AN INCONSISTENCY BETWEEN PROTEIN RESEMBLANCE AND TAXONOMIC RESEMBLANCE: IMMUNOLOGICAL COMPARISON OF DIVERSE PROTEINS FROM GALLINACEOUS BIRDS

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INTRODUCTION

The comparative study of proteins is being used with greater frequency to produce information concerning genetic differences among species. Such studies make it increasingly evident that there may not be a strict correlation between degree of genetic difference and degree of difference at the supramolecular or organismal level. This point can be illustrated by the results of comparing proteins from the Domestic Chicken (*Gallus gallus*) with those of the Domestic Turkey (*Meleagris gallopavo*) and the Ring-necked Pheasant (*Phasianus colchicus*). We present evidence from our studies at the protein level that appears to contrast with the evidence at the organismal level regarding the degree of resemblance among these species.

According to most ornithologists, the pheasant is more similar in anatomy and way of life to the chicken than is the turkey. This view is reflected in the system of bird classification which traditionally puts pheasants together with the chicken in the subfamily Phasianinae of the family Phasianidae, whereas the turkey is assigned to another family, Meleagrididae (Wetmore 1960). In the last two decades, however, evidence from hybridization experiments (Asmundson and Lorenz 1957; Olsen 1960; Poole 1963), chromosome studies (Stenius et al. 1962), electrophoretic patterns of protein mixtures (Sibley 1960, Sibley and Ahlquist 1972), immunological comparison of cell surfaces (Mainardi 1963), and the study of additional anatomical characters (Holman 1964, Hudson et al. 1966) has made it less certain that the turkey is as distant from the chicken as once thought. Consequently, Brodkorb (1964), Storer (1971), and Sibley and Ahlquist (1972) eliminated the family Meleagrididae and placed the turkey in the family Phasianidae. Nevertheless, these authors kept the turkey outside the subfamily Phasianinae. There seems to be unanimous agreement among ornithologists that the turkey shows less overall resemblance (at the supramolecular or organismal level) to the chicken than the pheasant does.

At the protein level, however, the similarities among these three species contrast with the organismal similarities noted above. It has been known for several years, from immunological comparison of lactate dehydrogenases (Wilson et al. 1963, Wilson and Kaplan 1964), ovalbumins (Wilson and Wachter 1969), and lysozymes (Wetter et al. 1953, Arnheim and Wilson 1967, Prager and Wilson 1971), that turkey proteins could be more similar to those of the chicken than were pheasant proteins. We have now compared additional chicken proteins with their turkey and pheasant counterparts. The comparisons were made by the micro-complement fixation test, which provides an estimate of the degree of amino acid sequence difference between related proteins (Prager and Wilson 1971, Prager et al. 1972, Wilson and Prager 1974, Champion et al. 1974). Altogether, nine proteins were investigated. Yet in no case was the turkey-chicken difference greater than the pheasant-chicken difference. This observation is at variance with expectations based upon the taxonomic system.

MATERIALS AND METHODS

Birds. Carcasses, sera, and eggs of Domestic Chickens (White Leghorns) and Domestic Turkeys were obtained commercially. Ring-necked Pheasant material was provided by the State Game Farm at Vacaville, California, and by the Massachusetts Audubon Society, Drumlin Farm, Lincoln, Massachusetts.

Proteins and antisera. Chicken proteins were purified and injected into New Zealand white rabbits in order to elicit production of antisera for use in micro-complement fixation experiments.

1. Serum albumin (MW = 69,000): This is the major protein in plasma and is composed of a single polypeptide chain. It is thought to be physiologically important because of its osmotic properties and its ability to bind certain small organic molecules (Wallace and Wilson 1972).

Chicken serum albumin was purified from a single White Leghorn rooster (Strain 99, Kimber Farms, Inc.,

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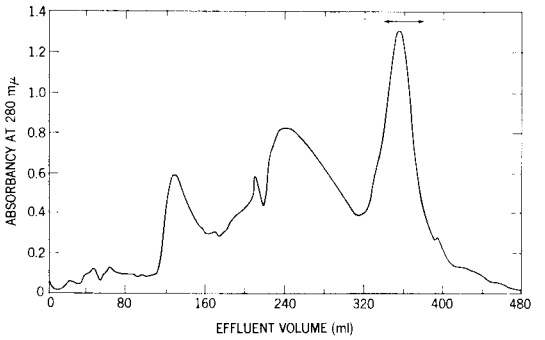

FIGURE 1. Initial purification of serum albumin by ion exchange chromatography of chicken serum on a column of DEAE-Sephadex (A-50) at pH 7.7. A continuous linear gradient of 0 to 0.3 M NaCl was used to elute the column. Fractions were collected and their absorbancy at 280 mμ measured. Fractions between the arrows were combined and designated DEAE pool.

