Polymorphism and Protein Evolution The Neutral Mutation-Random Drift Hypothesis

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Enzyme and Protein Diversity

In recent years many examples of what are conveniently referred to as enzyme and protein polymorphisms have been discovered in the course of surveys of human populations and of naturally occurring populations of other animal species. The term polymorphism is used in this context for any situation where members of a population can be sharply classified into several distinct phenotypes in terms of particular characteristics of the enzyme or protein, and where at least two of the phenotypes have an appreciable incidence (greater than 2%). Most of these polymorphisms can be attributed to the occurrence of two or more alleles each coding for a structurally distinct form of a polypeptide chain in the particular enzyme or protein. It is thought that the structural difference between the polypeptides usually amounts to no more than a single amino-acid substitution, and that the allelic difference originated in a single mutational event involving the change of only one base for another in the sequence of several hundred or thousand bases in the DNA of the particular gene. But direct evidence for this has so far only been obtained in a limited number of polymorphisms, and there are certainly some exceptions where the polypeptide products of the two common alleles differ by more than one amino acid, eg, the sheep haemoglobins A and B (Boyer *et al*, 1967).

Electrophoretic surveys of arbitrarily chosen enzymes and proteins have been carried out in various naturally occurring animal populations to see how often such polymorphisms occur. A number of very different species have been studied in this way. They include man (Harris, 1966 and 1969), *Drosophila pseudoobscura* (Hubby and Lewontin, 1966; Lewontin, and Hubby, 1966; Prakash, Lewontin, and Hubby, 1969), mouse (Ruddle *et al*, 1969; Selander, Hunt, and Yang,

1969; Selander and Yang, 1969), and the horseshoe or king crab *Limulus polyphemus* (Selander *et al*, 1970). The results indicate that two or more relatively common alleles giving rise to electrophoretic differences may exist at some 25–40% of loci coding for enzyme and protein structure.

The frequencies of the common alleles which make up the different polymorphisms vary considerably from case to case. However a useful index of the extent of the phenomenon in a particular population is obtained by estimating the average heterozygosity per locus. This is the sum of the proportion of individuals found to be heterozygous at each locus divided by the total number of loci studied, including of course both 'polymorphic' and 'non-polymorphic' loci. Studies on some 26 arbitrarily chosen loci in European and negro populations gave values in each population of just over 0.05 for the average heterozygosity per locus which could be detected by enzyme electrophoresis (Harris, 1970). However it was of interest that the contributions of different loci to this average sometimes varied quite widely between the two ethnic groups. Since it seems probable that at most only about one third of all possible structural changes in an enzyme produced by mutations would be detected electrophoretically, the results suggest that the true value for average heterozygosity per locus may be around 0.16. Put another way, one might expect that any single individual in these populations would be heterozygous at perhaps 16% of gene loci coding for enzyme structure. Less systematic data on some 30 other enzyme loci in man appear to lead to essentially the same estimate. Similar or higher estimates of average heterozygosity per locus have been obtained from electrophoretic studies of different enzyme and other proteins in naturally occurring populations of *Drosophila pseudoobscura*, *D. persimilis*, *Mus musculus*, *Peromyscus polionotus*, and *Limulus polyphemus* (Selander *et al*, 1970).

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It should be pointed out that these investigations have been largely confined to soluble enzymes and other proteins which can be examined electrophoretically. It is possible, for example, that structural proteins or membrane-bound enzymes may differ in this respect, though there is as yet little evidence one way or another on this point. Also of course only a very small number of different enzymes and proteins have been investigated compared with the many thousands that actually occur in an organism. So the estimates of the fraction of polymorphic loci or of average heterozygosity per locus must be regarded as very rough and tentative. Nevertheless it is clear that in each of several very different species enzyme and protein polymorphism is a not infrequent phenomenon. It is certainly very much more extensive than was generally thought possible only a few years ago.

