

REVIEW

**The neutral theory of molecular evolution:
A review of recent evidence¹⁾**

Motoo KIMURA

National Institute of Genetics, Mishima 411 Japan

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ABSTRACT

In sharp contrast to the Darwinian theory of evolution by natural selection, the neutral theory claims that the overwhelming majority of evolutionary changes at the molecular level are caused by random fixation (due to random sampling drift in finite populations) of selectively neutral (i.e., selectively equivalent) mutants under continued inputs of mutations. The theory also asserts that most of the genetic variability within species at the molecular level (such as protein and DNA polymorphism) are selectively neutral or very nearly neutral and that they are maintained in the species by the balance between mutational input and random extinction. The neutral theory is based on simple assumptions, enabling us to develop mathematical theories based on population genetics to treat molecular evolution and variation in quantitative terms. The theory can be tested against actual observations. Neo-Darwinians continue to criticize the neutral theory, but evidence for it has accumulated over the last two decades. The recent outpouring of DNA sequence data has greatly strengthened the theory. In this paper, I review some recent observations that strongly support the neutral theory. They include such topics as pseudoglobin genes of the mouse, α A-crystallin genes of the blind mole rat, genes of influenza A virus and nuclear vs. mitochondrial genes of fruit flies. I also discuss such topics as the evolution of deviant coding systems in *Mycoplasma*, the origin of life and the unified understanding of molecular and phenotypic evolution. I conclude that since the origin of life on Earth, neutral evolutionary changes have predominated over Darwinian evolutionary changes, at least in number.

1. INTRODUCTION

The most important development that has occurred during the last hundred years in the field of evolutionary studies, I believe, is the incorporation of Mendelian genetics. Actually, the breakthrough came with the introduction of

1) This is an extended version of my talk entitled "Molecular evolution and the neutral theory" presented at the Cold Spring Harbor Laboratory Centennial Symposium on *Evolution: From Molecule to Culture*, September 24–27, 1990. This is contribution number 1876 from the National Institute of Genetics, Japan.

the concept of genes, which led to the formulation of population genetics with its far-reaching influence in the field. This has enabled us to discuss the mechanism of evolution at the level of genetic factors.

A second revolution came with the incorporation of molecular genetics, which began with the Watson-Crick model in 1953. The resulting development of the new field of molecular evolution, from its start some two decades ago, has brought many surprises that were beyond imagination when studies of organic evolution were restricted to the level of visible phenotypic characters.

Let me mention a striking example. Comparative studies of DNA (or RNA) sequences show that they diverge irreversibly with time by accumulating molecular mutants at a steady pace, and that we can construct reliable phylogenetic trees without being misled by similarity due to convergent evolution. A recent triumph in such studies is the work by Miyata, Hasegawa, Osawa, and their group on the phylogeny of archaebacteria (Iwabe et al., 1989; Miyata et al., 1991; Hasegawa et al., 1990). Using DNA sequence data involving a few duplicated genes shared by all three primary kingdoms, i.e., eubacteria, eukaryotes, and archaebacteria, these authors presented definitive evidence showing that archaebacteria are not old but rather new. In other words, archaebacteria are more closely related to eukaryotes than to eubacteria. Hori and Osawa had already reached this conclusion through their extensive studies on phylogenetic relationship among major groups of organisms using the 5S ribosomal RNA (5S rRNA) sequences (see Hori and Osawa, 1987 for review). Because of this reason, Osawa and Hori (1979) proposed the new terminology "metabacteria" as more appropriate one to represent the group of bacteria that had previously been called "archaebacteria."

2. FEATURES OF MOLECULAR EVOLUTION

Generally speaking, there are two outstanding features that distinguish molecular evolution from phenotypic evolution. The first is the constancy of the rate, i.e., for each protein or gene region, the rate of amino acid or nucleotide substitutions is approximately constant per site per year (known by the term "molecular evolutionary clock"). The second is the "conservative nature" of the changes, i.e., functionally less important molecules, or portions of molecules, evolve faster than more important ones.

What is really remarkable about the first feature is that the rate constancy holds when measured in terms of physical years rather than in biological generations. More than two decades ago, I pointed out the possibility (Kimura, 1969a), especially if the neutral theory is valid, that hemoglobin and other molecules, of "living fossils" have undergone as many amino acid (and therefore DNA base) substitutions as corresponding molecules (genes) in more rapidly evolving species. Since then, much evidence corroborating this prediction has been found. For

Table 1. Amino acid differences between the α and β chains of hemoglobins in human and shark

Type of change	Human α vs. β	Shark α vs. β
0	62	50
1	55	56
2	21	32
3	0	1
Gap	9	11
Total	147	150

example, according to Romer (1968), the Port Jackson shark is a relict survivor of a type of ancestral shark which had numerous representatives in the late Paleozoic days, notably in the Carboniferous period (270–350 million years ago). Thus, this shark is well entitled to be called a living fossil. In Table 1, a result of comparison between the α and β chains of the hemoglobin of the Port Jackson shark (data from Fisher et al., 1977) is presented together with a similar comparison of α and β chains of humans (The numbers in the first column represents the type of amino acid differences that can be interpreted from the code table as being due to a minimum of 0, 1, 2 and 3 nucleotide substitutions). From the two sets of comparisons, it is clear that genes coding for the α and β chains of hemoglobin in this shark have diverged to roughly the same extent (or slightly more) as have the corresponding two genes in humans by accumulating random mutations since the origin of the α - and β -globin genes by duplication possibly some 500 million years ago.

