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Slow Evolution of Transferrin and Albumin in Birds According to Micro-Complement Fixation Analysis*

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Summary. Rabbit antisera were prepared to purified ovotransferrin from chicken (order Galliformes) and red-winged blackbird (order Passeriformes) and to purified serum albumin from chicken and rhea (order Rheiformes). Quantitative micro-complement fixation was used to compare these proteins immunologically with those of representatives of all 27 orders of birds. The average interordinal immunological distances were 123 units for transferrin and 53 units for albumin.

Extensive intraordinal comparisons of transferrin among 51 species within the order Galliformes and 33 species within the order Passeriformes were also carried out. Values ranging from 0–75 immunological distance units were found within each order.

Rabbit antisera to purified alligator albumin were also prepared and shown to react with representatives of all 27 orders of birds, the average immunological distance being 166 units.

When the data presented here are considered in relation to the fossil record of birds, it appears that transferrin and albumin have evolved more slowly in birds than in other vertebrates. If prevailing interpretations of the fossil record are correct, transferrin has evolved 2–4 times as fast in mammals and snakes as in birds, while serum albumin has evolved about 3 times as fast in mammals, iguanids, crocodilians, and frogs as in birds. Published immunological and sequence comparisons of lysozyme and cytochrome *c* are also consistent with a slower rate of evolution in birds than in other vertebrates. The implications of a general slowdown in the evolution of bird proteins are discussed.

Key words: Transferrin — Albumin — Micro-Complement Fixation — Protein Evolution — Evolutionary Rates — Birds — Crocodilians.

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The following abbreviations are used in this work: RWBB = red-winged blackbird; OT = ovotransferrin; EW = egg white; TE = tissue extract; ND = not done; MY = million years.

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Introduction

Both immunological and sequencing techniques show that the rate of protein evolution has been surprisingly constant in many vertebrates (Dickerson, 1971; Sarich and Wilson, 1973). Extensive micro-complement fixation data indicate that serum albumin, for example, has consistently evolved at essentially the same rate in mammals (Sarich and Wilson, 1967a, b; Sarich, 1969, 1970), iguanids and crocodilians (Gorman, Wilson, and Nakanishi, 1971), and frogs (Wallace, Maxson, and Wilson, 1971; Wallace, King, and Wilson, 1973). Similar experiments suggest that transferrin has likewise changed at a rather constant rate in both mammals (Sarich, 1973) and snakes (Mao and Dessauer, 1971).

The micro-complement fixation technique has now been used to measure the rate of albumin and transferrin evolution in birds. Although avian transferrins have been compared extensively by various chemical methods (for example, Feeney, Anderson, Azari, Bennett, and Rhodes, 1960; Clark, Osuga, and Feeney, 1963; Feeney and Komatsu, 1966; Osuga and Feeney, 1968; Feeney and Allison, 1969), the present study is the first involving micro-complement fixation. According to the results presented here, based on measurements of almost 400 cross-reactions, albumin and transferrin have evolved more slowly in birds than in other land vertebrates. We also review published evidence concerning the rates of evolution of additional proteins in birds relative to those in other vertebrate classes.

Experimental Procedure

Materials

Bird egg whites, sera, and tissue extracts were obtained from the long-established collection of A. C. Wilson. Tissue extracts were generally made using 0.25 M sucrose or 0.1 M Tris-HCl, pH 7.5. A few serum samples were diluted in isotris buffer (the buffer employed in micro-complement fixation), heated for 20 min at 55–60°, and centrifuged to eliminate anti-complementarity (Champion, Prager, Wachter, and Wilson, 1974) before doing the micro-complement fixation experiments. Commercial chicken serum transferrin and albumin were obtained from Pentex and chicken ovotransferrin from Nutritional Biochemicals. Reptilian materials were obtained and processed as described (Gorman *et al.*, 1971; Nakanishi, 1971). All samples were stored frozen at −10°.

Methods

Protein Purification. The 4 avian proteins used as immunogens were purified as described below.

Ovotransferrin (formerly known as conalbumin) was prepared from the egg white of chicken (*Gallus gallus*) and red-winged blackbird (*Agelaius phoeniceus*) by DEAE-column chromatography (Osuga and Feeney, 1968). A few other transferrins—used only for micro-complement fixation—were similarly purified. Ovotransferrin and avian serum transferrin appear in all measurable respects to be the same protein molecule, except for slight differences in the carbohydrate attached (Azari and Feeney, 1958; Williams, 1962, 1968). In particular, ovotransferrin lacks sialic acid, while serum transferrin contains it (Williams, 1962, 1968).

Serum albumin was purified from the serum of the common rhea (*Rhea americana*) by gel electrophoresis on a polyacrylamide slab under the conditions described in Sarich (1972). The gels were run for 3 hrs at 150 V and 75 mA. Chicken albumin was purified by ion-exchange chromatography and gel filtration, as described in Nolan, Brush, Arnheim, and Wilson (1974).

Alligator (*Alligator mississippiensis*) serum albumin was purified as described (Gorman *et al.*, 1971; Nakanishi, 1971).

Antisera. Antisera were made by injecting each purified avian antigen except chicken albumin into 3 or 4 New Zealand white rabbits over a period of 3 months. The initial injection consisted of 0.5 mg of protein in Freund's supplemented complete adjuvant (Prager and Wilson, 1971a). At 5 weeks an intravenous injection of 0.1 mg protein was given, while at 12 weeks 3 intravenous injections on alternate days were given. Bleeding, by cardiac puncture, was one week after the final injection.

Preparation of antisera to chicken serum albumin has been described in detail (Nolan *et al.*, 1974); antisera 7D3, 7D4, and 7B4 were used in the present report. The production of antisera to alligator albumin has been described (Gorman *et al.*, 1971); the antisera used were from 6-week and 6-month bleedings.

The rabbits used are identified by immunogen and rabbit numbers as follows: chicken OT, 978, 983, and 984; blackbird OT, 997–1000; rhea albumin, 970–972; chicken albumin, B and D; and alligator albumin, 2072, 2073, and 2076. Antisera were heated, stored, and pooled as described (Prager and Wilson, 1971a), except that pools were not made with the antisera directed toward chicken and alligator serum albumin. In these two cases, average values determined with 2 or more individual sera were generally used.

