COMPARATIVE EVOLUTION AT THE LEVELS OF MOLECULES, ORGANISMS, AND POPULATIONS

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1. Introduction

Nearly all modern biologists who study evolution agree in believing that the rate and direction of evolution is determined by interactions between different processes, all of which are significant and none of which can be neglected. A synthetic theory of evolution, in some form or another, is here to stay. Five processes are recognized to be of basic significance. Two of these, mutation and gene recombination, contribute to the genetic variability that exists in all populations of higher organisms. The other three, selection, chance variations or gene fixations, and reproductive isolation, tend to reduce genetic variability, but are most significant as determiners of the rates and directions of evolution.

In spite of general agreement that all of these processes exist and may be significant at least under some conditions, great disagreement exists as to their relative importance. These differences may be due in part to the fact that the relative importance of different processes differs considerably from one group of organisms to another. Furthermore, even in the same evolutionary line great differences exist with respect to the relative importance of mutation, recombination, selection and the effects of chance when different molecules or even parts of molecules are compared with each other. Consequently, agreement and understanding are most likely to be reached by intensive studies of comparative evolution. Comparisons must be made both with respect to the evolution of entire organisms that belong to different evolutionary lines, as well as with respect to the evolution of different individual molecules or molecular systems in organisms belonging to the same evolutionary line. In accordance with the subject of the present conference, the comparisons to be made in this paper will be largely at the molecular level. Nevertheless, analogies to the evolution of molecular systems and of organs will be made when relevant.

2. Neutral mutations at the molecular level

Knowledge of the genetic code makes unavoidable the conclusion that a certain proportion of mutations, consisting of nucleotide substitutions from one codon to another that is synonymous with it, have little or no effect on the phenotype, since they do not alter the amino acid sequence of the polypeptide chain that is the primary product of the gene [17], [23]. Some of these may affect the phenotype because the synonymous codons are complementary to different molecules of transfer RNA, and so may be subject to different mechanisms that regulate transcription [8], [34]. That all synonymous codons differ with respect to these regulatory mechanisms is, however, rather unlikely. Nevertheless, even mutations to codons that are completely synonymous with each other may in many instances alter the future mutational possibilities of the genotype [8], [34]. This is because the most synonymous nucleotide of triplet codons, that in the third position, may sometimes be synonymous for all possible substitutions, while in other instances substitution of a purine for a pyrimidine or vice versa causes the codon to code for an amino acid having very different properties. If, for instance a DNA codon complementary to the messenger codon GGC mutates to one that is complementary to GGG, the coding for glycine at that position will be unchanged. Nevertheless, the unmutated codon can mutate by a single change in its first nucleotide to the complement of either UGC (cysteine) or AGC (serine), which requires a two step change for the mutated codon; while the latter can mutate in a single step to the complement of UGG (tryptophan), which requires the two steps for the unmutated codon.

In addition, mutations to codons for amino acids having properties that are very similar to those specified by the unmutated codon, for example, GUU (valine) to GCU (alanine) are very unlikely to affect the phenotypic properties of many proteins, particularly large enzyme molecules, and so would be neutral in their effects on the phenotype. Since the relationships between properties of individual amino acids and the effects that they produce upon proteins into which they have become incorporated is highly complex and imperfectly understood [37], the limits of this class of neutral mutations are even harder to define than are the limits of those that are due to synonymy of codons. Nevertheless, although we may for a long time have difficulty in classifying individual mutations as neutral or significant, we can hardly deny the existence of phenotypically neutral mutations. The frequency of their occurrence may be fairly high. Although, as has been so clearly shown by Ayala, Powell, Tracey, Mourao, and Perez-Salas [2], natural populations contain a very large amount of genetic variability that can be detected at the molecular level, the total amount that they contain is probably much higher. One way in which this can be shown is by selecting artificially for increased frequency of certain rare abnormalities, as has been done by Huether [14] with respect to corolla structure in *Linanthus*. As will be discussed later, the relationship of this variability to neutrality and

random fixation of genes depends to a large extent upon the relative positions of adaptive and neutral genes upon chromosomes.

3. The importance of adaptive shifts and of accessory adaptive characters

Most of this paper will be devoted to comparing the relative importance of natural selection as compared to random deviations, both in different evolutionary lines and in different molecules. At the outset, therefore, we need to have clearly in mind the relationship between adaptation and evolutionary change. To the extent that selection guides the course of evolution, it does so not by accepting or rejecting individual mutations, but through the effects of an altered environment upon the adaptive values of constellations of genes. The adaptive value of a particular allele or mutant gene, therefore, cannot be treated mathematically or statistically as a constant. Adaptive values of genes vary widely in relation to both the external environment to which the population is exposed, and the internal environment of the cell, in which the primary product of each gene must perforce interact with the products of many other genes.

The issue at hand, therefore, is not whether frequencies of individual genes are altered by the action of natural selection or by chance variations, but whether alterations of entire genotypes represent adaptive shifts of interacting genes that form integrated systems, or whether genotypes are altered by random changes in the frequencies of genes that act independently of each other. The case for believing that most observable changes in phenotypes and their determinant genotypes represent adaptive shifts that are due to natural selection in the presence of altered interactions between populations and their environment has been well presented and richly documented by many evolutionists [9], [30], [36]. Further discussion of it here would be superfluous. The question that must be asked here is: to what extent do the observed changes in molecules, particularly changes in the amino acid sequences of proteins, represent adaptive shifts in response to changing environments, and to what extent have they resulted from neutral mutations and random drift?