As to the second feature, namely, the property that functionally less important molecules or parts of a molecule evolve faster than more important ones, Ohta and I proposed this as one of the 5 principles governing molecular evolution (Kimura and Ohta, 1974). This principle immediately aroused much opposition and criticism from the Neo-Darwinian establishment. This principle has now become a part of common knowledge among molecular biologists, even though very few of them appear to realize that it was derived from the neutral theory. A common practice of molecular biologists is to search for various signals by comparing a relevant region of homologous DNA sequences of diverse organisms and to pick out a constant or “consensus” pattern as functionally important, while disregarding variable parts as unimportant and irrelevant.

3. THE NEUTRAL THEORY

Ever since it was first proposed more than two decades ago (Kimura, 1968), the neutral theory of molecular evolution has been a target of severe criticism of various kinds. The theory looked heretical to the then prevailing neo-Darwinian

or the "Synthetic" view. I am glad and feel proud that the neutral theory has survived through the turbulent years, and that it has gained much strength now, thanks to steadily accumulating evidence in its support, particularly since the outburst of DNA sequence data starting some ten years ago.

Unlike the Darwinian theory of evolution by natural selection, the neutral theory claims that the overwhelming majority of evolutionary changes at the molecular level are not caused by Darwinian natural selection acting on advantageous mutants, but by random fixation of selectively neutral or very nearly neutral mutants through the cumulative effect of sampling drift (due to finite population number) under continued input of new mutations. In other words, the neutral theory emphasizes the predominant role that mutation pressure and random genetic drift play in evolutionary changes at the molecular level.

I would like to add here that by "selectively neutral" I mean selectively equivalent: namely, mutant forms can do the job equally well in terms of survival and reproduction of individuals possessing them. The neutral changes are often referred to as "evolutionary noise", but, I want to emphasize that this is a misnomer, because, neutral changes do not impair genetic information, even if the process of substitution is random. If the neutral theory is valid, a large fraction of evolutionary nucleotide substitutions occurring at functionally important parts of the genome are also selectively neutral, even though the probability of a new mutation that occurs those parts being selectively neutral is lower as compared with functionally less important parts. Thus, neutral evolution is by no means restricted to "junk" part of the genome. The neutral theory does not deny the role of Darwinian positive selection in determining the course of adaptive evolution, but it assumes that only a very small fraction of DNA (or protein) changes are adaptive and positively selected.

The neutral theory also asserts that most of the intraspecific variability at the molecular level, as revealed by protein and DNA polymorphisms, is selectively neutral, and is maintained in the species by the balance between mutational input and random extinction. In other words, protein and DNA polymorphisms represent a transient phase of molecular evolution (Kimura and Ohta, 1971).

A special feature of the neutral theory is that its underlying assumptions are sufficiently simple that population genetical consequences can readily be worked out mathematically, enabling them to be tested by observations and experiments: the neutral theory differs from other traditional theories of evolution in that it is quantitative.

4. POPULATION GENETICS OF NEUTRAL MUTATIONS

In considering the mechanism of molecular evolution from the standpoint of genetics, we must clearly distinguish between "mutation" at the individual level and "mutant substitution" at the population level. Without keeping these two

events conceptually distinct, discussion of evolutionary genetics lacks clarity. In this section, I shall present few formulae which are basic for the neutral theory to treat evolution and variation at the molecular level. First, let us consider the long-term process in which molecular mutants accumulate one after another at a given locus or site within the species. For selectively neutral mutations, we have the following formula for the rate of evolution per generation.

$$k_g = v_0, \quad (1)$$

Note that, in this equation, k_g represents the long term average per generation of the number of mutants that spread through the population and, v_0 is the rate of production of neutral mutants per locus (or site) per generation. This formula is based on the well-known property (Kimura, 1968; Crow, 1969) that, for selectively neutral mutants the rate of substitutions is equal to the mutation rate.

If we denote by v_T the total mutation rate (per locus or site), and if f_0 is the fraction of neutral mutations at the time of occurrence, so that $f_0 = v_0/v_T$, then Eq. 1 may be rewritten as

$$k_g = v_T f_0. \quad (2)$$

Advantageous mutations may occur, but the neutral theory assumes that they are so rare that they may be neglected from our consideration. Thus, $(1-f_0)$ represents the fraction of definitely deleterious mutants that are eliminated from the population without contributing either to evolution or polymorphism, even if the selective disadvantages involved may be very small in the ordinary sense. The above formulation has a remarkable simplicity in that the evolutionary rate (in the long term basis) is independent of population size and environmental conditions of each organism.

In actual data on molecular evolution, the rate of evolution is usually measured in terms of years (i.e., taking one year as the unit length of time) rather than in generations. Therefore, in order to check the theory with actual data we must modify Eq. 2 so that it gives the evolutionary rate per year.

$$k_1 = (v_T/g) f_0. \quad (3)$$

In this formula, g stands for the generation span (in years), and v_T/g is the total mutation rate per year.

