*A MOLECULAR TIME SCALE FOR HUMAN EVOLUTION**

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Abstract.—We discuss published molecular evidence concerning the relationship of man to African apes and Old World monkeys. Quantitative comparisons of their serum albumins, transferrins, hemoglobins, and DNA show that man is genetically much more similar to the African apes than to the Old World monkeys.

The amino acid sequences of hemoglobins from humans, chimpanzees, gorillas, and rhesus monkeys are consistent with the hypothesis that the probability of an amino acid substitution occurring in a given interval of time is the same for every hemoglobin lineage. This allows the use of these data as a hemoglobin evolutionary clock, just as we have previously done with the albumins. It is shown that concordance exists between the hemoglobin and albumin results and that both support the suggestion that the human lineage diverged from that leading to the African apes far more recently than is generally supposed. Considering both the albumin and hemoglobin data, we would set the most probable date at 4 to 5 million years.

Introduction.—In spite of the vast effort which has been devoted to the study of human evolution, there is still no measure of agreement as to the origin of man. Although gorilla and chimpanzee are generally regarded as man's closest living relatives, estimates of the time of divergence of the human lineage from that (or those) leading to these African apes have varied widely. Current estimates range from 4 million[1] to 30 million[2] years for the time of the origin of the hominid line. Thus, some investigators consider that African apes are scarcely more closely related in time to man than are the Old World monkeys (Fig. 1*A*), while others think that the relationship of apes to man is very close (Fig. 1*B*). The disagreements are due in part to the fragmentary nature of the fossil record which consists largely of teeth and jaws, and in part to the failure of traditional comparative anatomy to develop methods which would lead to agreement, even among anatomists, as to the evolutionary meaning of such data.

Molecular biology now offers new methods of estimating both degree of relationship and time of divergence among living species, thereby helping to circumvent the problems caused by few fossils and uncertain anatomical conclusions.

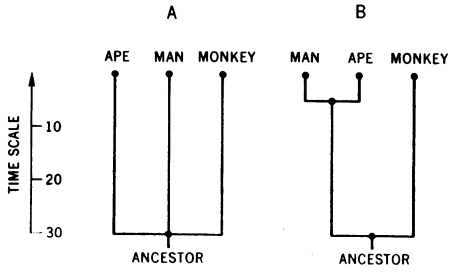

Fig. 1.—Alternative hypotheses as to the phylogenetic relationship of man to the African apes and the Old World monkeys.

Albumin and Transferrin.—Serum protein studies support the hypothesis of a close genetic relationship between man and the African apes. Human serum albumin is far more similar to ape albumins than to the albumins of the Old World monkeys, according to quantitative immunological techniques.[3, 4] Similar findings have been made with the transferrins of these primates.[5, 6] The albumin and transferrin data are summarized in Figure 2.

DNA Hybridization.—Martin and Hoyer[7] have done hybridization experiments with purified primate DNA's. Specifically, they tested DNA fragments from humans, chimpanzees, and rhesus monkeys for the ability to compete with human DNA fragments for binding to unfragmented human DNA embedded in agar. As shown in Figure 2, chimpanzee and human DNA's differed only slightly (9%) in their competitive abilities, whereas there was a relatively large difference (34%) between rhesus monkey and human DNA in competitive ability. This evidently means that there is considerably more base sequence homology between chimpanzee and human DNA than between rhesus monkey and human DNA. However, it should be recalled that this technique examines only that redundant fraction of the DNA which hybridizes readily.[8]

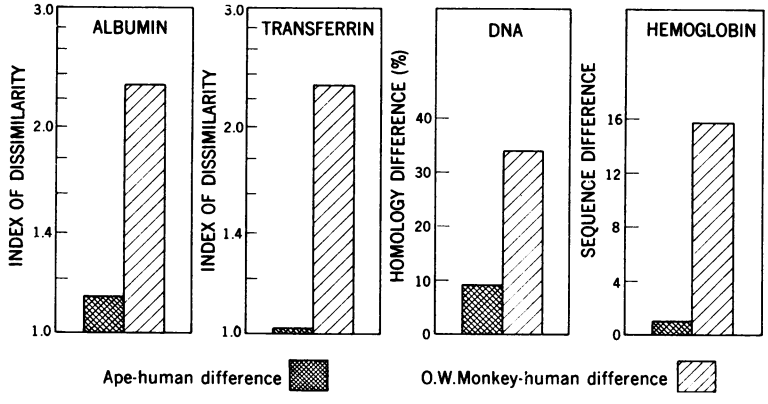

FIG. 2.—Quantitative comparisons of human macromolecules with those of African apes and Old World monkeys. The height of each bar is a measure of the difference between a human macromolecule and its counterpart in apes (cross-hatching) or monkeys (hatching). The albumin and transferrin results were obtained by the microcomplement fixation method; the albumin results are published,[3, 4] and the transferrin results were obtained with the antisera described by Wang *et al.*[5] The DNA results are taken from Martin and Hoyer;[7] we use the term "homology difference" to refer to the percentage of bindable radioactive human DNA fragments that the ape or monkey DNA was unable to compete with under these experimental conditions. The hemoglobin results are taken from Table 1.