Besides the alleles giving rise to these common polymorphic differences many more alleles determining distinctive variants have also been found to occur when particular enzymes or proteins have been examined by suitable procedures in sufficiently large numbers of individuals. Human adult haemoglobin α and β loci (Lehmann and Carrell, 1969), and the enzyme glucose-6-phosphate dehydrogenase (Kirkman, 1971) have for various reasons been studied from this point of view in more detail, and in very many more individuals than any other proteins, and at each of the loci concerned some 50 or more rare variants determined by different rare alleles have already been discovered. And the rate at which new examples are currently being reported suggests that many more remain to be identified. Other enzymes and proteins have been surveyed less extensively, but nevertheless in a variety of cases (eg, serum albumin, transferrin, phosphoglucomutase, phosphohexose isomerase, placental alkaline phosphatase, NADH diaphorase, etc) a series of different variants each evidently due to a different rare allele at the structural locus concerned have been found. In general it is now beginning to appear that as more individuals are examined for a particular enzyme or protein the number of different rare variants that are discovered progressively increases. Some of these rare variants give rise in either homozygous or heterozygous states to overt clinical abnormality, but many appear to be relatively harmless. Furthermore the great majority of rare variants found in population surveys do not appear when family investigations are carried out, to be the product of fresh mutations occurring in the immediately preceding generation. The occasional exceptions have usually been variants (such as unstable haemoglobins) where overt

clinical abnormality is seen in the heterozygous state.

It is usual to regard any allele with a frequency in the population of less than 10^{-2} as 'rare'. In fact the majority of rare alleles which have so far been found appear to have frequencies much less than this. In surveys involving 2000 to 10,000 individuals particular 'rare' alleles are often found in only one or two individuals. So their individual frequencies appear to be generally less than 10^{-3} and usually perhaps less than 10^{-4} . However, because quite a number of different 'rare' alleles can evidently occur at any one locus, they may contribute appreciably to individual heterozygosity (Hopkinson and Harris, 1971). Rough estimates derived from electrophoretic surveys of enzymes in human populations suggest that any single individual may be heterozygous for one or another rare allele at perhaps 0.3% of loci coding for enzyme structure.

Mutation, Selection, and Drift

Thus it now seems likely that at most gene loci coding for enzyme or protein structure many different alleles occur among the members of natural populations. At any particular locus the majority are probably very rare, but there must of course always be one allele which is very common, and often as we have seen there may be two.

The alleles which are present in populations today may be presumed to have arisen by separate mutations in individuals in earlier generations, some perhaps relatively recently, others in the more remote past. And in terms of classical theory one would expect that in general their incidence and distribution today will reflect the operation of natural selection over the course of many previous generations on the flux of mutant alleles being continuously generated by fresh mutations at a low but steady rate.

Natural selection will tend to eliminate from the population mutant alleles which have relatively deleterious effects on individuals who carry them, and tend to cause the spread of those which are advantageous. Since one would expect that a single random alteration in a complex structure like an enzyme protein is more often likely to impair than to improve its function, very many more of the mutants that occur at any particular locus are likely to be detrimental than advantageous. Thus the conclusion that the great majority of variants due to alleles at a single locus are rare, is in general keeping with this expectation.

But the discovery that at an appreciable

proportion of loci, two common alleles occur rather than just one was rather surprising, and its interpretation has engendered a great deal of discussion and controversy.

What can be called the classical view is that such polymorphisms are the consequences of differential selection. Many polymorphisms are thought to represent stable, balanced equilibria due to heterozygous advantage, frequency-dependent selection, or other processes. Others however may represent intermediary stages in gene evolution in which one allele is progressively replacing another on its way to fixation. Such situations are sometimes called 'transient polymorphisms'. But whatever the details of a particular situation it is usually assumed that the occurrence of two common alleles at any particular locus must have come about because of differential selection on the phenotypic differences they produce.

A quite different view of the matter has however recently been put forward, notably by Kimura (Kimura, 1968; Kimura and Ohta, 1971). This suggests that the enzyme and protein variants that make up these polymorphisms are selectively neutral and that the occurrence of two common alleles at many different loci is a simple consequence of random genetic drift. If correct, this hypothesis would seem to call for a considerable reappraisal of many long held concepts in population genetics and in evolutionary biology.

Molecular Evolution

The strongest evidence for Kimura's hypothesis comes from the mathematical analysis of the rates of molecular evolution of certain proteins such as cytochrome C and haemoglobin whose amino-acid sequences have been determined over a wide range of species.

