid	Peptides							
	T-7		T-8		T-9		T-11	
	BWQ	Ch	BWQ	Ch	BWQ	Ch	BWQ	Ch
Lysine					1.00(1)	<u>0</u>	1.00(1)	1
Histidine								
Arginine	1.00(1)	1	1.00(1)	1	-- (0)	<u>1</u>		
Cys(Cm)					0.93(1)	1	2.60(3)	3
Aspartic acid	2.87(3)	3	3.77(4)	4	2.30(2)	2	4.11(4)	4
Threonine	0.84(<u>1</u>)	<u>2</u>	1.83(2)	2			1.89(<u>2</u>)	<u>1</u>
Serine	1.78(<u>2</u>)	<u>1</u>	1.82(2)	2			2.85(<u>3</u>)	<u>4</u>
Glutamic acid	1.82(2)	2	1.04(1)	1				
Proline							1.07(1)	1
Glycine			1.98(2)	2	1.15(1)	1		
Alanine	0.96(1)	1					2.93(3)	3
Valine			1.07(<u>1</u>)	<u>0</u>			1.17(1)	1
Methionine								
Isoleucine			0.84(<u>1</u>)	<u>2</u>			1.81(2)	2
Leucine			0.76(1)	1			2.63(3)	3
Tyrosine			0.94(1)	1				
Phenylalanine	1.72(2)	2						
Tryptophan					1.80(2) ^a	2		
Net change, BWQ relative to chicken	+Ser -Thr		+Val -Ile		+Lys -Arg		+Thr -Ser	

^aCalculated spectrophotometrically, as indicated under "Amino Acid Composition."

with our cadmium-ninhydrin stain, the chlorination stain was also used.

Sequencing of Bobwhite Quail Peptides Containing Amino Acid Substitutions

T-7—Sephadex G-10 chromatography of a partial acid hydrolysate of T-7 yielded a fraction with an amino acid composition having 1 residue each of threonine, serine, glutamic acid, and alanine, and thus corresponding to the fragment consisting of residues 40 to 43, with the serine for threonine substitution. However, amino acid analysis showed also the presence of the equivalent of 1 residue each of aspartic acid and arginine. Electrophoresis at pH 2.0 revealed more than one band, including free aspartic acid and arginine. Despite the presence of these impurities, 55 nmoles of peptide were coupled to resin and degraded by the solid phase sequencing technique. The first degradative cycle indicated serine, suggesting that the serine for threonine substitution occurs at

position 40 rather than 43 in the lysozyme molecule. A dansyl determination on the same peptide also implied a substitution at position 40; serine and no threonine appeared, but several other spots were also evident.

To confirm these results, we coupled the parent T-7 tryptic peptide (30 nmoles) to resin and degraded it. The characteristic serine pattern (see "Experimental Procedure") appeared in the seventh cycle, revealing the serine for threonine substitution at position 40. No other changes from chicken T-7 were found. Fig. 4 presents the histogram for the degradation of T-7.

T-8—Sephadex G-15 chromatography of a partial acid hydrolysate of T-8 yielded a fraction with a composition corresponding to residues 53 to 59 in chicken lysozyme, except that (see Table I) the bobwhite fragment of course included a valine for isoleucine substitution (aspartic acid and asparagine following large hydrophobic residues are sometimes not cleaved during partial acid hydrolysis). Some excess aspartic acid was

TABLE II

Amino acid compositions of reduced, carboxymethylated bobwhite quail lysozyme tryptic peptides identical in composition to analogous reduced, carboxymethylated chicken lysozyme tryptic peptides

Lysine or arginine was set equal to 1.00 residue per molecule of peptide (except for the COOH-terminal T-18, which contains only leucine) and the values of the other amino acids computed in relation to them. The integral values then assumed for the bobwhite quail lysozyme peptides are given in parentheses and are identical to the values one obtains for the analogous chicken lysozyme peptides from the sequence (4, 5). The data for all peptides except T-17 are from peptides eluted from peptide maps; the data for T-17 are from the peptide isolated by chromatography on Dowex 50. BWQ, bobwhite quail; Ch, chicken.