Immunological Methods. Ouchterlony double diffusion, carried out as described by Arnheim and Wilson (1967) except that isotris buffer was used, served as a qualitative guide for the micro-complement fixation tests. Immunoelectrophoresis, done as described in Wallace and Wilson (1972), was used to assay the purity of the antisera obtained. Micro-complement fixation was conducted as described by Champion *et al.* (1974). For experiments involving anti-ovotransferrin, egg whites, sera, or tissue extracts (and in a few cases purified OT) were used as antigen sources; for studies with anti-serum-albumin, sera and tissue extracts served as antigen sources. The degree of antigenic difference in the micro-complement fixation test is given throughout in immunological distance units, which appear proportional to the degree of sequence difference between two homologous proteins (Prager and Wilson, 1971a, b; Wilson and Prager, 1974; Champion *et al.*, 1974).

Purity of Antisera. The antisera obtained were tested by techniques and criteria described in Arnheim and Wilson (1967). Immunoelectrophoresis was done with whole egg white or whole serum as the antigen source, as appropriate. A faint extra arc appeared with the antisera to chicken OT, while 2 additional arcs appeared with anti-RWBB OT. However, antibodies to antigens other than transferrin were so weak as not to interfere with the micro-complement fixation tests.

The antisera to rhea albumin showed one faint extra arc when tested against whole rhea serum and no extra arcs when tested against whole serum from 7 other avian species. The antisera to chicken albumin from rabbit D showed no contamination upon testing *vs.* whole chicken serum, while arcs to 2 contaminants appeared with the antiserum from rabbit B.

The anti-alligator sera were demonstrated to be pure by immunodiffusion, immunoelectrophoresis, and micro-complement fixation, as described (Gorman *et al.*, 1971).

Titers of the Antisera. The antiserum titers (for 75% peak micro-complement fixation with the homologous antigen) which we obtained toward the purified avian proteins were as follows, according to immunogen: chicken OT, 9600 (pool of 3 sera); blackbird OT, 3600 (pool of 4 sera); rhea albumin, 1000 (pool of 3 sera); and chicken albumin, 4000 (serum 7D3), 2600 (7D4), and 1100 (7B4). The anti-alligator albumin titers (1000–4000 for 50% peak fixation with the homologous antigen) have been described in detail (Gorman *et al.*, 1971).

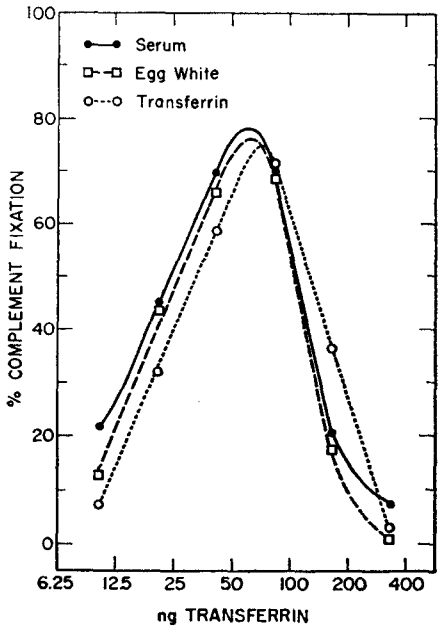

Fig. 1

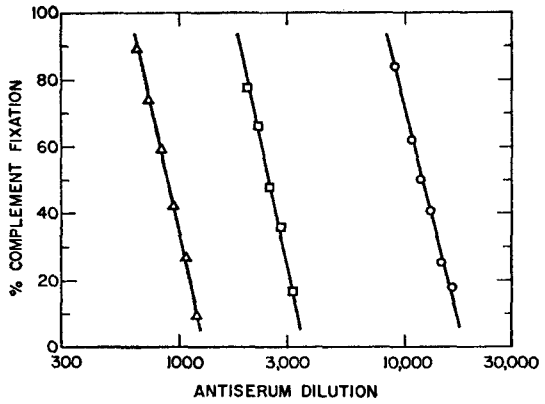

Fig. 2

Fig. 1. Micro-complement fixation by anti-chicken OT tested with chicken serum, egg white, and purified serum transferrin. Each tube contained 1 ml of a 1:9600 dilution of the antiserum pool. The nanograms of purified transferrin were determined from the absorbance at 280 mμ. The transferrin contents of serum and EW were assumed to be 1 and 25 mg/ml, respectively

Fig. 2. Dependence of complement fixation on antiserum concentration. The antiserum pool to chicken OT was tested at various concentrations for reactivity with the transferrins present in chicken serum (o), chachalaca egg white (□), and herring gull serum (△). The abscissa gives the antiserum dilutions (*i.e.*, reciprocal concentrations) used on a logarithmic scale, while the ordinate gives the maximum percentage of complement that is fixed at a given antiserum dilution. The equation $Y = m \log X + b$ relates antiserum concentration, X, to maximum percent complement fixed, Y. The slope, m, is the same within experimental error for the 3 antigens. The relative antiserum concentrations required to produce a given value of Y are 1 for chicken, 4.9 for chachalaca, and 13.2 for gull. The corresponding immunological distances, obtained by multiplying the logarithms of these numbers by 100, are 0, 69, and 112, respectively

Characterization of the Transferrin Immune System. Although albumin and lysozyme have been well characterized by comparative micro-complement fixation analysis (Sarich and Wilson, 1966; Arnheim and Wilson, 1967; Prager and Wilson, 1971a, b), transferrin has not. We considered it important to check on the validity of using micro-complement fixation to study transferrin evolution. As transferrin is a metalloglycoprotein, it is important to see whether variations in its carbohydrate or iron content affect its reactivity in micro-complement fixation tests.

Virtually identical complement fixation curves resulted when either whole chicken serum, whole chicken egg white, or purified chicken transferrin were tested with the

antiserum pool directed against purified chicken ovotransferrin, as illustrated in Fig. 1. Thus the presence of sialic acid in serum transferrin and its absence in OT appear not to influence the complement fixation results. We conclude that ovo- and serum transferrin can be used interchangeably in micro-complement fixation tests. This is consistent with genetic and biochemical evidence that these two proteins have identical primary structures (Williams, 1962, 1968).