Next, let us consider intraspecific variability. Assuming neutral mutations, the average heterozygosity per nucleotide site is given by the formula,

$$\bar{H}_{\text{nuc}} = 4N_e v_{0(\text{nuc})}, \quad (4)$$

where $v_{0(\text{nuc})}$ is the neutral mutation rate per nucleotide site per generation and N_e is the effective population size (or roughly the number of breeding individuals per generation). This result was first derived by using the "infinite site model" (Kimura, 1969b), assuming the equilibrium state in which mutational input and

random extinction of mutants balance each other. In fact, I have stated (Kimura, 1969b) that "the probability of a particular site being heterozygous for a selectively neutral mutant is $4N_e u$, where u is the mutation rate per site."

When we treat heterozygosity per gene (or protein) locus, rather than per individual nucleotide site, the infinite allele model of Kimura and Crow (1964) is more appropriate. Using this model, the average heterozygosity per locus is

$$\bar{H} = \frac{4N_e v_0}{4N_e v_0 + 1} \quad (5)$$

where v_0 is the mutation rate for selectively neutral alleles per locus per generation.

5. INTRASPECIFIC VARIABILITY AT THE MOLECULAR LEVEL

A straightforward prediction of the neutral theory is that those DNA regions evolving faster should show higher intraspecific variability (i.e., higher heterozygosity or polymorphism). This may be immediately seen by comparing Eq. 4 with Eq. 1. Data supporting this prediction have been obtained from recent studies on evolution and variation at the DNA level in *Drosophila* species. On the whole, synonymous and other silent sites (including those in introns) are much more polymorphic and also show higher evolutionary rates than non-synonymous (amino acid altering) sites. This was clearly shown by recent work of Brady et al. (1990) who compared *esterase-5* gene of *Drosophila pseudoobscura* with its homologue (*esterase-6* gene) of *D. melanogaster*. These authors came to the conclusion that the patterns of nucleotide substitutions and amino acid replacements between these genes are consistent with the neutral theory.

With respect to protein polymorphism, the study by Ward and Skibinski (1985) also supports strongly the prediction of the neutral theory. Using electrophoretic data for 42 proteins from over 200 invertebrate and 300 vertebrate species, they discovered a very strong correlation between genetic distance and heterozygosity among different proteins and concluded that the observed relationship between the two can be explained quantitatively by the neutral theory. I believe that quantitative investigations of intraspecific variability at the DNA sequence level in many species, including human species, will become increasingly important in the field of experimental population genetics. According to Kazazian et al. (1983), the average heterozygosity per nucleotide site ("nucleotide diversity, π " in the sense of Nei and Tajima, 1981) at the human β globin gene cluster region, consisting of about 50 kb of DNA, is approximately 0.002 in various human races. Extrapolating this to the whole human genome consisting of about 3 billion (3×10^9) nucleotide sites, we obtain the estimate that the average human individual is heterozygous at about six million nucleotide sites.

Under the neutral theory, heterozygosity per site is equal to $4N_e v_{0(\text{nuc})}$ as

shown by Eq. 4, where $v_{0(\text{nuc})}$ is the average neutral mutation rate per site per generation over the whole genome. The selective constraint may be very small for the majority of sites (for which f_0 may not be very different from unity), but the constraint must be rather strong in the coding region. I have estimated (Kimura, 1983a), using data on rare variant alleles at protein loci, that the fraction of neutral mutations (P_{neut}) at such loci is about 0.14 for a few organisms including humans.

Using this estimate for P_{neut} and assuming that the average coding region of each enzyme locus consists of 1,000 nucleotide sites, we can estimate the average heterozygosity per enzyme locus with respect to electrophoretically detectable alleles. Let v_E be the neutral mutation rate for electrophoretically detectable alleles per locus per generation. Then we can estimate $4N_e v_E$ as follows.

$$4N_e v_E = 0.002 \times 1000 \times 0.75 \times (1/3) \times 0.14 = 0.07,$$

where 0.75 is the approximate probability that a random base change within a codon leads to an amino acid change, and 1/3 is a rough estimate for the probability of a random amino acid change leading to a change in electric charge of the protein. By using Eq. 5 and substituting $4N_e v_E$ for $4N_e v_0$ in this equation, we obtain $\bar{H}_E \approx 0.065$, where \bar{H}_E is the average heterozygosity per locus with respect to electrophoretically detectable alleles. This agrees well with the corresponding observed value, $\bar{H} = 0.067$ that was obtained by Harris and Hopkins (1972) as the average heterozygosity per locus for electrophoretically detectable alleles in European populations.

Among various species of fruit flies, *Drosophila melanogaster* has been studied most extensively. The estimated value of heterozygosity per nucleotide site is $\bar{H}_{\text{nuc}} = 0.006$ for alcohol dehydrogenase (*Adh*) gene region (Kreitman, 1987), $\bar{H}_{\text{nuc}} = 0.012$ for *white* locus region (Langley and Aquadro, 1987), $\bar{H}_{\text{nuc}} = 0.007$ for *Notch* locus region (Schaeffer et al., 1988), $\bar{H}_{\text{nuc}} = 0.003$ for *rosy* locus region and $\bar{H}_{\text{nuc}} = 0.002$ for 87 A heat shock locus (Aquadro et al., 1988). The average of these estimates turns out to be $\bar{H}_{\text{nuc}} = 0.006$, which is 3 times higher than the corresponding estimate obtained for man. If this value is representative for the whole genome, and if we apply the same calculation as we did above, we obtain $\bar{H}_E = 0.17$ for the expected heterozygosity per locus with respect to electrophoretically detectable alleles in *D. melanogaster*. On the other hand, the corresponding observed value $\bar{H}_E = 0.102 \pm 0.014$ (see Aquadro et al., 1988), is somewhat smaller than expected, but not very much. The situation appears to be more complicated and puzzling when a similar comparison is made between *D. melanogaster* and *D. simulans*. The latter species shows a slightly lower (or nearly equal) heterozygosity with respect to allozyme variability, but it shows much higher heterozygosity per nucleotide site at the *rosy* region, that is, $\bar{H}_{\text{nuc}} = 0.019$ (For details, see Aquadro et al., 1988). If this is representative of the whole genome of *D. simulans*, and if this applies to the first and second

positions of the codons, then the expected heterozygosity per allozyme locus is $\bar{H}_E=0.40$, inconsistent with actual observations. More studies will be needed to estimate \bar{H}_{nuc} for other gene regions of this species.

