BLOOD PROTEINS AND PRIMATE PHYLOGENESIS: IMMUNOCHEMICAL AND ELECTROPHORETIC TECHNIQUES APPLIED TO SYSTEMATIC SEROLOGY

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Introduction

In charting the course of primate evolution, the primary data we have to work with comes from taxonomy (Simpson 1959). Usually, characters of gross anatomy are used to derive phylogenetic relationships. Taxonomists have discovered that some morphological traits are exceptionally good indicators (Cain and Harrison 1958). Empirically, a few characters have proven to be constant within taxa, but different between taxa at any significant level. Such anatomical traits as teeth and skeletal material have proven to be most useful in classification. Living animals also provide superficial characters, useful as an anatomical basis for identification.

This is usually adequate for the identification of specimens into already defined classifications, but it is not adequate for making a biologically significant classification in the first place (Simpson 1962). Classification should, in principle, and as far as possible, consider all determinable characters which can reasonably be assumed to be at least partially under genetic control. A taxonomic panacea has been searched for, which will, in itself, indicate taxonomic relationships. In primates, the basic cranium or ear region has been supposed to be more resistant to evolutionary change than other characters. However, some primates show more variation in basicranial characters than in dentition (Simon and Russell 1960). Anatomical evidence for classification is now being supplemented by histology, biochemistry, genetics, and now behavioral studies, all of which help us to develop a more complete picture of the phylogenetic relationships.

General Remarks on Protein Evolution

Of course, the ultimate in classification would be the complete DNA code. At the present, we must be content to study only partial evidence and infer from this, genetic and evolutionary relationships. In the last few years, advances in biochemical techniques have allowed the more extensive studies of blood proteins which promise to be welcome additions to current taxonomic techniques.

The study of proteins as a new area, bearing on evolutionary processes, as well as taxonomy, has steadily risen in respectability with the development of better analytical techniques. Proteins are intimately connected with genetic relationships in that primary gene function consists in directing the synthesis of polypeptide chains which make up proteins (Zuckerkandl and Pauling 1962). The structural specificity of proteins is now known to be very closely related to the code of information in genetic material (Anfinsen 1959, Ingram 1961, Crick 1961). Deletion, insertion, and substitution of information constitute mutations that may culminate in evolutionary change. The immunochemical techniques have the advantage of being able to distinguish gradual changes in protein structure that are not discernible to

the eye of a taxonomist, changes which are minor and cumulative during the course of evolution (Goodman 1962b). This is an important concept which has elicited much discussion among taxonomists (Simpson 1959a, Fiedler 1956).

The similarities by which a distant genetic relationship is determined must be those characteristics of a common ancestor which both descendants have retained throughout their own independent evolution. Convergent or parallel evolutionary trends may be responsible for the similarity in some traits when an important function is to be preserved or evolved with similar modifications to meet the requirements of fitness in a selective environment. In general, however, the less significant a structure or substructure is in terms of its selective advantage, the wider will be the variation expected in its expression (Williams 1962). This is not only true among related species, but also among individuals of the same population (Allison 1959). In this way, a sizable pool of viable variants may be accumulated in a population until such a time as selective factors favor the fixation of a particular allele, or until certain heterozygotes evolve with selective advantage significant enough to favor a limited number of alleles which govern a structure.

These concepts should be applicable to the evolution of protein homologues just as they are to the evolutionary changes in the more classic morphological traits. However, we still cannot accurately describe every protein in terms of function related to structure (Scheraga 1959). While the properties of many proteins are known, their function in the organism may not be known. And even if the function is known, at least in part, the process by which the protein accomplishes its function is usually not known. We also do not have a complete picture of the three dimensional conformation, the functional form, of any protein, though the covalent structures of a few important ones are known (Crick 1958). A further limitation in the study of protein evolution is an absence of a fossil record.

