A SEROLOGICAL ANALYSIS OF SOME ANATID CLASSIFICATIONS

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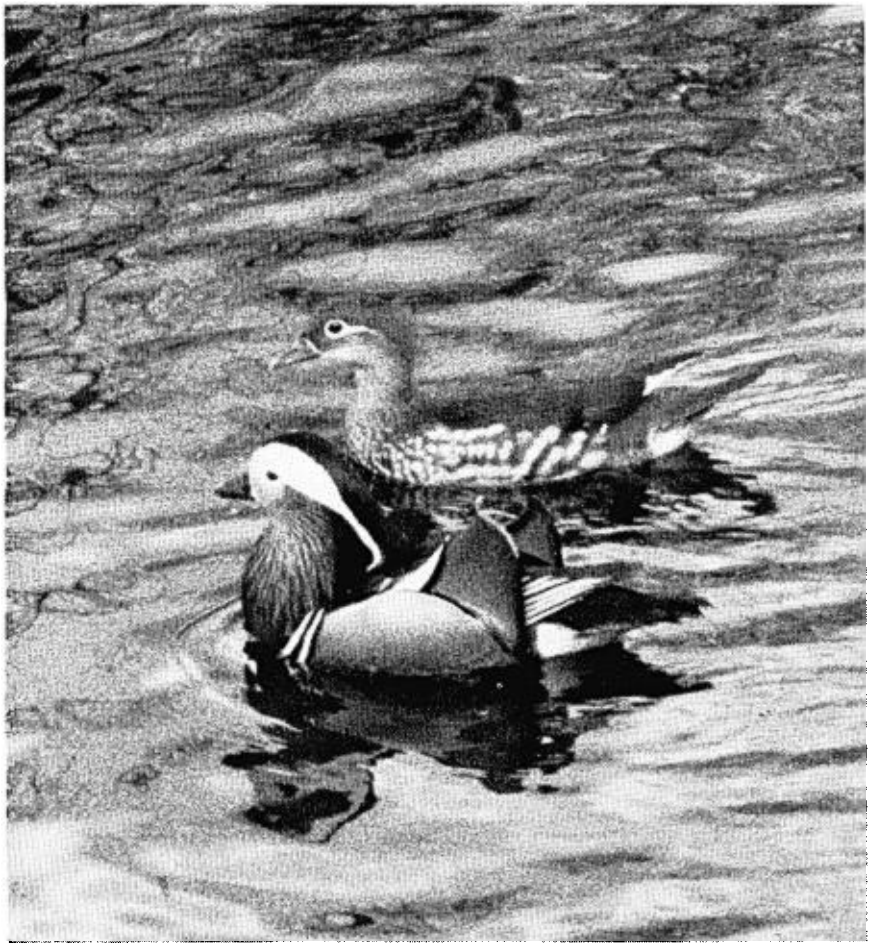
THE recent classification of the ducks by Delacour and Mayr (1945) is in some respects in conflict with the older systems of Phillips (1922–1926) and of Peters (1931). Delacour and Mayr instituted, on the basis of ecological preferences, behavior, and the plumage of the downy young, a new tribe, Cairinini (the Perching Ducks), for the Spur-winged Goose (*Plectropterus gambiensis*), Muscovy (*Cairina moschata*), Wood (*Aix sponsa*) and Mandarin (*A. galericulata*) ducks, among others. The older classifications had associated the Wood and the Mandarin ducks with the river ducks of the genus *Anas*. The genus *Aix* had been relegated to that position in the revisions of Salvadori's subfamily Plectropterinae (1895) by Phillips and Peters. In addition, Peters removed the Muscovy Duck from the same subfamily and placed it within the Anatinae, diametrically opposed to the position of the Wood and Mandarin ducks. Between the genera *Cairina* and *Aix* in Peters' Checklist, 13 genera were interposed, including the type genus *Anas*.

