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EARLY DEVELOPMENT OF THE NEUTRAL THEORY

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Introduction

The neutral theory of molecular evolution has become well known and widely discussed. As defined by Jukes and Kimura [1], it postulates that nucleotide substitutions inherently take place in DNA as a result of point mutations followed by random genetic drift. In the absence of selective constraints, the substitution rate reaches the maximum value set by the mutation rate, for example, about 5×10^{-9} substitutions per site per year. Rates slower than this occur when constraints are imposed by natural selection.

This brief communication will review the history of the neutral theory during the 1960s, when it was proposed. Before this, genetic drift was often discussed; but not until sequences of amino acids in proteins became known was it possible to perceive neutral changes in molecular evolution. In 1961, differences in amino acid content of total protein in bacteria were found to be related to base composition of DNA. This will be discussed below. Later, sequences of nucleic acids became available and provided further evidence for neutral changes.

Two proposals were made independently, one through biochemistry and one through population genetics.

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Biochemical Approach

Long before the nature of protein synthesis and structure was known, it was generally accepted that enzymes had a small "active center" that combined with the substrate with which the enzyme reacted. Most of the large molecule of the enzyme, according to this concept, furnished bulk rather than specificity. In the 1950s, Sanger and his colleagues discovered, by studies with insulin, that proteins were made of polypeptide chains with amino acids in a definite order [2]. They also discovered, by amino acid replacements, that insulins from different vertebrates differed from each other. For example, an alanine at one position in bovine insulin is replaced by threonine in human insulin. Nevertheless, bovine insulin can be used to treat human patients, so that this replacement is neutral in its effect.

In 1966, I wrote a textbook [3] in which I simplistically outlined the neutral theory.

> If we consider as an example the two cytochrome c molecules found respectively in dogs and horses, it will be noted that these differ in about 10 of the amino acids in a chain of 104. The question arises, are the two molecules splendidly tailored to the different requirements specified by "dogfulness" and "horsefulness," have they evolved to conform to these two different requirements, or have the two cytochromes passively been carried along as dogs and horses evolved separately from a common ancestor? In the latter case, it is undoubtedly quite probable that separation of the two species would be followed by changes in the genes that in time would result in differences in the two cytochrome c molecules. We do not know whether a horse could get along just as well with dog cytochrome c as with its own.[1] To cite another example, often quoted, the alpha chains of human and gorilla hemoglobins differ only with respect to seemingly inconsequential substitutions of glutamic acid by aspartic acid at site 23. It taxes the imagination severely to infer that this minuscule difference serves to distinguish the oxygen-transporting properties of the two hemoglobins in a manner that conforms with differences in the respective needs of the two species, so that the single amino acid difference has become fixed by natural selection. . . . The changes produced in proteins by mutations will in some cases destroy their essential functions *but in other cases the change allows the protein molecule to continue to serve its purpose.* [Emphasis added]

DIRECTIONAL MUTATION PRESSURE

In 1961, Noboru Sueoka showed that the amino acid composition of the total proteins in a bacterial species was related to the percentage of A+T in its DNA [4]. The AT:GC ratio in DNA is controlled by directional mutation pressure [5]. As reported originally by Chargaff [6], bacteria, in contrast to vertebrates, had a wide species-dependent range of DNA base composition. The A+T content of DNA in *Mycobacterium tuberculosis* was much lower than in *Hemophilus influenzae.* Sueoka [4] found that "high AT organisms" such as *Bacillus cereus* were higher in isoleucine, lysine, phenylalanine, tyrosine, aspartic acid plus asparagine (Asx), and glutamic acid plus glutamine (Glx) than were high GC organisms, such as *Micrococcus lysodeikticus,* which in contrast were higher in alanine, arginine, glycine, and proline. This result was reported before anything was known about the genetic code.

Sueoka was aware of the implication of his results for neutrality, because he said [7] "When the GC content of two organisms differs appreciably, it is unlikely that a protein formed in one cell will be similar in primary structure to any found in the other. This also applies to enzymes of identical function with the exception that the active site may be similar *but the dispensable parts of the molecule will be quite different*" [emphasis added]. When the code became known, it was found that isoleucine, lysine, phenylalanine, and tyrosine (but not Asx and Glx) had codons in which the first two positions consisted of A and T. Alanine, arginine (CGN codons), glycine, and proline had codons with C and G in the first two positions. Sueoka did not publish further on the subject of directional mutation pressure until 1988.