Therefore, it appears that the evolution of proteins can only be studied in terms of divergent trends which resulted in the loss of ancestral characteristics. The immunochemical corollary of the progressive loss of ancestral conformations is that antigenic determinants become progressively more characteristic of a species with the passage of time, leading to our concepts of specificity of serological and immunochemical reactions (Williams and Wemyss 1962). Nevertheless, it is the ancestral conformation retained, or modified, only in part that permits the comparison of antigenic homologues from different modern species. It is important to note that though the structure of the protein was evolved through the selection of modifications that improved fitness, antigenic determinants reflect only changes in the ancestral conformation. For the most part, the antigenic capacity of the protein did not play a part in the selection process.

The ability of a protein to combine with an antibody is a structural characteristic, but it is not a biological function of many proteins, as far as we know (Perlman and Diringer 1960). Each protein has surface configurations which are responsible for its primary functional activity, and hence are called its active centers. Such centers are responsible for the hormonal, enzymatic, antigenic, or other properties of the protein (Porter 1953). In higher animals, ancillary configurations would also be present which would enable it to preferentially cross certain membrane barriers or to enter certain cells. Also, the remaining surface of the protein must be sterochemically

contoured so as not to interfere with the specific molecular associations of the functional configurations of other proteins (Pauling and Corey 1951).

Serological Techniques

With these general remarks about proteins, we may now proceed to examine the results of protein studies which have proved useful in the analysis of primate phylogenesis. The studies of primate blood proteins are far from complete. This is partially due to the recent invention of more sensitive techniques (starch gel electrophoresis, paper chromatography, agar-gel diffusion, and elaborate fractionation of sera) which render previous data such as the classic precipitation studies by Nuttall and Mollison subject to review and revision. However, we find these works still cited in the literature.

The first studies which made use of the comparative serology as a technique for primate classification were by Nuttall (1904), Mollison (1926), and Wolf (1933, 1939). They used immunochemical precipitation techniques to investigate the serological affinities of man. Their results were in agreement with the general zoological classification of major primate groups, but many problems concerned with the systematics of the primates were left unsolved. These early studies used whole serum as the immunizing preparation, as it was not technically possible to fractionate the 20 or more protein fractions now discernible. It was not possible to identify the proteins showing degrees of species specificity, since the serological procedures of the time, as contrasted with present agar-gel precipitation techniques, were incapable of separating the reactions of various antigens in a mixture.

A preliminary discussion of the modern techniques would now be in order. There are two basic analytical approaches to the study of soluble proteins. One is centered on the antigenic properties of protein homologues, while the second separates proteins on the basis of electrophoretic charge and molecular size. The reactions which result in a precipitate being formed as an antibody-antigen complex can take place in agar-gel (Wilson 1958, Cuchterlony 1953) or in solution (Nuttall 1904, Goodman & Vulpe 1961, Wolf 1933). The resultant complexes leave precipitin lines in agar-gel and measurable precipitates in solution which can be evaluated to demonstrate the structural similarities of the proteins involved.

The Structural Similarities of the Proteins Involved

Electrophoretic Studies of Primate Blood Proteins

Electrophoresis separates plasma or serum proteins on the basis of electric charge and molecular size. It is used with proteins in agar, paper chromatography and in starch gels, along with various combinations of these techniques (Picard 1960, Poulik and Smithies 1958) or these methods. Perhaps the most advanced of these is the two dimensional starch gel electrophoresis. The proteins are first electrophoretically separated on paper, and then attached to a starch gel, and the proteins are separated again. When the starch gel is finally dyed, the proteins thus separated form a distinct pattern. This allows us to compare the serum pattern of one serum sample against the overall pattern of other samples. The various serum proteins such as albumins, Gamma globulins, etc., separate out into 19 to 25 components. Each species presents a total serological pattern which can often be used to differentiate it from other species.

For example, Goodman (1962) identified the three sub-species of the species Lemur fulvus, genus Lemur: Lemur fulvus fulvus, Lemur fulvus albifrons, and Lemur fulvus rufus, which are quite similar and can be distinguished from Lemur variegatus and Lemur catta. The latter two species can also be distinguished from each other. In the Lorisidae, Perodicticus, and Galago (belonging to different subfamilies) there are sharply divergent patterns, whereas Galago crassicaudatus and Galago senegalensis (two species of the same genus) show patterns which are virtually indistinguishable.