For example, the amino-acid sequence of the cytochrome C obtained from some 30 different species has now been determined (Nolan and Margoliash, 1968). In the sequence of the 100 or so amino acids which occur in each of these proteins, about 30 positions appear to be invariant, and the others differ to varying extents in the amino-acid substituents which occur. These amino-acid differences may be assumed to have arisen during the course of evolution by single base change mutations, and by comparison of the amino-acid differences between each pair of proteins it is possible to estimate the minimal number of mutations which would have been required for their divergence from their nearest common ancestor. From such data it is then possible to construct the most

likely phylogenetic tree which would have given rise with the minimum number of mutational changes to the amino-acid sequences in the proteins as they are found to occur in the different species today (Fitch and Margoliash, 1967). The evolutionary tree so constructed turns out to resemble quite closely the phylogenetic relationships obtained in the orthodox way using morphological and other standard taxonomic criteria. In this case however the phylogenetic tree is entirely deduced from the amino-acid sequences of a single polypeptide chain and can therefore be taken to represent the evolution of a single gene.

Using estimates derived from palaeontology of the time that has elapsed since various groups of species diverged from a common ancestor, it is possible from such data to estimate and compare the rates of molecular evolution by amino-acid substitution in particular proteins and in different lines of evolutionary descent (Kimura, 1969; King and Jukes, 1969). Thus for cytochrome C the average rate of amino-acid substitution per amino-acid site per year appears to have been about 4×10^{-10} . Not surprisingly the rate varies from protein to protein. For example, the rate for haemoglobin appears to have been about $2\frac{1}{2}$ times greater than for cytochrome C, and the rate for fibrinogen peptide A about 4 times that for haemoglobin.

But it is claimed that for any one protein, the rate of evolutionary change by amino-acid substitution has been remarkably uniform over widely divergent lines of descent (Kimura, 1969). Thus the average rate of amino-acid substitution in haemoglobin is estimated as about 10^{-9} per amino-acid site per year, and it appears that this rate has been essentially the same in the evolutionary lines leading for example to the α haemoglobin chains of the carp and of man since they diverged from a common ancestor some 350–400 million years ago; in the lines leading to the α or β chains of various mammals such as man, mouse, rabbit, horse, pig, and cow since they diverged from a common ancestor some 80 million years ago; and indeed in the lines leading to the human α and β chains since they originated by gene duplication at a time estimated to have been about 450 million years ago.

Kimura argues that this apparent constancy of evolutionary rate in a protein can only be plausibly accounted for by supposing that the evolutionary changes are due to the fixation in different species of particular mutations which while resulting in amino-acid substitutions in the protein cause no significant change in its functional properties, and which are therefore selectively neutral. The term non-Darwinian evolution has sometimes been

used to describe such a process (King and Jukes, 1969).

Kimura has shown that if one defines the rate, k , of mutant substitution in evolution as the long term average of the number of mutants that are substituted per unit time, then under the neutral mutation-random drift hypothesis, $k = u$, where u is the mutation rate per gamete for neutral mutants at this locus per unit time (Kimura, 1968). It follows that the apparent uniformity of rate of amino-acid substitution per year in a particular polypeptide chain is readily accounted for, provided that the substitutions are indeed selectively neutral and that rate of mutation of the gene to such neutral alleles has been the same in different species and in different lines of descent.

Only a proportion of all the possible mutants that may arise are assumed to be neutral, and the differences in rates of amino-acid substitution in different proteins such as haemoglobin and cytochrome C, can be explained by supposing that a different fraction of mutants are neutral in each case, according to the structural and functional characteristics of the different proteins.

An intriguing and unexpected consequence of the hypothesis is that for any given protein the neutral mutation rate per year should be the same in different species. But mutation rates are usually considered in terms of the rate per gene per generation rather than the rate per gene per year. Since generation times vary widely from species to species, it seems necessary on this hypothesis to assume that they are proportional to mutation rate. Thus if in the mouse the generation time is taken as 6 months and in man as 20 years, the hypothesis implies that the mutation rate per gene per generation is about 40 times greater in man than in mouse.