Before discussing this question, we must explore the consequences of recognizing that adaptive shifts based upon simultaneous alterations in the frequency of many genes, are far more significant than are alterations in the frequency of individual genes acting independently. For this purpose, we should like to extend the concept of homeostasis from animal physiology, where it was originally developed by Cannon [6] and from genetic variation in populations, for which it was developed by Lerner [27], to evolutionary variation in related groups of organisms. The basic principle of homeostasis, that can be applied at all three of these levels, is that certain essential functions, or processes can be kept constant over a wide variety of conditions by adaptive alterations of secondary or accessory characteristics.

A good example of evolutionary homeostasis is presented by the evolution of molecules and structures associated with photosynthesis in plants. This is shown in Table I. The basic molecules for the process are those of chlorophyll. The

TABLE I

ADAPTIVE DIFFERENCES ASSOCIATED WITH PHOTOSYNTHESIS AND LEVELS OF THE EVOLUTIONARY HIERARCHY AT WHICH THEY OCCUR

Structure affected	Level at which differences occur
Chlorophyll molecule	Kingdom
Plastid pigments	Phylum
Fine structure of chloroplast	Phylum, class, order (family, genus, species)
Cellular organization	Order, family (genus, species)
Leaf anatomy	Order, family, genus, species
Leaf size and shape	Genus, species, local population

principal molecule, chlorophyll *a* has been constant and unchanged throughout the evolution of plants, so that the only significant difference at the level of these molecules is that between the photosynthetic bacteria and other photosynthetic organisms. On the other hand, secondary chlorophyll molecules (*b* and *c*), as well as the molecules that constitute the accessory pigments of plastids, such as carotene and xanthophyll, and the special pigments found in various algae, vary in chemical structure from one phylum to another, but are usually constant within phyla. At a higher level of organization, that of the fine structure of chloroplasts, most variation is at the level of phyla or classes, but an outstanding exception is the specialized kind of chloroplast that exists in certain tropical grasses and other plants, and is associated with a different pathway of photosynthesis [26]. In one example, this difference with respect to fine structure and metabolic pathway exists between species of the same genus, *Atriplex* [4]. Finally, variation with respect to gross characteristics that are accessory to photosynthesis, such as leaf anatomy and leaf shape, exists mostly between different genera of the same family, between species of a genus, between races of the same species, or even between different leaves on the same plant.

Consequently, neither the great constancy of a structure or molecule in an evolutionary line nor its excessive variability can by itself be regarded as evidence for its adaptiveness or nonadaptiveness. On the other hand, adaptive variability at the level of populations and species is more likely to occur with respect to accessory functions rather than basic metabolic processes.

4. Comparisons of variations in different molecules

The principle of evolutionary homeostasis may explain the differences in rate of evolution of different molecules, that have already been discussed in this conference. Cytochrome *c*, which now may be regarded as a classic example of

evolutionary stability, catalyzes one of the most basic processes of cellular metabolism, that is equally important for all cellular organisms, and takes place at a site that is well protected from variations in either the external or the internal, cellular environment. Ferredoxin, which is somewhat more variable [5] catalyzes a reaction that is accessory to the main reaction of photosynthesis, but is closely associated with it. Hemoglobin, which is comparable in variability to ferredoxin, but may show significantly more variation, is also an accessory molecule, since its function is to transport oxygen through the vertebrate body. Finally, a much greater amount of variation at the level of family and genus is found in the fibrinopeptides [10], [31]. These molecules, since they are functionally associated with blood clotting, are definitely accessory, and in addition are particularly likely to interact with the external environment of the animal.

The question of adaptive shifts *versus* neutral mutations and random drift requires particular attention with respect to fibrinopeptides, for two reasons. In the first place, both King and Jukes [23] and Ohno [32] have maintained that their variations are neutral. Secondly, among the molecules in which variation of amino acid sequences has been recorded at the phyletic level, they are the only ones that are neither enzymes nor molecules with enzymelike functions, such as hemoglobin. The latter fact is important to those who wish to correlate phylogenies based upon phenotypes with those that are based upon changes in enzymes and similar molecules. The phenotype of an animal or plant is based chiefly upon the nature of two kinds of protein molecules. The first kind are those associated with regulation of growth: enzymes responsible for the biosynthesis of hormones and other regulators of gene action, growth and differentiation, as well as the proteins to which active hormones are frequently bound [15]. The second kind are the various structural proteins, such as collagen, actin and myosin of muscles, the proteins of nerve cells, keratin, chitin, cell wall proteins of plants, and many others. Many of these proteins differ widely in amino acid composition from the metabolic proteins that have been studied phylogenetically, and we cannot assume *a priori* that rates and degrees of adaptiveness in these different classes of molecules are necessarily the same. Possibly, the situation presented by the fibrinopeptides may foreshadow what will be found when other nonenzyme proteins are analyzed comparatively.