Ernst Freese [8] discussed the differences in DNA base ratios for different organisms in terms of their evolution from a common ancestor. He explained this by assuming that (a) most base pairs in DNA can undergo changes that have no or only an insignificant effect, (b) for "each DNA species one kind of basepair, e.g., G–C, has been altered more frequently than the other one [i.e., A–T] resulting in a shift of the base ratio." He pointed out that "most of the DNA base pairs can be changed with no, or an insignificant, selective advantage. . . . The evolution of functional properties, by natural selection is merely superposed on the evolution of mean base composition, but does not control it."

GENETICAL BEGINNINGS

James Crow reviewed his ideas for the infinite allele model [9], proposed by him in correspondence in 1958, and outlined by Kimura and Crow [10] as follows:

> It has sometimes been suggested that the wild-type allele is not a single entity, but rather a population of different isoalleles that are indistinguishable by any ordinary procedure. With hundreds of nucleotides, each presumably capable of base substitutions and with additional permutations possible through sequence rearrangements, gains, and losses, the number of possible gene states becomes astronomical. It is known that a single nuceotide substitution can have the most

[1] In "Promising Human Tests of New Blood Substitute" (*San Francisco Chronicle,* p. A4, June 9, 1990, Thomas H. Maugh describes the use of bovine hemoglobin to replace human hemoglobin "because cow hemoglobin is very similar to its human counterpart." This would be a commercialization of the neutral theory!

drastic consequences, but there are also mutations with very minute effects and there is the possibility that many are so small as to be undetectable. It is not the purpose of this article to discuss the plausibility of such a system of isoalleles, or the evidence for or against. Instead, we propose to examine some of the population consequences of such a system if it does exist. The probability seems great enough to warrant such an inquiry.

SILENT SUBSTITUTIONS IN GENES

By 1965, the nature of degeneracy in the code had been discovered; an amino acid could have a code or codes ending with G or C, or with A or U, as a nonspecific "degenerate" base. Accordingly, I pointed out [11] that an amino acid sequence

Ala Gly Val Thr Ser Leu Gly Lys Ile Ala

could be coded by

GCG GGC GTG ACC TCC CTC GGG AAG ATC GCC

with 73 percent G+C, or by

GCT GGT GTT ACT TCT CTT GGT AAA ATT GCA

with a base composition of about 40 percent G+C.

This showed that directional mutation pressure could potentially produce many silent (presumably neutral) changes without changing the amino acid content of a protein. The same pressure exerted on nonsilent sites could also change the amino acid composition without impairing protein function [4, 11], but the changes in amino acid content occurred at a rate of only about 15 percent of the changes in silent nucleotide sites ([12]; see also [13]).

In summary, the nature of the universal genetic code shows that many changes in codons can take place, mostly in the third position of codons, without changing amino acid assignments. These would be neutral changes, and directional mutation pressure could favor such changes. An additional group of neutral changes would take place when amino acids were replaced in evolution without changing protein function.

The Process of Evolution

I realized that the idea of neutral amino acid replacements in proteins was inimical to the firmly selectionist opinions of classical evolutionists and geneticists, and also that the science of population genetics was necessary to explain the spread of evolutionary changes through a species. Accordingly, I asked for help from a friend, Jack Lester King, who

was a population geneticist. We challenged statements by G. C. Simpson, who said [14] that "it . . . seems highly improbable that proteins, supposedly fully determined by genes should have nonfunctional parts . . . or that molecules should change in a regular but nonadaptive way." To which we said that "Natural selection is the editor, rather than the composer, of the genetic message. One thing the editor does *not* do is to remove changes that it is unable to perceive." Emil Smith had said [15] that each amino acid in a single protein "must have a unique survival value in the phenotype of an organism." We said "we think life is not so inflexible," and we noted that "the rate of non-Darwinian [i.e., neutral] evolutionary change is a function only of the rate of occurrence of neutral mutations and is independent of population size." We also pointed out the evolutionary importance of normally occurring errors in DNA replication. Our conclusion was that "the stream of spontaneous alterations in DNA, continuously fed into the genetic pool, should include far more acceptable changes that are neutral than changes that are adaptive."