The close relationship of *Cairina moschata* and *Aix sponsa*, as repostulated by Delacour and Mayr, was supported by Yamashina (1952), who compared the shape, number, and relative lengths of the anatid macrochromosomes in gametogenesis. *Cairina moschata* and *Aix sponsa* in all features resembled one another more closely than either resembled any other of the river ducks. However, because of similarity in shape and number of chromosomes, these two genera were retained in the supergenus *Anas* as a closely related group. A divergence in the number of macrochromosomes in the Mandarin Duck, with the associated changes in relative lengths and shape, caused Yamashina to isolate this duck in the monotypic supergenus *Dendronessa*. The supergenus is a taxonomic category roughly equivalent to the tribe. This action was contrary to the grouping of the Wood and the Mandarin ducks in the single genus *Aix* as done by Delacour and Mayr and other workers.

These conflicts concerning the validity of the Muscovy–Wood Duck complex, of the relationships *inter se* and also with the tribe Anatini have been resolved by the present study. A serological analysis was proposed to validate the most natural classification of the ducks, and incorporated in the methods were four major innovations in the field of systematic serology. As antigens, three separate protein systems (ovalbumin of the egg, serum albumin and serum gamma globulin of the blood) were analyzed to correct *inter se* for variation in any one system. Secondly, each protein used had been purified to provide a single chemically-defined antigen, instead of the usual moiety of antigens used in other investigations. Thirdly, the precipitated antigen–



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antibody complexes were graded directly on the basis of the precipitated protein, and the per cent relatedness expressed as a fraction of the homologous cross-reaction's precipitate. Lastly, the usual method of cross-reaction estimation was supplemented by absorption titrations to determine the amounts of antigens shared in common by any two birds.

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Materials and Methods

The proteins that constituted the central material of this investigation were obtained from whole eggs and blood samples taken from six anatid representatives: *Anser anser* (Embden and Toulouse varieties of the Common Goose), *Anas platyrhynchos* (Mallard), *A. platyrhynchos* (domestic Pekin), *Aix sponsa* (Wood Duck), *A. galericulata* (Mandarin Duck), *Cairina moschata* (Muscovy).

The ovalbumin protein of the egg-white was prepared in crystalline form by the method of Kekwith and Cannan (1936); to reduce amounts of contaminating proteins, the ovalbumins were redissolved and recrystallized four times. Ovalbumins prepared by this method have been demonstrated by Heidelberger and Kendall (1935) to behave as single antigens in their reactions with rabbit antisera.

Crystalline serum albumin was prepared and recrystallized four times by the method of Adair and Robinson (1930), with the sole modification of substitution of sodium sulfate for ammonium sulfate as the salting agent. The serum gamma globulins were removed from the globulins first salted out from the serum, and then purified by Kendall's (1937) technique. This latter protein was not crystallized, but after proper purification was maintained in aqueous solution at 0°C. for two weeks, after which it was prepared anew from fresh serum. This precaution helped to reduce experimental error due to the use of auto-denatured proteins.

Standard 0.1 per cent protein solutions were prepared, checked with micro-Kjeldahl nitrogen determinations, used for antibody formation, and later for the titrations of those antisera in the cross-reactions and in the absorption tests. When inciting the production of antibodies, the rabbits were injected with these standardized solutions in the following manner:

First week	First day	5 cc. intravenous
	Second day	1 cc. subcutaneous
	Third day	1 cc. subcutaneous
Second week	First day	3 cc. intraperitoneal
	Second day	1 cc. subcutaneous
	Third day	1 cc. subcutaneous
Third week	First day	3 cc. intraperitoneal
	Second day	1 cc. subcutaneous
	Third day	1 cc. subcutaneous

The rabbits were then bled on the first day of the fourth week, and after separation of the serum from the clot, the antibody-containing antiserum was sterile-filtered and stored at 0°C. until used.

Relationships between these anatid species were determined first by the extent of cross-reactions of the anti-protein antisera in the presence of the homologous and the heterologous antigen solutions. This was effected by the addition of 0.1 cc. of the specific anti-protein antiserum to 0.5 cc. of that antigen solution, the latter in serial dilution from 1:1000 to 1:1024000. The reaction volume was adjusted then to 1.0 cc. with the addition of 0.4 cc. of 0.9 per cent saline; the test antigen–antibody solutions then were incubated at 4°C. for 24 hours, and at the end of that time, the precipitated antigen–antibody complexes were graded. The grading was performed visually, with six grades: 0, ±, +, ++, +++, ++++. These grades represent actual reaction levels, since they were checked frequently with micro-Kjeldahl nitrogen determinations on the precipitated antigen–antibody proteins after washing; the range values for these respective grades overlapped very little.