We also compared the complement fixation behavior of transferrins differing markedly in iron content. The iron content of purified transferrins was measured by the absorption at 465–470 mμ (Feeney and Komatsu, 1966). Any residual iron in an aliquot of commercial chicken OT was removed by lowering the pH to about 4 with acetic acid and then gradually increasing the pH to 7 by dialysis at 4° against distilled water at pH 5–7. In an adaptation of published procedures (for example, Bates, Billups, and Saltman, 1967; Aisen, Leibman, Pinkowitz, and Pollack, 1973), another aliquot of OT, 3×10^{-4} M in binding sites, was brought to approximately 75% iron saturation by extensive dialysis at 4° against isotris buffer containing 2.5×10^{-3} M ferrous ammonium sulfate and citric acid (1:1) and sufficient bicarbonate to readjust the pH to 7. NaOH was used to achieve a final pH of 7.4. In subsequent micro-complement fixation tests with anti-chicken OT, the iron-saturated and apo-OT did not differ.

We conclude that species differences detected by microcomplement fixation tests are more likely to be in the protein part of transferrin than in carbohydrate composition or iron content.

Whenever a new immune system is used for studies of protein evolution, it is important to examine the dependence of the height of the complement fixation curve on antiserum concentration. If the dependence is similar for heterologous and homologous antigens, then it is valid to express degrees of antigenic difference as units of immunological distance (Champion *et al.*, 1974). That this requirement is fulfilled is shown in Fig. 2. The slopes of the 3 lines are the same within experimental error. The method of calculating the immunological distances between the homologous antigen and the 2 heterologous antigens is presented in the figure legend.

Results

Interordinal Distances

Transferrin. The average avian interordinal value measured was 123 immunological distance units. Table 1 presents these results in detail. As indicated at the end of the table, the average value measured with anti-chicken OT was 130 units, while with anti-blackbird OT it was 115 units. Some of these cross-reactions had previously been detected by less sensitive techniques (Wetter, Cohn, and Deutsch, 1953; Miller and Feeney, 1964).

Serum Albumin. The average interordinal immunological distance measured for avian serum albumin was 53 units, less than half the value for transferrin. The data, given in detail in Table 2, indicate an average value of 54 with anti-rhea albumin and of 51 with anti-chicken albumin.

Intraordinal Distances

Intraordinal surveys were carried out with anti-chicken sera to both transferrin and albumin; the results are presented in Table 3. The serum

Table 1. Immunological distances among avian transferrins[a]

Species	Immunological distance	
	Measured with anti-chicken OT	Measured with anti-RWBB OT
Struthioniformes		
Ostrich, *Struthio camelus*, EW	234	224
Rheiformes		
Rhea, *Rhea americana*, EW	117	115
Casuariiformes		
Cassowary, *Casuarius aruensis*, EW	152	151
Emu, *Dromiceius novae-hollandiae*, EW	151	136
Apterygiformes		
Kiwi, *Apteryx australis*, EW	139	137
Tinamiformes[b]		
Tinamou, *Crypturellus cinereus*, EW	114	171
Tinamou, *Eudromia elegans*, EW	126	180
Gaviiformes		
Loon, *Gavia immer*, TE	111	93
Podicipediformes[b]		
Grebe, *Aechmophorus occidentalis*, EW	97	85
Grebe, *Podiceps cristatus*, EW	99	87
Sphenisciformes		
Penguin, *Pygoscelis adeliae*, EW	132	77
Procellariiformes		
Albatross, *Diomedea immutabilis*, EW	137	93
Petrel, *Daption capensis*, EW	127	96
Pelecaniformes		
Snake bird, *Anhinga anhinga*, EW	124	118
Ciconiiformes		
Heron, *Ardea cocai*, EW	136	116
Flamingo, *Phoenicopterus ruber*, TE	143	~123
Anseriformes[b]		
Duck, *Anas platyrhynchos*, Serum	97	110
Goose, *Anser anser*, Serum	91	109
Falconiformes		
Buzzard, *Buteo lagopus*, EW	130	84
Falcon, *Falco sparveria*, EW	122	94
Galliformes		
Chachalaca, *Ortalis vetula*, EW	69	134
Megapode, *Leipoa ocellata*, EW	64	105
Megapode, *Megapodius freycinet*, Serum[c]	75	106
Grouse, *Bonasa umbellus*, EW	32	154
Guinea fowl, *Numida meleagris*, EW	53	157
Turkey, *Meleagris gallopavo*, Serum	27	192
Chicken, *Gallus gallus*, Serum[d]	0	139
Chicken, *Gallus gallus*, EW	0	136
Pheasant, *Lophura nycthemera*, EW	50	163
Pheasant, *Crossoptilon auritus*, EW	33	176
Pheasant, *Syrmaticus ellioti*, EW	43	176
Pheasant, *Syrmaticus reevesi*, EW	41	175
Pheasant, *Phasianus colchicus*, Serum	32	166

Table 1 (continued)

Species	Immunological distance	
	Measured with anti-chicken OT	Measured with anti-RWBB OT
Pheasant, *Chrysolophus amherstiae*, EW	33	177
Peafowl, *Pavo cristatus*, EW	33	152
Partridge, *Alectoris graeca*, EW	34	139
Francolin, *Francolinus clappertoni*, EW	27	136
Quail, *Coturnix coturnix*, EW	35	140
Quail, *Lophortyx californica*, EW	61	174
Quail, *Colinus virginianus*, EW	61	172
Gruiformes		
Rail, *Rallus limicola*, EW	138	84
Charadriiformes		
Sandpiper, *Actitis macularia*, EW	135	117
Gull, *Larus argentatus*, Serum	112	93
Murre, *Uria lomvia*, EW	114	100
Columbiformes[b]		
Dove, *Zenaidura macroura*, Serum	90	93
Pigeon, *Columba livia*, EW	112	76
Psittaciformes[b]		
Parrot, *Psittacus erithacus*, EW	84	88
Parakeet, *Melopsittacus undulatus*, EW	101	96
Cuculiformes		
Cuckoo, *Coccyzus americanus*, EW	193	99
Coucal, *Centropus bengalensis*, EW[e]	136	127
Touraco, *Tauraco hartlaubi*, TE	175	137
Strigiformes		
Owl, *Asio otus*, EW	135	98
Caprimulgiformes		
Goatsucker, *Caprimulgus parvulus*, EW	111	94
Apodiformes		
Swift, *Chaetura pelagica*, EW	100	94
Hummingbird, *Selasphorus sasin*, EW	106	96
Coliiformes		
Mousebird, *Colius* sp., TE	116	83
Trogoniformes		
Trogon, *Aphaloderma narina*, EW	163	177
Coraciiformes		
Kingfisher, *Megaceryle alcyon*, EW	133	110
Piciformes		
Woodpecker, *Colaptes auratus*, EW	148	122
Passeriformes		
Robin, *Turdus migratorius*, OT	135	8
Starling, *Sturnus vulgaris*, Serum	143	9
Finch, *Carpodacus mexicanus*, EW	133	8
Sparrow, *Zonotrichia leucophrys*, Serum[f]	130	4
RWBB, *Agelaius phoeniceus*, OT[d]	138	0
RWBB, *Agelaius phoeniceus*, EW	137	—3
Blackbird, *Agelaius tricolor*, Serum	136	—2
Blackbird, *Xanthocephalus xanthocephalus*, OT	139	5