Hemoglobin.—We now wish to point out that further evidence for a close genetic relationship between man and African apes is provided by a third protein, hemoglobin. For several years, sequence information has been available for the hemoglobins of man,[9] the chimpanzee,[10] and the gorilla,[11] as well as of several nonprimates such as the horse.[12] Only recently was the hemoglobin sequence of a monkey elucidated.[13] Table 1 records the differences between the amino acid sequences of these proteins.[14] Clearly, the sequence difference between the hemo-

globins of apes and man is very small compared with that between the rhesus monkey and man.

The closeness of the ape–human resemblance in hemoglobin sequence is further illustrated if we compare it to the donkey–horse difference. The donkey and horse are so closely related that they are classified in the same genus (*Equus*). It is well known that they can readily produce viable hybrids. Donkey hemoglobin appears to differ in sequence from horse hemoglobin by at least one or two amino acid residues.[16] According to the sequence criterion, men and African apes are as similar as the donkey and horse.

TABLE 1. *Comparison of amino acid sequences of hemoglobins.*

Species compared	Number of amino acid differences	Mutational* distance
Man vs. chimpanzee	0	0
Man vs. gorilla	2	2
Monkey vs. man	12	15
Monkey vs. chimpanzee	12	15
Monkey vs. gorilla	14	17
Horse vs. man	43	52
Horse vs. chimpanzee	43	52
Horse vs. gorilla	45	54
Horse vs. monkey	43	52

* The minimum number of base substitutions required to account for the observed amino acid substitutions, calculated by the method of Jukes, and Fitch and Margoliash.[15]

Evolutionary Relevance.—The question of what the consistent molecular similarity between the African apes and man means in phyletic terms is, however, equivocal. Phylogeny *A* (Fig. 1) would be compatible with these results only if molecular evolution had been retarded in the ape and human lineages relative to that in the monkey lineage. Phylogeny *B* (Fig. 1) would be indicated if molecular evolution had proceeded at approximately the same rate in all three lineages. In order to evaluate the evolutionary or phylogenetic significance of the consistent close resemblances between the informational macromolecules of apes and man, then, it is necessary to obtain information about the relative amounts of molecular evolution in the monkey, ape, and human lineages, i.e., to perform regularity tests.

Regularity Test.—Since we have already shown that albumin evolved in a regular fashion with respect to time in primates,[4, 17] and as there are insufficient data to apply a regularity test in the transferrin and DNA cases, the hemoglobin data remain to be rate-tested. The relative amounts of hemoglobin evolution that have occurred in the monkey, ape, and human lineages can be calculated from Table 1 data, using horse hemoglobin as a reference point. The only assumption made in this calculation is that the common ancestor of monkeys, apes, and man lived more recently than did the common ancestor of primates and horses—an assumption favored by numerous lines of zoological and paleontological evidence. The time of divergence of the horse lineage from that leading to primates is estimated by paleontologists to be about 75 million years.[18] Now, Table 1 shows that the four primate hemoglobins are about equally distinct in sequence from that of the horse. Therefore, the hemoglobins of monkeys on the one hand, and

those of the apes and man on the other, have changed to about the same extent since these species last shared a common ancestor.[19]

These results are neither unique nor surprising. Others have already recognized that protein molecules often appear to have evolved in a regular fashion with respect to time.[20] The bulk of the available sequence information is consistent with the hypothesis that for any given protein, such as hemoglobin, the probability of an amino acid substitution occurring in a given interval of time is the same in every lineage.[4, 20, 21] Such regularity is difficult to explain in terms of natural selection. Evolutionary biologists are thus being forced to consider the possibility that the spread of selectively neutral mutations accounts for much of molecular evolution. A mathematical model explaining how such a process could occur has appeared.[22]

Regardless of the reason for this regularity in protein evolution, however, its existence opens the exciting possibility that protein molecules can be used as evolutionary clocks.[23]

The Clock Approach.—Since regularity appears to have characterized the evolution of ape, human, and monkey hemoglobins and albumins, the close similarity of the ape and human proteins must imply that these species diverged from one another much more recently than did monkeys from apes and man, as shown in phylogeny *B* (Fig. 1). According to immunological criteria, the albumins of chimpanzee, gorilla, and man are only about one sixth as different from one another as they are from the albumin of the rhesus monkey.[4] It then follows from the regularity test[17] that the African apes and man diverged about six times more recently than did rhesus monkey and man. As the time of divergence between men and monkeys (*Hominoidea* and *Cercopithecoidea*) can scarcely be greater than about 30 million years, we have calculated that the lineages leading to man and the African apes diverged about 5 million years ago.[4]