For this reason, the data on fibrinopeptides of artiodactyls presented by Mross and Doolittle [31] have been analyzed further by one of us (Stebbins). Two questions were asked: (1) Are amino acid substitutions randomly distributed over the peptide chains or are they localized in particular regions? (2) If the substitutions are divided into two groups, either (a) conservative, with little effect on the properties of the peptide, or (b) radical, with potentially large effects, are the relative distributions of these two kinds of substitutions random or nonrandom? The classification into conservative and radical was made according to the criteria set forth by Smith [37] and followed his arrangement of residues into groups (his Table I). For example, the substitution of arginyl for lysyl, both of them positively charged and hydrophilic, is conservative, as

is also aspartamyl for glutamyl and alanyl for valyl. On the other hand, substitutions of glutamyl for lysyl, of aspartyl for glycyl, or of seryl for valyl, were all classified as radical, and would be expected to affect greatly the properties of the peptide, unless concealed by other residues when the peptide assumed its tertiary structure.

The classification of Sneath [38] is similar to that of Smith but more quantitative. Using Sneath's correlation coefficients for pairs of amino acids, substitutions between pairs of residues having coefficients higher than 0.600 are conservative, and between pairs having coefficients lower than 0.600 are radical.

The results of this analysis were unequivocal (Figure 1). The statement of Mross and Doolittle, that substitutions are unequally distributed, was amply confirmed. Moreover, the high concentration of substitutions is at the amino terminal end of the peptide, at which end are also concentrated the various deletions that have taken place during phylogeny. In addition, the radical substitutions were concentrated in those sites having the largest number of substitutions, while the conservative ones are mainly scattered over regions in which few or no substitutions have become established.

These results must be considered in the context of the extensive studies that various workers have made on the physiological action of fibrinopeptides. For example, Bayley, Clements, and Osbahr [3] found that bovine fibrinopeptide B and human fibrinopeptide A given in minute concentrations to rabbits, dogs and lambs caused pulmonary hypertension, decreased effective pulmonary blood flow, and had several other effects on the circulatory system.

Even more significant is the evidence reviewed by Chandrasekhar and Laki [7], indicating that the residues present near the amino terminal end of fibrinopeptide A, particularly at position 13, influence the rate at which thrombin splits the molecule. Moreover, they conclude (p. 127): "that thrombin can strictly discriminate the amino acid residue at position 13 only if a serine residue occurs at position 16 or if an aspartic acid is located at position 18." On the basis of these conclusions, we postulate that the radical substitutions recorded in Figure 1 for positions 12 to 19 in fibrinopeptide A and for positions 18 to 21 in fibrinopeptide B took place during artiodactyl evolution in association with adaptive shifts. We believe that they are associated with alterations in the accessory adaptive properties of the fibrinogen molecule and its derivatives, which have aided in the acquisition by these very different animals of their various physiological and ecological properties.

5. Adaptiveness *versus* chance variation with respect to enzymes and similar molecules

The next question to ask is: Can the concept of evolutionary homeostasis, as outlined at the beginning of this paper, be applied to the evolution of amino acid sequences in enzymes and other molecules having similar properties? Based upon available evidence, this question can tentatively be answered in the

