A COMPARATIVE ELECTROPHORETIC STUDY OF AVIAN PLASMA PROTEINS

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The search for data indicative of taxonomic relationships has led to the study of many characters. In addition to comparative morphology some systematists have utilized ecology, behavior, serology, and biochemistry as clues to the degrees of genetic relatedness among organisms. Because protein molecules are primary gene products it is logical to assume that comparisons among homologous proteins from different organisms should provide useful systematic data. The rationale behind this approach to systematics has been discussed by several authors including Sibley (1960, 1962, 1964, 1965, 1967), Zuckerkandl and Pauling (1965), and Dessauer (1969).

The plasma proteins are an obvious choice for investigation because they are easy to collect and because a great deal is known about their properties and functions. Plasma is the fluid portion of blood in which the blood cells are suspended. It is a complex mixture of proteins, carbohydrates, lipids, steroids, and free ions whose composition varies with sex, age, starvation, season, etc. (Moore 1948; Clegg et al. 1951; Vanstone et al. 1955; Dessauer and Fox 1956; Saito 1957b). The protein constituents of plasma, while often quantitatively variable, usually show a high degree of qualitative species specificity when examined by any standard biochemical technique (Morris and Courtice 1955; Zweig and Crenshaw 1957; Drilhon et al. 1958; Woods et al. 1958; Sulya et al. 1961). Some of these studies were based upon serum, the fluid portion of the plasma which is extruded from a blood clot. Plasma thus contains the blood proteins involved in clotting while serum lacks them.

Most of the research on plasma proteins has dealt with human material although there has been a great deal of work on other mammalian species and the domestic fowl, *Gallus gallus*.

The major protein components of plasma are albumin, the alpha-, beta-, and gamma-globulins, and various subfractions thereof. The nomenclature of the various components is usually determined by their electrophoretic mobilities with reference to normal human plasma. Thus, albumin is the fastest fraction, alpha-globulin the next fastest and gamma-globulin the slowest. The identification of plasma proteins under different conditions can be difficult (Espinosa 1961; Beaton et al. 1961). Up to 70 different proteins have been found in normal human plasma (Dessauer and Fox 1964) while Baker et al. (1966) found 40 electrophoretic bands in pheasant serum, 14 of which were identifiable. The review by Putnam (1960) provides information on the chemical composition of plasma.

In paper electrophoresis at *p*H 8.6 the fastest component in human plasma is albumin. It has a molecular weight of about 69,000 and is isoelectric at *p*H 4.7 (Phelps and Putnam 1960). It may be assumed that the values for other mammals and for birds are similar (Phelps and Putnam 1960:169). Plasma albumin has the same chemical properties as alpha livetin of egg yolk (Williams 1962a) but it has no known specific biological functions (Foster 1960).

There are several alpha-globulins, which presumably have different functions. The best known are the hemoglobin-binding haptoglobins and the copper-binding ceruloplasmin. Human haptoglobin has a molecular weight of 85,000 and it is isoelectric at *p*H 4.1, while ceruloplasmin has a molecular weight of 151,000 and is isoelectric at *p*H 4.4 (Phelps and Putnam 1960).

There are several discrete beta-globulins, most of which have unknown functions. The best known of these are the transferrins. Transferrin, also called siderophilin, is an iron-binding protein with a molecular weight of approximately 90,000; it is isoelectric at *p*H 5.9 (Phelps and Putnam 1960). It has been found that the protein moiety of the transferrin molecule is identical to that of the conalbumin of egg white. They differ only in their

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carbohydrate prosthetic groups (Williams 1962b). Fibrinogen, the blood-clotting protein, is another beta-globulin. It is a large molecule with a molecular weight of 341,000 and an isoelectric point of *p*H 5.8 (Phelps and Putnam 1960).

The electrophoretically slowest group of plasma proteins are the gamma-globulins. However, they frequently have electrophoretic mobilities which overlap those of the beta-globulins, making identification difficult. Unlike the alpha- and beta-globulins which break up into discrete fractions, gamma-globulins migrate in a broad area over a given range of the electrophoretogram regardless of the technique used. Biochemical tests confirm that gamma-globulin is a series of different molecules isoelectric in a continuous range from *p*H 5.7 to 7.3 and having molecular weights near 170,000 (Phelps and Putnam 1960; Porter 1960). Most of the antibody properties of plasma are associated with the gamma-globulin fraction. Considering the wide range of antibodies carried by most animals, the difficulty of chemically defining the gamma-globulins can be understood.