Table 1 (continued)

Species	Immunological distance	
	Measured with anti-chicken OT	Measured with anti-RWBB OT
Blackbird, *Xanthocephalus xanthocephalus*, Serum	135	4
Blackbird, *Euphagus cyanocephalus*, OT	143	6
Cowbird, *Molothrus ater*, Serum	134	0
AVERAGE INTERORDINAL DISTANCE[g]	130	115
STANDARD DEVIATION	29	35

[a] Species are listed according to orders in the first column. The antigen sources for the micro-complement fixation tests are also given. For orders in which more than one representative has been tested, each species is a member of a different family within the order, except as indicated in Footnotes b–f below.
[b] Both species are members of the same family.
[c] Member of the same family as *Leipoa ocellata*.
[d] This and all remaining species within the order are members of the same family.
[e] Member of the same family as the cuckoo.
[f] Member of the same family as the finch.
[g] In cases in which more than one representative of an order was tested, the average for the entire order was computed. Thus each order received equal weight.

albumin values are about 60% as great as the transferrin values, consistent with similar observations in our interordinal surveys. The values within the family Phasianidae range almost as high as those outside the superfamily Phasianoidea.

The results of an intraordinal survey conducted with anti-blackbird sera to transferrin are given in Table 4. In contrast to the Galliformes, the Passeriformes exhibit a smaller range of values within the family (Icteridae) to which the species from which the immunogen was derived belongs.

Avian-Crocodilian Distances

A further test of the rate of bird protein evolution could be made because of the cross-reactivity of avian and crocodilian serum albumins. The average bird-alligator distance, as detailed in Table 5 with antisera to alligator albumin tested *vs.* all bird orders, is 166 units. The reciprocal test—anti-chicken serum albumin *vs.* crocodilians—gave values of 160 (alligator) and 175 (caiman), for an average value of 168 units, in excellent agreement with the 166 in Table 5.

Discussion

Rates of Transferrin and Albumin Evolution

From the average interordinal distances given above and available fossil data, one can estimate the average rates of transferrin and albumin

Table 2. Immunological distances among avian serum albumins[a]

Species	Immunological distance	
	Measured with anti-rhea serum albumin	Measured with anti-chicken serum albumin[b]
Struthioniformes		
Ostrich, *Struthio camelus*, TE	46	65
Rheiformes[c]		
Rhea, *Rhea americana*, Serum, TE	0	ND
Rhea, *Pterocnemia pennata*, TE	0	70
Casuariiformes		
Cassowary, *Casuarius bennetti*, TE	41	51
Emu, *Dromiceius novae-hollandiae*, Serum	43	59
Apterygiformes		
Kiwi, *Apteryx australis*, TE	17	49
Tinamiformes[d]		
Tinamou, *Crypturellus cinereus*, TE	43	58
Tinamou, *Crypturellus soui*, TE	ND	58
Tinamou, *Eudromia elegans*, TE	ND	61
Tinamou, *Nothura maculosa*, TE	ND	88
Tinamou, *Nothoprocta ambigua*, TE	ND	79
Gaviiformes		
Loon, *Gavia immer*, TE	39	40
Podicipediformes		
Grebe, *Podiceps auritus*, TE	58	53
Sphenisciformes[c]		
Penguin, *Spheniscus mendiculus*, TE	38	ND
Penguin, *Eudyptula minor*, TE	ND	45
Procellariiformes		
Albatross, *Diomedea nigripes*, TE	37	24
Pelecaniformes		
Pelican, *Pelecanus occidentalis*, TE	ND	45
Cormorant, *Phalacrocorax phalacrocorax*, TE	ND	65
Snake bird, *Anhinga anhinga*, TE	74	ND
Ciconiiformes		
Heron, *Ardea herodias*, TE	89	70
Stork, *Ibis leucocephalus*, TE	ND	56
Ibis, *Plegadis falcinellus*, TE	ND	51
Flamingo, *Phoenicopterus ruber*, TE	59	47
Anseriformes[e]		
Screamer, *Anhima cornuta*, TE	ND	45
Swan, *Cygnus olor*, TE	ND	36
Goose, *Nettapus coromandelianus*, TE	ND	37
Duck, *Aix galericulata*, TE	ND	30
Duck, *Anas platyrhynchos*, Serum, TE	58	30
Duck, *Anas laysanensis*, TE	ND	30
Duck, *Anas bahamensis*, TE	ND	30
Falconiformes		
Vulture, *Cathartes aura*, TE	43	ND
Osprey *Pandion haliaetus*, TE	ND	40

Table 2 (continued)