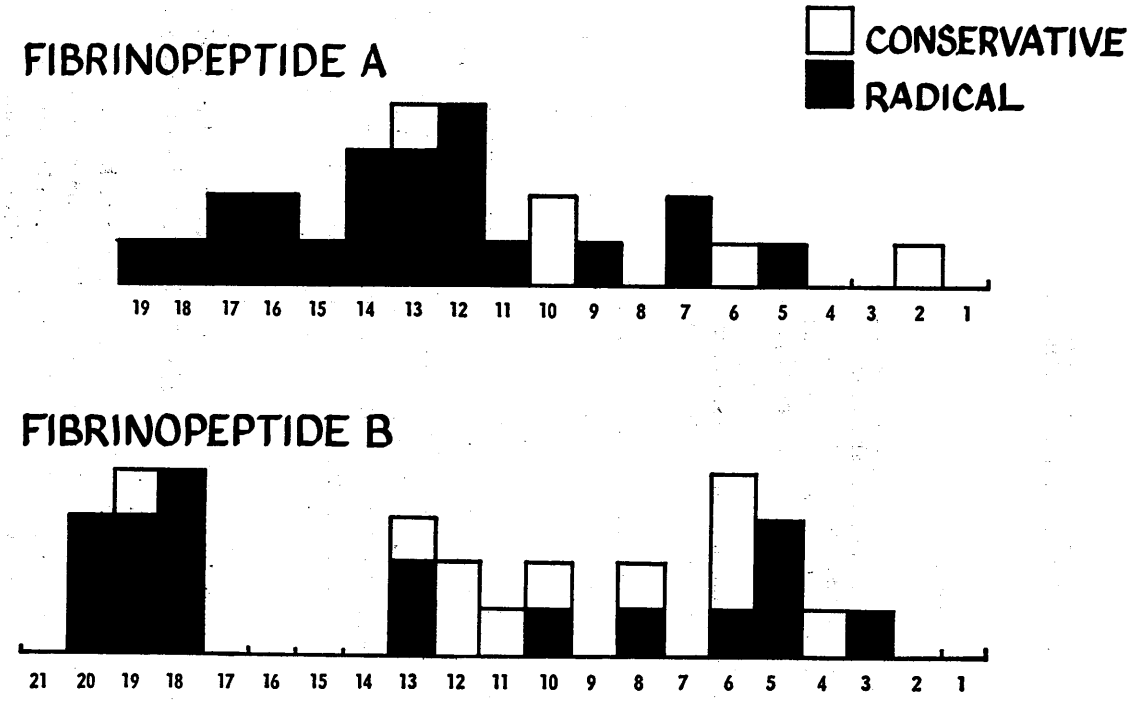

FIGURE 1

Diagram showing the numbers and positions of amino acid substitutions that occurred in fibrinopeptides A and B during the evolution of artiodactyls, as recorded by Mross and Doolittle ([31], Tables III and IV and Figure 3). White, open squares represent conservative substitutions; black squares represent radical substitutions. The numbers represent positions of residues from the position of the split by thrombin (1 = arginine in all peptides) and the amino terminal end of the peptide. Further explanation in the text.

affirmative. Moreover, basic functions as well as accessory adaptations are normally carried out by different parts of the same molecule. The different kinds of adaptations that an enzyme molecule can possess are listed in Table II. The

TABLE II

ADAPTIVE CHARACTERISTICS OF ENZYMES AND PROTEINS WITH SIMILAR METABOLIC FUNCTIONS

Basic to function
Configuration of active site
Configuration of binding sites
Accessory to function
Position of principal residues (cys) that determine tertiary structure
Position of residues that can form alpha helices
Position of residues that affect subunit association
Number and position of residues that affect solubility
Number and position of residues that affect hydrophobic properties
Related to enzyme activation, inhibition, and interactions
Position and configuration of allosteric site or sites
Number and positions of residues capable of forming hydrogen bonds

list of accessory functions was compiled largely from the review by Smith [37].

Based upon the principle of evolutionary homeostasis, we would expect residues or sequences of residues that are present at active or binding sites of an enzyme to be extremely conservative, since all mutations causing radical substitutions would be deleterious and rejected by selection. That this is the case is generally recognized and has already been stated by several of the speakers in this conference. On the other hand, differences of interpretation exist with respect to substitutions at regions other than the active and the binding sites. Hence, we must ask the question: To what extent are these substitutions associated with adaptive shifts in accessory properties of the molecules, and to what extent are they neutral substitutions in "spacer" regions that have no adaptive function?

This question has no simple answer. In order to illustrate its complexity, we would like to suggest an analogy with accessory adaptations at the level of macroscopic organs, and to present an example of adaptational complexity at the molecular level in a bacterial enzyme.

A widespread feature of accessory adaptations in organs of higher plants is that many alternative pathways exist for achieving the same adaptation. If, for instance, the impact of a new environment increases the adaptive value of increased seed production, the population can respond by either (1) an increase in the number of flowers per plant, (2) an increase in the number of gynoecial units or "carpels" per flower, or (3) an increase in the number of seeds per capsule. If the latter kind of adaptation takes place, it can be accomplished either by increasing the length of the capsule, its width or its thickness, or by various combinations of these changes. Hence, the same kind of selective pressure,

acting simultaneously upon different populations that are isolated from each other, can cause them to diverge, if they respond at the outset in different ways. Which of the possible responses will be made may be determined by chance, depending upon the kinds of mutations that appear first, or that happen to be present in the gene pool. On the other hand, the adaptive structures that already exist may play the decisive role, since the most acceptable mutational changes will be those that cause the least disturbance of the developmental pattern [39].

There is good reason to believe that accessory regions of enzyme molecules, that are responsible for such characteristics as tertiary structure, temperature optima, solubility and interaction with other enzymatic processes would respond in a similar fashion to changes in either the external or the internal cellular environment. This is strongly suggested by the evidence that Yanofsky and his associates have obtained on the tryptophan synthetase enzyme in *Escherichia coli*. Figure 2 is a summary of some of their results [41]. They were obtained by analyzing a succession of mutations from full function to the auxotrophic condition that requires tryptophan in the medium, followed by reverse mutations to partial or full function.

The diagram shows that four different combinations of residues, involving substitutions at three different sites in two different peptides, can give full function. Two combinations at these same sites give partial function, and two of those obtained are completely nonfunctional. One could reason from these results that adaptive shifts to full function under a different set of environmental conditions could likewise be accomplished by several different combinations of mutational changes. The data obtained by Hardman and Yanofsky [13] suggest that the adaptive properties at this site affect substrate binding.

The probable existence of these complex alternative pathways of adaptation warns us that we must be very careful in assuming that a particular amino acid substitution is neutral unless we have positive evidence that this is so. In particular, we must avoid committing what may be called "the fallacy of omniscience." This fallacy, which has often been committed in the past by evolutionists and taxonomists who are comparing macroscopic characters of organisms, runs about as follows: "I can't see what adaptive value this character difference could have, therefore, it is inadaptive and was not influenced by natural selection." The fallacy here lies in the implication that the author of the statement knows everything that can be known about the adaptiveness of the organisms concerned. With respect to macroscopic organisms this is usually not true. With respect to enzyme action *in vivo*, about which our knowledge is still very rudimentary, it probably is never true. Evolutionists are, therefore, unable to prove, by the process of elimination, the null hypothesis that amino acid substitutions in certain portions of enzyme molecules are neutral and do not affect the function of the whole molecule.