The hypothesis that the differences in amino-acid sequence of a particular protein in different species are simply the consequence of neutral mutations which have been fixed by random genetic drift, is of course exactly the opposite of the classical view which would ascribe the observed evolutionary changes in protein structure to the operation of natural selection. On this view the structure of a particular protein in a given species has been selected because it is best adapted to meet the particular features of the external environment in which the species lives, and also to meet the special internal environment which depends on the activities of all the other genes present.

According to Kimura and Ohta (1971) the rate of mutant substitution in evolution by natural selection is given by

$$k = 4N_e s_1 u$$

where N_e is the effective population number of the species, s_1 is the selective advantage of the mutant, and u is the rate at which advantageous mutants are produced per gamete per unit time. They argue that in order for the rate of amino-acid substitution to have remained constant per year over diverse lines such as the lines leading to carp and human haemoglobins from a common ancestor, it would be necessary to assume under natural selection that the three different parameters N_e , s_1 , and u were so adjusted that their product remained constant. And they consider that this is highly implausible, particularly over lines leading to such species as carp and man, which have been separate for some 350–400 million years, during which time many marked differences at the phenotypic level have evolved.

In this connexion it should be pointed out that Kimura is not arguing that natural selection has been of no importance at all in evolution. He is however saying that surrounding any adaptive changes that may occur because of natural selection, there is a very great deal of random noise from neutral change, and that the differences in amino-acid sequence of a particular protein in different species are largely, if not entirely, the product of such neutral substitutions.

Polymorphism: A Phase of Molecular Evolution?

Now whether these differences between species are the products of neutral mutations, or whether they are the result of natural selection, it is clear that they must have occurred in any given case by the progressive replacement of a pre-existing gene by a particular mutant which has spread through the species and eventually become fixed as the characteristic type. So at any one time one might expect to find evidence for such partially completed replacements occurring. The obvious candidates for this are of course the enzyme and protein polymorphisms.

A very large number of different alleles may in principle be generated by separate mutations within the confines of a single gene. Thus from a typical gene containing a DNA sequence say 900 bases long and coding for a polypeptide chain of 300 amino acids, as many as 2700 different alleles may be generated by single base change mutations alone, since each base could be altered to one of three others in different mutational events. From what is known about the genetic code and about the amino-acid composition of proteins one would expect that some 70% or so of these mutant alleles

would determine a structurally altered protein which differs from the original by a single amino-acid substitution. An indeterminate number of other mutations involving deletions, duplications, or other kinds of rearrangement of parts of the base sequence of the gene may also occur. Kimura suggests that although many of the possible structural variants of a protein which may be generated by random mutation are detrimental and therefore tend to be eliminated by natural selection, there will be some which are effectively neutral in the sense that they do not produce any functional change in the organism which would affect its viability or fertility and hence its ability to contribute offspring to the next generation. The persistence of such neutral mutants in subsequent generations will be largely a matter of chance, and the majority will in fact be lost. But very occasionally one of them will spread by random drift and eventually become the predominant allelic form. Such a process would however require a very large number of generations before it were complete (about $4N_e$ generations), and so for quite long periods of time there would be two common alleles co-existing in the species.

The argument is supported by an elegant mathematical analysis (Kimura and Ohta, 1971) which appears to show that the degree of heterozygosity resulting from polymorphism which has been observed in different animal populations, is of a magnitude consistent with that expected from the rates of amino-acid substitution in protein evolution, on the assumption that both the intraspecific polymorphic differences and the interspecific amino-acid substitutions are selectively neutral. Thus the hypothesis suggests that protein polymorphism and protein evolution should be regarded not as separate phenomena, but as two aspects of a single process, that is random frequency drift of neutral mutants in finite populations.

Selective Differences

The crucial question of course is whether or not selective differences between the different phenotypes which make up the enzyme and protein polymorphisms actually exist. Such selective differences if they occur should in general be manifest by differences between the phenotypes in the extent to which they contribute offspring to the next generation. The differences might arise for example because the phenotypes differ in their relative viabilities in early life or in their relative fertilities when adult.

