The Neutral Theory of Molecular Evolution

It holds that at the molecular level most evolutionary change and most of the variability within a species are caused not by selection but by random drift of mutant genes that are selectively equivalent

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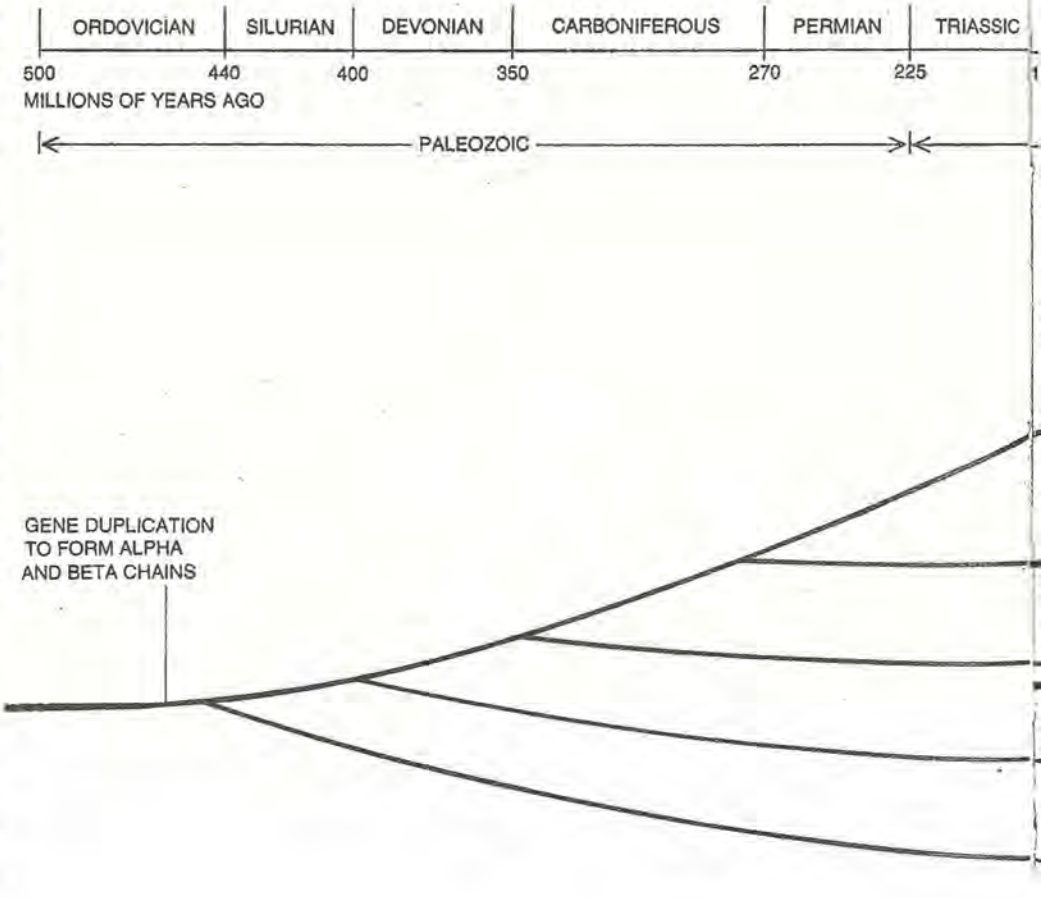
The Darwinian theory of evolution through natural selection is firmly established among biologists. The theory holds that evolution is the result of an interplay between variation and selection. In each generation a vast amount of variation is produced within a species by the mutation of genes and by the random assortment of genes in reproduction. Individuals whose genes give rise to characters that are best adapted to the environment will be the fittest to survive, reproduce and leave survivors that reproduce in their turn. Species evolve by accumulating adaptive mutant genes and the characters to which those genes give rise.

In this view any mutant allele, or mutated form of a gene, is either more adaptive or less adaptive than the allele from which it is derived. It increases in the population only by passing the stringent test of natural selection. For more than a decade now I have championed a different view. I believe most of the mutant genes that are detected only by the chemical techniques of molecular genetics are selectively neutral, that is, they are adaptively neither more nor less advantageous than the genes they replace; at the molecular level most evolutionary changes are caused by the "random drift" of selectively equivalent mutant genes.

The Evolution of Darwinism

The controversy between the neutralist view and the "panselectionist" assumption arises from the way the modern "synthetic" theory of evolution has itself evolved. When Darwin formulated his original theory, the mechanisms of inheritance and the nature of heritable variations were not known. With the rise of Mendelian genetics in this century the way was opened for efforts to supply a genetic base for Darwin's insights. This was achieved largely through the elucidation by H. J. Muller of the fundamental nature of the gene and through the methods of population genetics developed mainly by R. A. Fisher, J. B. S. Haldane and Sewall Wright. On their foundation subsequent studies of natural populations by Theodosius Dobzhansky, paleontological analyses by George Gaylord Simpson, the "ecological genetics" of E. B. Ford and his school and other investigations built a large and impressive edifice of neo-Darwinian theory.