At the same time, interpretations of enzyme structure and function must guard against the reverse fallacy, which would necessarily ascribe an adaptive significance to amino acid substitutions, without definite evidence in favor of it.

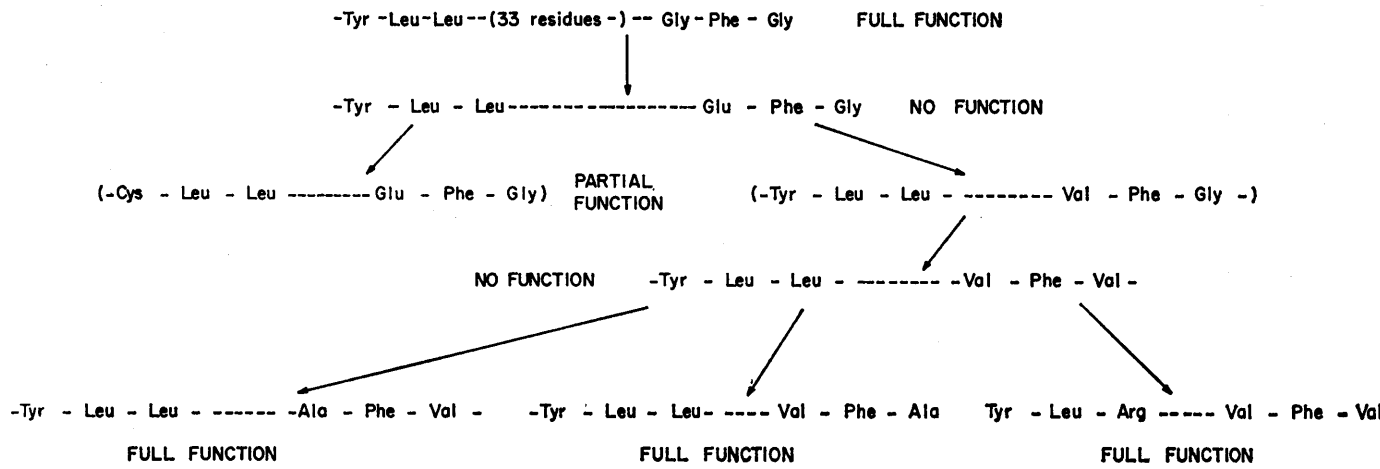

FIGURE 2

Functional combinations of residues at a single site of the tryptophan synthetase molecules. Data from Yanofsky, Horn, and Thorpe [41].

In the present state of our knowledge, molecular evolutionists must resign themselves to saying more often those three very difficult words: "I don't know."

Any distinction between hypotheses of evolution by random differentiation as opposed to natural selection must then take a more complex form. In particular, advocates of the neutrality hypothesis offer evidence that the observed facts of protein variation both within and between species, are consonant with an assumption of random variation, while at the same time being irreconcilable with a selective hypothesis. We will review separately the quality of these *positive* arguments for neutrality and the *negative* ones against selection.

6. Proposed positive arguments in favor of neutral amino acid substitutions

The first of these positive arguments, advanced by King and Jukes [23], is that the proportion of amino acid sites that have 0, 1, 2, $\cdots$, n substitutions in variants of globins, cytochromes c, and the variable ($S-$) regions of immunoglobins follow the Poisson distribution. This argument, however, is based upon an erroneous assumption, which includes the "fallacy of omniscience," as has been pointed out by Clarke [8]. Since they recognize that some sites of enzyme molecules have great adaptive significance, they arbitrarily divide the polypeptide chains into "adaptive" or "invariant" regions and "nonadaptive" or "variant" regions. Clarke shows that the distribution of residues and substitutions in the latter regions is not random. Moreover, the assumption of King and Jukes, that only the invariant sites are concerned with adaptation includes a second, hidden assumption that is surely unwarranted. This is that the action of enzymes is an all-or-none affair under any set of environmental conditions, and that it cannot be gradually modified in adaptation to shifting conditions of the external or the internal environment.

The second argument, advanced by the same authors, is that when the average amino acid frequencies are tabulated among 53 vertebrate polypeptides, their frequencies agree remarkably well with those to be expected from random permutations of nucleic acid bases. From this they conclude that the average amino acid composition of proteins reflects, in a neutral fashion, the genetic code. Even if this interpretation is correct, however, one cannot conclude from it that amino acid substitutions in all or even in the majority of proteins have been largely neutral. The 53 proteins are in all probability a nonrepresentative sample, as has been pointed out by Clarke [8]. In structural proteins, the deviation from frequencies expected on the basis of the code is very great. More important, however, the interpretation represents a gross misunderstanding of the selective argument. No one suggests that on the average over a heterogeneous collection of proteins and species, particular amino acids should be in excess or deficiency. Indeed, if there were some general necessity for a particular amino acid to be very frequent in most proteins, the code probably would have evolved to take account of this physicochemical necessity. Even if the selective hypothesis were

true in the most extreme form for every amino acid position in every protein, on the average for many unrelated proteins and distantly related species, the gross frequency of amino acids would represent the codon frequencies. Stone walls in Vermont reflect, on the average, the naturally occurring frequencies of stones of different sizes (Vermonters being sensible and frugal), but different walls have different size distributions depending upon whether they are rough boundary markers, pasture fences, farm plot fences, or garden fences. Moreover, when a stone falls out, only some stones will fit into the hole. This is not the only case when the advocates of the neutrality hypotheses have been confounded by the law of large numbers.