Fremont, California). Blood was obtained by wing-vein bleeding with heparin (2%, v/v). The samples were cleared of blood cells by centrifuging at 13,000 × g for 20 min at 4°C and were then dialyzed overnight against a standard buffer (0.1 M Tris, adjusted to pH 7.7 with hydrochloric acid) at 4°C. Approximately 7 ml of the dialyzed sample were added to a column of DEAE-Sephadex (A-50) equilibrated with standard buffer. A continuous linear gradient of 0–0.3 M NaCl (250 ml 0.1 M Tris, pH 7.7 and 250 ml 0.1 M Tris, pH 7.7, plus 0.3 M NaCl) was used to elute the column. Four-milliliter fractions were collected and fractions 84–95 were pooled (arrows, fig. 1). The protein in the pooled fractions was precipitated with 90% saturated ammonium sulfate and applied to a Sephadex G-100 column. Fractions 17–33 were collected and pooled (2.4 ml fractions) (arrows, fig. 2A) and then precipitated with 90% saturated ammonium sulfate and dialyzed overnight against the standard buffer. The standard buffer had been used to elute the column. This material was then added to a second DEAE-Sephadex (A-50, pH 7.7) column which was eluted under the same conditions as the first except that the total volume used was 600 ml and 3-ml fractions were collected. Fractions 150–163 were combined (arrows, fig. 2B). The albumin present was precipitated, dialyzed against a potassium phosphate buffer (0.05 M phosphate, pH 5.3), and then added to an SE-Sephadex (C-50) column equilibrated with the phosphate buffer. The column was developed with the same phosphate buffer, and fractions 14–18 were collected (2.7-ml fractions) (arrows, fig. 2C). All columns were 2.2 × 35 cm. A single type of albumin was detected by horizontal starch gel electrophoresis at pH 6.0 and 8.6, in agreement with the findings of McIndoe (1962); and a single protein was also detected using immunoelectrophoresis, MC'F and the Ouchterlony technique. Various other properties of chicken albumin are described elsewhere (Wallace and Wilson 1972).

Antisera to chicken albumin were made in two rabbits. Rabbit D was initially injected with 0.3 mg of purified albumin in Freund's complete adjuvant and then at 5 weeks with 0.3 mg of albumin intravenously. Bleeding from the ear vein was done at 6 weeks (serum D3) and 7 weeks (serum D4). Rabbit B was initially injected with partially purified albumin in Freund's complete adjuvant and then with 0.5 mg of highly purified albumin, intravenously, at 7 weeks. Serum B1 obtained at 3 weeks was used in another study (Nakanishi et al. 1969), while serum B4 obtained at 11 weeks was used in the present study. The results given below are averages of the results obtained with D3, D4, and B4.

2. Ovalbumin (MW = 46,000): This is the principal protein of egg white and contains a single polypeptide chain to which an oligosaccharide is attached (Feeney and Allison 1969). Ovalbumin is believed to function primarily in the nourishment of the embryo (Sibley 1960). Chicken ovalbumin, 5× crystallized, was obtained from Pentex, Inc., and further purified by chromatography on carboxymethyl cellulose. Three rabbits (Nos. 92, 93, and 94) were immunized with the Pentex product and two rabbits (Nos. M19 and M20), with the more highly purified protein. The immunization procedure was that of Prager and Wilson (1971). The period of immunization was 4 months for the first group of rabbits and 7 months for the second group. The antisera were pooled in inverse proportion to their MC'F titers. The results given are those for the pool.

3. Ovotransferrin (MW = 76,000): This is an egg-white protein, formerly known as conalbumin, which binds iron and appears to consist of one long polypeptide chain to which two oligosaccharide chains are attached (Feeney and Allison 1969). Antisera to purified chicken ovotransferrin were prepared by immunization of rabbits for 3 months, as described by Prager et al. (1974). The cross reaction data given below are taken from Prager et al. (1974).

4. Lysozyme (E.C. 3.2.1.17; MW = 14,400): This egg-white protein is a bacteriolytic enzyme consisting of a single polypeptide chain, 129 amino acid residues long. The same protein also occurs in the lysosomes of chicken granulocytes (Hindenberg et al. 1974). Chicken, turkey, and pheasant lysozymes were prepared by Prager and Wilson (1971). Since antisera to each one of the three proteins were available, the results given here are the averages of the reciprocal tests reported by Prager and Wilson (1971).

