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Hemoglobins A and A_2 in New World Primates: Comparative Variation and Its Evolutionary Implications

Abstract. *Hemoglobin A_2 ($\alpha_2\delta_2$) in New World primates represents about 1/160 to 1/16 of total hemoglobin and, by virtue of this low proportion, is presumed to be functionally unimportant. Nonetheless, A_2 exhibits genetic polymorphism by electrophoresis in three out of five genera, whereas the major component, hemoglobin A ($\alpha_2\beta_2$), is electrophoretically invariant. Moreover, in four genera, including man, the evolutionary accumulation of mutations has been greater in δ than in β. Such findings suggest that both polymorphism and evolutionary changes can accrue to an effectively functionless and thus selectively nearly neutral gene.*

Some investigators have maintained that demonstrable genetic polymorphism in man (*1*) and *Drosophila* (*2*) is too extensive (*2*) and mutations accumulate too rapidly (*3*) to be easily accounted for by natural selection alone. Consequently it has been supposed that most mutations have little adaptive importance, that is, they are nearly neutral (*3*, *4*), and that much of evolution has proceeded through such mechanisms as genetic admixture and drift. Darwinism is not without defenders (*5*) who argue that the way in which selection operates may have been misjudged and that the rate of adaptive evolution may considerably exceed Haldane's earlier estimate (*6*). Others suggest that natural selection can, in fact, support moderately extensive genetic polymorphism (*7*). Prakash, Lewontin, and Hubby (*8*) have found essential constancy of gene frequencies for a large variety of randomly selected polymorphic proteins in several well-separated, natural populations of *Drosophila*. Such similarity of gene frequencies in different populations is most easily explicable by selection. On the other hand, a large body of molecular evidence analyzed by King and Jukes (*9*) indirectly suggests that most changes in proteins stem from neutral mutations and represent non-Darwinian evolution rather than natural selection. In this connection, direct evidence from the study of variation in nearly functionless genes is lacking, since no very good objects of study have been available. In this report we describe contemporary and evolutionary variation in a *pair* of similar proteins, the adult hemoglobins A and A_2, for which there is substantial physiologic basis for supposing that one member of the pair (A_2) has little or no functional significance and is thus nearly or entirely neutral in adaptive importance.

The distinctive chains of hemoglobins A and A_2 in man presumably arose through gene duplication (*10*). Hemoglobin A_2 appears only in man, apes, and New World primates (*11*). It has not been detected in Old World monkeys. In man, A_2 usually forms less than 3 percent of total hemoglobin while in New World primates it ranges between 0.6 to 6.0 percent (*12*) (Fig. 1 legend). We endeavor to show from comparisons of peptide compositions that the distinctive gene for A_2 arose in an ancestor common to man and New World primates. The low proportion of A_2 in all species suggests that it has not been a major component at any time since the evolutionary divergence of these species. Moreover, there is no evidence that the intracellular proportions of A and A_2 differ substantially from the proportions found in whole blood. The ratio of A to A_2 in man remains approximately constant in postnatal life (*13*), and the distribution of A_2 is essentially homogeneous in erythrocytes of adults (*14*). With these facts as background, we presume (i) that hemoglobin A_2 has little or no functional importance disproportionate to its concentration, (ii) that the functions of adult hemoglobin are principally or entirely served by the major component, hemoglobin A, and therefore, (iii) that A_2 is essentially functionless and thus the δ gene characteristic of A_2 ($\alpha_2\delta_2$) is either neutral in the face of natural selection or, at least, much more nearly so than the β gene characteristic of A ($\alpha_2\beta_2$). If these presumptions are correct, then in man, for example, the present-day adaptive importance of δ should be 3/97, that is, $\sim 1/30$ that of β; thus adaptive variation of δ might be expected to be substantially less than that of β.