Four species of the genus Macaca showed a high degree of similarity except in the positioning of protein transferrin and for quantitative variations in the amounts of various components. In contrast, the hominoid patterns show striking divergencies from each other (Goodman et al. 1958). Boyer and Young (1960) have also commented on the Beta-globulin polymorphism in chimpanzees. Goodman, on the basis of these results, argues against the suggestion of some taxonomists of placing the gorilla and chimpanzee in the same genus. His results also indicate that man shows more similarity to the gorilla and chimpanzee than to the gibbon or orangutan. The constellation of components surrounding the albumin are similar, but not identical, in man, gorilla, and chimpanzee, and different in gibbon and orangutan.

Buettner-Janusch (1961) also used the starch-gel electrophoresis technique; however, instead of studying serum, he breaks down the hemoglobins and transferrins. He finds that Prosimian hemoglobin, in contrast to the other primates, shows a relatively large amount of pigment that is alkali resistant. In the Anthropoidea, alkali resistant hemoglobin vanishes from the embryo at the age of three months, providing further support of the embryological principle that ontogeny recapitulates phylogeny. Buettner-Janusch finds no reason to suppose that the alkali-resistant property of a hemoglobin has any functional significance.

He finds the hemoglobins of non-primates to be quite as heterogeneous as those of man (Cumley and Irwin 1943, Blumberg 1960). Buettner-Janusch shows his results from samples of hemoglobin coming from various individuals of a single population of Perodicticus potto. The variety is interesting, because it indicates apparent polymorphism of hemoglobin within one small forest population found in East Africa. However, Goodman (personal communication) expresses doubts as to the validity of this study; technical difficulties may have been involved.

Lemur hemoglobins from Lemur fulvus fulvus also show definite polymorphism. Buettner-Janusch believes that genetically controlled traits such as those of hemoglobins are not neutral in respect to natural selection. Hemoglobin has the important physiological function of oxygen transport. This may be the clue to the discovery of selective factors leading to differentiation of the molecule among primates.

The transferrins also have shown a considerable heterogeneity (Buettner-Janusch 1961). Transferrins are one of a number of transport proteins in plasma. They function in taking iron from one tissue compartment to another. In order to demonstrate transferrin, plasma is reacted with Fe^{59} and autoradiographs are made from the gels after electrophoresis. The findings indicate that all baboon transferrins are inherited as simple autosomal alleles, based on serum samples from inbred colonies of Papio doguera and Papio ursinus. The wide

variations in transferrin phenotypes found among species of primates promise to be very useful in the analysis of the population genetics of these animals.

Where sufficient numbers of samples from clearly defined troops of Kenya baboons are available, the variety of transferrins is proving useful in characterizing genetically different groups within larger populations. Eventually, it may be possible that the transferrins will provide more data bearing on phylogeny. The use of the immunological approach may enable a detailed analysis of possible continuous differentiation of this protein within the primates.

Zuckerkandl, Jones, and Pauling (1960) analyzed hemoglobins by using paper chromatography and found that primate and human hemoglobins are very similar, especially gorilla, chimpanzee, and human. In fact, adult human hemoglobin peptide patterns actually differ more from human fetal patterns than from adult gorilla and chimpanzee patterns. The preceding results were obtained by using paper chromatography.

However, the earlier antigenic approach used by earlier investigators, Mollison (1926) and Nuttall (1904), with refinements, has proved to be of considerable value in phylogenetic studies, especially now that purified serum fractions can be used to make antisera, allowing comparisons of fractions instead of only whole serum.