Amino acid	Peptides																
	T-1	T-1+2 ^a	T-2	T-3	T-4	T-5	T-6	T-10	T-12	T-13	T-14	T-15	T-16	T-17	T-17+18	T-18	
Lysine	1.00 (1)	0.89 (1)		1.00 (1)			1.00 (1)		1.00 (1)			1.00 (1)					
Histidine						0.95 (1)											
Arginine		1.00 (1)	1.00 (1)		1.00 (1)	1.00 (1)		1.00 (1)		1.00 (1)	1.00 (1)		1.00 (1)	1.00 (1)	1.00 (1)		
Cys(Cm)				1.00 (1)		2.04 (2)	0.94 (1)					0.73 (1)		1.00 (1)	0.63 (1)		
Aspartic acid							1.31 (1)			3.12 (3)	0.89 (1)		1.10 (1)				
Threonine								0.88 (1)					1.04 (1)				
Serine							1.14 (1)	0.93 (1)		1.22 (1)			1.23 (1)				
Glutamic acid				0.88 (1)													
Proline								0.95 (1)									
Glycine		0.96 (1)	1.12 (1)			1.07 (1)	2.31 (2)	1.02 (1)		2.33 (2)			1.17 (1)	1.05 (1)	1.08 (1)		
Alanine				2.77 (3)			2.33 (2)			2.14 (2)			1.15 (1)				
Valine		0.77 (1)	0.96 (1)				1.16 (1)			2.02 (2)			1.22 (1)				
Methionine				0.86 (1)													
Isoleucine										1.19 (1)							
Leucine				0.85 (1)		1.00 (1)	0.95 (1)			0.58 (1)			0.77 (1)		0.92 (1)	1.00 (1)	
Tyrosine						0.65 (1)	0.96 (1)										
Phenylalanine		0.93 (1)	0.87 (1)														
Tryptophan							^b (1)			^b (2)			^b (1)				

^aT-1+2 was contaminated with up to 0.4 residue of a number of other amino acids per residue of arginine.

^bDetermined qualitatively with the Ehrlich stain. "+" denotes a positive reaction. See text for discussion of the tryptophan in T-6, T-13, and T-16.