In view of such presumptions and in light of the findings of Prakash *et al.* (*8*) that polymorphisms are probably maintained by selection, we were somewhat surprised to find considerable heterogeneity of A_2 in a variety of New World primates (Fig. 1). Three minor hemoglobin types—A_2, W_2, and Y_2—appeared in 106 spider monkeys (*Ateles*). Hemoglobin W_2 occurred only in *Ateles geoffroyi*; the gene frequency in 52 animals was 0.35. Hemoglobin Y_2, although present in three *A. geoffroyi*, was more common in *A. fusiceps* where δY_2 gene frequency in 39 animals was 0.17. Both W_2 and Y_2 appeared in animals from several sources, and thus the common occurrence of these variants is not simply attributable to sampling of closely related individuals. The nature of Y_2 is pertinent; it represents a change from

glutamic acid to lysine at δ-6 and is consequently homologous to the human β variant, hemoglobin C. Homozygosity of C is moderately deleterious, whereas Y_2 homozygotes did not exhibit any erythrocytic or systemic abnormalities. The apparent benignity of Y_2—in contrast to human C—may of course depend on more than concentration and, for example, might reflect other differences between *Ateles* δ and human β (Fig. 2), which modify the effect of lysine at position 6. Structurally distinctive A_2 variants were also found in three other genera (Fig. 1). Seven animals with A_2/Z_2 phenotype were found among 100 squirrel monkeys (*Saimiri sciureus*), three A_2/U_2 among 27 titis (*Callicebus moloch*), and one A_2/V_2 among 65 owl monkeys (*Aotus trivirgatus*). We interpret the A_2 heterogeneity in *Ateles, Saimiri,* and *Callicebus* as true genetic polymorphisms; that is, the frequencies of variant alleles exceed expectation from recurrent mutation alone. The single variant in *Aotus* may represent no more than the chance detection of a solitary mutant. Only one genus of those examined, the tamarins represented by 35 *Saguinus mystax* and 10 *S. nigricollis*, failed to exhibit electrophoretic heterogeneity of A_2. The appearance of five structurally distinctive A_2 variants in an aggregate of five genera contrasts sharply with hemoglobin A for which only one electrophoretically distinguishable variant (an α mutant in a single *Ateles*) was found in a total of 343 individuals.

Further comparisons of variation in β and δ developed from amino acid analysis of tryptic peptides (*15*). Results are given in Fig. 2 where β and δ positions that have the same residue in all species are omitted and only those positions exhibiting evident mutations are shown. The uncertainties attached to position assignments (Fig. 2 legend) stem from lack of sequence analysis in individual peptides. This lack does not substantially impede our conclusions. Residues at two positions, 116 and 117, have particular pertinence to our subsequent arguments. At these positions all β chains, including that of man, are histidine-histidine while all δ chains are arginine-asparagine. This suggests that the archetypal duplication producing the β and δ genes (*10*) occurred in an ancestor common to both man and New World primates. If this supposition is correct, then the differences between β and δ chains at positions 116-117 are the residua of mutations that

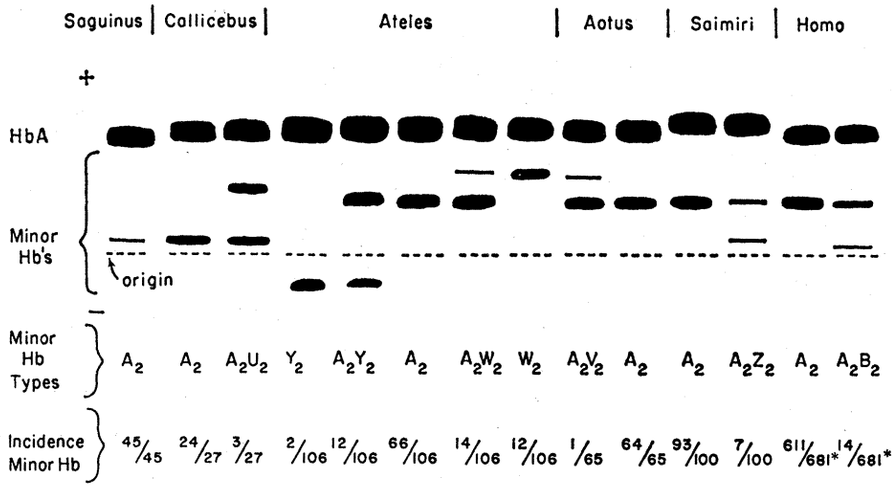

Fig. 1. Diagram of benzidine stain of starch-gel electrophoresis (*12*) of hemoglobin from whole hemolyzates. In all instances the δ nature of unusual minor components was suggested by the relative diminution of the wild-type A_2 component. Confirmation of the δ origin of all minor variants was obtained by column chromatography (*22*). Diagram approximately reflects the following differences in total percentage of A_2: *Ateles,* 6.0 percent (60); man, 2.6 percent; *Saimiri,* 2.4 percent (3); *Aotus,* 2.2 percent (2); *Callicebus,* 2.0 percent (2); *Saguinus nigricollis,* 1.0 percent (5); and *Saguinus mystax,* 0.6 percent or less (3) where number of determinations is indicated parenthetically. The incidence values at the bottom of figure represent the number of individuals with indicated phenotype/total individuals. Asterisk (*) indicates incidence in Amercan Negroes (*23*).