Recently, Satta et al. (1990) reported that in *Drosophila melanogaster* the estimated nucleotide diversity (π) in mitochondrial DNA (mt DNA) is $\pi=0.0022$. As mentioned above, for nuclear DNA, the corresponding value is $\pi=0.006$, which happens to be the same as the estimated value for *Adh* gene. According to Satta et al. (1990), the evolutionary rates of nucleotide substitutions are roughly equal between the mt DNA and the *Adh* gene in this species. To analyse these observations, they proposed the following formula which can readily be derived from the neutral theory (by noting Eq. 4).

$$E(\pi_n)/E(\pi_m)=4(\mu_n/\mu_m).$$

In this formula, $E(\pi_n)$ and $E(\pi_m)$ are the expected values of the nucleotide diversity for nuclear and mitochondrial genes respectively, while μ_n and μ_m stand for the mutation rates per site for nuclear and mitochondrial genes. This formula is based on the consideration that the mitochondrial genome is haploid and maternally transmitted, so that the effective population size of mitochondrial genomes is only 1/4 as large as that of diploid nuclear genome. Taking into account the possible statistical errors involved, the observed ratio 0.0022/0.006 or 0.37 may not differ significantly from the theoretical value, i.e. 1/4, as predicted from the neutral theory.

A similar comparison of π values may be made for human mitochondrial and human nuclear genes. According to Brown (1983), the evolutionary rate of mt DNA is approximately ten times as great as that of single-copy nuclear DNA in primates. Thus, we can assume $\mu_n/\mu_m=0.1$ since the evolutionary rate is equal to the neutral mutation rate if the neutral theory is valid. Assuming this ratio, and putting $E(\pi_n)=0.002$, we obtain, from Satta et al.'s formula, $E(\pi_m)=0.005$. This value is not very different from the observed nucleotide diversity which is roughly in the range 0.3~0.4% (Horai, 1991).

6. RECENT DATA SUPPORTING THE NEUTRAL THEORY WITH RESPECT TO EVOLUTIONARY CHANGES AT THE MOLECULAR LEVEL

The first truly favorable evidence for the neutral theory was the finding that synonymous base substitutions, which do not cause amino acid changes, occur almost always at much higher rates than non-synonymous, that is, amino-acid-altering substitutions. It has also been found that evolutionary base substitutions at other "silent" sites, such as introns, occur at comparably high rates. These and other observations suggest that molecular changes which are less likely to be subjected to natural selection occur more frequently in evolution and therefore show higher evolutionary rates. This is easy to understand from the

neutral theory, because such changes are more likely to be non-deleterious (i.e., selectively neutral) and therefore f_0 in Eq. 2 is larger for them. On the other hand, these observations do not fit nicely with the neo-Darwinian, or synthetic, theory of evolution, which claims that the speed and direction of evolution are almost completely determined by natural selection (see, for example, Stebbins 1966, p. 29). Note that if positive selection is the driving force of evolution, one should expect that synonymous and other silent changes should show lower evolutionary rates than amino-acid-altering changes, because natural selection acts on the phenotype of the organism, in the determination of which the structure and function of proteins play a decisive role.

More than a decade ago, I predicted (Kimura, 1977), from the neutral theory (i.e., using Eq. 2), that the maximum evolutionary rate is set by the mutation rate ($k_g \leq v_T$) and that the maximum rate is attained when all the mutations are selectively neutral (i.e., when $f_0=1$). A dramatic example vindicating this prediction emerged a few years later. It was the discovery of very high evolutionary rates for pseudogenes (or "dead" genes) that have lost their function. This was first shown clearly by Miyata and Yasunaga (1981) who analysed the evolu-

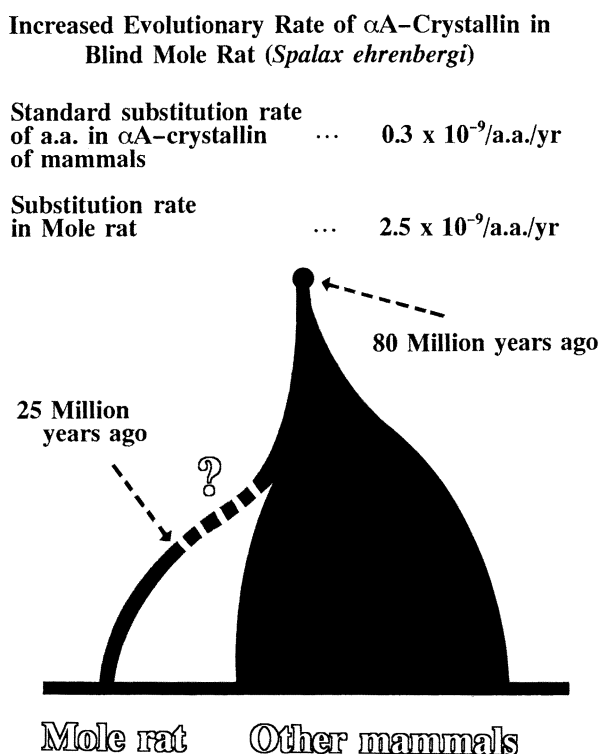


Fig. 1. An increased evolutionary rate of the eye lens protein, α A-crystallin, in the blind mole rat *Spalax ehrenbergi*.