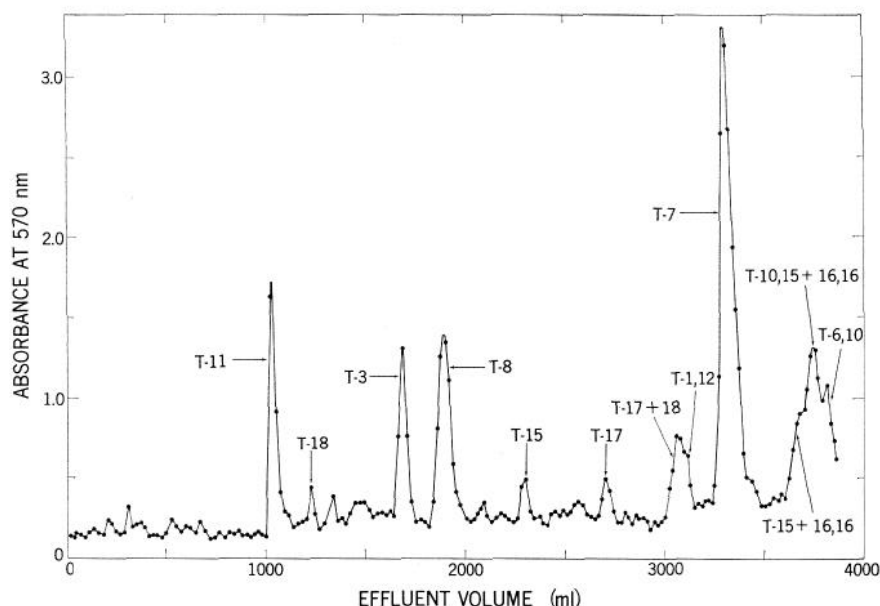


FIG. 3. Dowex 50-X4 chromatography of a tryptic digest of reduced, carboxymethylated bobwhite quail lysozyme. The absorbance at 570 nm of aliquots analyzed by the quantitative ninhydrin technique following alkaline hydrolysis is indicated; control tubes gave an absorbance of 0.15 to 0.20.

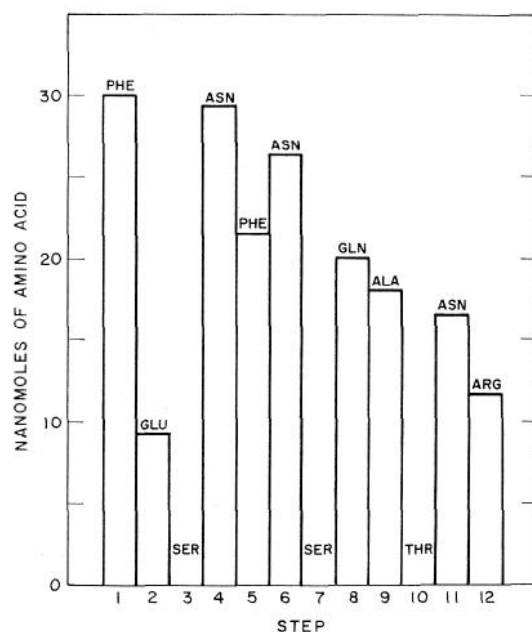


FIG. 4. Histogram of solid phase degradation of bobwhite quail tryptic peptide T-7. Serine was identified by the characteristic pattern described (see "Experimental Procedure"), whereas threonine was assigned by the failure to recover any amino acid in the 10th cycle (see "Experimental Procedure").

present; contamination by the other amino acids present in the parent T-8 was negligible. Electrophoresis at pH 2.0 revealed one major band and then a fainter one where free aspartic acid would be found. Fourteen nanomoles of this peptide were coupled to resin; four degradative cycles gave the sequence Tyr-Gly-Val-Leu, establishing the presence of valine in place of isoleucine at position 55 and not at position 58. An additional piece of data was obtained by pH 2.0 electrophoresis of the unchromatographed partial acid digest, elution of the bands, and determination of their amino acid compositions.

One fragment contained equal amounts of serine and arginine, thus confirming a serine at position 60 (since arginine must be COOH-terminal due to trypsin's specificity).

T-9—In view of trypsin's specificity and the compositional analysis (Table I) indicating a lysine for arginine substitution, no further work was needed to establish the existence of lysine at position 68 in bobwhite quail lysozyme.

T-11—Three-hundred nanomoles of the 23-residue T-11 (the longest tryptic peptide) coupled to resin were degraded through 23 cycles. The yield at Step 23 was 24 nmoles of lysine, and at this point the degradation was about 35% out of phase. Serines were detected at the 8th, 12th, and 13th cycles (corresponding to positions 81, 85, and 86, respectively, in the whole molecule), but not at the 18th cycle (position 91). A small aliquot of resin-peptide saved from prior to the 18th coupling was degraded through one cycle and the thiazolinone hydrolyzed in 50% hydriodic acid as described above. α -Aminobutyric acid was produced, confirming the occurrence of threonine in the bobwhite quail in place of serine in the chicken at position 91 in the lysozyme molecule. No additional changes (which of course would have to be intrapeptide interchanges) relative to chicken T-11 were found among these 23 residues.