5. Lactate dehydrogenases (E.C. 1.1.1.27; MW = 140,000): We worked with two different forms of this enzyme, M_4 and H_4 lactate dehydrogenase, each of which is composed of four identical subunits. Although these two proteins both catalyze the same reaction, they differ from each other considerably in chemical structure and are encoded by separate genes. They are intracellular enzymes that are vital components of the energy-yielding metabolic pathways of birds.

Chicken heart (H_4LDH) and muscle (M_4LDH) lactate dehydrogenases were prepared by the technique of Pesce et al. (1964). The anti-H_4 antisera used for this study were 175B5 and 202A4, which were obtained from two rabbits immunized by the method of Prager and Wilson (1971) for a period of 7 and 12 weeks, respectively. The results given are the averages of the values obtained with the two antisera. The anti-M_4 sera used were 7B5 and 11B6 obtained from two rabbits immunized for 9 and 16 weeks, respectively. The properties of some of these antisera have been described elsewhere (Wilson et al. 1964; Wilson and Kaplan 1964; Rose and Wilson 1966; Bailey and Wilson 1968).

6. Triose phosphate dehydrogenase (E.C. 1.2.1.12; MW = 140,000): Triose phosphate dehydrogenase (TPD) consists of four identical subunits and is a key enzyme in carbohydrate and energy metabolism. The immunological information obtained by Allison and