tionary rate of a pseudo α -globin gene in the mouse, followed by a more elegant statistical analysis by Li et al. (1981). What is really remarkable and interesting in these studies is that the rates of substitution are equally high in all three codon positions. The estimated rate in globin pseudogenes is about $k=5\times 10^{-9}$ substitutions per nucleotide site per year. This is roughly twice as high as the rate of synonymous substitutions in the normal α -globin gene.

A similar and equally interesting example suggesting neutral evolution is the recent observation that the evolutionary rate of the eye lens protein, α A-crystallin, has been much enhanced in the blind mole rat, *Spalax ehrenbergi* (Hendriks et al., 1987). This animal is adapted to a burrowing, subterranean way of life, and possibly has been for the last 25 million years according to fossil evidence (see Fig. 1). It is completely blind, although the crystallins are still expressed in the atrophied lens cells. According to these authors, α A-crystallin is generally a slowly evolving protein, with the average replacement rate of 0.3×10^{-9} per amino acid site per year in rodents and other vertebrates, but in the mole rat lineage, this rate has increased several fold namely, about 2.5×10^{-9} per amino acid site per year. This means that, although the rate is much increased, its maximum estimate is still only 1/5 of the observed rate of the pseudo-globin gene. This is quite understandable, because in this animal, the α A-crystallin gene is still expressed (i.e., transcribed and translated), and even if the eyes are no longer used for visual purposes, vestigial amounts of the protein still exist in the body. Therefore, some selective constraint should still remain, and the evolutionary rate must not exceed the normal synonymous rate. This expectation, based on the neutral theory, is quite consistent with actual observations. It is a pity that these authors, in their discussion, resort to the concept of constraints due to developmental programs, which I believe is a rather meaningless concept in considering the mechanism of evolution from the standpoint of genetics (For a critique of the concept of developmental constraints, see Stebbins and Hartl, 1988).

Generally speaking, if the neutral theory is valid, mutation pressure plays a predominant role in molecular evolution. In recent years, much evidence corroborating this has been added. One of the most remarkable examples demonstrating this is the very rapid evolutionary changes observed in RNA viruses, which are known to have very high mutation rates: genes of RNA viruses show evolutionary rates roughly a million times as high per year as those of DNA organisms. This was studied by Saitou and Nei (1986), who analysed data on evolution and polymorphism of influenza A virus genes. Like other RNA viruses, this virus is known to have a mutation rate approximately one million times higher per year than DNA organisms. Therefore, the observed high evolutionary rate fits nicely to the expectation of the neutral theory. It is also remarkable that, in this case, synonymous substitutions predominate over non-synonymous substitutions, similar to what has been found in genes of DNA-

containing organisms. An extremely high substitution rate and the clocklike progression of substitutions in influenza A virus were also reported by Hayashida et al. (1985). A more recent analysis of data on human influenza A virus evolution by Gojobori et al. (1990) confirmed the existence of very clear, clocklike progression of base substitutions, in which the rate of synonymous substitutions (13.1×10^{-3} /site/year) is about 3.5 times as high as that of non-synonymous substitutions (3.6×10^{-3} /site/year). These observations can readily be explained by the neutral theory by noting that in Equation 2, the value of v_T is about a million times higher in the RNA genome than in the DNA genome while the values of f_0 remain roughly the same.

In fruit flies, even if the evolutionary rate of nucleotide substitutions is not as dramatically high as in RNA viruses, it is nevertheless very high as compared with the substitution rates in mammals. Through statistical analysis of the sequence data of alcohol dehydrogenase (*Adh*) and heat shock protein (hsp 82) genes, Moriyama (1987) obtained the result that the rate of synonymous (or silent) substitutions in *Drosophila* lineages is roughly 10^{-8} per site per year, which is about twice as high as that of rodents and ten times higher than in higher primates. She showed that, in this case also, synonymous substitutions much predominate over nonsynonymous (i.e., amino acid altering) substitutions. Note that the generation span of the fruit flies is some two hundred times shorter than that of man. So, if we assume that the average generation span of *D. melanogaster* is 0.1 year, the rate of neutral mutation rate per site per generation is roughly 10^{-9} . Using Eq. 4, and taking $\bar{H}_{\text{nuc}} = 0.006$, we obtain $N_e = 1.5 \times 10^6$ as the effective population size of *D. melanogaster*. More recently, Sharp and Li (1989) obtained a slightly higher estimate for the substitution rate, that is 1.6×10^{-8} per site per year, at "silent sites" in *Drosophila*. If we use this value, the effective population size turns out to be slightly less, that is, about $N_e = 0.9 \times 10^6$.