In summary, four amino acid substitutions, serine for threonine₄₀, valine for isoleucine₅₅, lysine for arginine₈₈, and threonine for serine₉₁, have been found in bobwhite quail lysozyme relative to chicken lysozyme. Any additional substitutions we failed to detect, by assigning on the basis of identical compositions of the tryptic peptides from the two species those residues whose positions we did not explicitly determine either by sequencing or by the specificity of trypsin's action, would have to be intrapeptide interchanges, which are quite unlikely within these rather short tryptic fragments.

Table III summarizes for reference in the "Discussion" the number of amino acid sequence differences and also the minimal mutation distances for all those animal lysozymes of known sequence.

TABLE III

Sequence and mutational distance comparison for animal lysozymes of known sequence

The number of amino acid sequence differences between any two lysozymes appears in the upper right-hand section of the matrix, while the minimal mutation distance (37) appears in the lower left-hand section. The sequence data are from References 4, 5 (chicken), Prager *et al.*, this work (bobwhite quail), 6 (Japanese quail), 8 (turkey), 7 (Duck II), and 9, 9a (human).

Species compared	Chicken	Bobwhite quail	Japanese quail	Turkey	Duck II	Human
Chicken.....		4	6	7	22	53
Bobwhite quail...	4		10	11	23	54
Japanese quail...	7	11		10	25	55
Turkey.....	7	11	10		22	54
Duck II.....	27	28	29	27		54
Human.....	71	72	73	72	70	

DISCUSSION

Interpretation of Immunological Difference Between Bobwhite Quail and Chicken Lysozyme

Lysozyme evolution seems in general to have been primarily a surface phenomenon (12, 38). As shown in Table IV, only 13% of the positions at which amino acid replacements have been detected during lysozyme evolution in birds appear to be buried, if it is assumed that the three-dimensional structures of these molecules are identical. Good evidence for this possibility comes from the fact that, although human and chicken lysozyme differ in amino acid sequence at 53 positions, their conformations are essentially identical, most of the amino acid substitutions having occurred at external or superficial positions in the molecule (38).

It is therefore remarkable that in our comparison of the chicken and bobwhite quail lysozymes three of the four amino acid replacements found occur at buried locations. X-ray studies indicate that the side chains of threonine₄₀, isoleucine₅₅, and serine₉₁ are buried in the interior of chicken lysozyme, whereas the arginine₆₈ side chain is fully external (14, 15). Since the replacements we have detected at these four positions are each chemically conservative, the assumption that bobwhite quail lysozyme retains the same conformation as chicken lysozyme is reasonable.

Interactions between antibodies and globular protein antigens are also primarily a surface phenomenon. The unexpectedly high proportion (75%) of buried differences between chicken and bobwhite quail thus explains the unexpectedly strong immunological cross-reactions observed (1, 2, 12, 13). Antibodies directed toward either of these two bird lysozymes would not be expected to react with residues at positions 40, 55, and 91 nor to detect amino acid substitutions at these positions.

The immunological difference between chicken and bobwhite quail may be due entirely to the arginine-lysine interchange at position 68. Evidently residue 68 is part of an antigenic determinant. This observation is consistent with other reports (39-44) indicating the presence of an antigenic determinant in the "loop" peptide region of lysozyme consisting of residues 64 to 83 with a disulfide bridge between residues 64 and 80 (Reference 40). The "loop" region could of course contain

TABLE IV

Location of amino acid replacements among bird lysozymes

All possible pairs of bird lysozymes are compared as to the total number of amino acid substitutions and their locations between members of the pairs. "External" means fully exposed to solvent, "surface" means partly exposed, and "internal" means not exposed to solvent. The information in Columns 3, 4, and 5 is obtained by assuming that the lysozymes of bobwhite quail, Japanese quail, turkey, and Duck II have the same conformation as the chicken enzyme and that the amino acid residues substituted for those in the chicken have the same degree of exposure to solvent as has been described for all the amino acid side chains in chicken lysozyme by Browne *et al.* (14). The assignments of side chain accessibility have been essentially confirmed by Lee and Richards (15). BWQ, bobwhite quail; JQ, Japanese quail.