The third argument, that has been advanced not only by Jukes and King, but also by several of the speakers at this conference, is that when the numbers of amino acid substitutions in a group of related organisms are compared with the times when the ancestors of these modern forms are believed to have diverged from each other, a high correlation is found between numbers of substitutions and presumable ages of divergence. Based upon these correlations, uniform rates of amino acid substitutions are inferred. Admittedly, however, these "rates" are only approximately uniform, and even with the relatively small sample that is now available, striking exceptions can be found, such as the rapid rate of substitution in guinea pig insulin [8]. Moreover, some data suggest that these inequalities of rate are greater when we compare organisms that have diverged relatively recently than when comparisons are made between divergences that occurred long ago [29]. An example is the chart of differences with respect to hemoglobin prepared by Kimura and Ohta [22] for this Symposium. The figures for mammals belonging to different orders differ from each other by a factor of up to 65 per cent (17 in mouse-human *versus* 28 in mouse-rabbit) but the differences between each of these mammals and the fish (carp) are much more similar. If this situation exists generally, we can conclude that the so-called "constancy" of rates over millions of years is nothing but the law of large numbers. In the intervening times, there have been many speedings-up and slowings-down of the rate of amino acid substitution differentially in different lines as within the mammals. On the average, over vast stretches of time, however, we expect that different phylogenetic lines will have similar *average* numbers of substitutions in molecules having similar basic functions. The entire argument is based upon a confusion between an average and a constant. This point is clearly illustrated by fossil evidence. Simpson [36] shows that many phyletic lines have both fast and slow periods of evolution such that the average rate gives no index to the detailed history of the line. There are even periods when many independent lines simultaneously show very high rates of extinction and speciation, as in the Pleistocene, yet no one would suggest that the similarity in taxonomic rates of such lines indicates randomness!

A fourth "permissive" argument for neutrality concerns the variation *within* species as revealed by recent studies of "allozyme" variation at various structural gene loci [33], [35]. Typically, within any population of *Drosophila*, mice,

or man, among other species, between one quarter and one half of a random sample of loci is found to be polymorphic, and 10 to 15 per cent of the loci are heterozygous in a typical individual. The question is whether such variation is a result of the accumulation of mutations subject to random variation in frequency in finite populations, or whether one needs to invoke some selective balance to explain the maintenance of the variation. Kimura [18] has asked how much variation is to be expected in a population at equilibrium under recurrent mutation and loss of alleles from genetic drift. If one assumes that each mutation is unique, then in a population of size N and a mutation rate to new alleles of u, the effective number of alleles (the reciprocal of the probability that two alleles in the population are identical) is, to a close order of approximation $n_e = 4Nu + 1$. Kimura, in several publications, makes use of "reasonable" values of N and u to show that the observed allelic frequencies in populations are consonant with this prediction. The difficulty with this formula is its remarkable behavior with respect to the two parameters N and u, whose values we can only guess to an order of magnitude. Provided that Nu is of smaller order than unity, the effective number of alleles is 1, irrespective of N and u. On the other hand, if Nu is of larger order than unity, the number of effective alleles is very much larger than anything ever observed. Only when Nu is of order 1 is there any interesting sensitivity of n_e to the actual values. Thus, all that can be said from evidence on allozyme polymorphism is that if these polymorphisms are neutral, Nu is smaller than unity for most loci and about order 1 for a few (the esterase locus in *D. pseudoobscura*, for example). This theory is then *too* permissive.

The great power, and therefore weakness as a testable hypothesis, of the random theory can also be seen in another aspect of the observations on genic polymorphism. The simplest form of the random theory predicts that although each population of a species will have about the same value of n_e for each locus, the particular alleles that are in high or low frequency in a population will be random, so that allele frequencies will vary considerably from population to population. The contrary is observed, in *D. pseudoobscura* [33] and in *D. willistoni* [2]. These species display a remarkable similarity in allele frequencies in widely separated populations. Such an observation would seem to rule out the random theory, but a small change saves it. If there is a small amount of migration from one population to another, there will be virtually no differentiation between populations under a pure drift model. If m is the migration rate between neighboring populations, then if Nm is of order 1 or greater, differentiation will be effectively prevented. But if $Nm = 1$, that is the same as saying that one or less migrant individual is exchanged per generation! Such tiny migration rates are in practice unmeasurable, or rather, could never be ruled out by any observation. Thus, the random theory, augmented by a small but unmeasurable migration rate, is so powerful a prediction that no observation can be in contradiction to it. That is what we mean by a theory so powerful that it is weak as a testable hypothesis. In the terms used by Popper, the neutral theory is "empirically

void" because it has no set of potential falsifiers. All observations can be made consonant with it. For that reason, a different quality of evidence is necessary for distinguishing a selective from a neutral theory. In recognition of this necessity, the advocates of the neutral theory have tried to show that a selective theory is *contradicted* by the evidence, so that the neutral theory is confirmed by elimination.