7. DISCUSSION

According to the neutral theory, the rate of mutant substitutions in evolution is equal to the neutral mutation rate (see Eq. 1), and this rate is independent of the population size and environmental conditions. On the other hand, the intraspecific variability at the molecular level (i.e., heterozygosity per nucleotide site) is directly proportional to the effective population size (see Eq. 4). Because of its simplicity, the neutral theory enables us to test its validity against actual observations more directly than any other rival theories. The real biological world, however, is very complicated, containing some disturbing or complicating factors that make actual observations sometimes depart from the neutrality predictions. The neutral theory assumes that the mutations can be classified into two distinct groups, namely, the completely neutral class (with the fraction f_0) and the definitely deleterious class (fraction $1 - f_0$). If, as Ohta (1974, 1976) proposed,

the majority of “neutral mutations” are, in reality, very slightly deleterious rather than strictly neutral, the evolutionary rate is higher in smaller populations than in larger populations. This is because a very slightly deleterious mutant behaves as if selectively neutral when $N_e s'$ is much smaller than unity, where s' (>0) is the selection coefficient against the mutant, and N_e is the effective population size, while it may be effectively selected against if $N_e s'$ is larger than unity. Recently, Ohta and Tachida (1990) applied the concept of “near neutrality” to explain the puzzling observation regarding nucleotide versus allozyme heterozygosities in *D. similans*, on which I have mentioned in one of the above sections. These authors assume that mutations causing protein polymorphisms are subject to stronger selection on the average (such as $N_e \bar{s}'=0.5$) compared with mutants that involve in DNA polymorphisms (such as $N_e \bar{s}'=0.1$), even if the absolute sizes of the selection coefficient (denoted by s') are very small in the ordinary sense. Taking into account the fact that *D. similans* has a much larger panmictic population than *D. melanogaster* which has the subdivided population structure, these authors claim that the contrasting pattern of polymorphisms in these two species can be understood by assuming nearly neutral mutations. Whether such very slightly deleterious mutations are really prevalent in nature or not, I think, remains to be investigated for many genes in various organisms.

Generally speaking, observations are increasing showing that patterns of evolution and variation at a large fraction of nucleotide sites is consistent with the prediction of the neutral theory (Aquadro et al., 1988; Brady et al., 1990; Bowcock et al., 1991). By applying the neutral theory, we can estimate the total mutation rate due to base substitutions per genome per generation in man. For this purpose we use the estimated value of the evolutionary rate of the mouse globin pseudogene (i.e., $k_1=5 \times 10^{-9}$), which must be equal to the total mutation rate per nucleotide site per year in the mouse. We also take into account the observation (Li and Tanimura, 1987) that the rate of synonymous substitutions in rodents is about 7 times higher than that in higher primates. Since the human genome consists of about three billion (3×10^9) nucleotide sites, if we assume that the average generation span is 20 years for human lineage, we can obtain the total mutation rate per genome per generation as follows.

$$V_T = (3 \times 10^9) \times (5 \times 10^{-9}) \times 20 \div 7,$$

or approximately $V_T=43$, which means that the total number of new mutations per generation due to base substitutions amount to 43 per gamete, and twice as many per zygote.

This is a very high value when we compare this with the traditional estimates of the genomic mutation rate (i.e., much smaller than unity). From the consideration of genetic load (see Crow and Kimura 1970, pp. 297–312), the mutational load becomes intolerably high, unless the great majority (say, 99% or more) of them are selectively neutral (i.e., non-deleterious).

If we make a similar calculation as above, utilizing the estimate $P_{\text{neut}}=0.14$ and accompanying calculations that led to the relation $4N_e v_E=0.07$ in section 5, we can obtain the estimate for the rate of neutral mutations per locus per generation in man with respect to electrophoretically detectable alleles as follows.

$$v_E=(5 \times 10^{-9}) \times 1000 \times 0.75 \times (1/3) \times 0.14 \times 20 \div 7,$$

i.e., $v_E=0.5 \times 10^{-6}$. Then, using the relationship $4N_e v_E=0.07$, we obtain $N_e=3.5 \times 10^4$ as the representative effective population size of humans during the last million years or so. Note that the effective population size can be much smaller than the actual population size (i.e., the number of heads), particularly because population bottlenecks must have occurred rather frequently in the history of human evolution (see Crow and Kimura, 1970, pp. 345–365, on “effective population number” under various conditions).

Let us now examine the problem of the molecular evolutionary clock. This is the clock-like progression of mutant substitutions in the course of evolution at the molecular level. There has been a great deal of controversy on this topic, particularly in relation to the validity of the neutral theory in explaining the mechanism involved. From the standpoint of the neutral theory, this feature of molecular evolution can be explained by assuming that v_T/g (mutation rate per year) remains about the same (constant) among diverse lineages and over time for a given gene or protein, for which f_0 is assumed to be constant.

One problem which immediately arises is that traditional mutation studies on “visible” and viability traits (including lethals) strongly suggest that the spontaneous mutation rate per generation, but not per year, is roughly equal among different animals whose generation spans are very different. This observation has been used repeatedly to criticize the neutral theory. For example, Gillespie (1989) claims that the actual data on the rates of molecular evolution do not appear to exhibit the degree of dependence on the generation time as predicted by the neutral theory. It now appears, however, that many of these “visible” and “viability” mutations are caused or controlled by transposons and insertion sequences (see, for example, Rubin, 1983; Mukai and Yukuhiro, 1983; Green, 1988). On the other hand, it is likely that errors in DNA replication and repair are the main causes of DNA base changes responsible for molecular evolution. Thus, the mutation rate for nucleotide substitutions may depend on the number of cell divisions in the germ lines, particularly in the male line (see Miyata et al., 1987), and this will make the mutation rate for nucleotide substitutions roughly proportional to year. Experimental studies on this subject are much needed.