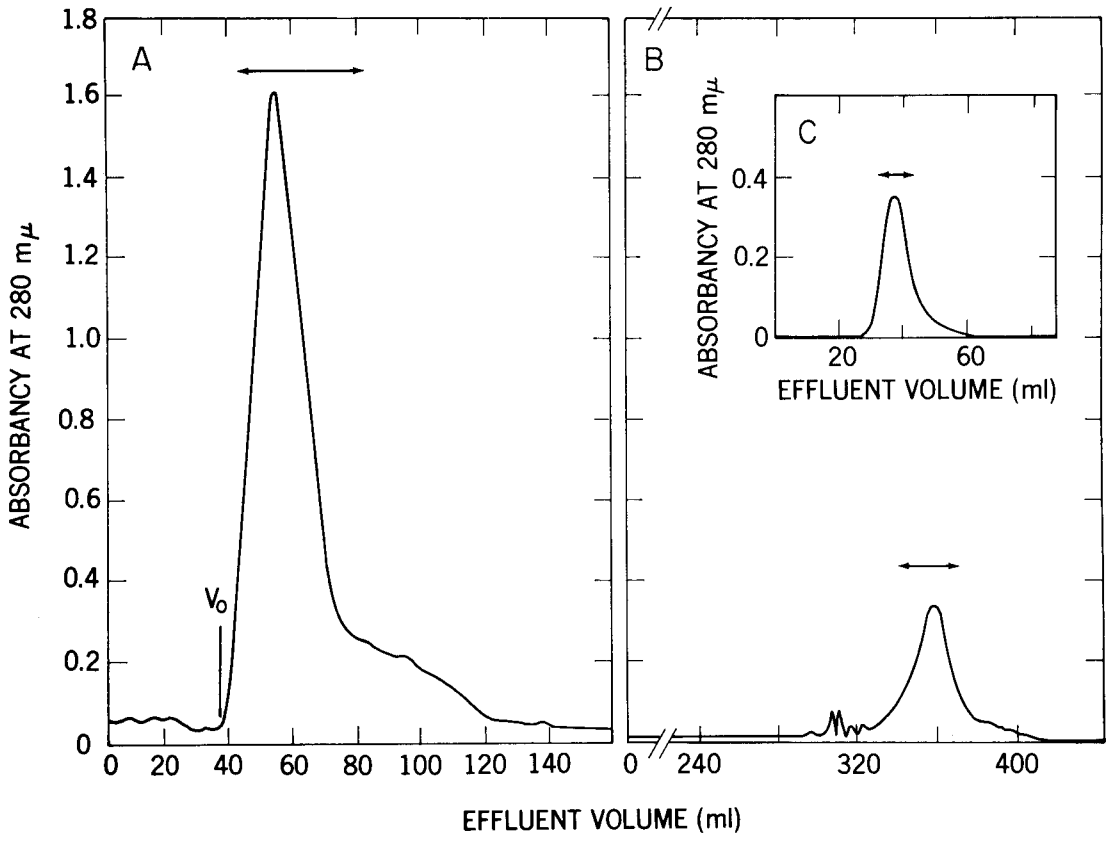

FIGURE 2. Further purification of chicken serum albumin in three successive column steps. (A) Gel filtration: The DEAE pool (see fig. 1) was concentrated and applied to a column of Sephadex G-100. The column was eluted with the pH 7.7 buffer. Fractions between the arrows were combined and designated Sephadex pool. (B) Ion exchange chromatography: the Sephadex pool was concentrated, dialyzed, and applied to a column of DEAE-Sephadex (A-50) at pH 7.7. Elution was performed with a linear salt gradient like that described in figure 1. Fractions between the arrows were combined and designated second DEAE pool. (C) Ion exchange chromatography: The second DEAE pool was concentrated, dialyzed, and applied to a column of SE-Sephadex (C-50), equilibrated with the pH 5.3 buffer. The column was eluted with the same buffer. Fractions between the arrows were combined and used for purity tests and immunization.

Kaplan (1964) and Wilson et al. (1964) was used in this study.

7. Malate dehydrogenases (E.C. 1.1.1.37; MW = 68,000): Mitochondrial malate dehydrogenase (M-MDH) is restricted to mitochondria where it functions in the Krebs cycle, whereas supernatant malate dehydrogenase (S-MDH) occurs in the cytoplasm where it may function in several metabolic pathways (Greville 1969). Although the two types of MDH are similar and are composed of two identical subunits with molecular weights of about 34,000, the M-MDH and S-MDH subunits differ considerably in amino acid composition and are coded by different genes (Greville 1969). Antisera to purified chicken M-MDH and S-MDH were prepared by Kitto and Kaplan (1966) and kindly supplied by Dr. Kitto.

Purity of immune systems. Antisera and immunizing antigens were tested for purity by most of the criteria exemplified by Arnheim and Wilson (1967). Although traces of antibodies to impurities in the immunizing antigens were found in most antisera, we demonstrated in every case that the complement fixation titer of such antibodies was far lower than the titer of antibodies to the major component. Hence, antibodies to impurities could not interfere with the cross-reaction tests described here.

Micro-complement fixation (MC'F). Experiments were performed according to the technique of Arnheim and Wilson (1967). For experiments involving the dehydrogenases, the complement fixation buffer contained bovine serum albumin, Fraction V (1 mg/ml). The reaction times at 5°C were standardized at between 18 and 21 hr, depending upon the protein.

The degrees of antigenic difference between chicken, turkey, and pheasant protein measured by these tests are expressed in immunological distance units. These units were defined by Prager and Wilson (1971), who also showed that immunological distance (y) was related to percentage difference in amino acid sequence (x) by the equation $y \simeq 5x$. Although such a relationship has been demonstrated most clearly for bird lysozymes, recent evidence indicates that the relationship also obtains for serum albumins, myoglobins, azurins, tryptophane synthetases (α-subunit) and hemoglobins (Champion, Prager, Sarich, and Wilson, unpubl. data).

RESULTS

The MC'F data presented in table 1 are impressive in their broad correspondence. Among the nine proteins tested, three (S-MDH, M-MDH,

TABLE 1. Immunological comparison of chicken proteins with turkey and pheasant proteins.

Protein	Immunological distance from chicken		
	Turkey	Pheasant	Difference
Secreted proteins			
Serum albumin	19	23	+4
Ovalbumin	39	82	+43
Ovotransferrin	27	32	+5
Lysozyme	18	28	+10
Intracellular proteins			
M_4 lactate dehydrogenase	4	6	+2
H_4 lactate dehydrogenase	22	43	+21
Triose phosphate dehydrogenase	0	0	0
Mitochondrial malate dehydrogenase	0	0	0
Supernatant malate dehydrogenase	0	0	0

and TPD) were found to be immunologically identical in all three species. Of the remaining six proteins, the chicken protein differed antigenically from the corresponding protein of both the turkey and pheasant. The chicken-turkey immunological distance did not differ significantly from the chicken-pheasant distance in three of the six cases (serum albumin, ovotransferrin, and M_4-LDH); immunological distance measurements are subject to an error of about ± 2 units. There were three cases (ovalbumin, lysozyme, and H_4-LDH) in which the chicken-turkey distance was definitely smaller than the chicken-pheasant distance. In no case was a pheasant protein more similar to its chicken counterpart than was the corresponding protein from the turkey.

It is noteworthy that secreted proteins, on the average, gave greater immunological distance values than did intracellular proteins (table 1). Evidently, secreted proteins evolve faster than intracellular proteins. The ovomucoid data of Wetter et al. (1953) are also consistent with this observation. The significance of this observation will be discussed elsewhere.