All of these fractions have been found in many other animals but they constitute varying proportions of the total plasma and often have different electrophoretic properties. Few tests of identity have been made for homologous proteins in different animals, with the exception of the transferrins and haptoglobins.

ONTOGENY OF THE PLASMA PROTEINS

Amin (1961) found that, with a few exceptions, all of the plasma proteins present in the adult fowl can be found in 10-day old embryos and that the changes occurring during this period are primarily quantitative, not qualitative. Brandt et al. (1951), Heim and Schechtman (1954) and Vanstone et al. (1955) all noticed a gradual increase in total plasma protein during embryogenesis, with albumin maintaining a constant percentage composition. The alpha- and beta-globulins either increase or decrease in relative concentrations before hatching (Brandt et al. 1951; Heim and Schechtman 1954). All investigators agree that gamma-globulin does not appear until the last stages of incubation or the first few days after hatching. Vanstone et al. (1955) also reported a prealbumin in the 14-day old embryo which had disappeared by the seventh day after hatching.

Brandt et al. (1951) reported the appearance of an additional, fast-moving component in the serum of the laying hen which could be duplicated in capons by the injection of diethylstilbestrol, a female sex hormone. Moore (1948), Clegg et al. (1951), and Common et al. (1953) concluded that the electrophoretically observed differences in plasmas were due to conjugated lipids. That these differences are not due to the hormones themselves is indicated by the difficulty in finding estradiol and estrone in fowl plasmas (O'Grady and Heald 1965).

Accompanying the appearance of these seemingly new fractions is a marked increase in total protein. Shortly after ovulation has terminated, total protein returns to its former level and the components assume their pre-ovulatory pattern. Similar findings have been made for snakes (Dessauer et al. 1956; Dessauer and Fox 1959), the lamprey eel (Thomas and McCrimmon 1964), the coho salmon (Vanstone and Ho 1961), and certain elasmobranchs (Saito 1957c).

In addition, studies by Saito (1957b), Sibley and Johnsgard (1959) and Thomas and McCrimmon (1964) have shown that starved animals have decreased levels of albumin, and that disease can change the amounts of other fractions. The sources of non-genetic variability have been discussed by Engle and Woods (1960) and Petermann (1960).

COMPARATIVE STUDIES OF PLASMA PROTEINS

There have been many comparative studies of plasma proteins and most of them fall into two classes: studies of individual variation within a single species, and surveys of several, generally unrelated, species. Much of this work has been reviewed by Engle and Woods (1960).

Comparative studies on fish have revealed that fresh-water teleosts have a high concentration of albumin, whereas salt-water teleosts have abundant prealbumin (Saito 1957a). Elasmobranchs apparently lack albumin but have relatively large amounts of the gamma-globulins (Saito 1957a; Drilhon and Fine 1959; Drilhon 1959). A similar condition has been found in gars, some clupeids, and lung fishes (Sulya et al. 1961; DeBont and Paulus 1964). In the cyclostome *Myxine*, transferrin is the dominant component (Manwell 1963). All investigators agree that there is a high degree of pattern-specificity for each species examined (Drilhon et al. 1958), but Creyssel et al. (1964) found a transferrin polymorphism in the carp (*Cyprinus carpio*).

Species specificity also seems to be the rule in amphibians and reptiles (Crenshaw 1962; Dessauer and Fox 1964) and Hebard (1964) maintains that much can be learned about species formation in closely related forms (see also

Cei and Bertini 1961; Fox et al. 1961; Dessauer et al. 1962; Crenshaw 1965; Coates and Twitty 1967; Coates 1967). However, serum patterns seem to be of questionable value as a source of information about relationships among the higher categories (Dessauer et al. 1962; Hebard 1964; Dessauer 1966).

Baker and Hanson (1966) examined 11 closely related species of geese and found no basis for distinguishing among the species although there were small differences separating the two genera involved. Other investigators have found polymorphisms in the albumin of domestic fowl (McIndoe 1962), and turkeys (Quinteros et al. 1964), in the transferrins of chickens (Ogden et al. 1962), and in the prealbumins of pheasants (Baker et al. 1966). All of these polymorphisms appear to be simple Mendelian differences. Beckman et al. (1963) found similar conditions in several species pairs and their natural hybrids. Bush (1967) examined the developmental and populational variation in several enzymes and other blood proteins in the House Sparrow (*Passer domesticus*).