Grades	*Limits*	*Repetitions*	*Average*
±	0.002–0.030 mgm. N	69	0.016
+	0.030–0.070	77	0.049
++	0.066–0.116	91	0.092
+++	0.110–0.160	49	0.138
++++	0.156–0.304	37	0.236

The reaction values so determined were plotted with respect to antigen dilution. The areas under the heterologous reaction curves were expressed as a per cent relative to the area subtended by the homologous reaction curve. In the first three tables, the results of the cross-reactions of each antiserum with the heterologous and homologous antigens are given. In order, they represent the crystalline ovalbumin, the crystalline serum albumin and the serum gamma globulin systems.

To determine more precisely the common stocks of antigens, the specific anti-protein antisera were treated with the heterologous proteins individually to remove all antibodies reactant with the absorbing protein.

To 3.0 cc. of antiserum in a 15 cc. centrifuge tube was added 3.0 cc. of undiluted stock antigen solution (1:1000) and the mixture was incubated at 4°C. for 24 hours. After centrifugation, the supernatant serum was poured off into a sterile container, and

the pellet of precipitated antigen–antibody complex was discarded. This volume of 1:1 diluted antiserum was sufficient to use for simultaneous experiments with the homologous and the five heterologous proteins.

The residual antibodies of the diluted antiserum could react then with the homologous protein when added; the extent of the reaction (total amount of precipitate formed) was taken as an inverse measure of antigenic similarity of the protein pair (the absorbing protein and the titrating protein) being examined. If the homologous titration after absorption were high, few antibodies had been removed and hence there was very little similarity in the antigenic groups of the two proteins. Conversely, if the homologous titration following absorption produced only a slight precipitate, there was a high degree of antigen similarity denoted.

Tables 4 to 7 present the results of the anti-ovalbumin antisera after absorption by four of the five heterologous proteins, the data from the Mallard ovalbumin absorption experiments being omitted because of the extreme similarity to the protein absorption results for the Pekin. In each of these series, the homologous ovalbumin absorption with titrations provided experimental controls, as well as controls to indicate the complete nature of the absorption by each heterologous antigen.

Results and Discussion

In Table 1, the results are given from the reactions of the antisera prepared with the crystalline ovalbumins, when titrated with the homologous and the heterologous ovalbumin solutions. The precipitations by the anti-Embden Goose antisera gave similar results for the Pekin and the Mallard ducks (87 per cent, 81 per cent), and for the Wood and the Mandarin ducks (79 per cent, 79 per cent) also. Ovalbumins of the latter species pair reacted less strongly than the former species set, but each member of the set with an equivalent amount of antibodies. The position of the value for the Muscovy precipitation was intermediate, between those values for the *Anas* group, and for the Wood and the Mandarin ducks.

Antisera against the Pekin and the Mallard ovalbumins gave complementary values when titrated with the other's antigen; these results were utilized to determine the extent of variation within a single species, since the Pekin and the Mallard ducks are both members of the species *Anas platyrhynchos*.

Table 1

Cross-Reactions of Crystalline Ovalbumins and the Anti-Ovalbumin Antisera

	Titrating Antigens					
Antisera	Embden	Pekin	Mallard	Wood	Mandarin	Muscovy
a-Embden	100	87	81	79	79	85
a-Pekin	80	100	89	67	66	69
a-Mallard	87	94	100	81	81	99
a-Wood	75	87	87	100	88	94
a-Mandarin	57	62	64	97	100	93
a-Muscovy	54	70	77	98	91	100

In the anti-Pekin and anti-Mallard antisera the values determined for the precipitation of the Wood and Mandarin ovalbumins were almost identical (67 per cent, 66 per cent; 81 per cent, 81 per cent). As with the anti-Embden Goose antisera, the Muscovy protein gave a value intermediate between the homologous and the Wood–Mandarin set of values.