From the hemoglobin data it is not possible to obtain a precise date for the ape–human divergence, as a statistically reliable number of amino acid differences between ape and human hemoglobins have not yet accumulated. The average rate of evolutionary change among mammalian hemoglobins is only one amino acid replacement per approximately 3.5 million years.[20, 21] Thus, species that diverged 3 million to 4 million years ago would be expected to differ in hemoglobin sequences by approximately two amino acid replacements. As pointed out above,[19] about 17 amino acid replacements would be expected between the hemoglobins of species that diverged from each other 30 million years ago, and the ape and Old World monkey data are consistent with this expectation (Table 1). However, the ape–human difference is only zero to two amino acid residues and, consequently, a much more recent divergence time is indicated.

Although the calculation of an exact date from the hemoglobin data is unwarranted for statistical reasons, the results are obviously compatible with the albumin date of 5 million years or less.[24] More important, the results are incompatible with the divergence time of 20–30 million years that has recently been suggested by some paleontologists.[2] If the probability model of protein evolution that was outlined above is applied to this case, then it can be calculated with the Poisson distribution that there is less than one chance in 10^5 that a sequence

difference of zero to two residues could result when the divergence time was 30 million years ago, and from one chance in a hundred to less than one chance in a thousand (depending on the use of the 0 or 2 differences in the calculation), if the date were 15 million years. The same considerations can be applied to the independently derived albumin data,[25] thus making the combined probability that any date for the hominid-pongid divergence could approach 30 million or even 15 million years vanishingly low. Indeed, our calculations indicate that it is difficult to consider seriously any date in excess of 10 million years for the origin of the hominid lineage. On the other hand, a divergence time of 4 to 5 million years is highly probable according to the protein clock approach.

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[14] Hemoglobin is composed of two kinds of polypeptide chains, α and β, whose amino acid sequences are determined by two unlinked genes. Evolutionary change has gone on in both the α- and β-chains. The species differences shown in Table 1 are the sums of the differences in both chains.

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[19] A more detailed argument for regularity might go as follows. The hemoglobins of man and rhesus monkey are equally different from that of the horse; thus, the 15 mutational events separating these hemoglobins must be apportioned equally between the two lines. The minimum eight mutational events along the lineage leading to man after the divergence of the

Old World monkey line clearly occurred prior to the separation of the human and African ape lineages. These results imply that the hemoglobins of the African apes and man share a far more recent common ancestor than do those of the monkeys and apes. To put it another way: man, gorilla, and chimpanzee share a common ancestral lineage along which at least eight mutational events resulting in hemoglobin amino acid substitutions occurred after its separation from that leading to the Old World monkeys. Since the time that man, chimpanzee, and gorilla have had independent lineages, on the other hand, a total of only two such substitutions have occurred along the three lineages.

Now one might still argue that the eight mutational events prior to the ape–human divergence occurred over a very short period of time, thus still leaving this divergence relatively early in time. Therefore, we must consider the evidence for regularity in molecular evolution in general and hemoglobin evolution in particular.

In our previous albumin studies we showed that primate albumins had evolved in a regular fashion throughout their history.[17] The hemoglobin data discussed above are consistent with this finding of regularity in that the major primate lineages have changed to much the same degree (8–10 mutational events) since their separation. In addition, and perhaps of more relevance, is a calculation based on a date of 30 million years for the ape–Old World monkey separation assumed on the basis of nonmolecular evidence. The average rate of evolutionary change among mammalian hemoglobin lineages is one amino acidr eplacement per approximately 3.5 million years.[20, 21] Data in Table 1 suggest that a similar rate applies to primates. For example, species which separated 30 million years ago would be expected to have hemoglobins separated by about 17 mutational events (60/3.5 = 17). This figure is in excellent agreement with the values of 15, 15, and 17 for the differences between Old World monkey, and ape, and human hemoglobins given in Table 1. In addition, the mean 53 mutational events separating primate and horse hemoglobins should indicate a time of separation of $(53 \times 3.5)/2$ or $\sim$90 million years. Again this is in reasonable agreement with expectations based on the fossil record.

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[23] E. Zuckerkandl and L. Pauling[20] first pointed out the possibility of using proteins as evolutionary clocks.

[24] The agreement between the albumin data and partial sequence data for primate hemoglobins has been noted by Jukes and Cantor.[21]

[25] Two entirely different proteins, hemoglobin and serum albumin, have provided concordant estimates of the age of the human lineage. This was in fact to be expected as the times of divergence between the hemoglobin and albumin lineages must be the same as the times of divergence between the species themselves. In this sense the history of each protein is necessarily the same as the history of the species. In principle, therefore, and subject only to statistical limitations, the information at a single protein locus should be sufficient for elucidating the relative times of divergence of the modern species containing this protein.