DISCUSSION

The close molecular resemblance between chicken and turkey demonstrated by the nine proteins in this study is also evident from data on six other proteins. Micro-complement fixation experiments showed that liver glutamate dehydrogenase, muscle aldolase, and the major component of hemoglobin may be identical in the two species (Wilson et al. 1964). Amino acid sequence studies showed that turkey glucagon (Sundby et al. 1972) and cytochrome *c* (Dayhoff 1972) were identical with their chicken counterparts. Finally, turkey insulin chains (A and B) were identical in amino acid sequence with their chicken counterparts (Dayhoff 1972). In none of the six cases has the corresponding pheasant protein been investigated by these techniques, although it has been shown that pheasant and chicken hemoglobins are identical electrophoretically (Brush 1967). However, as the turkey-chicken difference is zero in all these cases, the pheasant-chicken difference could not be smaller.

In addition to the 15 proteins already discussed, we wish to consider ovomucoid (Feeney and Allison 1969). A rabbit antiserum to chicken ovomucoid gave 18% and 23% cross-reactions, respectively, with turkey and pheasant ovomucoids (Wetter et al. 1953). As the antiserum came from a single rabbit immunized for only 5 weeks, we consider these two values indistinguishable.

A further, albeit weak, line of macromolecular evidence comes from annealing studies with the repeated fraction of DNA from these species. Although interpretation of experiments with repeated DNA sequences is uncertain, it is noteworthy that there is a slightly closer resemblance between turkey and chicken than between pheasant and chicken repeated DNA sequences (Schultz and Church 1971).

To sum up, the available DNA and protein evidence is consistent with the idea that the turkey-chicken genetic distance is just as small as the pheasant-chicken distance.

The macromolecular findings contrast with the taxonomic practice of placing the turkey outside the subfamily Phasianinae, which includes the chicken and pheasant. This contrast does not induce us to advocate any changes in current schemes of galliform classification to take account of the protein evidence. We consider it premature to discuss the systematic problems that would be raised if one were to assign the turkey to the subfamily Phasianinae. Our reasons follow.

First, it is not self-evident that protein evolution and organismal evolution proceed in

unison. There is increasingly good evidence that protein evolution and organismal evolution can proceed independently. Indeed, protein evolution may not be at the basis of evolutionary changes in anatomy and way of life. Genetic changes in regulatory systems may account for much of organismal evolution (Wilson et al. 1974). This kind of genetic change is not detectable by current methods of protein comparison. We therefore adopt a cautious attitude toward interpretation of the contrasting results obtained by the protein and organismal approaches. It is quite likely in our opinion that the turkey lineage has experienced little protein evolution but much organismal evolution. This could explain why turkey proteins are not much different from their chicken counterparts, whereas in anatomy and way of life, the turkey differs considerably from the chicken.

If, on the one hand, ornithologists decide to continue basing bird classification primarily on overall resemblance in anatomy and way of life, it may well be appropriate to maintain the turkey in a distinct subfamily or family. If, on the other hand, it is decided, as recommended by Cracraft (1972), to base classification purely on phylogenetic (i.e., cladistic) relationships, then future protein studies may require modification of the turkey's taxonomic position. There is now much evidence that protein studies can reveal approximate phylogenetic relationships among species. However, the protein results shown in table 1 are inadequate for construction of a phylogenetic tree because they were derived solely by use of antisera to proteins of one species (the chicken). In order to construct a phylogenetic tree from micro-complement fixation data, it is essential to use antisera prepared against proteins from several species, including in this case not only the turkey and pheasant but also other species of galliform birds. This work is now proceeding in our laboratories. The data obtained will hopefully permit a cladistic analysis of relationships among these species, similar to the cladistic analyses already made on primates (Sarich and Wilson 1967, Wilson and Sarich 1969), carnivores (Sarich 1969, 1973), and ranid frogs (Wallace et al. 1973). If the turkey lineage is found not to have experienced slow molecular evolution and to be cladistically as close to the chicken lineage as the pheasant lineage is, the possibility of changes in classification will then merit discussion.

SUMMARY

Rabbit antisera to nine proteins purified from the Domestic Chicken were tested for reactivity with their counterparts in the Domestic Turkey and Ring-necked Pheasant by means of the micro-complement fixation technique. The proteins were chosen for reasons of convenience as well as diversity in size, subunit composition, and biological function. In every case the turkey protein reacted as well as or better than the pheasant protein. This result is at variance with expectations based on the prevailing system of classification, which places the turkey outside the subfamily that includes the chicken and pheasant. Although this contrast between protein resemblance and taxonomic resemblance may indicate a need to revise the classification, it may indicate simply that evolutionary changes in anatomy and way of life can proceed independently of protein evolution.

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