Species compared	No. of positions having amino acid substitutions				Per cent internal substitutions
	Total	External	Surface	Internal	
Chicken vs BWQ.....	4	1	0	3	75
Chicken vs JQ.....	6	5	1	0	0
Chicken vs Turkey.....	7	5	1	1	14
Chicken vs Duck II.....	22	13	8	1	5
BWQ vs JQ.....	10	6	1	3	30
BWQ vs Turkey.....	11	6	1	4	36
BWQ vs Duck II.....	23	12	8	3	13
JQ vs Turkey.....	10	9	0	1	10
JQ vs Duck II.....	25	17	7	1	4
Turkey vs Duck II.....	22	13	7	2	9
All birds.....	31	19	8	4	13

more than one determinant. Our suggestion of immunological activity associated with residue 68 could thus more narrowly define the position of a determinant in this region.

Sequence-Immunology Correlation

In Fig. 5A we plot immunological cross-reactivity *versus* total number of amino acid sequence differences for all possible pairs of bird lysozymes (*cf.* Fig. 8 of Reference 12). All the points involving the bobwhite quail enzyme lie significantly below the line—that is, toward unexpectedly high cross-reactivity values. This is expected, since, as shown in Table IV, the per cent of internal changes for pairs involving the bobwhite quail is disproportionately high, especially when the total number of sequence differences is small. In Fig. 5B we plot cross-reactivity *versus* the sum of external plus surface changes only, thereby eliminating the "hidden" changes. The bobwhite quail points no longer fall systematically below the line, and the line is in general a better fit for all the available data. The contrast between Figs. 5A and 5B is not surprising, considering that antigen-antibody interactions would not be expected to respond to changes in the interior of a molecule if the over-all conformation is the same for the bird lysozymes under consideration. X-ray crystallographic studies of all those bird lysozymes sequenced would of course be desirable to verify the validity of this assumption.