Immunodiffusion Studies of Primate Blood Proteins

The antigenic approach has been used to provide a very comprehensive measure of the relative degrees of structural correspondence for each protein type among the various primates. This is particularly true if the analysis is carried out with a variety of antisera to a particular protein type, for then, the species' specific properties of the surface configuration will affect the measurements (Wolfe 1933). For example, if a species related to man, such as the rhesus monkey, is immunized with a human protein, only a small proportion of the surface configuration of the protein will be antigenic, and there will tend to be more species specific configurations. On the other hand, if a species unrelated to man, such as the chicken, is immunized with human protein, a large portion of the surface configuration of the protein may be antigenic, and the antibodies to some of these (configurations which have been stable over long periods of evolution) will even cross react with antigens in non-primate mammals (Goodman 1957).

Various precipitation studies have consistently demonstrated the close serological relationships of man to the anthropoid apes, his intermediate relationship to Cercopithecoidea, then to Ceboidea, and more distantly to the Prosimians (Boyden 1958, Goodman 1960, Kramp 1956, Nuttall 1904). Although the exact position of man within Hominoidea has not been decided, a large body of data clearly demonstrates that man is more closely related to the African apes; gorilla and chimpanzee, than to the Asiatic apes; gibbon and orangutan.

Using extremely sensitive immuno-diffusion plates (Wilson 1958), Goodman (1960) examined the crossreaction of sera of various primates to chicken, monkey, and rabbit antisera of the various human serum proteins. With chicken antisera to human ceruloplasmin and chicken and rabbit antisera to human transferrin, gorilla and chimpanzee appeared to be identical

with man, whereas gibbon and orangutan diverged from man. With rhesus monkey antisera to human albumin, gorilla and man were very similar; chimpanzee diverged slightly from man, and gibbon and orangutan clearly diverged from man and from each other. The orangutan albumin appears to be the most divergent of the hominoid albumins. With a capuchin monkey antiserum to human serum, gibbon and orangutan showed moderate deviations from man, whereas gorilla and chimpanzee showed only a slight divergence from man, the divergence of the gorilla being the smallest. With rabbit antiserum to Alpha 2 macroglobulin, only the gorilla appeared to be identical with man; chimpanzee, gibbon, and orangutan showed small divergencies. However, with chicken and rabbit antisera to human Gamma globulin, only the chimpanzee appeared to be very similar to man; gorilla, orangutan, and gibbon were increasingly divergent.

From these results, Goodman infers that the Asiatic apes are more distantly related to man than the African apes, and also that each of these apes has diverged from the others, suggesting that the phyletic separations in the Hominoidea are fairly ancient. To be more specific, the phyletic separation of chimpanzee and gorilla may have been just as distant a separation as chimpanzee and man. Accordingly, gibbon and orangutan may have been separated as long as gibbon and man. Evidence that gibbon and orangutan show more ancient phyletic separation from man than gorilla and chimpanzee is provided by the cross reactions of a chicken antiserum to chimpanzee serum. This antiserum distinguished the chimpanzee from the orangutan and the gibbon, but not from the gorilla or man. Zuckerkandl et al. (1960) demonstrated that the primary structure of adult hemoglobin is almost identical in man, chimpanzee, and gorilla, but diverging somewhat in the orangutan; the gibbon was not examined. This gives additional weight to the evidence in support of the close phyletic relationships of man, chimpanzee, and gorilla within Hominoidea, according to Goodman, although others disagree (Reed 1960, Simpson 1959b). Goodman (1962) argues that these serological findings call for a revision of the taxonomy of the Hominoidea, since in current schemes, gorilla and Pan are placed with Pongo rather than with Homo. One solution would be to broaden the Hominidae to include Gorilla and Pan as well as Homo. Hylobates would be in the Hylobatidae, and Pongo would remain in the Pongidae. Goodman feels that the fact that the African apes are far more terrestrial in their mode of life than the Asiatic apes may further support these conclusions concerning the phylogeny of man.