At any rate, from the standpoint of the neutral theory, a universally valid and exact molecular evolutionary clock would exist only if, for a given molecule, the mutation rate for neutral alleles *per year* (v_0/g) were *exactly equal* among all organisms at all times (which are rather unlikely in nature). Thus, any deviation from the exact equality of neutral mutation rate per year makes the molecular

clock less exact. Note that if successive mutant substitutions cause the fluctuation of "neutral space" (Takahata, 1987) so that f_0 changes with time, this also contributes to make the molecular clock less exact, even if the total mutation rate (v_T) remains the same (see Takahata, 1987). Clearly, from these considerations, the variance of the evolutionary rates among different lineages for a given molecule may tend to become larger than expected from the simple Poisson distribution, as often noted in actual observations.

Gillespie (1984, 1986) utilizes such observations showing deviation from the Poisson distribution to criticize the neutral theory, and he claims that a model of evolution, which he calls the "episodic model" can fit the data better. His model is based on the idea that molecular evolution is episodic, with short bursts of rapid substitution being separated by long periods of no substitutions. According to him, each environmental change presents a challenge to the species that may be met by amino acid substitutions caused by positive natural selection. It seems to me that Gillespie's theory is highly unrealistic in that it assumes that the number of episodes in different lineages, which must experience different environments, follow the same probability distribution. I also feel grave doubt about the validity of his assumption that natural selection acts in such a way that the number of mutant substitutions per episode follows the same probability distribution for all episodes in all lineages. In his theory, natural selection is invoked arbitrarily to fit the data, while neglecting all the effects of the mutation rate, population size and selective constraint. If it turns out that difference of evolutionary rates among lineages is mainly caused by differences of v_T/g , i.e., mutation rate for base substitutions per site per year, Gillespie's "episodic clock" theory will break down completely. He also claims (Gillespie, 1987) that molecular evolution and polymorphism are essentially uncoupled, contrary to the claim of the neutral theory. According to him, polymorphism at the molecular level is caused by a randomly fluctuating environment. I would like to point out that, as compared with a theory containing a number of arbitrary parameters, the neutral theory is much simpler and is amenable to refutation if it is really wrong.

If the neutral theory applies not only to evolution at silent sites but also to that at the amino acid altering sites, this must mean that in general the neutral space of a protein is reasonably wide. In other words, a replacement of amino acid within a protein often preserve the activity of that protein unaltered. In fact, according to a recent review by Bowie et al. (1990), extensive studies by many investigators revealed that proteins are surprisingly tolerant of amino acid substitutions, and that proteins can keep essentially the same structure and function against changes in the amino acid sequence. Similarly, Perutz (1983), who made a detailed stereochemical examination of amino acid substitutions among vertebrate haemoglobins in relation to species adaptation, came to the following conclusion: adaptations leading to response to new chemical stimuli have evolved by only a few (one to five) amino acid substitutions in key positions, while most of

the amino acid replacements between species are functionally neutral. He says that the evidence supports the neutral theory.

The predominant role played by mutation pressure in molecular evolution, as dictated by the neutral theory, has become increasingly evident from recent studies. A remarkable example demonstrating this is the very rapid evolutionary change observed in genes of RNA viruses known to have very high mutation rates. As pointed out already, this can readily be explained by noting that in Eq. 2 the value of v_T is about a million times higher in RNA genomes than in DNA genomes.

One of the most interesting examples suggesting mutation-driven neutral evolution is the deviant coding system recently discovered by Osawa's group (Muto et al., 1985; Yamao et al., 1985) in *Mycoplasma capricolum*. In this bacterial species, UGA, a stop codon in the standard code table, codes for tryptophan instead of UGG, the ordinary codon for tryptophan. This organism is characterized by a very high A+T content (75%) in its genomic DNA. It was pointed out by Jukes (1985) that the A/T directed mutation pressure, that is, predominantly high mutation rate from G/C to A/T over the reverse direction (as may be caused by modifications in the DNA polymerase system), can explain the evolution of this codon. According to him, the A/T directed mutation pressure can lead to the replacement of UGA (as a stop codon) by UAA (another stop codon), followed by a change in the anticodon of one of the duplicated copies of the tRNA gene for tryptophan from CCA to UCA. What is important is that Osawa's group actually detected duplicated copies existing side by side in the genome. After these changes, there is nothing to hinder the gradual replacement of UGG by UGA as the major codon for tryptophan under A/T directed mutation pressure. Such a "capture of a stop codon" must have been brought about by a series of neutral changes. As a population geneticist, I must emphasize here that directional mutation pressure can be a cause of molecular evolution only when the changes involved are selectively neutral.