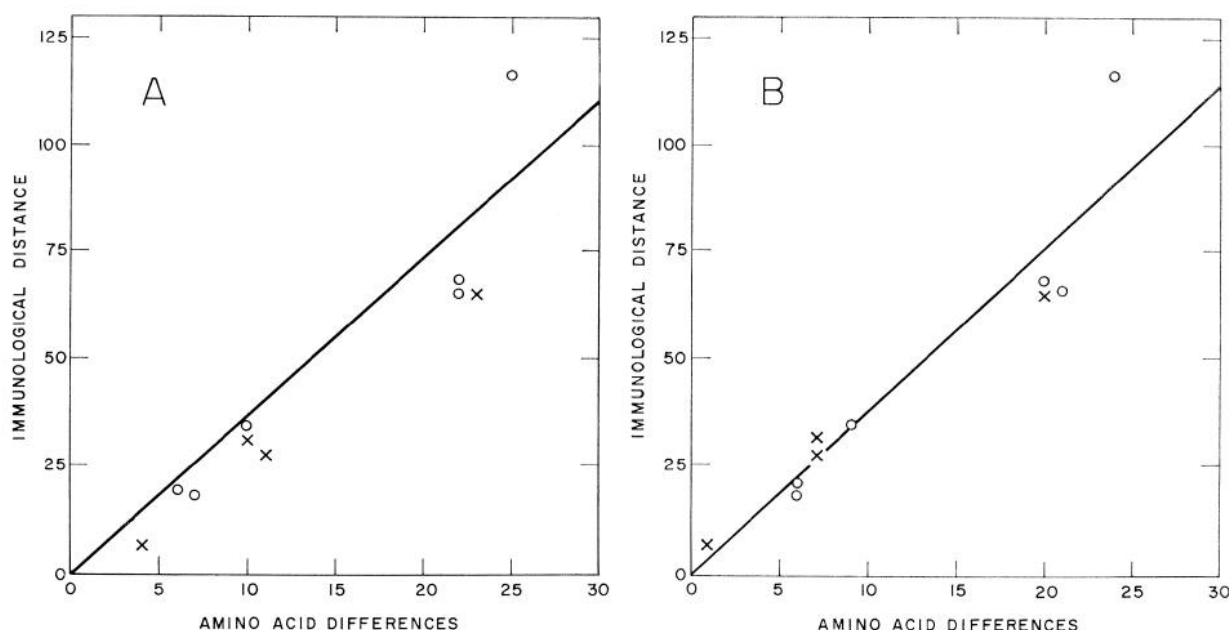


FIG. 5. Antigenic difference *versus* number of amino acid differences for bird lysozymes of known sequence. Cross-reactions were measured by the micro-complement fixation technique with antisera to every lysozyme and are taken from Table VI of Reference 12. The degree of antigenic difference is expressed as the immunological distance (12). The symbol X denotes cross-

reactions involving bobwhite quail lysozyme. In A the total number of amino acid substitutions is plotted; the line is drawn, as in Fig. 8 of Reference 12, without reference to the bobwhite quail data. In B the sum of only external plus surface substitutions (see Table IV) is plotted.

say, the Japanese quail *versus* chicken reaction, and consequently fewer antigenic determinants in common, the complete abolition of one determinant could have a fairly strong effect on the immunological cross-reactivity. Furthermore, since these four substitutions in the Japanese quail are not conservative ones, some conformational change caused by them cannot be ruled out. If there should be a determinant and/or a conformational change involving these 4 residues which are uniquely different in the Japanese quail, we could thus explain the weak Japanese quail-duck immunological cross-reaction.

Lysozyme Evolution

It is possible to construct a phylogeny for all those homologous lysozymes of known sequence (Fig. 6). There is certainly no dispute with classical taxonomy as to the relationships of human, duck, and the phasianoid birds (to which the chicken, bobwhite quail, Japanese quail, and turkey belong) as a group. However, there is disagreement as to the relationship of the members *within* the phasianoid group, and we therefore address ourselves to this question below.

Phylogenetic relationships among the phasianoid bird lysozymes sequenced to date can also be derived by methods such as that of Doolittle and Blombäck (48) and compared with the phylogenetic relationships among the organisms from which the lysozymes were obtained (Fig. 7). (Although ornithologists have not published actual phylogenetic diagrams for these birds, the taxonomic system (3) supports phylogeny B in favor of the lysozyme phylogeny A.) For the phylogenies of Figs. 7A and 7B, we have used Doolittle and Blombäck's method (48) to indicate the amino acid changes which probably occurred along each lineage in order to generate the lysozyme sequences present in the four birds today. The key difference between Figs. 7A and 7B is in the position of the bobwhite quail lineage. In

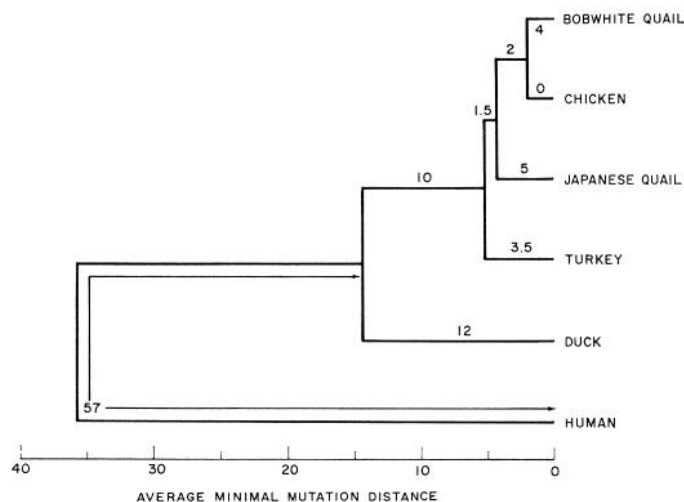


FIG. 6. Lysozyme phylogenetic diagram based on sequence. The minimal mutation distances (37) tabulated in Table III have been used, and the amounts of change along each line have been computed as described by Sarich (46), as was done for Fig. 10A of Reference 12. (In Reference 12 this method of calculation was erroneously attributed to the rather similar method of Fitch and Margoliash (47).)