In 1968, King and I sent our manuscript "Non-Darwinian Evolution" to *Science*. It was rejected by both reviewers. One of them said that the idea was obviously false, an opinion that we later heard many times. The other said that the idea was obviously true and therefore trivial, so that publication was unwarranted. This second opinion was quite intriguing because it reflected a biochemical appraisal of protein function, which says that enzymes have a small active center containing a few key amino acids, and the rest of the molecule supplies bulk, size, and other general properties that are more or less nonspecific. This larger region can accommodate many changes without losing its structure and function. In general, therefore, biochemists have no problems with the concept of neutral changes in evolution, while geneticists are resistant to the idea. Our manuscript [16] was accepted after we appealed against its rejection.

The term "non-Darwinian evolution" was later called "emotion-laden" [17]. When Darwin had proposed his theory of evolution, he had been regarded as sacrilegious, but he has long since been apotheosized, so that the term "non-Darwinian" was, in 1969, considered blasphemous. Our article was intentionally provocative and challenging to the established ideas of evolution solely through natural selection, and we drew much criticism. In 1983, our 1969 publication was chosen as a "citation classic" by *Current Contents* [18].

KIMURA 1968

Kimura's first publication had the nonprovocative title "Evolutionary Rate at the Molecular Level." His article [19] starts by pointing out the

existence of the molecular evolutionary clock in amino acid sequences of hemoglobin (mammals), cytochrome *c* (birds vs. mammals) and triose phosphate dehydrogenase (mammals). He then assumed that the entire genome evolves at the same rate as these examples. (However, King and Jukes [16] pointed out that "probably not much more than 1 percent of mammalian DNA codes for proteins.") Kimura then calculated the rate of base pair substitution within a mammalian genome as one every 2 years. He then contrasted this high rate with Haldane's calculations based on genetic load, which arrive at a much slower rate; Kimura therefore concluded that "most mutations produced by nucleotide replacement" must be almost neutral in natural selection, and that they occur "at the rate of roughly 0.5 per year per gamete" (0.5×10^{-9} per nucleotide site per generation), and, again, he assumed that "the entire genome could produce more than a million . . . enzymes." He estimated a higher mutation rate in *Drosophila* than "in man," and he ended by emphasizing "the great importance of random genetic drift due to finite population number in forming the genetic structure of biological populations."

Unquestionably, Kimura's 1968 publication had a great effect on evolutionary theory [18]. The article was particularly useful in pointing out that "Calculation of the cost based on Haldane's formula shows that if new alleles produced by nucleotide replacements" occur in a population at the rate of one every 2 years, then "the substitutional load becomes so great that no mammalian species could tolerate it." The way out of this impasse was that neutral changes could be fixed with a probability equal to their initial frequency, leading to the conclusion that the mutation rate for neutral substitutions could be as high as 5×10^{-10} per nucleotide site per generation. This, as noted below, was more than 100 times Haldane's estimate.

Elsewhere [20], Kimura has described in detail the genesis of his proposal as follows:

> I would like to tell you now how I came to propose the neutral theory of molecular evolution (Kimura 1968). This was not on the main line of collaborative work with Dr. Crow, but rather it was my spontaneous creation in Mishima.
>
> In 1967, I asked Tomoko Ohta, who had just joined my group as a postdoctoral fellow, to read relevant articles in the book "Evolving Genes and Proteins" (ed. Bryson and Vogel, 1965) and to supply me with some estimates of the rate of amino acid replacements in the actual course of evolution. This she did very efficiently. When I extrapolated the amino acid rates to the whole DNA of the mammalian genome, I was surprised to note that it amounted to at least one base substitution every two years. I realized that this rate is more than a hundred times higher than the corresponding estimate previously given by Haldane (1957), based on his concept of "cost of natural selection," that in the standard rate evolution one mutant substitution occurs every 300 generations on the average. This led me to consider seriously the possibility that at the molecular level mutant substitutions in evolution were mainly caused by random fixation of selectively neutral or nearly neutral alleles. I had great respect for Haldane's insight, and I accepted his concept of cost, or the substitutional load as I later called it (Kimura 1960), believing that it enables us to estimate the amount of selection involved in adaptive evolution. At any rate a large fraction of mutations due to base substitutions did not seem to me to be selected efficiently. Also, it occurred to me that most of the alleles responsible for enzyme polymorphisms which had started to be revealed shortly before that time were selectively neutral and that they were maintained in the population by the balance between mutational input and random extinction. Here, my previous work with Jim [Crow] on the number of alleles maintained in a finite population was helpful.
>
> To strengthen my case, I examined what fraction of new mutations ought to be advantageous if such a high substitution rate of mutants were sustained by natural selection. The result also appeared to collaborate [corroborate] my conclusion. In addition, I worked out, in collaboration with Maruyama, the substitutional load when the population size is finite (see Kimura and Maruyama 1969), and I confirmed that the finite population size introduces only a minor modification to the original Haldane formulation. Since more detailed arguments underlying these concepts were given in my book on the neutral theory (Kimura 1983), I shall not repeat them here.
>
> I only want to mention what a great influence I received from H. J. Muller's writings on evolution, without which I could never have been able to consider molecular evolution at this early stage of development. In fact, my desire to develop a theory of population genetics by incorporating the then new knowledge of DNA had gradually grown in my mind by reading reprints of Muller's papers regularly mailed to me in Mishima. This would not have happened unless Muller learned of my existence through Dr. Crow. In addition, the theory of genetic load in population genetics which Jim was then playing the leading role in developing (Crow 1958, Kimura and Crow 1964), was very helpful to me to arrive at my conclusion.
>
> At the time when I wrote my first paper on the neutral theory which was published in *Nature* in 1968, I was accustomed to the "panselectionist" way of thinking, and emotionally I had difficulty in believing the preponderance of selectively neutral allele. Only scientific reasoning compelled me to assume selective neutrality of alleles involved. This paper was also exceptional in that I submitted it without waiting for Dr. Crow to comment on it and to correct the English. I sent my manuscript to the *Nature* office reasonably soon after I completed it, although I sent preprints to Jim and Dr. Wright. Dr. Wright sent me a very long letter commenting on it, and I did not hear on it from Jim for quite a while. Probably, Jim, like myself, considered this paper no more than an interesting piece of work. Certainly, I did not imagine that it would lead to such heated controversies, and that my subsequent.debates with antineutralists would give me so much anxiety.

From this, it is evident that Kimura was unaware of my publications in 1965 and 1966. He arrived at the neutral theory by a different route. In the years ensuing, Kimura has written extensively on the neutral theory (e.g., [21]), especially on its relation to population genetics. We have sometimes shared our ideas, and in 1984 we wrote on the neutral theory under our joint authorship [1].

The "Clock"

The term "molecular evolutionary clock" was introduced by Zuckerkandl [22] to signal his perception that sequences of amino acids in hemoglobin molecules accumulated differences at the same rate that the corresponding species of organisms diverged from each other during evolution. The clock is therefore an adjunct of the neutral theory, which takes the clock a step further by pointing out that such amino acid replacements become fixed at a uniform rate by genetic drift. The 61.0 percent difference between alpha and beta hemoglobins is almost the same, within ±4.5 percent, whether the comparison is made for the same vertebrate or for different vertebrates [23]. The fact that this divergence has maintained constancy is a striking demonstration of both the clock and the neutral theory. For who would have expected the alpha and beta chains of sharks, fish, frogs, snakes, and mammals to show almost identical rates of divergence unless the replacements were produced by a process that was independent of speciation? All but two of the amino acid residues in hemoglobin are subject to evolutionary replacement [24]. Perutz has commented, "the structural evidence suggests that most of the amino acid replacements between species are neutral or nearly so, caused by random drift of selectively equivalent mutant genes, and that adaptive mechanisms generally operate by a few replacements in key positions."