Similarly, the reaction of the Muscovy ovalbumin with the antisera against the ovalbumins of the Wood and the Mandarin ducks showed an intermediate position between the Wood–Mandarin set of values and those of the Pekin–Mallard set. The complementary cross-reactions of the Wood and the Mandarin ducks demonstrated, as did the complementary titrations of the Pekin–Mallard set, an equal amount of serological divergence. When these antisera were titrated with the Pekin and Mallard ovalbumins, the reaction values were almost identical (87 per cent, 87 per cent; 62 per cent, 64 per cent) for these antigens, and greater in extent than those for the goose antigen (75 per cent, 57 per cent).

The antisera produced with the Muscovy ovalbumin yielded the greatest heterologous response with the Wood (98 per cent) and the Mandarin (91 per cent) ducks, appreciably less with the Pekin (70 per cent) and the Mallard (77 per cent), and least with the ovalbumin antigen of the goose (54 per cent).

On the basis of these data, it would be justified to represent the linear order of relationship as follows:

Embden Goose	Pekin–Mallard	Muscovy	Wood–Mandarin
(*Anser anser*)	(*Anas platyrhynchos*)	(*Cairina moschata*)	(*Aix sponsa–A. galericulata*)

In Table 2, the values are given for the cross-reactions of the serum albumin proteins with those antisera formed against the serum albumins of the Toulouse Goose, the Pekin, the Mallard and the Muscovy ducks. Here, the heterologous cross-reaction values for the anti-Toulouse antisera indicated a greater degree of divergence in the component parts of the serum albumin

TABLE 2

CROSS-REACTIONS OF THE CRYSTALLINE SERUM ALBUMINS AND THE ANTI-SERUM ALBUMIN ANTISERA

	Titrating Antigens					
Antisera	Toulouse	Pekin	Mallard	Wood	Mandarin	Muscovy
a-Toulouse	100	57	36	36	36	27
a-Pekin	48	100	81	53	55	57
a-Mallard	46	86	100	70	46	63
a-Muscovy	49	59	53	84	86	100

antigen complex than was indicated for the ovalbumin antigen in Table 1. This was true for all of the subsequent reactions in this table, the average heterologous reaction being smaller than that in the anti-ovalbumin system.

Use of the Toulouse Goose serum proteins, and the summation of those results with those from the Embden Goose ovalbumin can be defended by the data of these experiments. The goose, *Anser anser*, has been incorporated in this analysis as a reference point, far removed from the Cairinini and the Anatini. In addition, it has been determined that subspecific differences in heterologous cross-reactions tend to diminish or vanish as the taxonomic source of the inciting antigens for the testing antisera becomes more remote. Since in all non-goose antisera, the source of the inciting antigens was sufficiently distant, the author felt justified in the assumption of virtual identity in serum proteins for the two domestic strains of *Anser anser*. However, this holds only in those tests made with an antiserum developed against a bird of distant origin.

The serum albumins of the Pekin and the Mallard ducks still reacted as closely related sets of antigens with all antisera. The Wood and the Mandarin serum albumins behaved similarly. In addition, the Muscovy protein, in two reactions out of three with different types of heterologous antisera, gave an intermediate value between those of the Pekin–Mallard set and the Wood–Mandarin set. The data derived from these tests do support the postulated order of relatedness.

The cross-reaction values of the serum gamma globulins and the respective antisera are given in Table 3. These results showed a further confirmation for the high degree of similarity in protein structure of the two forms of *Anas platyrhynchos*. With the anti-Pekin antisera, the Mallard serum gamma globulin gave the greatest heterologous response; with the anti-Muscovy antisera, the values derived from the heterologous titrations by the Pekin and Mallard proteins were identical.

The amounts of precipitates yielded by the heterologous proteins of the Wood and the Mandarin ducks repeated, for the third time, the pattern of two distinct forms with an apparent underlying similarity in antigenic structure. The serum gamma globulin of the Muscovy duck gave evidence of closest association with the Wood–Mandarin set, both in the homologous and in the heterologous antisera tests. However, its position relative to the genera *Anas* and *Aix* was given by only one heterologous antiserum type; these results did corroborate the earlier findings of the anti-ovalbumin and the anti-serum albumin antisera.