Other primate groups have been examined by immunochemical techniques by Paluska and Korinek (1960) using anti-macaque sera. Their results have demonstrated a greater degree of antigenic correspondence between Cercopithecoids and Hominoids (man and chimpanzee) than between the Cercopithecoidea and Ceboidea (capuchin monkey). Goodman has also confirmed with chicken anti-macaque sera that Cercopithecoids show a closer correspondence to Hominoidea than to Ceboidea. Using chicken anti-spider monkey serum, it was noted that catarrhine primates (man and stump-tailed macaque) yield much larger cross reactions than lorisiform and lemuriform lower primates (galago and lemur). This data supports the argument for a taxonomic classification which implies a closer phylogenetic relationship of the Ceboidea to the catarrhine primates than to the Lemuriformes and Lorisiformes, even though paleontological evidence (Simpson 1949) points to the separation of the proto-ceboids from the proto-cercopithecoids and proto-hominoids at the Prosimian grade of phylogenetic development in the Paleocene or early Eocene.

Comparisons also have been carried out by Goodman with chicken anti-potto and anti-lemur sera (Goodman 1963). In each instance the lorisids, galago, loris and potto, and the lemurids, lemur showed more antigenic correspondence to the other than either to the tupaiids or to members of Anthropoidea. This suggests that the phyletic separation between the lorisiform Prosimians and the Malagary Prosimians was more recent than the phyletic separation of the common ancestor of the two groups from the progenitors of Anthropoidea. The cross reaction data also indicate that within Prosimii, no special phylogenetic relationship exists between the Tupaioidea and the Lemuroidea.

Immunological Selection and Primate Evolution

At this point, it might be interesting to examine a theory which considers the process of protein evolution not only on the basis of natural selection, but with reference to immunological systems of primates. This theory is proposed by Goodman (1961) as an explanation for not only the evolution of blood proteins, but for the evolution of more advanced brains in the higher primates.

One of the most important findings of the comparative analysis carried out with chicken and rabbit antisera is that certain protein types evolve at a much slower rate than others. Albumin shows a great similarity among the Hominoidea. The Cercopithecoid albumins showed somewhat moderate divergencies from the Hominoid albumins and also some divergencies among themselves (samples from the Colobinae and Cercopithecinae, each showing some configurations similar to man that the other lacked). The Ceboid albumins also developed significant cross reactions. It was not until lemurid, lorisid and tupaiid albumins were examined that marked divergencies from the albumins of Anthropoidea were noted.

In contrast to the albumins, Gamma globulin shows distinct antigenic differences among the members of the Hominoidea and very little similarity among the catarrhine and platyrrhine primates (see tables, Goodman 1963). This points to the conclusion that Gamma globulin has been evolving at a much more rapid rate than has albumin during the evolution of Anthropoidea. In a study described by Boyden (1958), antisera were produced to purified human serum albumin as well as to whole human serum. The differences between human serum and the non-human primate in the degree of precipitation measured turbidimetrically were somewhat less with the antisera to albumin than with the antisera to the whole mixture of serum proteins. Boyden interpreted this to mean that serum albumin is more conservatively evolved than the serum globulins.

According to Goodman, not only were the blood proteins under the influence of the immunological system, but also the entire progressive evolution of the primates. The environment, by selecting for an increasingly efficient placenta in mammals, decreased genetic plasticity and favored adaptive variations which had to be expressed in the postnatal development of the organism. More efficient placentas increased the contact between fetal and maternal blood systems which meant that a mutant protein which expressed itself in the fetus would be subjected to immunological attack by the maternal blood system. Prolonged gestation and intimate contact between maternal and fetal blood systems would favor genetic homozygosity. Since higher mammals

show the preceding traits, it follows that a species at a high morphologic grade of phylogenetic development (i.e. placental mammal) may have more truly primitive genes in its characteristic genotype and more ancient antigenic patterns than a species at a low morphologic grade (rigidly separated maternal and fetal circulations); a primitive species, therefore, shows greater genetic plasticity. In addition, primitive species with shorter gestation periods allow for more generations which also may quicken the pace of environmental selection.