If the neutral theory is valid so that the great majority of evolutionary changes at the molecular level are controlled by random genetic drift under continued input of mutations, it is likely that selectively neutral changes have played an important role in the origin of life and also in phenotypic evolution. For many years, the origin of life has been one of the most fascinating but puzzling problems in science. However, with the development of molecular genetics, there is a good hope that substantial progress will be made in near future. Recently, the well-known physicist, Dyson (1985), proposed a new theory on the origin of life, in which stochastic processes involving neutral evolution play a crucial role. According to his theory, which he modestly calls "a toy model," an active protein evolved first in an Oparin type primitive cell through a process similar to random frequency drift in a finite population. The theory assumes also that the RNA gene emerged later in the cell as a parasite. What made me much pleased is that

in developing his theory, Dyson found both the main idea of the neutral theory and my diffusion equation method very useful. Irrespective of whether his new theory is valid or not, it seems to me that chance in the form of random frequency drift must have played some very important role in the process leading to the origin of life, which is intimately related with molecular evolution. Similarly, Cairns-Smith (1986) invoked the "common ancestor effect" to explain the problem of why organisms use (mainly) L-amino acids. In his paper, he considers random fixation of organisms with L-amino acids starting from a mixture of two types of organisms, one using L-amino acids and the other using D-amino acids. He treats this problem similarly to that of random fixation of one of the two selectively neutral alleles in a finite population.

Finally, I would like to discuss briefly the problem: how can we understand evolution at two levels, that is, molecular and phenotypic, in a unified way? It is generally believed that, in contrast to the neutralist view of molecular evolution, evolutionary changes at the phenotypic level are almost exclusively adaptive and caused by Darwinian positive selection. However, I think that even at the phenotypic level, there must be many changes that are so nearly neutral that random drift plays a significant role, particularly with respect to "quantitative characters." I have shown (Kimura, 1981) that if a large number of segregating nucleotide sites each with a small effect are involved in a quantitative character subject to stabilizing selection, the average selection coefficient per mutant becomes exceedingly small. Under such a condition, extensive neutral evolution can occur through random drift. Note that stabilizing selection is the most prevalent type of selection in nature.

In considering the problem of progressive evolution, we should not forget the possibility that gene duplication and subsequent accumulation of new mutants in the duplicated genes must have played a very important role. As a kind of mutation, gene duplication is constantly fed into the population, and many of them may have so little deleterious effect that they become fixed in the population through neutral evolution, that is, by random drift under "gene duplication pressure." Because of substantial reduction of selective constraint after gene duplication, some of the mutants that are definitely deleterious and that would have been rejected before duplication can now reach high frequency by random drift. Such a store of new mutants may contain a few variants that will turn out to be useful for adaptation to a new environment (see Ohta 1988 for review on duplicated genes and their roles in evolution).

Paleontological studies have revealed that big evolutionary changes usually result from exploitation of a new ecological niche by a species. In this connection, I would like to emphasize the importance of a condition that I call "liberation from selective constraint" as a main cause of macroevolution. If a species is confined for a long time to a constant ecological niche with all other available niches being occupied by other species, further adaptive shift become impossible.

Only when new vacant niches are presented will the possibility be open for macroevolution to occur. This is clearly shown by spectacular evolution at the early Cambrian (often called "Cambrian explosion"), where large scale evolutionary experiments were performed in the then new way of life as multi-cellular organisms (Conway Morris, 1989). Recently, Gould (1989), in his book entitled "Wonderful Life," tells the fascinating story about many bizarre animals that lived in the Cambrian sea, as revealed by the studies of the Burgess Shale fossils. Similarly, explosive diversification of mammals in the early Cenozoic, immediately following the extinction of the then dominant dinosaurs, is well-known. What I want to emphasize is that relaxation of natural selection is the prerequisite for new evolutionary progress. In other words, "liberation from selective constraint" enables extensive neutral evolution to occur, creating new variants²⁾, some of which turn out to be useful in a new environment.

Based on these considerations, I recently proposed (Kimura 1990) a hypothesis which I called the "four-stage scenario" theory of macroevolution. According to this theory, macroevolution consists of the following four steps. (i) Liberation from the preexisting selective constraint. (ii) Sudden increase or boom of neutral variations under relaxed selection. In this stage, gene duplication in addition to point mutation must play a very important role in producing genetic variations.³⁾ Needless to say, their fate is largely determined by random drift. (iii) Realization of latent selection potential of some of the neutral mutants, which I have termed (Kimura, 1983b) Dykhuizen-Hartl effect. In other words, some of the accumulated neutral mutants (at the phenotypic level) turn out to be useful in a new environment, which the species then exploits. (iv) Intergroup competition as well as individual selection lead to extensive adaptive evolution creating a radically different taxonomic group adapted to a newly opened ecological niche.

If the above theory turns out to be essentially correct, the importance of the neutral theory as an evolutionary paradigm will be much enhanced. No one would be able to say then that neutral changes are by definition not concerned with adaptation, and therefore the neutral theory is biologically not very important: such a criticism has often directed to the neutral theory until recently.

In conclusion, I would like to claim that since the origin of life on Earth, neutral evolutionary changes, as propelled by random drift under mutational pressure, must have played a most important role in evolution. In other words, neutral evolutionary changes have predominated over Darwinian evolutionary changes, at

2) Dr. W. B. Provine who kindly went over this paper at the manuscript stage has suggested the term "nonadaptive radiation" to represent this process.

3) Recently, Hood and Hunkapiller (1991) suggested that the immunoglobulin gene superfamily emerged along with metazoan organisms and that it may have been one of the powerful driving forces of metazoan evolution. I believe that it is a very attractive and plausible hypothesis, because, in order to form metazoa (i.e., multicelled organisms), functions related to molecular cell-surface recognition (as performed by the superfamily) are essential.

least in number, throughout the whole history of life on Earth.

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