The following set of tables (4–7) for the reactions of the anti-ovalbumin antisera allowed confirmation in part of the relationship order as postulated on the basis of the first three data sets. Each anti-ovalbumin antiserum was treated with a heterologous antigen to remove all antibodies common to the

TABLE 3

CROSS-REACTIONS OF PURIFIED SERUM GAMMA GLOBULINS AND THE ANTI-SERUM GAMMA GLOBULIN ANTISERA

Antisera	Titrating Antigens: Toulouse	Pekin	Mallard	Wood	Mandarin	Muscovy
a-Pekin	42	100	96	44	45	49
a-Muscovy	29	54	54	80	79	100

inciting and the absorbing antigens. The residual antibodies, detected by a homologous titration following this procedure, were used as an inverse measure of the degree of divergence that the protein-synthesizing systems had undergone.

In the Table 4, the titration with the homologous Pekin ovalbumin after absorption with each antigen showed that there was no reaction with the Pekin-absorbed antisera—a control, indicating complete removal of all antibodies. The minimal positive value (21 per cent) was given by those sera absorbed by the Mallard ovalbumin, which was able to remove most of the antibodies, but not all. The Wood and the Mandarin proteins were less efficient absorbers, and the Muscovy-absorbed antisera contained the greatest amount of residual antibodies.

Hence, the order of relationship is repostulated as:

Anser *Anas* *Aix* *Cairina*

following from this set of data. With all absorbing antigens, the Pekin and Mallard set of ovalbumins reacted in equal amounts. Similarly, it was true also for the reactions of the Wood and the Mandarin ducks, when their oval-

TABLE 4

ANTI-PEKIN DUCK OVALBUMIN ANTISERA ABSORBED WITH ALL OVALBUMIN ANTIGENS AND TITRATED WITH ALL ANTIGENS[1]

Antigens Titrating	Cross Reactions	Absorbing Antigens: Embden	Pekin	Mallard	Wood	Mandarin	Muscovy
Embden	80	0–1	0	12	16	36	41
Pekin	100	25	0	21	40	46	58
Mallard	89	23	0	0–1	35	44	53
Wood	67	10	0	6	0	9	29
Mandarin	66	7	0	6	8	0	28
Muscovy	69	8	0	5	6	8	0

[1]In this table, the antisera are those made against the Pekin ovalbumin. The values for the cross-reactions are given on the extreme left; each column to the right gives the reaction value for that antiserum after absorption by the antigen at the top of the column, with the titrating antigen on the left side.

TABLE 5

ANTI-WOOD DUCK OVALBUMIN ANTISERA ABSORBED WITH ALL OVALBUMIN ANTIGENS AND TITRATED WITH ALL ANTIGENS

Antigens Titrating	Cross Reactions	Absorbing Antigens					
		Embden	Pekin	Mallard	Wood	Mandarin	Muscovy
Embden	75	0	1	1	0	0	0
Pekin	87	1	0	0	0	0	4
Mallard	87	7	1	0	0	0	7
Wood	100	34	34	39	0	9	23
Mandarin	88	26	21	26	0	0	15
Muscovy	94	15	9	17	0	0	0

bumins were used as the titrating antigens.

A further elaboration of the fundamental similarity of Wood and Mandarin ducks in their ovalbumin antigen complexes is found in Tables 5 and 6, where the reactions of the absorbed antisera for these ovalbumins are presented. When the complementary absorptions were made (that is, anti-Wood Duck antisera absorbed with Mandarin ovalbumin, and anti-Mandarin antisera absorbed with Wood Duck ovalbumin), the only antibodies remaining in solution were those specific for the inciting antigen, and in all other titrations no heterologous antigen reactions were observed. Similarly, after absorption by the other heterologous ovalbumins, the Wood Duck and Mandarin ovalbumin titrations followed identically with a slight increment for the homologous always found. This had been noted in the reactions of the Pekin–Mallard set of anti-ovalbumin antisera.