arose after gene duplication but before the divergence of ancestral lines. Antithetical to this notion is the appearance of glycine at both β-5 and δ-5 in all New World primates, and the contrasting appearance of proline at these positions in man. Such discordance supports the contrary presumption of separate duplicatory origins of β and δ in each of two stem lines. This latter possibility becomes more remote in light of other pairs of changes, such as *Saimiri* β-6 and δ-6, *Saimiri* β-9, and *Ateles* δ-9, wherein the same amino acid substitution seems to have occurred twice. Like these examples, the disparities between men and monkeys at position 5 may have arisen through separate mutations in β and δ. In this connection it is noteworthy in a list (Fig. 2) with comparatively few overall differences that there are numerous positions (12, 46, 50, 125, and 126) where the same codons have apparently been subject to repeated, albeit different, mutations (*16*). While we are not entirely pleased with the mechanism of repetitious mutation as the explanation for the findings at position 5 and can conceive of alternatives (*17*), we nonetheless believe that the results at positions 116-117 distinctly favor the supposition of a single origin for β and δ genes in a common ancestor.

Once given a single common origin of β and δ, it is possible to reconstruct an approximation of the relevant positions in the archetype chain (top line, Fig. 2) as they existed before gene duplication. Archetype assignments are essentially unambiguous at all positions except 5, 12, 116, and 117. Reasonable assignments can be made at positions 12 and 117 through use of brown lemur β (*18*) and rhesus β (*19*) sequences (*20*). The archetypal residues at the remaining positions, 5 and 116, are indeterminate and arbitrarily shown as doublets. It is next possible to calculate for each species the minimum number of changes that have accumulated in each gene since its inception. The range for the minimum number of accumulated amino acid changes in each chain, based on differences from the archetype, is shown for each species on the right in Fig. 2. Beside these values are the minimum number of nucleotide changes, derived from genetic code (*21*), necessary to account for the observed amino acid changes. By hypothesis, the time course of variation is the same in both genes and in all species; thus overall rates of change in the interval since β-δ duplication may be compared. It is evident, in each species, that the total number of accumulated changes in δ tends to exceed that in β. This phenomenon is particularly noticeable in the two closely related species of tamarins (*Saguinus nigricollis* and *S. mystax*) whose β chains are identi-

Position	5	6[5]	9	10	12	16	21	22	46[6]	47[7]	50	51[8]	70[9]	86	87	116	117	121	125	126	130	139	Minimum Total Changes From Archetype: Amino Acids	Minimum Total Changes From Archetype: Nucleotides
Archetype	Pro Gly	Glu	Ser	Ala	Thr	Gly	Asp	Glu	Gly	Asp	Thr	Pro	Ala	Ala	Gln	Arg His	His	Glu	Gln	Val	Tyr	Asn		
Human β	(PRO)	Glu	Ser	Ala	Thr	Gly	Asp	Glu	Gly	Asp	Thr	Pro	Ala	Ala	(THR)	(HIS)	His	Glu	(PRO)	Val	Tyr	Asn	2-4	3-6
Ateles β[1]	(GLY)	Glu	Ser	Ala	(ALA)	Gly	Asp	Glu	(SER)	Asp	Thr	Pro	Ala	Ala	Gln	(HIS)	His	Glu	Gln[15]	(LEU)	Tyr	Asn	3-5	3-6
Saimiri β	(GLY)	(ASP)	(ALA)	Ala	Thr	Gly	Asp	Glu	(ASN)	Asp	Thr	Pro	(THR)[14]	Ala	Gln	(HIS)	His	Glu	Gln[15]	Val	Tyr	Asn	4-6	5-8
Saguinus β[2]	(GLY)	Glu	Ser	(THR)[12]	Thr	Gly	(GLU)	Glu	(SER)	Asp	(ASN)	Pro	Ala	Ala	Gln	(HIS)	His	Glu	Gln[15]	Val	Tyr	Asn	4-6	4-7
Human δ	(PRO)	Glu	(THR)	Ala	(ASN)	Gly	Asp	(ALA)	Gly	Asp	(SER)	Pro	Ala	(SER)	Gln	(ARG)	(ASN)	Glu	Gln	(MET)	Tyr	Asn	7-9	7-10
Ateles δ[3]	(GLY)	Glu[10]	(ALA)	Ala	(ALA)	Gly	Asp	Glu	Gly	(ALA)	Thr	Pro	Ala	Ala	Gln	(ARG)	(ASN)	Glu	Gln[15]	Val	(PHE)	(THR)	6-8	6-9
Saimiri δ	(GLY)	(ASP)	Ser	Ala	(ALA[13])	(SER[13])	Asp	Glu	Gly	(ALA)	(SER)	(ALA)	Ala	Ala	Gln	(ARG)	(ASN)	Glu	Gln[15]	Val	(PHE)	(THR)	9-11	9-12
Saguinus nigricollis δ[4]	(GLY)	Glu[11]	Ser	Ala	(ALA[13])	(SER[13])	Asp	Glu	Gly	(ALA)	(SER)	Pro	Ala	Ala	Gln	(ARG)	(ASN)	Glu[11]	(ARG)	Val	(PHE)	(THR)	8-10*	8-11