Species	Immunological distance	
	Measured with anti-rhea serum albumin	Measured with anti-chicken serum albumin[b]
Galliformes[d]		
Chicken, *Gallus gallus*, Serum, TE, albumin	53	0
Pheasant, *Phasianus colchicus*, Serum, TE	71	28
Peafowl, *Pavo cristatus*, Serum, TE	76	34
Gruiformes		
Crane, *Balearica regulorum*, TE	ND	47
Trumpeter, *Psophia crepitans*, Serum, TE	50	28
Finfoot, *Podica senegalensis*, TE	ND	43
Coot, *Fulica americana*, TE[f]	ND	49
Rail, *Rallus longirostris*, TE	ND	33
Rail, *Rallus limicola*, TE	ND	36
Rail, *Porzana carolina*, TE	ND	40
Gallinule, *Porphyrio porphyrio*, TE	ND	31
Charadriiformes		
Jacana, *Jacana spinosa*, TE	ND	30
Oystercatcher, *Haematopus ostralegus*, TE	ND	38
Plover, *Pluvialis dominica*, TE	ND	39
Lapwing, *Vanellus* (*Belonopterus*) *chilensis*, TE[g]	ND	39
Sandpiper, *Tringa ochropus*, TE	ND	28
Avocet, *Recurvirostra americana*, TE	ND	31
Phalarope, *Phalaropus fulicarius*, TE	ND	46
Thick-knee, *Burhinus capensis*, TE	ND	46
Pratincole, *Glareola maldivarum*, TE	ND	39
Seedsnipe, *Thinocorus rumicivorus*, TE	ND	40
Skua, *Catharacta antarctica*, TE	ND	36
Gull, *Larus argentatus*, Serum	39	29
Tern, *Larosterna inca*, TE[h]	ND	31
Dovekie, *Plautus alle*, TE	ND	31
Auk, *Alca torda*, TE[i]	ND	33
Columbiformes[e]		
Dove, *Zenaidura macroura*, Serum	58	ND
Pigeon, *Columba livia*, Serum	ND	52
Psittaciformes[e]		
Parrot, *Psittacus erithacus*, TE	38	ND
Parakeet, *Brotogeris jugularis*, TE	ND	45
Cuculiformes		
Cuckoo, *Coccyzus americanus*, TE	53	41
Touraco, *Tauraco hartlaubi*, TE	80	56
Strigiformes		
Owl, *Strix varia*, TE	42	39
Caprimulgiformes		
Poorwill, *Phalaenoptilus nuttalli*, TE	69	55
Apodiformes		
Swift, *Aëronautes saxatalis*, TE	64	39
Coliiformes		
Mousebird, *Colius* sp., TE	75	65
Trogoniformes		
Trogon, *Trogon melanus*, TE	50	40

Table 2 (continued)

Species	Immunological distance	
	Measured with anti-rhea serum albumin	Measured with anti-chicken serum albumin[b]
Coraciiformes		
Kingfisher, *Megaceryle alcyon*, TE	59	ND
Kingfisher, *Halcyon chloris*, TE[j]	ND	63
Hoopoe, *Upupa epops*, TE	ND	63
Piciformes		
Toucan, *Ramphastos toco*, TE	ND	70
Woodpecker, *Colaptes auratus*, TE	71	ND
Passeriformes		
Crow, *Corvus brachyrhynchos*, TE	ND	82
Starling, *Sturnus vulgaris*, Serum	ND	83
Blackbird, *Agelaius tricolor*, Serum	77	ND
Sparrow, *Zonotrichia leucophrys*, Serum, TE	ND	86
AVERAGE INTERORDINAL DISTANCE[k]	54	51
STANDARD DEVIATION	16	14

[a] Species are listed in the first column as in Table 1 (Footnote a). Footnotes c–j below indicate those cases in which more than one representative of a family within an order has been tested.
[b] Values obtained with antiserum 7D3 and some checked with antiserum 7B4.
[c] Both species are members of the same family.
[d] All species are members of the same family.
[e] All species except the screamer are members of the same family.
[f] This and all remaining species within the order are members of the same family.
[g] Member of the same family as the plover.
[h] Member of the same family as the gull.
[i] Member of the same family as the dovekie.
[j] Member of the same family as *Megaceryle alcyon*.
[k] Computed as described in Footnote g to Table 1.

evolution in birds. The bird fossil record is consistent with an average interordinal divergence time of about 100 million years (Fisher, 1967; Brodkorb, 1971; Prager, Arnheim, Mross, and Wilson, 1972; Cracraft, 1973). Hence the average rate of transferrin evolution in birds has been 123/100, *i.e.*, 1.2 units of immunological distance per MY. In contrast, the immunologically determined rate of mammalian transferrin evolution (Sarich, 1973) is 2.6 units/MY, about twice as great as in birds. In snakes (Mao and Dessauer, 1971) the rate of transferrin change appears to be nearly 4 times as great as in birds—4.7 units/MY.

An analogous interordinal calculation for serum albumin gives a rate of change of 0.53 unit/MY for birds. As summarized by Wallace and Wilson (1972), the immunologically determined rate of evolution for albumin in mammals, reptiles, and frogs has been about 1.7 units/MY, 3 times the rate in birds.

Table 3. Immunological distances within the order Galliformes relative to chicken[a]

Species	Immunological distance	
	Trans-ferrin	Serum albumin[b]
Cracoidea		
Chachalaca, *Ortalis vetula*, EW, serum	69	47
Curassow, *Crax globulosa*, TE	71	47
Megapodioidea		
Megapode, *Leipoa ocellata*, EW	64	ND
Megapode, *Megapodius freycinet*, Serum	75	59
Phasianoidea		
Tetraonidae		
Grouse, *Dendragapus obscurus*, TE	23	ND
Grouse, *Canachites canadensis*, TE	23	29
Ptarmigan, *Lagopus mutus*, EW	27	ND
Grouse, *Bonasa umbellus*, EW, TE	32	29
Prairie chicken, *Tympanuchus cupido*, TE	26	29
Phasianidae		
Phasianinae		
Chicken, *Gallus gallus*, Serum, albumin, TE	0	0
Chicken, *Gallus gallus*, EW	0	—
Jungle fowl, *Gallus gallus*, EW	3	ND
Jungle fowl, *Gallus sonnerati*, EW	0	ND
Jungle fowl, *Gallus varius*, TE	6	8
Pheasant, *Lophophorus impeyanus*, TE	33	18
Pheasant, *Lophura nycthemera*, EW	50	ND
Pheasant, *Lophura swinhoei*, EW	40	ND
Pheasant, *Lophura edwardsi*, TE	ND	27
Pheasant, *Crossoptilon auritus*, EW	33	ND
Pheasant, *Crossoptilon manchuricum*, TE	ND	27
Pheasant, *Syrmaticus ellioti*, EW	43	ND
Pheasant, *Syrmaticus reevesi*, EW	41	ND
Pheasant, *Phasianus colchicus*, Serum, TE	32	23
Pheasant, *Chrysolophus amherstiae*, TE	32	27
Pheasant, *Chrysolophus pictus*, TE	32	ND
Peacock pheasant, *Polyplectron chalcurum*, TE	32	21
Peacock pheasant, *Polyplectron germaini*, TE	32	ND
Peafowl, *Pavo cristatus*, EW, TE	33	34
Peafowl, *Pavo muticus*, TE	ND	34
Perdicinae		
Snow cock, *Tetraogallus himalayensis*, TE	25	ND
Partridge, *Ammoperdix griseogularis*, TE	23	ND
Partridge, *Alectoris graeca*, EW	34	ND
Partridge, *Alectoris barbara*, TE	32	ND
Francolin, *Francolinus clappertoni*, EW	27	ND
Francolin, *Francolinus erckeli*, TE	ND	30
Partridge, *Perdix perdix*, TE	36	22
Quail, *Coturnix coturnix*, EW, TE	35	18
Quail, *Coturnix delagorguei*, TE	41	23
Quail, *Coturnix (Excalfactoria) chinensis*, TE	ND	21
Quail, *Synoicus ypsilophorus*, TE	32	ND
Quail, *Perdicula asiatica*, TE	32	ND
Quail, *Perdicula erythrorhynchos*, TE	35	ND
Partridge, *Caloperdix oculea*, TE	50	ND