The position of the Muscovy ovalbumin as representative of the species showed that, in respect to the total sum of different antigens, this duck was the farthest from the *Anas* type. Its ovalbumin possessed the least share of common antigens with *Anas*, but formed a close-knit complex with the Wood

TABLE 6

ANTI-MANDARIN DUCK OVALBUMIN ANTISERA ABSORBED WITH ALL OVALBUMIN ANTIGENS AND TITRATED WITH ALL ANTIGENS

Antigens Titrating	Cross Reactions	Absorbing Antigens					
		Embden	Pekin	Mallard	Wood	Mandarin	Muscovy
Embden	57	0	11	14	0	0	0–1
Pekin	62	14	0	0–1	0	0	4
Mallard	64	16	0–1	0	0	0	4
Wood	97	29	30	29	0	0	16
Mandarin	100	37	39	39	13	0	33
Muscovy	93	24	26	25	0	0	0

TABLE 7

ANTI-MUSCOVY DUCK OVALBUMIN ANTISERA ABSORBED WITH ALL OVALBUMIN ANTIGENS AND TITRATED WITH ALL ANTIGENS

Antigens Titrating	Cross Reactions	Absorbing Antigens					
		Embden	Pekin	Mallard	Wood	Mandarin	Muscovy
Embden	54	0	13	14	17	20	0
Pekin	70	34	0	3	18	31	0
Mallard	77	41	8	0	24	28	0
Wood	98	55	29	35	1	20	0
Mandarin	91	59	25	29	7	1	0
Muscovy	100	75	47	60	61	53	0

and the Mandarin ducks. This was not only true in its cross-reactions, but also in the step-wise reductions shown by each of the three species of Cairinini as the different absorbing antigens were used (*cf.* Tables 5 to 7). With the anti-serum albumin and the anti-serum gamma globulin antisera after absorption, the same order of relationship was found.

In the cross-reactions of all three protein types, the antigens of the Muscovy Duck appeared to be intermediate in reaction extent between those of *Anas* and *Aix*. However, the absorption titrations clearly demonstrated that the antigenic complex of *Cairina* was qualitatively more differentiated from that of the *Anas* group, than were the antigen complexes of *Aix* when compared to *Anas*. The findings of the absorption tests inferred that a greater number of bio-synthetic changes had occurred within the phyletic line of the Muscovy Duck, and that the usual cross-reaction techniques were not sufficiently sensitive to demonstrate these changes. In addition, the data revealed a relationship between *Aix* and *Cairina*, not so close as within the subspecific set of the Pekin and the Mallard ducks, or as within the species set of the Wood Duck and the Mandarin, but closer than that relationship of *Anas* and *Cairina* or *Anas* and *Aix*.

Hence, the validity of the original concept of the perching ducks as a natural taxonomic category, as presented by Salvadori and modified by Delacour and Mayr, has been strengthened by these findings from the application of serological techniques. Those revisions by Phillips, Peters, and Yamashina do not reflect the most natural placement of the ducks within the limits of the Cairinini. The latter classifications of the Anatidae showed, with particular reference to the perching ducks, the use of nonobjective criteria or undue reliance on a single taxonomic feature.

SUMMARY

Analysis of recent classifications of the ducks by refined serological techniques has validated the conclusions of Delacour and Mayr, in their uniting

the genera *Cairina* and *Aix* in the single tribe Cairinini. To better the existing standard serological techniques, three separate protein systems were studied through the cross-reactions of the purified crystalline proteins with the testing antisera. Comparison of the final data derived from each set of experiments with those of the other sets served to reduce error possibly resulting from chance variations in single systems. As a check upon the cross-reaction as a true measure of species relatedness, absorption of the antisera by different heterologous antigens in the same protein species, followed by homologous titration, gave a measure of the common stocks of antigens shared. In certain cases, notably those of the Muscovy Duck proteins, the cross-reaction values indicated a much closer relationship than existed on the basis of common antigen stocks. Hence, the sole use of cross-reaction data requires that caution be exercised in their interpretation.

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