The great diversification of the eutherian mammals which began in the Cretaceous agrees with these conclusions. From an embryological standpoint (Hamilton et al. 1952, Nace 1957) and on immunological grounds, we can deduce that the eutherian mammals had a primitive allantoic placenta with a very simple non-deciduate epitheliochorial arrangement in which several avascular layers separated the fetal and maternal blood vessels. No doubt this primitive placenta functioned with low efficiency in the transport of metabolites, but also blocked the entrance of fetal isoantigens into the maternal circulation. Furthermore, in these small mammals the period of intra-uterine existence, gestation, was probably on the order of three weeks, and during much of this time, embryonic development would occur in the lumen before the functional placenta was established. Since a two week incubation preceeds a primary immune response, and approximately a one-week period preceeds a secondary challenge, genetic variability could be expressed in the embryo and still escape the selective action of maternal immunizations (Brambell 1958). In the Cretaceous mammals isoantigens could exist not only among proteins which differentiate late in fetal development, but also among molecular structures which differentiate early, thus favoring variations of genotypes.

Progressive evolution can be correlated with the evolution of more efficient placentas. The monotremes are egg layers. Marsupials show only a rudimentary placental connection between embryo and mother, and marsupial young are born in an almost larval state after a brief gestation period. The placental mammals vary markedly in their gestation processes. According to Le Gros Clark (1959) the functional efficiency of placentation in contemporary primates correlates with the level of phylogenetic development of the members of the order. The intimacy of the embryo with maternal blood progressively increases in the following series: prosimians, platyrrhine monkeys, catarrhine monkeys, and finally anthropoid apes and man. The differentiation of the placenta becomes more rapid and the vascularization of the chorion more precocious. The hominoid embryo no longer undergoes its initial development in the lumen of the uterus, as in all lower primates; while it is still quite minute, it becomes submerged in the uterine wall. Hemochorial placentas in which the chorion containing fetal blood vessels comes into direct contact with the maternal blood are found uniformly in the Anthropoidea.

Thus, ontogenetically late appearing proteins, such as Gamma globulin, could evolve at a relatively rapid rate, since in this case, divergence would not be selected against by maternal immunizations. The lineages responsible for phylogenetic advances of the primates were those in which a marked prolongation of gestation and an elaboration of the hemochorial placenta, with its intimate apposition of fetal and maternal blood streams occurred at a comparatively early stage of eutherian phylogenesis, according to Goodman's theory. Further, the external environment of these, progressively evolving, could only select for traits which expressed themselves late in ontogenesis or even

postnatally. The functionally advanced placentas also allowed for elaboration of the primate brain since the phylogenetically new parts of the cerebral cortex require a much richer supply of nutrients and oxygen than the paleobrain. The selective pressure bearing on the adaptive functions of the hemochorial placenta is the utility of late penetrance.

The dynamics of Goodman's theory are as follows: Early in the progressive evolution of the primates, the hemochorial placenta developed, providing the metabolic basis for a continuing progressive elaboration of the brain. In turn, this cerebral evolution, which correlated with increasing body size, allowed the phylogenetically advancing primates to invade broader ecological domains (Washburn and Avis 1958). But this leads to a contradiction. On the one hand, a broader environmental zone selected for genetic heterozygosity and variation. On the other hand, the maternal immunological system selected for a state of genetic homozygosity. This contradiction was resolved by selection for genetic codes which delayed maturation until the postnatal phase of ontogeny. The expression of variation began to center on the late maturing molecular structures. This goes along with the fact that man, who shows the greatest cerebral development and occupies the broadest environmental zone, is also the most retarded mammal in his maturational processes.

What can be said about the relative value of such studies? George Gaylord Simpson (1962) makes some important comments:

> Proteins and hemoglobins are only a few steps removed from the basic genetic message in DNA, certainly much closer to it than any characters of gross anatomy. . . . It is, therefore, certain, not only that these various data give evidence as to genetic and phylogenetic relationships among populations and taxa, but also that their evidence must be significant. . . . We need more data of this sort and also their complex coordination with all the older information. It cannot be assumed that precipitation reactions are correlated directly with degrees of phylogenetic separation. Goodman's own further considerations on serum protein evolution seem to exclude so simple a solution. A potential taxonomic contribution of the first magnitude is here under way, but not yet quite in our grasp.

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