HEMOGLOBIN AS A TARGET OF MUTATIONS

The molecules of alpha (or beta) hemoglobin, or myoglobin, in all vertebrates have the same tertiary structure (fig. 1) formed by folding of a polypeptide chain of about 140 to 146 amino acids [17]. This structure consists of eight helical regions that enclose a central cavity from which water is expelled by juxtaposition of hydrophobic side chains of amino acids. The eight helices are folded back and forth to form a pocket that contains a heme group that serves to carry oxygen. Hydrophilic amino acids are spread over the surface of the structure. Despite this specialized structure, practically every species of vertebrate that has been examined has an alpha hemoglobin different from all the others, as shown by differences in the sequence of 141 amino acids (the primary structure). There are two possible reasons for this variation: the first (the selectionist explanation) is that each of the 141 amino acids "must have a unique survival value in the phenotype of the organism, the phenotype being manifested in the structures of the proteins" [15]. If this were true, it would be unlikely that there would be a steady taxonomic divergence, measured in percentage of replaced amino acids, throughout the vertebrate phylum from sharks to mammals. The differences would

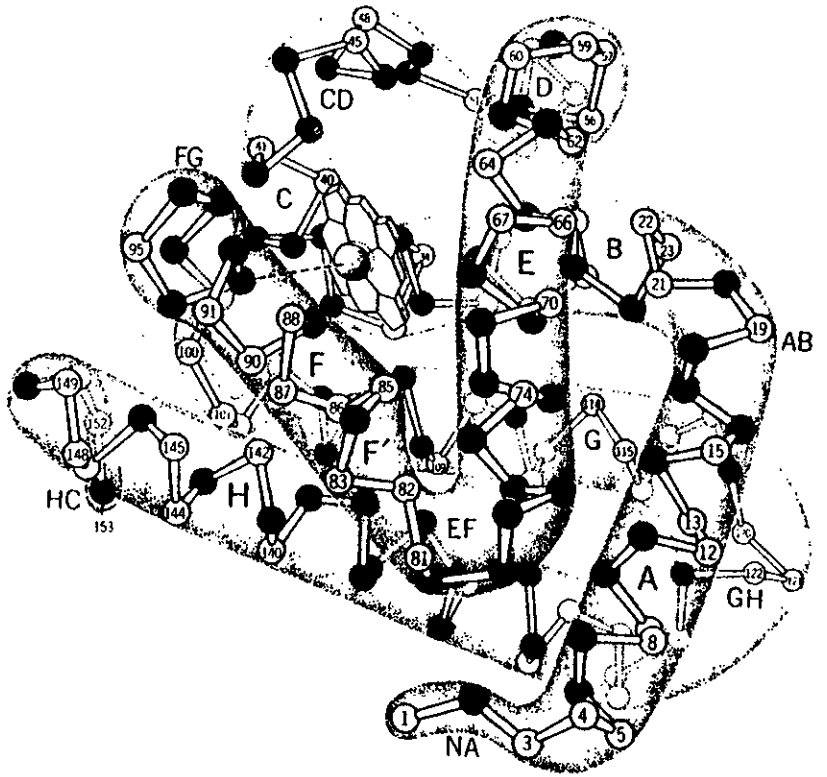

FIG. 1.—Tertiary structure of globin molecule (shown as myoglobin). The single capital letters designate the alpha helices and the paired letters (such as *CD*) show their connecting regions. The numbered circles are variable residues in 55 mammalian myoglobin sequences. Reprinted with permission from Dickerson and Geis (Ref. 17).

more likely express individuality rather than phylogeny, and would be scattered irregularly. Also there is no selectionist reason why alpha hemoglobin should change so much (see [24]; Perutz points out that only a very few replacements have been identified as adaptive).

The second explanation derives from the neutralist proposal [16] that "protein molecules are subjected to incessant probing as a result of point mutations . . . most proteins contain regions where substitutions (replacements) of many amino acids can be made without producing appreciable changes in protein function." These mutations are produced mainly by errors in DNA replication plus the effect of environmental mutagens, so they occur essentially at random and at a constant rate. This explains why point mutations occur at different locations in homologous proteins of various species, so that a steady evolutionary divergence of alpha hemoglobins takes place. The differences in amino acid