Table 3 (continued)

Species	Immunological distance	
	Trans-ferrin	Serum albumin[b]
Partridge, *Rollulus roulroul*, TE	50	ND
Partridge, *Ptilopachus petrosus*, TE	38	ND
Partridge, *Bambusicola thoracica*, TE	13	ND
Spurfowl, *Galloperdix spadicea*, TE	29	ND
Odontophorinae		
Quail, *Callipepla squamata*, EW	61	ND
Quail, *Lophortyx douglasii*, EW	67	ND
Quail, *Lophortyx gambelii*, EW	61	ND
Quail, *Lophortyx californica*, EW, serum	61	27
Quail, *Colinus virginianus*, EW, TE	61	32
Capueira, *Odontophorus capueira*, TE	56	24
Quail, *Cyrtonyx montezumae*, TE	73	21
Numididae		
Guinea fowl, *Numida meleagris*, EW	53	ND
Guinea fowl, *Acryllium vulturinum*, TE	56	26
Meleagrididae		
Turkey, *Meleagris gallopavo*, Serum, albumin, TE	27	19
Turkey, *Meleagris ocellata*, TE	27	20

[a] The species are listed according to superfamilies and families and for the Phasianidae according to subfamilies.
[b] The values given are averages for antisera 7D3 and 7D4.

Similar estimates emerge from our *intra*ordinal data taken in conjunction with the fossil record. Using 40 MY (Brodkorb, 1964; Prager *et al.*, 1972) as the time of divergence of the chicken lineage from that leading to other phasianoids and the average immunological distance values from Table 3 for phasianoids (except for those within the genus *Gallus*) *vs.* chicken, we obtain a rate of phasianoid transferrin and albumin evolution, respectively, of 0.97 and 0.63 unit/MY. Using 70 MY (Cracraft, 1973) as the time of divergence of the Megapodioidea from the other gallinaceous birds and the values from Table 3 for chicken *vs.* the megapodes, we obtain a similar rate for transferrin evolution, 1.0 unit/MY, and a somewhat greater rate (based on only one value) for albumin, 0.84 unit/MY. Intraordinal and interordinal rates are thus in reasonable agreement. Insufficient fossil data exist for estimation of the rate of transferrin evolution within the order Passeriformes.

Another line of evidence that avian serum albumin evolution has been slow is presented in Table 6. As the table shows, the rate of albumin evolution among crocodilians is 1.4 units per MY, yet the average rate since crocodilians and birds diverged is only 0.78 unit/MY. Thus a slowdown must have occurred in the avian lineage, lines c and d in Fig. 3, since the

Table 4. Immunological distances among transferrins within the order Passeriformes relative to RWBB[a]

Species	Immunological distance
Suborder Eurylami	
Eurylaimidae—Broadbill, *Calyptomena viridis*, TE	57
Suborder Tyranni	
Pipridae—Manakin, *Chiroxiphia linearis*, TE	49
Tyrannidae—Flycatcher, *Pitangus sulphuratus*, TE	45
Pittidae—Pitta, *Pitta brachyura*, TE	68
—Pitta, *Pitta reichenowi*, TE	65
Suborder Passeres	
Hirundinidae—Swallow, *Iiridoprocne bicolor*, TE	33
Corvidae—Crow, *Corvus brachyrhynchos*, Serum	38
Paradisaeidae—Bird of paradise, *Paradisaea rubra*, TE	33
Paridae—Chickadee, *Parus atricapillus*, TE	24
Sittidae—Nuthatch, *Sitta carolinensis*, TE	24
Certhiidae—Creeper, *Certhia familiaris*, TE	28
Chamaeidae—Wren-tit, *Chamaea fasciata*, TE	18
Troglodytidae—Wren, *Troglodytes aëdon*, TE	24
Mimidae—Thrasher, *Toxostoma rufum*, TE	13
Turdidae—Robin, *Turdus migratorius*, OT	8
Sylviidae—Kinglet, *Regulus satrapa*, TE	31
Muscicapidae—Bald crow, *Picarthartes gymnocephalus*, TE	22
Bombycillidae—Waxwing, *Bombycilla cedrorum*, TE	19
Sturnidae—Starling, *Sturnus vulgaris*, Serum	9
Nectariniidae—Sunbird, *Nectarinia famosa*, TE	37
Vireonidae—Vireo, *Vireo gilvus*, TE	23
Drepaniidae—Laysan finch, *Psittirostra cantans*, TE	16
Parulidae—Warbler, *Mniotilta varia*, TE	6
—Warbler, *Dendroica striata*, TE	10
—Ovenbird, *Seirus aurocapillus*, TE	6
Icteridae—RWBB, *Agelaius phoeniceus*, OT	0
—RWBB, *Agelaius phoeniceus*, EW	—3
—Blackbird, *Agelaius tricolor*, Serum	—2
—Blackbird, *Xanthocephalus xanthocephalus*, OT	5
—Blackbird, *Xanthocephalus xanthocephalus*, Serum	4
—Blackbird, *Euphagus cyanocephalus* (Homozygote)[b], OT	5
—Blackbird, *Euphagus cyanocephalus* (Heterozygote)[b], OT	7
—Cowbird, *Molothrus ater*, Serum	0
Fringillidae—Finch, *Carpodacus mexicanus*, EW	8
—Sparrow, *Zonotrichia leucophrys*, Serum	4
Ploceidae—Sparrow, *Passer domesticus*, TE	14

[a] The species are listed according to suborders and families. As in preceding tables, the antigen sources are also indicated.