By the early 1960's there was a general consensus that every biological character could be interpreted in the light of



PHYLOGENETIC TREE displays the evolutionary relations among seven vertebrates and shows how and when their lineages have diverged from one another over geologic time. The table at the right shows the extent to which an important protein, the alpha chain of hemoglobin, differs in the seven animals; specifically it gives the number of differences in the sequence

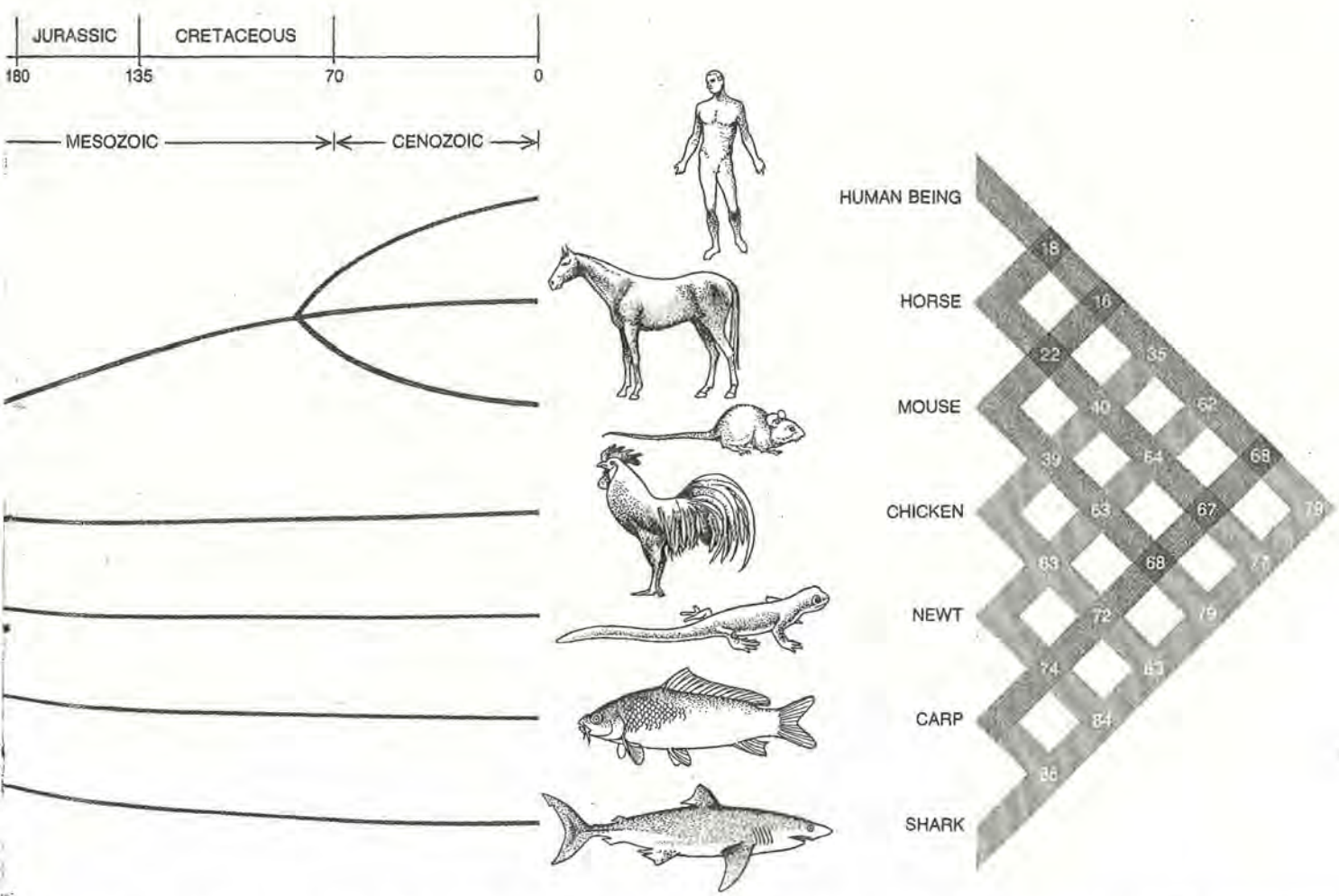
adaptive evolution through natural selection and that almost no mutant genes were selectively neutral. As Ernst Mayr stated the case in 1963, "I consider it... exceedingly unlikely that any gene will remain selectively neutral for any length of time." A great deal was said by many workers about how genes interact, how gene pools of species are organized and how gene frequencies in populations change in the course of evolution. These conclusions, however, were necessarily inferences based on observations at the phenotypic level: the level of the form and function arising from the operation of genes. There was no way of knowing what actually goes on in evolution at the level of the internal structure of the gene.