7. Positive and negative evidence on selection

The history of evolutionary science during the last half century has demonstrated clearly the fact that an understanding of the processes of evolution can best be acquired by examining first actual evolutionary events at the level of populations and gene frequencies, and synthesizing from this firm base theories and concepts about the broader aspects of macroevolution, rather than by beginning with attempts to construct all encompassing phylogenies. We believe that this basic principle of studying evolution upward from the population rather than downward from a postulated phylogeny must be applied to molecules as well as to organisms. Because of this fact, data on the distribution of allozymes and other variants of protein molecules has the greatest possible relevance to biochemical evolution.

As we have said, a large amount of data have been accumulated on the variability of homologous proteins, particularly allozymes, in both natural and artificial populations. Good samples have been presented in this conference by Drs. Ayala [2] and Allard [1]. The opinion of these authors, that most of this variation is adaptive or at least associated with adaptation and maintained by natural selection, is shared by the majority of the workers in this field.

Nevertheless, direct evidence for this point of view is still scanty. The selection experiments with populations of barley, described by Dr. Allard, constitute an example. Another is the distribution of allozymes of serum esterase in populations of catastomid fishes, in which an allozyme having a more southerly distribution, in a warmer climate, has a higher temperature optimum *in vitro* than the predominant allozyme of populations adapted to cooler climates [24], [25].

Indirect evidence that points toward the adaptiveness of molecular polymorphism in populations is, however, much more extensive. Dr. George Johnson [16], in a manuscript that he has kindly let us read before publication, has discovered some very interesting correlations and parallelisms between molecular and phenetic evolution in a group that is now becoming a classic example of rapid evolution in response to environmental diversity: the Hawaiian species of *Drosophila*. In the first place, after comparing the number of allozyme types per locus in species inhabiting four different islands (Hawaii, Maui, Oahu, Kauai), he has found an indirect correlation between this number and the number of different species found on each island. Interestingly enough, these numbers are not correlated with the sizes of the islands, as would be predicted on the basis of some models that ecologists have constructed, but with ecological diversity.

By far the largest amount of indirect evidence for the selective basis of molecular polymorphism, however, comes from the consistent observation that in nearly every species studied certain polymorphisms occur in a similar fashion over wide stretches of territory. As previous speakers have already pointed out, the only possible explanations for this constancy are (1) that it is maintained by selection, or (2) that it is due to extensive and continuous migration between widely separated populations. In at least some of the examples, this latter explanation can be ruled out, as Drs. Ayala and Allard have explained.

The crux of the evidence on selection, however, is of the *negative* variety. That is, it is contended by Kimura, Crow, King, and others that any selective hypothesis that is not trivially different from a neutral theory contains certain irresolvable contradictions. In particular, it is maintained that the differential production of offspring necessary to account either for rates of gene replacement in evolution or for the maintenance of observed polymorphism in a balanced state, is vastly greater than any real populations can actually afford. This is the problem of "genetic load," but it might equally well be regarded as the problem of "fitness variance." If selection is operating with respect to a particular locus, such that different genotypes have, on the average, different numbers of offspring, two results follow. First, the mean rate of offspring production of a segregating population will be less than the rate in a population consisting of only the most fit genotype. The difference between this mean rate and the rate for the best genotype is the *genetic load* associated with selection at that locus. Second, there is a certain variation in reproductive rate among genotypes which is reflected in the *variance in fitness* in the population. Both of these quantities are necessarily limited in any real population by the biological facts of the reproductive cycle of a species. While it is not certain how many fertilized eggs a human female could produce in a lifetime, it is obviously less than, say, 1,000, and this puts an absolute ceiling on the variance in fitness possible in a human population. What is at issue is whether a selective hypothesis applied to known rates of gene substitution in evolution and the standing genic polymorphism, would require more fitness variance than is biologically possible.

We will not repeat here the evidence, so well presented by Kimura in this Symposium, and by Haldane [12], Kimura [18], [19], [22], and Kimura and Crow [20], [21] to the effect that evolutionary rates and degrees of polymorphism are too high to be consonant with a selective hypothesis. Rather, we will examine briefly some of the suppositions inherent in these models that make them rather less convincing than is usually assumed.

First, the argument about genetic load is based upon a model that is clearly incorrect. It assumes that genes segregate independently from each other, and so neglects the very powerful effects of linkage over short distances [11]. If it is really true that at least one third of the genome is segregating in *Drosophila*, then the average recombination between segregating loci is on the order of one in a thousand and the result is that strongly linked interacting blocks will be built up. Before we can discard selection on the basis of genetic load, we must

examine more thoroughly the whole theory of linked loci. When our present understanding of linkage is coupled with more sophisticated understanding of gene interaction, as for instance the possibility that many genes are maintained by selection for an intermediate optimum, the argument against the adaptive nature of a large genetic load fails to be convincing.