In its simplest form Kimura's hypothesis appears

at first sight to be incorrect, since there seems no doubt that at least certain polymorphisms involve significant differences in the extent to which different phenotypes contribute to the next generation. The best known example is the sickle-cell haemoglobin polymorphism in African populations (Allison, 1964). Here it has been shown that the heterozygotes are less susceptible than other individuals to the more serious consequences of malaria, so that in conditions where malaria is endemic they have a better chance of surviving to adult life and so becoming parents. The selective difference that this confers is evidently the reason for the high incidence that this particular mutant has come to assume in Africa, despite its lethal effects in homozygotes who develop the severe disease, sickle-cell anaemia. Another probable example of such a selective differential is provided by the glucose-6-phosphate dehydrogenase polymorphism in Mediterranean populations. Here certain genotypes are predisposed to develop the disease Favism and this must limit at least to some extent their average contribution to the next generation. The effect is thought to be balanced as in the sickle-cell case by a better viability of heterozygotes under malarial conditions (Luzzatto, Usanga, and Reddy, 1969).

However, it would I think be argued by Kimura and his colleagues that these are special cases and that their occurrence does not necessarily destroy the general argument that most enzyme polymorphisms are selectively neutral. And it is true that in the majority of the enzyme and protein polymorphisms that are known to occur in man and in other species there is as yet little direct evidence to suggest that the several genotypes differ in either viability or effective fertility.

However, selectionists can reasonably contend that only extremely small selective differences would have been needed to produce and subsequently maintain most polymorphisms, and that the selective differences which can be detected in such cases as the sickle-cell and glucose-6-phosphate dehydrogenase polymorphisms simply represent extreme examples of the general rule. In order to detect directly the very small selective differences which might in most cases be expected it would certainly in humans be necessary to carry out population and demographic studies of a very considerable size and complexity, and so far only a few relatively limited investigations of this sort have been attempted. The same general problem arises in analogous studies in naturally occurring animal populations. Thus, in general, the absence of direct evidence for viability or fertility differences

between individuals of the several phenotypes in most polymorphisms though consistent with the hypothesis of neutrality, is not at present particularly strong evidence in its favour.

An important source of difficulty in such investigations is that in general one would expect that selection will be directed at complex phenotypic characteristics dependent on many different enzymes and proteins acting together. It may therefore be necessary to compare the relative fitnesses of multiple combinations of phenotypes of different enzymes and proteins, rather than rely simply on comparisons between the common phenotypes for each enzyme or protein taken separately. This is likely to enhance greatly the difficulty of obtaining unambiguous evidence for selective differences in terms of viability or effective fertility.

Functional Differences

However, even small selective differences in viability or fertility if they occur must be the consequence of functional differences between the particular enzymes or proteins determined by the common alleles involved in a polymorphism. Thus, while on the neutralist hypothesis the enzyme products of the common alleles should be functionally the same, on the selectionist hypothesis they should be in some degree functionally different.

One way of trying to assess such possible functional differences is to measure the level of activity of the enzyme in the different phenotypes. In theory a very large number of different variants of a particular enzyme protein may be generated by separate mutations and one might expect that while some of them will not be associated with any change in activity, others will show such a change. So one can argue that on the neutralist hypothesis the enzyme variants that make up the common polymorphisms should usually be variants which show no change in activity, whereas on the selectionist view differences in activity should frequently be found.

In fact differences in enzyme activity have been demonstrated in a number of polymorphisms originally detected by electrophoresis. Red-cell acid phosphatase in man is a good example. The enzyme appears to be peculiar to the red cell and differs in its structure and properties from the acid phosphatases found in other tissues. In European populations several electrophoretically distinct phenotypes are seen and it has been shown that they are due to the occurrence of three common alleles determining structurally distinct forms of

the enzyme protein (Hopkinson, Spencer, and Harris, 1963 and 1964). But the differences in structure which cause the electrophoretic differences also result in differences in activity. On average the level of acid phosphatase activity in red cells of homozygous type B individuals is about 50% greater than in type A individuals, and the heterozygous type BA individuals show intermediate levels. Similar differences are found in the other types.