Meanwhile the mathematical theory of population genetics was becoming quite sophisticated (which is rather unusual in biology). Particularly noteworthy was the theoretical framework provided by the manipulation of partial differential equations called diffusion equations. Diffusion models enable one to describe the behavior of mutant alleles by considering the random changes resulting from random sampling of gametes (germ cells) in reproduction as well as the deterministic changes caused by mutation and selection. Although the diffusion-equation method involves approximation, it yields answers to important but difficult questions that are inaccessible by other methods, such as: What is the probability of fixation for a single mutant appearing in a finite population and having a certain selective advantage, that is, what is the probability that it will eventually spread through the entire population?

The applicability of this method to gene changes in evolution, however, remained rather limited for some time. The reason is that population genetics deals with the concept of gene frequencies (the relative prevalence of various alleles within a population), whereas conventional studies of evolution were conducted at the phenotypic level, and there was no direct way of connecting the two sets of data unambiguously. That obstacle was removed with the advent of molecular genetics. It became possible to compare, in related organisms, individual RNA molecules (the direct products of genes) and proteins (the ultimate products) and so to estimate the rate at which allelic genes are substituted in evolution. It also became possible to study the variability of genes within a species. At last the time was at hand for applying the mathematical theory of population genetics to find out how genes evolve. One might have expected that the principle of Darwinian selection would prove to prevail at that fundamental level. Indeed, many evolutionary biologists found what they expected to find, and they have tended to extend panselectionism to the molecular level.

The Neutral Theory

The picture of evolutionary change that actually emerged from molecular studies seemed to me, however, to be quite incompatible with the expectations of neo-Darwinism. One of my salient findings with regard to evolution was that in a given protein the rate at which amino acids (the subunits of proteins) are substituted for one another is about the same in many diverse line-



of amino acids that constitutes the chain. The hemoglobin molecule has two alpha chains and two beta chains, which originated through the duplication of a single gene some 450 million years ago. The table reflects the approximate uniformity (predicted by the neutral theory) of the rate of evolution of a given protein in very different organisms. The number of amino acid differences is roughly 20 when any of the three mammals are compared with one another, and it is approximately 70 when the carp is compared with any of the three mammals.

TYPE OF CHANGE	HUMAN ALPHA V. HUMAN BETA	CARP ALPHA V. HUMAN BETA
NO CHANGE	62	61
ONE-NUCLEOTIDE	55	49
TWO-NUCLEOTIDE	21	29
GAP	9	10
TOTAL	147	149

NUMBER OF DIFFERENCES between the amino acid sequences of the alpha chain and the beta chain of human hemoglobin is compared with the number of differences between the sequences of the alpha chain in the carp and the human beta chain. The column at the left categorizes the amino acid sites according to whether there is no change, a change due to a minimum of at least one nucleotide substitution or at least two substitutions in the genetic code at each site, or a "gap": an addition or a deletion of an amino acid. The numbers are similar whether one compares the chains in the same species or in the two species, suggesting that alpha chains in two lineages have accumulated mutations at about same rate for 400 million years.

ages. Another finding was that the substitutions seem to be random rather than having a pattern. A third finding was that the overall rate of change at the level of DNA, the actual genetic material, is very high, amounting to the substitution of at least one nucleotide base (DNA subunit) per genome (total genetic complement) every two years in a mammalian lineage. As for the extent of variability within a species, electrophoretic methods for detecting small differences among proteins suddenly disclosed a wealth of genetic variability; the proteins produced by a large fraction of the genes in diverse organisms were found to be polymorphic, that is, they were present in the species in variant forms. In many cases the protein polymorphisms had no visible phenotypic effects and no obvious correlation with environmental conditions.