For example, Lewontin [28] found that selection for an intermediate optimum caused profound linkage disequilibrium, even for genes that are quite far apart on the chromosome, yet the variance of fitness in the equilibrium population was quite small. The conclusions of Franklin and Lewontin are clearly on the conservative side since they were based on a very weak model of gene interaction. Bringing in optimum model selection will magnify these linkage effects many times and thus reduce the genetic load associated with gene substitution and polymorphism. The meaning of these findings is that genes are not substituted in evolution independently of each other, but in correlated blocks.

A second and important objection to genetic load arguments is that they are based on a special and unrealistic model of population growth and regulation. While it is part of the model that the mean reproductive rate of a segregating population is lower than that of a population made up only of the best genotype, this assumption is, in general, false. Because of nongenetic causes of mortality and the existence of an elastic "ecological load," there is usually no effect on population size or growth rate from the segregation of selected genes [40], until there is a complete saturation of the available reproductive excess. With the exception of a few species on the verge of extinction, there is no evidence of such a saturation. Such an argument does not deny that a saturation of the "load space" is possible if genetic load were high enough.

There is a third perspective that does not seem to have been appreciated by the supporters of evolution by random drift. When gene frequencies change over the course of time, there is a variance in fitness of genotypes *a fortiori*. This can best be appreciated in a haploid population. Suppose we observe a gene replacement in such a population over the entire course of its substitution, beginning at a very low frequency and finally reaching the frequency of 1. The gene will have increased and decreased during the evolution of the population, but since it eventually went to fixation, there was an average positive rate of increase of the allele. Now the question is: "How, from this sample path can one distinguish selection from random drift?" The answer is: "In no way." The total variance in "fitness" and the total integrated "genetic load" can be *tautologically* calculated from the sample path, since at every generation the change in gene frequency *tautologically* defines the net selection at that generation. Now it might be objected that an erratic path is evidence of drift, while a monotone path to fixation indicates selection. But this misses the point. First, of course, there is no requirement that the environment be uniform over the history of the population. More important, the total integrated load calculated for an erratic path is *greater* than for a monotone one. The longer the gene frequency bounces around in an unfixed state, the greater the eventual total load, as tautologically

defined. This is because, *a posteriori*, it is impossible to know the difference between a "selective" and a "neutral" sample path. This is because a selection coefficient, if it has any biological meaning, must be defined from the expectation of gene frequency change over replicated populations simultaneous in time. It cannot be defined from the expectation of gene frequency change over a *time* ensemble for one population, since then all processes are selective *a fortiori*.

In a diploid population, one might imagine a change in genotype composition, even though family size, that is, number of individuals per generation, remained constant. In each successive generation, a different sample of segregates from heterozygotes might survive and reproduce. This, however, is never the case. Even on a pure random model with a Poisson distribution of offspring number, half of the change in gene frequency arises from variance in family size between genotypes and half from variance in composition among families of heterozygotes. Thus, half of the genetic variance in a diploid population changing its gene frequency will be ascribable *a posteriori* to selection, without any evidence about the numbers of offspring expected in replicate populations that are subject to selection. Thus, it is hard to see how, given a certain rate of gene substitution, one avoids the problem of genetic load simply by postulating a "neutral model." Selection, in its tautological sense, does not thereby disappear unless it is also proposed that there is no variance in offspring number between genotypes and all the variance arises from segregation in heterozygotes. But that would require a device for producing negative correlations between families, so that even the sampling variance between genotypes can be suppressed. No one has yet suggested this as a possibility.

8. Conclusion

In our opinion, the relative position of population geneticists and molecular evolutionists who are dealing with the comparative molecular structure of proteins and nucleic acids can be stated about as follows. Both groups agree that some evolutionary changes have been due to adaptive shifts mediated by natural selection, while other changes have been the result of chance events. The principal differences between the two groups are the three following ones.

(1) Most geneticists who have studied actual populations believe that the effects of chance are minor relative to those of selection, and can be regarded as a kind of "evolutionary noise." Many comparative molecular evolutionists, on the other hand, believe that most changes have been brought about by neutral mutations randomly fixed.

(2) Most evolutionists who have studied macroevolutionary changes from the point of view of comparative morphology, physiology, and ecology believe that these changes represent chiefly adaptive shifts mediated by natural selection, while many comparative molecular evolutionists believe that at the molecular level most macroevolutionary changes are the result of chance events, and that natural selection has acted chiefly to stabilize highly adaptive molecules,

certain amino acid sequences that are parts of molecules, and, presumably, highly adaptive phenotypes.

(3) Finally, many evolutionists believe that the greatest progress will be made by working upward from an understanding of actual populations of organisms living in natural environments or known and controlled conditions in the laboratory, while others prefer to make inferences and extrapolations from comparisons between distantly related organisms, using various mathematical devices to make these extrapolations plausible.

From this summary, we believe that the nature of evolutionary processes is not yet resolved. However, the pathways toward a resolution are available. We believe that many exciting new results will be obtained by both groups in the near future. As long as pathways of communication remain open, as they have been at this conference, a resolution of the differences is bound to come. Most likely it will be accomplished by younger scientists, whose opinions have not yet become congealed.

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