Fig. 2. Differences between hemoglobin β and δ chains from primates. Positions where all chains are similar are omitted. Except where noted, results are based on amino acid (*24*) composition of tryptic peptides from single individuals (*15*). Distinction between acids and amides was made by peptide electrophoresis. Animal sequences were inferred through homology with corresponding β and δ sequences from man (*18*). Archetype represents probable reconstruction of residues as they existed before gene duplication producing β and δ in a common ancestor of all species shown. Archetype assignments are discussed in text. Differences from archetype are encircled. Superscripts indicate the following. 1, Identical in *A. belzebuth* and *A. geoffroyi*; 2, identical in *S. mystax* and *S. nigricollis*; 3, *A. geoffroyi* A_2-δ; identical, except δ-6, in Y_2-δ from both *A. geoffroyi* and *A. fusiceps*; 4, two-animal pool; 5, position 6 or 7; 6, position 46 or 56; 7, position 47 or 52; 8, position 51 or 58; 9, position 70 or 76; 10, Lys in Y_2; 11, Asp in *S. mystax*; 12, position 10 or 13; 13, position 12 or 16; 14, Ala/Thr heterozygote in one of four animals; 15, position 124 or 125. * Sequence of *S. mystax* δ is incomplete and thus total differences from archetype can be assessed only for *S. nigricollis*.

cal but whose δ chains differ by at least two residues (Fig. 2 legend). It might be inferred from this last finding and from the considerable electrophoretic heterogeneity of A_2 that enhanced variation of δ is a comparatively recent phenomenon. It is apparent from Fig. 2 that quite the opposite is true. In all New World primates identical and, in general, unique changes from the archetype exist at δ-12, -47, -130, and -139. These changes presumably occurred after the divergence from man's ancestors but before taxonomic divergence of the New World animals. In contrast, most of the changes in monkey β apparently occurred after New World speciation. Indeed, in the period since divergence of the New World primates from one another β changes exceed those in δ in every instance, except possibly the only partially characterized δ chain of *S. mystax*. While it may be that major concentrations of A_2 existed before New World speciation and thereby rendered A_2 less neutral than presumed from its present low level, we continue to regard this sort of assumption, discussed earlier, as unlikely. This set of events would necessitate two independent regulatory mutations, one in monkeys and one in man, both of which set A_2 to roughly the same low level.

Interpretations of our findings hinge in part upon the validity of the proposal that δ is selectively much more nearly neutral than β. This proposal may be rejected, and if one's faith in natural selection as the only arbiter of evolution is strong enough, the existence of a larger number of changes in δ than in β (Fig. 2) might suggest that, in some unknown manner, the functional and thus the overall adaptive importance of A_2—even at concentrations 1/160 that of A—exceeds that of A. We discount this extreme interpretation as implausible and note that Old World primates tolerate the apparent absence of A_2. We cannot, however, formally exclude selection as the source of our findings since it is possible that δ has come to vary more than β through a combination of (a) selective constraints upon β and (b) comparative freedom of δ, by virtue of its lesser concentration, to escape such constraints and undergo adaptive Darwinian evolution. In this respect, our initial presumptions may have been unrealistic insofar as they were based on the supposition that selection would operate in the same direction—albeit to a different extent—on two similar genes.