General comparative surveys of mammalian plasmas, such as those by Blumberg et al. (1960), Auernheimer et al. (1960), Lawrence et al. (1960), Riou et al. (1962), and Johnson (1968) as well as the earlier reports reviewed by Engle and Woods (1960), also indicate a high degree of species specificity with the greatest differences being found between the most distantly related species. Comparisons among many individuals of the same species almost invariably reveal polymorphisms in either transferrins (Goodman and Poulik 1961; Buettner-Janusch et al. 1961; Ashton and Ferguson 1963; Cooper and Sharman 1964), or haptoglobins (Blumberg 1960). Fried (1963) has found that albumins from different species may have similar electrophoretic mobilities but very different amino acid compositions. Van Tets and Cowan (1966) found a large amount of confusing polymorphism in the serum proteins of deer (*Odocoileus*).

METHODS AND MATERIALS

Specimens of blood were collected using a 10 percent (w/v) solution of ethylenediaminetetraacetate (EDTA) as an anticoagulant. The plasma was isolated by centrifugation and frozen immediately.

Electrophoretic comparisons, using vertical starch-gel equipment, were made with a discontinuous system of buffers. The starch-gel buffer, *p*H 7.95, was composed of 0.046 *M* tris(hydroxymethyl)aminomethane, 0.007 *M* citric acid, 0.005 *M* lithium hydroxide, and 0.019 *M* boric acid. The bridge buffer, *p*H 7.98, was composed of 0.05 *M* lithium hydroxide and 0.19 *M* boric acid (Smithies 1955, 1959; Ashton and Braden 1961; Ferguson and Wallace 1961).

During this study we have examined the starch-gel electrophoretic patterns of the plasma proteins of approximately 1500 specimens and 450 species of birds. These represent 106 of the 171 living families and 25 of the 27 living orders of birds recognized by Wetmore (1960). This sample should be adequate to determine whether or not the electrophoretic patterns of avian plasma proteins vary in a manner which is consistent with and indicative of taxonomic groupings. To be useful in the determination of genetic relationships, a taxonomic character must show consistent similarities among closely related species and consistent differences between species less closely related. In the present study many species from many families were sampled repeatedly to determine the amount of variation present in taxa of different categorical levels.

The evaluation of the similarities and differences among the patterns was accomplished by inspection, giving due allowance for variation in the concentration of samples and the effects of denaturation, polymorphism, and other taxonomically non-significant variability. The presence and absence of stained bands or areas and the relative mobilities of the major components were given principal weight in the assessment of the patterns.

RESULTS

Our comparisons reveal a high degree of individual variation superimposed upon what seems to be a common basic pattern. In other words, once we eliminate the taxonomically

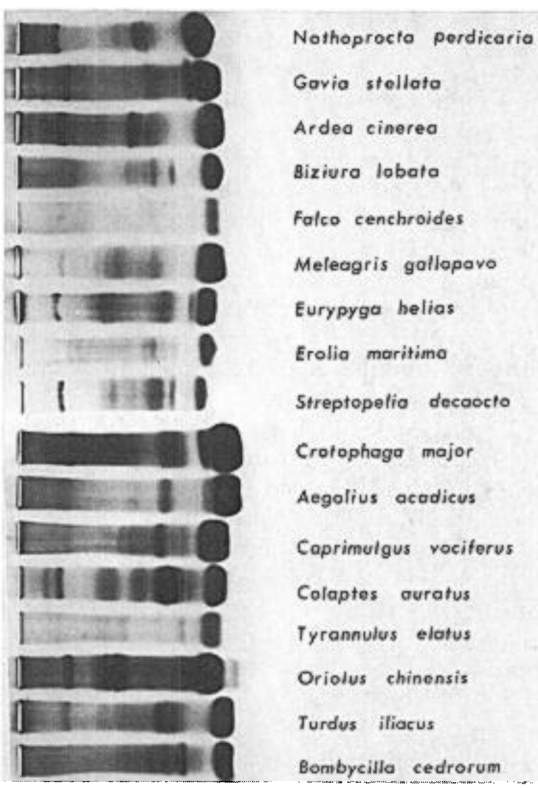

FIGURE 1. Starch gel electrophoresis patterns of the plasma proteins of 17 species of birds representing 14 orders.