The precise role of this particular acid phosphatase in red cell metabolism is not known, and there are no obvious differences in health or viability between individuals of the different phenotypes. But it would seem rash to assume that the differences in activity are not reflected in some way in red cell metabolism and they therefore play no part at all in determining the biological fitness of individuals of the different types.

Similar activity differences have been demonstrated in other enzyme polymorphisms originally detected electrophoretically. Besides this there are a number of polymorphisms which were originally identified precisely because of such quantitative differences. I have attempted to list all the enzyme polymorphisms so far detected in major human populations in the Table and to

TABLE
ENZYME POLYMORPHISMS IN MAN

Polymorphisms where Quantitative Differences between the Common Phenotypes have been Found	Polymorphisms where as yet no Quantitative Differences have been Reported
Glucose-6-phosphate dehydrogenase	Adenosine deaminase
Red-cell acid phosphatase	Peptidase D (prolidase)
Phosphogluconate dehydrogenase	Pancreatic amylase
Adenylate kinase	Pepsinogen
Placental alkaline phosphatase	Glutamate-oxalate transaminase
Peptidase A	Phosphoglucomutase
Peptidase C	Locus <i>PGM</i> ₁
Galactose-1-phosphate uridyl transferase	Locus <i>PGM</i> ₃
Glutathione reductase	
Liver acetyl transferase	
Red cell NADase	
Salivary amylase	
Serum cholinesterase	
Locus <i>E</i> ₁	
Locus <i>E</i> ₂	
Alcohol dehydrogenase	
Locus <i>ADH</i> ₂	
Locus <i>ADH</i> ₃	

classify them according to whether or not quantitative differences in activity have been demonstrated between the common phenotypes. Out of the 23 gene loci involved, evidence for quantitative differences have been obtained in 16. In most of the other cases the point has not yet been closely

examined. So such quantitative differences appear to be a not uncommon phenomenon among the enzyme polymorphisms.

It should be noted that enzyme activity determined by assay *in vitro* using standard techniques is usually likely to be a very crude and somewhat insensitive indicator of activity *in vivo*, because the assay has generally to be carried out with quite unphysiological substrate concentrations and in an ionic environment often very different from that obtaining intracellularly. So subtle differences which may be important *in vivo* may often be missed.

The finding that activity differences are by no means uncommon tends to favour the view that the polymorphisms are the consequence of natural selection rather than of random drift. However, the argument is far from conclusive because in the absence in most cases of any clear understanding of how such differences might affect the organism as a whole, it can still be argued that the quantitative differences observed may cause no significant selective differential so that the alleles concerned are effectively neutral.

The question of possible functional differences between the structurally distinct versions of particular proteins such as cytochrome C and haemoglobin in different species is also important to the argument, since in its simplest form the neutral mutation-random drift hypothesis of molecular evolution implies that the amino-acid differences which they exhibit do not have any significant effect on their functional properties. In the case of cytochrome C the failure to observe significant differences in reaction rates of bovine cytochrome C oxidase with the cytochrome Cs obtained from a variety of species and differing from bovine cytochrome C by up to 27 amino acids out of a sequence of 104 (L. Smith cited by Margoliash, Barlow, and Byers, 1970) seemed to argue in favour of the view that these amino-acid substitutions were neutral in their effects. But more recently evidence has been obtained for differences in their ion binding properties which may be relevant to mitochondrial ion transport, and possibly of important functional and selective significance (Margoliash *et al*, 1970).

In the case of haemoglobin, functional activity as expressed for example in its oxygen affinity depends both on the structure of the protein and on the particular ionic environment in the red cell which may also differ from species to species. An interesting example which illustrates the difficulty of assuming that the structural differences in the protein are of no functional significance is provided by the demonstration that the oxygen affinity of

sheep haemoglobin freed from small molecules is similar to that of human haemoglobin only when the latter is complexed with 2-3 diphosphoglycerate, a substance which is virtually absent from the red cells of ungulates, but which is present in most other animals including man where it has been shown to be of major importance in the regulation of oxygen release by haemoglobin (Benesch, Benesch, and Yu, 1968; Benesch and Benesch, 1969).