The one unexpectedly weak cross-reaction shown in Figs. 5A and 5B involves Japanese quail *versus* duck. Investigation of the three-dimensional structure of chicken lysozyme (45) indicates that the side chains of residues 19, 21, 102, and 103 are in close tertiary juxtaposition. These residues are all external (14, 15) and are uniquely different in the Japanese quail compared to the other birds under consideration. Thus, in the Japanese quail *versus* duck reaction, where there is already a significant number of sequence differences compared to, let us

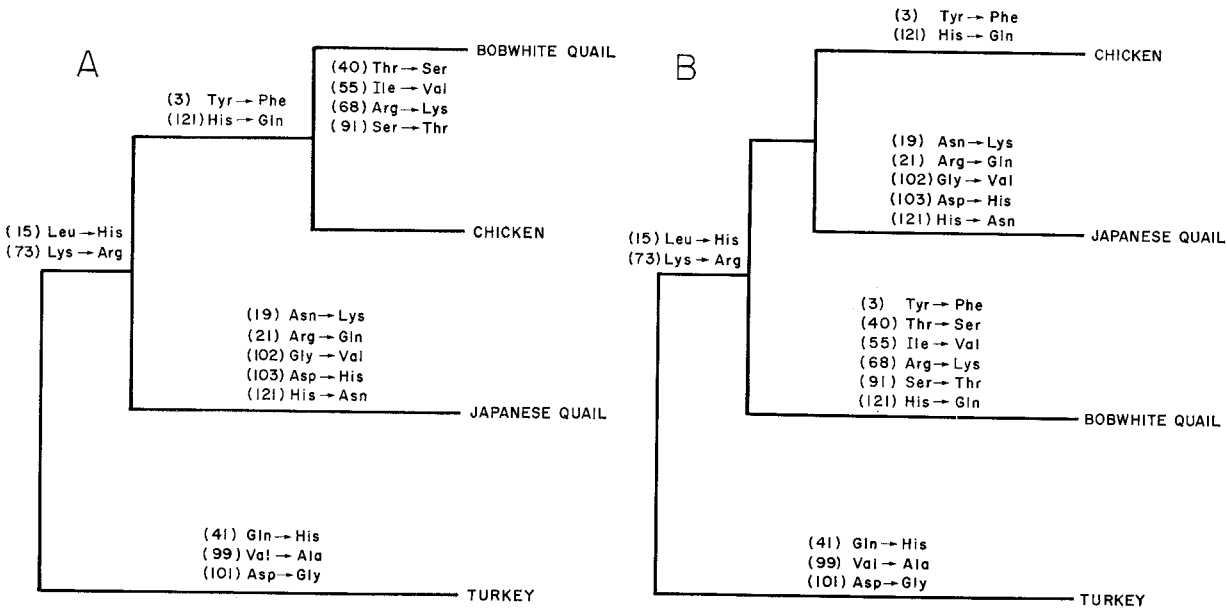


FIG. 7. Phylogeny of phasianoid birds based on lysozyme sequence (A) and traditional taxonomy (B) (3). The amino acid substitutions (by position in the molecule) postulated to have occurred in the lysozyme molecule along each lineage were determined as done by Doolittle and Blombäck (48) and are given for

each phylogeny. Minimal mutation distances were used and reference made to the sequences of Duck II and human lysozymes to determine for each position the amino acid most likely to be ancestral to those found in the phasianoid birds.

order to reconcile the molecular phylogeny with the nonmolecular one, it would be necessary to postulate that the same two amino acid replacements (at positions 3 and 121) had occurred coincidentally in both the chicken and bobwhite quail lineages. Furthermore, phylogeny B is less economical because the total number of changes needed would be two more than in phylogeny A. As the morphological evidence that the American quail lineage is phylogenetically outside the group that includes the chicken and Japanese quail is very weak (49, 50), phylogeny A ought to be seriously considered by ornithologists.