[b] Described in Brush (1970).

Table 5. Immunological distances between alligator and avian serum albumins[a, b]

Species	Immunological distance
Struthioniformes—Ostrich, *Struthio camelus*, TE	171
Rheiformes—Rhea, *Pterocnemia pennata*, TE	148
Casuariiformes—Emu, *Dromiceus novae-hollandiae*, Serum	162
Apterygiformes—Kiwi, *Apteryx australis*, TE	156
Tinamiformes[c]—Tinamou, *Nothoprocta ambigua*, TE	168
—Tinamou, *Crypturellus cinereus*, TE	176
Gaviiformes—Loon, *Gavia immer*, TE	152
Podicipediformes—Grebe, *Podiceps auritus*, TE	157
Sphenisciformes—Penguin, *Eudyptula minor*, TE	154
Procellariiformes—Albatross, *Diomedea nigripes*, TE	157
—Petrel, *Oceanites oceanicus*, TE	168
Pelecaniformes—Pelican, *Pelecanus occidentalis*, TE	170
—Cormorant, *Phalacrocorax phalacrocorax*, TE	186
Ciconiiformes—Heron, *Ardea herodias*, TE	177
—Ibis, *Plegadis falcinellus*, TE	172
Anseriformes—Screamer, *Anhima cornuta*, TE	174
—Duck, *Anas platyrhynchos*, TE	164
Falconiformes—Vulture, *Cathartes aura*, TE	174
—Osprey, *Pandion haliaetus*, TE	169
Galliformes—Megapode, *Megapodius freycinet*, Serum	174
—Chicken, *Gallus gallus*, Serum	163
—Quail, *Coturnix coturnix*, TE[d]	161
—Turkey, *Meleagris ocellata*, TE	161
Gruiformes—Rail, *Rallus limicola*, TE	161
Charadriiformes—Phalarope, *Phalaropus fulicarius*, TE	158
—Dovekie, *Plautus alle*, TE	148
—Auk, *Alca torda*, TE[e]	149
Columbiformes—Pigeon, *Columba livia*, Serum	163
Psittaciformes—Parakeet, *Brotogeris jugularis*, TE	157
Cuculiformes—Cuckoo, *Coccyzus americanus*, TE	171
—Touraco, *Tauraco hartlaubi*, TE	173
Strigiformes[c]—Owl, *Otus asio*, TE	171
—Owl, *Strix varia*, TE	176
Caprimulgiformes—Poorwill, *Phalaenoptilus nuttalli*, TE	152
Apodiformes—Swift, *Aëronautes saxatalis*, TE	161
Coliiformes—Mousebird, *Colius* sp., TE	~183
Trogoniformes—Trogon, *Trogon melanus*, TE	166
Coraciiformes—Kingfisher, *Megaceryle alcyon*, TE	183
Piciformes—Woodpecker, *Centurus pucheranti*, TE	~180
Passeriformes—Bird of paradise, *Paradisaea rubra*, TE	~181
—Sparrow, *Zonotrichia leucophrys*, Serum	>185
AVERAGE IMMUNOLOGICAL DISTANCE[f]	166
STANDARD DEVIATION	11

Table 5 (continued)

[a] Measured with anti-alligator serum albumin. The immunological distance values are from Nakanishi (1971) and are generally the average values for the 6-week and 6-month bleedings of rabbit 2073.

[b] Species are listed as described in Footnote a to Table 1. Cases in which more than one representative of a family within an order has been tested are indicated in Footnotes c–e below.

[c] Both species are members of the same family.

[d] Member of the same family as the chicken.

[e] Member of the same family as the dovekie.

[f] Computed as described in Footnote g to Table 1.

Table 6. Immunological comparison of crocodilian and avian serum albumins measured with anti-alligator (*Alligator mississippiensis*) albumin

Species	Immunological distance	Divergence time, millions of years	Rate of albumin evolution, units per million years
Caiman[a]	70[b]	50[c]	1.4
Crocodile[d]	96[b]	70[c]	1.4
Birds—27 orders[e]	166	213[f]	0.78

[a] *Caiman sclerops*.

[b] From Gorman *et al.* (1971).

[c] Based on Romer (1966) and Sill (1968).

[d] *Crocodylus niloticus*.

[e] The average immunological distance calculated in Table 5 is given.

[f] An average of the value of 200 suggested by Walker (1972) and of 225 suggested by Ostrom (1973).

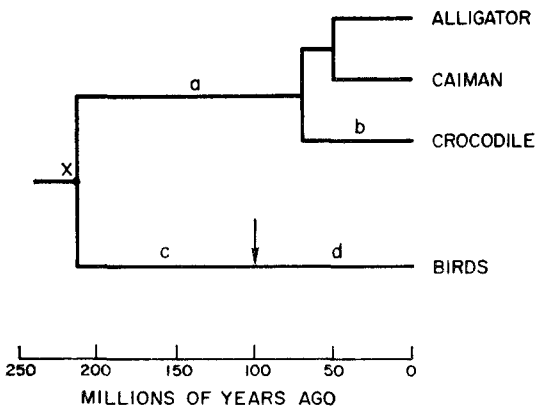

Fig. 3. Times of divergence among crocodilians and birds based on non-molecular evidence. The arrow indicates the average divergence time of bird orders from each other according to the fossil record (Fisher, 1967; Brodkorb, 1971; Prager *et al.*, 1972; Cracraft, 1973). The sources for the remaining divergence times are given in Table 6

divergence at point X of birds and crocodilians. It is of course possible that part of the overall slowdown is attributable to a slowdown along line a (as well as along lines c and d) in Fig. 3, prior to the divergence of crocodilians one from the other during the time represented by line b.