In any case, the pivotal question be-

comes: at what level of concentration does a minor member of a pair of duplicate gene products become sufficiently functionless so as to be invisible to selection? We are inclined to believe that level has been reached in the case of A_2 where the contribution of this component to overall hemoglobin concentration is quantitatively less than day-to-day variation in total concentration. Nonetheless, even if the variation of δ sequence and the contemporary polymorphism of A_2 are largely or entirely attributable to non-Darwinian evolution, it is still necessary to posit selective constraints upon β in order to explain the lesser variation in β than δ (Fig. 2). These constraints upon β have apparently differed in several evolutionary epochs in that detectable variation of β among the New World primates studies has only occurred since the taxonomic divergence of these species. The fact that δ variation is less in the latter interval than in an earlier time suggests, on the presumption of δ neutrality, that the postspeciation interval among the New World primates examined has been shorter in duration than the prespeciation period.

Despite the uncertainties attached to our presumptions, and thus to our interpretations, we regard the situation of duplicate genes with products of major and minor concentration as one that provides a potentially useful model for assaying the relative influences of Darwinian and non-Darwinian factors in evolution.

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16. Our finding of an unusual number of sites (7 of 21) with two or more changes in closely related chains and species (Fig. 2) is at variance with the random distribution of about 484 changes in globin obtained from a less closely related group of animals (*9*). If hypermutability is a property of particular codons rather than particular positions, then this phenomenon would tend to be self-eliminating and would not be observed when data from very diverse species are compared.
17. Alternative explanations for the findings at position 5 include (i) a complex succession of nonhomologous recombinations between β and δ, and (ii) homozygosity for a mutation affecting the amino acid acceptor site of transfer RNA used as this position. In either case, the mutation would need to have taken place after divergence of man and New World primates from the stem line.
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20. Threonine occurs at β-12 and histidine at β-117 in both brown lemur (*18*) and rhesus (*19*). It is believed that these residues have been preserved in both animals since their divergence from the primate stem line. These residues are assigned to the archetype (Fig. 2), since it is supposed that the ancestors of lemurs diverged before and those of rhesus after the branching between the progenitors of man and New World primates.
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24. The following abbreviations are used: Ala, alanine; Arg, arginine; Asn, asparagine; Asp, aspartic acid; Gln, glutamine; Glu, glutamic acid; Gly, glycine; His, histidine; Leu, leucine; Lys, lysine; Met, methionine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Tyr, tyrosine; Val, valine.
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Lateral Hypothalamic Stimulation: Inhibition of Aversive Effects by Feeding, Drinking, and Gnawing

Abstract. *The opportunity to engage in feeding, drinking, and gnawing behavior facilitated by localized hypothalamic stimulation can delay the onset of the aversive effects of the stimulation and may completely suppress them. This suggests that the aversive effects of the stimulation are due to the excessive arousal of a drive.*

Electrical stimulation of some brain sites gives rise to both rewarding and punishing effects (*1–3*). Short durations of stimulation are rewarding, while longer durations become punishing. Cats and rats with electrodes in these parts of the brain typically make one response to turn on the stimulation, wait for a few seconds, and then very quickly make another response to shut it off. The on-off sequence may be repeated over 80 times during a 10-minute period (*4*). This pattern of intracranial self-stimulation is usually interpreted as indicating that the stimulation is initially rewarding (as indicated by the fact that the animals turn it on), but soon becomes aversive (thus motivating the animals to turn it off) (*5*).

While there have been a number of studies concerned with the effects of stimulation parameters, such as intensity (*3, 6*), on the preferred duration of stimulation, there is a lack of experiments attempting to analyze the nature of the aversive process itself. One clue as to the aversive quality of the stimulation may be gleaned from an observation of the responses elicited by the stimulation. Electrodes whose stimulation gives rise to both rewarding and punishing effects frequently lie in and around the lateral hypothalamic area. This is the same area whose stimulation facilitates a variety of motivational activities (*1, 7*), such as feeding, drinking, gnawing, biting, sexual behavior, grooming, wheel-running, exploration, and various components of maternal behavior (nest building and pup retrieval).

Many of these behaviors induced by brain stimulation have been shown to be