In order to gain a precise understanding of the mechanism of evolution at the molecular level, it is important to have accurate information concerning the rates at which proteins have been evolving. First, we note in Fig. 7 (and Fig. 6) that lysozyme has not evolved at a strictly uniform rate. For example, more change has occurred in the quail lineages than in the chicken lineage, whether we consider phylogeny A or B. The absolute rate at which lysozyme has been evolving can be estimated if we consider the sequence differences among bird lysozymes in relation to fossil evidence regarding the divergence times of the lineages leading to the modern species. The phasianoid group of birds is represented by fossil fragments dating back to the lower Oligocene period, approximately 30 to 36 million years ago (49). Although their fossil record is not so well known as that of some other vertebrate groups, it seems probable that the lineages leading to the phasianoid birds whose lysozymes have been studied split apart about 40 million years ago. Since the average sequence difference between the lysozymes of the chicken, turkey, and quails examined to date is 8, we calculate that the average time required for one amino acid sequence difference to accumulate between the lysozymes of two phasianoid species has been 5 million years.

The duck belongs to a different order (Anseriformes) and is representative of a lineage that can be traced back to Eocene times, about 50 million years ago (49, 51). The time of diver-

TABLE V
Evolutionary rate of lysozymes

From the amino acid sequences of lysozymes from the organisms listed in Table III, one can derive the unit evolutionary period, i.e. the average time required for a 1% difference in amino acid sequence to arise between lysozymes. For each category of comparison the average percentage difference in amino acid sequence for all possible comparisons is listed as *n*. The corrected per cent change, *m*, is corrected for multiple hits at the same site according to the formula used by Margoliash and Fitch (53) and by Dickerson (54).

$$m/100 = -\ln [1 - (n/100)]$$

The unit evolutionary period is then obtained by dividing *m* into the divergence time.

Comparison	Per cent change, <i>n</i>	Corrected per cent change, <i>m</i>	Average divergence time, millions of years	Unit evolutionary period, millions of years
All pairs of phasianoid birds.	6.2	6.4	40	6.2
Duck <i>versus</i> all phasianoid birds.	17.8	19.6	~100	~5.1
All birds <i>versus</i> man.	41.8	54.0	300	5.6

gence of the duck lineage from the phasianoid lineage is very uncertain, however. One hundred million years is perhaps a reasonable guess, as avian paleontologists feel that bird orders probably diverged from a common ancestor in Cretaceous times, 65 to 130 million years ago (51, 52). Since the duck lysozyme sequence differs from those of phasianoid birds by approximately 23 amino acid replacements, we calculate that lysozyme differences have accumulated at the rate of one substitution every 3 to 6 million years, in approximate agreement with the 5 million year figure derived above.

These figures may be converted, as recommended by Margoli-

ash and Fitch (53) and by Dickerson (54), into unit evolutionary periods, *i.e.* the time in millions of years required for a 1% difference in amino acid sequence to occur between two divergent lineages. The unit evolutionary period for phasianoid lysozymes is found to be about 6.2 million years (Table V). This figure is very close to that of 5.6 million years calculated by comparing human and bird lysozymes, which diverged 300 million years ago (*cf.* Reference 54). However, preliminary immunological data² as well as the comparison of the compositions of baboon and human milk lysozymes (55) suggest that the rate of lysozyme evolution among primates may be greater than among birds.

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² E. M. Prager and A. C. Wilson, unpublished observations.