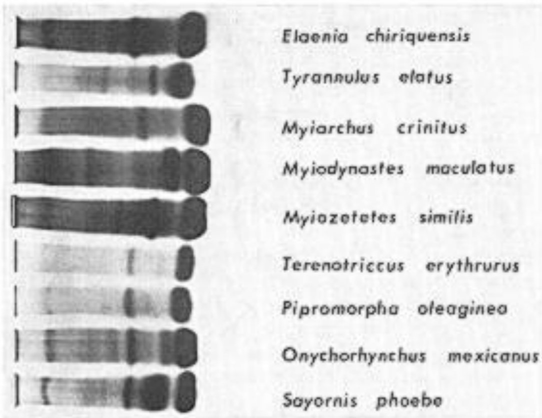

FIGURE 2. Electrophoretic patterns of the plasma proteins of nine species of the family Tyrannidae.

non-significant variation from the patterns, we find that they are remarkably similar in all birds. The patterns in figure 1 were chosen to illustrate this point. Thus we do not find a taxonomically useful situation involving consistent similarities among closely related groups and consistent differences between distantly related ones. Instead we find that the patterns of different species in a single, apparently natural family, are sometimes quite different from one another, and that similar patterns recur in species representing different families. For example, figure 2 illustrates the considerable variation among the plasma protein patterns of 9 species of tyrant flycatchers (Tyrannidae). The egg-white protein patterns (Sibley, in press) and hemoglobins (Sibley et al., in prep.) of the Tyrannidae do not show such variation. On the contrary, these two protein systems indicate that the Tyrannidae are not only related to one another but to other families of New World non-oscine birds as well. The superficial variation in the plasma protein patterns of the Tyrannidae in figure 2 is almost as great as in the entire Class Aves.

It thus appears that the variations in the electrophoretic patterns of avian plasma proteins do not correspond to the accepted families and orders. Because many other characters, including the egg-white proteins and hemoglobins, do show such correspondence in the majority of cases we are compelled to conclude that the observed qualitative and quantitative variation is due to taxonomically non-significant factors. Among these, presumably, are age, sex, condition of health, degree of starvation, reproductive condition, and perhaps others. The transferrins and ovalbumins, as noted above, are prone to exhibit genetic polymorphisms which add to the variability.

From the electrophoretic patterns alone it is not possible to determine the degree of similarity among the plasma proteins of different groups of birds but it is our impression that there is a basic pattern composed of the main components. Some of these vary in quantity in different individuals but all are present in normal patterns. This basic pattern seems to be about the same in all groups of birds. At least we cannot detect consistent similarities and differences which are readily correlated with taxonomic groupings. The variation superimposed upon this basic pattern mainly reflects the physiological functions of the plasma proteins and is consonant with the known or suspected roles which these substances play in the life of the organism.

Our findings agree with those of Dessauer et al. (1962), Hebard (1964), Dessauer (1966) and Voris (1967) based on the serum proteins of reptiles and amphibians, those of Johnson (1968) on mammalian serum proteins, and those of Kartashev et al. (1966) on avian serum proteins. They parallel the results obtained by Sibley and Brush (1967) from a study of avian eye lens proteins.

Although it seems clear that the electrophoretic patterns of avian plasma proteins are unlikely to provide evidence of the relationships of the higher categories of birds, it should not be assumed that they are devoid of useful taxonomic information. Among others, Coates (1967) and Coates and Twitty (1967) have shown that intraspecific and possibly intrageneric relationships can be inferred from the electrophoretic patterns of plasma proteins. Studies at the population level (Gorman and Dessauer 1965) and problems involving hybridization (e.g., Dessauer et al. 1962; Beckman et al. 1963) have also found such patterns highly informative.

A promising approach for future studies of plasma proteins is the comparison of the peptides produced by specific proteases, such as trypsin. Plasma albumin and other major fractions may yet yield information of value in the higher category systematics of birds. Such studies will be preparatory for eventual comparisons of the complete amino acid sequences of such molecules.

SUMMARY

Comparisons of the starch gel electrophoretic patterns of the plasma proteins of approximately 450 species of birds revealed a high degree of individual variation superimposed upon a common basic pattern. No obvious correlations between pattern types and taxo-

nomic groups were found. Some of the variation is apparently due to such factors as sex, season, age, condition of health, and polymorphism. Although the one-dimensional electrophoretic patterns of avian plasma proteins do not provide useful information pertaining to higher category relationships, they may be useful at the lower levels.

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