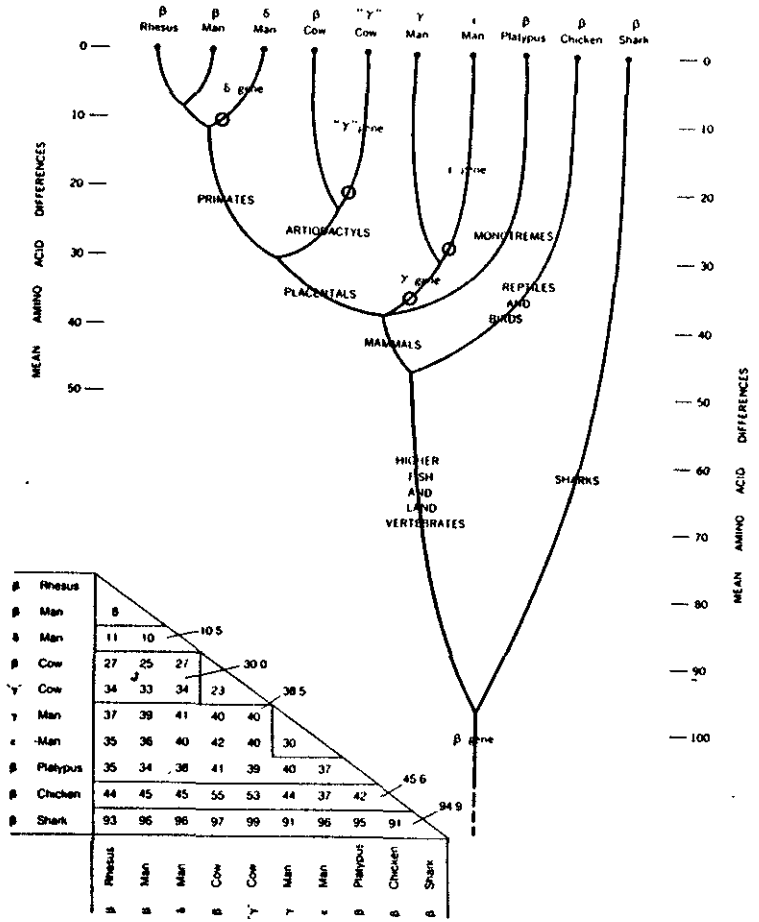

FIG. 2.—Evolutionary tree for beta family of hemoglobins. Reprinted with permission from Dickerson and Geis (1983).

sequences between alpha hemoglobins of placental mammals and of kangaroo average 22 percent; monotreme 30 percent, chicken 30 percent, snake 39 percent, newt 47 percent, carp 49 percent, shark 58 percent.

There are local perturbations in evolutionary rates, but these tend to "smooth out" during prolonged time. This is shown strikingly in shark data for beta hemoglobin; see figure 2. The data from figure 2 are in table 1, which shows that the differences of shark from the other beta hemoglobins have the lowest coefficient of variability in the series. In the absence of a "clock," the shark differences would be expected to be hitgher, rather than lower, in variability. Sharks diverged from the line of descent to the other species about 450 million years ago.

TABLE 1

AMINO ACID DIFFERENCES

		β Rhesus	β Man	δ Man	β Cow	"γ" Cow	γ Man	ε Man	β Platypus	β Chicken	Average	Coefficient of Variability
β	man	8	...	...	...	...	...	...	...	...	...	...
δ	man	11	10	...	...	...	...	...	...	...	10.5	...
β	cow	27	25	27							30.0 ± 4.1	.137
"γ"	cow	34	33	34								
γ	man	37	39	41	40	40						
ε	man	35	36	40	42	40					38.5 ± 2.4	.062
β	platypus	35	34	38	41	39	40	37				
β	chicken	44	45	45	55	53	44	37	42		45.6 ± 5.8	.127
β	shark	93	96	96	97	99	91	96	95	91	94.9 ± 2.7	.028

NOTE.—Amino acid difference between globin molecules listed in figure 2, with averages, SD, and coefficients of variability.

Discussion

From this and other concordant examples, I conclude that the neutral process of evolution explains the differences in hemoglobins and gives rise to the molecular evolutionary clock.

Many authors have objected to the neutral theory and the molecular evolutionary clock because substitutions and replacements often are not completely neutral, or because the clock runs at different speeds at different times. But the real choice is between panselectionism versus near-neutrality, and it seems to me, for the reasons stated above, that the neutral process accounts for many findings in molecular evolution, including the clock, and that panselectionism cannot supply nearly as satisfactory an explanation.

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