As the above evidence for slow protein evolution is strictly immunological, it might be asked whether the immunological approach could be misleading. We consider this unlikely because the ability of the microcomplement fixation method to estimate degree of sequence difference has now been tested on various proteins of known degree of sequence difference, including lysozymes (Prager and Wilson, 1971a; Prager *et al.*, 1972; Wilson and Prager, 1974), myoglobins, azurins, and tryptophan synthetases (Champion *et al.*, 1974). There are also unpublished data on albumins of known sequence difference[1]. In all cases, there is an approximately linear relationship between immunological distance (y) and percent sequence difference (x) of the form $y=kx$. The constant k is usually about 5, but it may be as high as 7 or 8 for some immune systems. The variations in k seem too small to account for the 3-fold difference in rates of immunological evolution between birds and other vertebrates.

Rates of Evolution of Other Proteins

The slowdown phenomenon is not unique to the albumins and transferrins of birds. Bird lysozymes have evolved more slowly than primate lysozymes by a factor of 2 as computed on the basis of available sequence data. Among Galliformes lysozymes (Wilson and Prager, 1974), an average of 10 amino acid replacements have occurred over 40 MY, which corresponds to an evolutionary rate of 0.25 replacement/MY; similarly, between the orders Galliformes and Anseriformes (Wilson and Prager, 1974), 25 replacements/100 MY, or 0.25 replacement/MY, have occurred. Baboon and human lysozymes differ by 14 amino acid substitutions (Hermann, Jollès, Buss, and Jollès, 1973). Since these species diverged only 30 MY ago (Simons, 1972), the rate of lysozyme change in primates has been 0.47 replacement/MY, twice the rate in birds. Immunological data (Hanke, Prager, and Wilson, 1973; Wilson and Prager, 1974) strongly support these relative rates based on sequence comparison.

Cytochromes *c* in birds (Nolan and Margoliash, 1968; Dayhoff, 1972; Augusteyn, 1973) have also evolved roughly 2 times as slowly as in mammals. As shown in Table 7, amino acid sequence information is available for birds of 5 orders. The average interordinal difference is 3.8 amino acid replacements, which corresponds to an average evolutionary rate of 0.038 replacement/MY. By contrast, the average interordinal difference for the cytochromes *c* of placental mammals is 6.1 replacements (Dayhoff, 1972). As the orders of placental mammals diverged about 75 MY ago (Lillegraven,

1 J. R. Brown, V. M. Sarich, A. Bennett, A. C. Wilson; Unpublished observations.

Table 7. Amino acid sequence differences among bird cytochromes *c*[a]

Species compared	Emu	Penguin	Duck	Chicken	Pigeon
Emu	0	2	3	2	6
Penguin		0	3	2	6
Duck			0	3	5
Chicken				0	6
Pigeon					0

[a] The number of amino acid sequence differences between any 2 avian cytochromes *c* is given. Sequence data are from Nolan and Margoliash (1968), Dayhoff (1972), and Augusteyn (1973).

1969), the average rate of cytochrome evolution in placental mammals—0.081 replacement/MY—is about 2 times that estimated for birds.

Sequence data are available for insulin (Dayhoff, 1972; Markussen and Sundby, 1973), glucagon (Dayhoff, 1972; Sundby, Frandsen, Thomsen, Kristiansen, and Brunfeldt, 1972), and hemoglobin (Dayhoff, 1972; Matsuda, Maita, Mizuno, and Ota, 1973) for a very small number of birds. Fragmentary micro-complement fixation comparisons are also available for hemoglobins (Wilson, Kaplan, Levine, Pesce, Reichlin, and Allison, 1964; Prager and Wilson, 1971b; Sarich, 1972), lactic dehydrogenases (Wilson and Kaplan, 1964; Wilson *et al.*, 1964; Salthe and Kaplan, 1966; Gorman *et al.*, 1971), and triose phosphate dehydrogenases (Wilson *et al.*, 1964) in both birds and other groups. However, the information on these proteins is in our opinion too incomplete to allow accurate determination of the relative rates of evolution of each of these proteins in birds and in other vertebrates. At present we can only say that in birds they have evolved at rates equal to or slightly less than those of their homologues in other vertebrates.

Implications of the Evolutionary Slowdown

One should consider the possibility that the slowdown is an artifact caused by use of an erroneous time scale. Could the time scale assumed for interordinal and intraordinal evolution of birds be too long by a factor of two or three? It would be very hard to reconcile the bird fossil record with this possibility. Furthermore, the comparisons of crocodilian and bird albumins, which also reveal a slowdown, make no assumptions concerning the times of avian inter- or intraordinal splitting. The crocodilian-bird approach to rate measurement assumes only a time of divergence of the lineage leading to birds from that leading to crocodilians. There is very strong fossil evidence that this time was at least 200 MY ago (Walker, 1972; Ostrom, 1973). Hence, the slowdown is tentatively regarded as real.

Stochastic models do not predict that a particular group of organisms would experience a general evolutionary slowdown at the molecular level. It is therefore intriguing that those proteins for which the best comparative information exists, namely transferrin, albumin, lysozyme, and cyto-

chrome *c*, all seem to have evolved more slowly in birds than in other vertebrates. We should now address ourselves to the problem of ascertaining whether additional proteins also exhibit this phenomenon. It is also important to find out whether the slowdown is evident at the DNA level. Annealing techniques (Kohne, 1970) would enable this to be done. If the slowdown applies to most of the bird genome, the mechanism involved would be worth investigating. A slowdown could, in principle, be due to a low rate of either the occurrence or the fixation of mutations. The factor responsible would presumably be one that is peculiar to birds, *e.g.*, high body temperature or low DNA content (Sparrow, Price, and Underbrink, 1972). Mutation rates may be lower in organisms with low DNA content per cell (Abrahamson, Bender, Conger, and Wolff, 1973).

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Note Added in Proof. Our finding that iron-saturated and apo-OT behave identically in quantitative micro-complement fixation tests is consistent with the findings of Faust and Tengerdy (1971). They report that these two molecules act the same in tests measuring primary antibody binding as well as in the quantitative precipitin technique, although they differ in behavior in the secondary phase of the antigen-antibody reaction [Tengerdy, C., Azari, P., Tengerdy, R. P.: Nature **211**, 203 (1966); Faust, C. H., Jr., Tengerdy, R. P.: Immunochemistry **8**, 211 (1971)].

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