In its simplest form Kimura's hypothesis would suggest that if one could entirely replace the haemoglobin present in human red cells by haemoglobin obtained from say the mouse or the rabbit, or even the carp, no functional disability would result. Such an experiment is not of course feasible, but essentially the same kind of comparison may be provided in the particular cases where the long-term evolutionary products of a gene duplication occur together in a single species. For example Kimura (1969) has argued that the rate of amino-acid substitution in the evolution of the α and β chains of human haemoglobin since they originated from a common ancestral gene by duplication, has been the same as the rates of amino-acid substitution occurring in the subsequent divergence of evolutionary lines leading to structurally different α chains in different species, and also in the lines leading to the structurally different β chains. This is considered as strong evidence for the view that the various amino-acid differences between these polypeptide chains are the consequence of neutral mutations. If so one might have expected that the α and β chains of haemoglobin would be functionally interchangeable. That this is not the case however is readily seen from a comparison of the properties of the normal Hb A tetramer $\alpha_2\beta_2$ and the abnormal tetramer Hb H which has four normal β chains but no α chain (Jones, *et al*, 1959) and which is formed in individuals in whom α chain synthesis is specifically restricted (Weatherall, 1965). Hb H is very much less stable than Hb A and readily precipitates. It has an oxygen affinity 10 times greater than the Hb A, and its oxygen dissociation curve shows no evidence of haem-haem interaction (Benesch *et al*, 1961). This and other evidence makes it clear that the molecular evolution of the α and β chains has been shaped to a significant degree by natural selection, and that the emergence of the tetrameric form of haemoglobin with two α and two β chains is not purely a fortuitous consequence of neutral mutations and random drift.

Slightly more than 50% of the homologous sites in the human α and β chains differ in their amino-acid substituents and in addition there are several

sites represented in one chain but not in the other which are presumably the consequence of the fixation of mutations causing deletion or additions to the ancestral DNA sequences. It remains to be determined to what extent the specific differences at the various sites contribute to the marked overall difference in the functional properties of the two chains. If it should turn out that only a small proportion of the substitutions were significant in this respect then the neutral mutant-random drift hypothesis would not be too seriously eroded. But most protein chemists would probably regard this result as unlikely.

The β and γ haemoglobin chains which are presumed to have derived from a somewhat more recent gene duplication raise another interesting point. They are both formed in the normal individual but at different times. Synthesis of γ chains occurs in fetal life and gives place to β chain synthesis at the end of gestation, so that while the principal haemoglobin in the red cells of the fetus is Hb F ($\alpha_2\gamma_2$) in postnatal life it is Hb A ($\alpha_2\beta_2$). The two chains differ in about one quarter of their amino-acid substituents. If these differences are all to be attributed to neutral substitutions, then it would seem necessary to conclude that the development shift from γ to β chain synthesis at birth is also fortuitous and of no functional significance.

Concluding Remarks

The neutral mutation-random drift explanation of polymorphism and protein evolution brings together areas of biology as diverse as the mathematical theory of population genetics, the nature of structure-function relationships of enzymes and proteins, the interpretation of field studies of polymorphic variation, and the significance of species differences. It calls for a new look at many long held concepts in each of these different fields, and not surprisingly it has provoked considerable controversy.

A variety of other arguments besides those already mentioned have been deployed both for and against the hypothesis. See for example, Boyer *et al* (1969); King and Jukes (1969); Clarke (1970a and b); Smith (1970a and b); Richmond (1970); Fitch and Markowitz (1970); Jukes and King (1971); Kimura and Ohta (1971). These arguments cite phenomena of many different kinds derived from a wide range of species. But critical data by which the hypothesis can be assessed is still very limited, and it is often far from clear whether a particular example should be regarded as a special case or as something which illustrates a general rule.

However, although the arguments which have been put forward are still in many respects inconclusive, they have served to clarify some of the key issues involved in the controversy and have exposed a number of quite basic problems which had tended to become somewhat neglected.

Clearly much more extensive and critical data needs to be collected, and there is obviously also a need for the development of new and more sensitive methods for investigating from the functional point of view both the enzyme and protein polymorphic variation within species, and the differences between species.

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