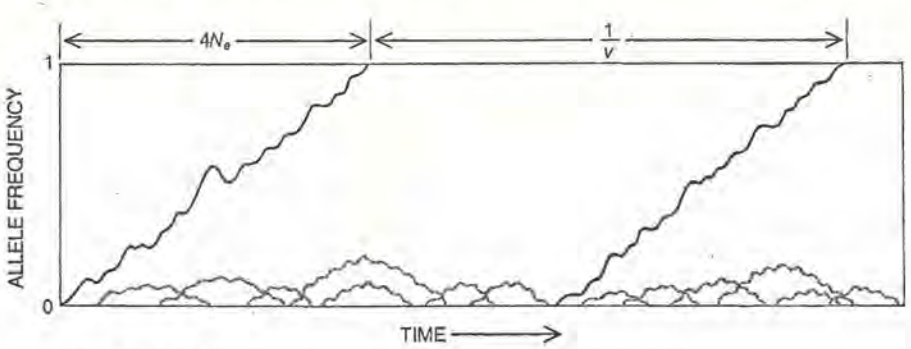
In 1967, as I considered these puzzling observations, I decided they suggested two things. One was that a majority of the nucleotide substitutions in the course of evolution must be the result of the random fixation of neutral or nearly neutral mutants rather than the result of positive Darwinian selection. The other was that many protein polymorphisms must be selectively neutral or nearly so and must be maintained in a population by the balance between mutational input and random extinction. I presented these thoughts at a meeting of the Genetics Club in Fukuoka in November, 1967, and in a short paper in *Nature* the following February. In 1969 strong support came from a paper in *Science* by Jack Lester King, now of the University of California at Santa Barbara, and Thomas H. Jukes of the University of California at Berkeley. They had arrived at the same ideas on molecular evolution (although not on protein polymorphisms) independently, and they presented cogent supporting data from molecular biology.

The papers suggesting a neutral theory were severely criticized by evolutionists who believed the new molecular data could be understood in the light of orthodox neo-Darwinian principles, and the neutralist-selectionist controversy continues today. The essential difference between the two schools of thought can be appreciated by comparing their differing explanations of the evolutionary process by which mutant genes come to be substituted in a species. Every substitution involves a sequence of events in which a rare mutant allele appears in a population and eventually spreads through the population to reach fixation, or a frequency of 100 percent. Selectionists maintain that for a mutant allele to spread through a species it must have some selective advantage (although they admit that an allele that is itself neutral may occasionally be carried along by "hitchhiking" on a gene that is selected for and with which it is closely linked, and may thus reach a high frequency).

Neutralists, on the other hand, contend that some mutants can spread through a population on their own without having any selective advantage. If a mutant is selectively equivalent to preexisting alleles, its fate is left to chance. Its frequency fluctuates, increasing or decreasing fortuitously over time, because only a relatively small number of gametes are "sampled," out of the vast number of male and female gametes produced in each generation, and are therefore represented in individuals of the next generation [*see illustration on opposite page*].

In the course of this random drift the overwhelming majority of mutant alleles are lost by chance, but a remaining minority of them eventually become fixed in the population. If neutral mutations are common at the molecular level and if the random drift is continuous over a long time (say millions of generations), the genetic composition of the population will change significantly. For any neutral mutant that appears in a population the probability of eventual fixation is equal to its initial frequency. The average length of time until fixation (excluding alleles that are lost) is four times the "effective" population size, or $4N_e$. (The effective size of a population is approximately equal to the number of breeding individuals in one generation, and it is usually much smaller than the total number of individuals in the species.)

The neutral theory, I should make clear, does not assume that neutral genes are functionless but only that various alleles may be equally effective in promoting the survival and reproduction of the individual. If a mutant allele encodes variant amino acids in a protein, the modified protein need function only about as well as the original form; it need not be precisely equivalent. In higher organisms particularly, homeostasis counteracts external environmental changes just as it does internal physiological changes; fluctuations in the environment do not necessarily imply



MUTANT ALLELES (variant genes) arise in a population at random. Their frequency fluctuates; in time most of them disappear (*gray lines*), but some of them spread through the population to fixation: a frequency of unity, or 100 percent (*black lines*). Population-genetic studies show that for a neutral allele that is destined for fixation the average number of generations until fixation is four times the effective population size, or $4N_e$. The average number of generations between consecutive fixations is equal to the reciprocal of the mutation rate v.

comparable fluctuations in the Darwinian fitness of mutant genes.

Some criticisms of the neutral theory arise from an incorrect definition of "natural selection." The phrase should be applied strictly in the Darwinian sense: natural selection acts through—and must be assessed by—the differential survival and reproduction of the individual. The mere existence of detectable functional differences between two molecular forms is not evidence for the operation of natural selection, which can be assessed only through investigation of survival rates and fecundity. Moreover, a clear distinction should be made between positive (Darwinian) selection and negative selection. The latter, which Muller showed is the commoner form, eliminates deleterious mutants; it has little to do with the gene substitutions of evolution. A finding of negative selection does not contradict the neutral theory. Finally, the distinction between gene mutation in the individual and gene substitution in the population should be kept in mind; only the latter is directly related to molecular evolution. For advantageous mutants the rate of substitution is greatly influenced by population size and degree of selective advantage (as I shall show below) as well as by the mutation rate.

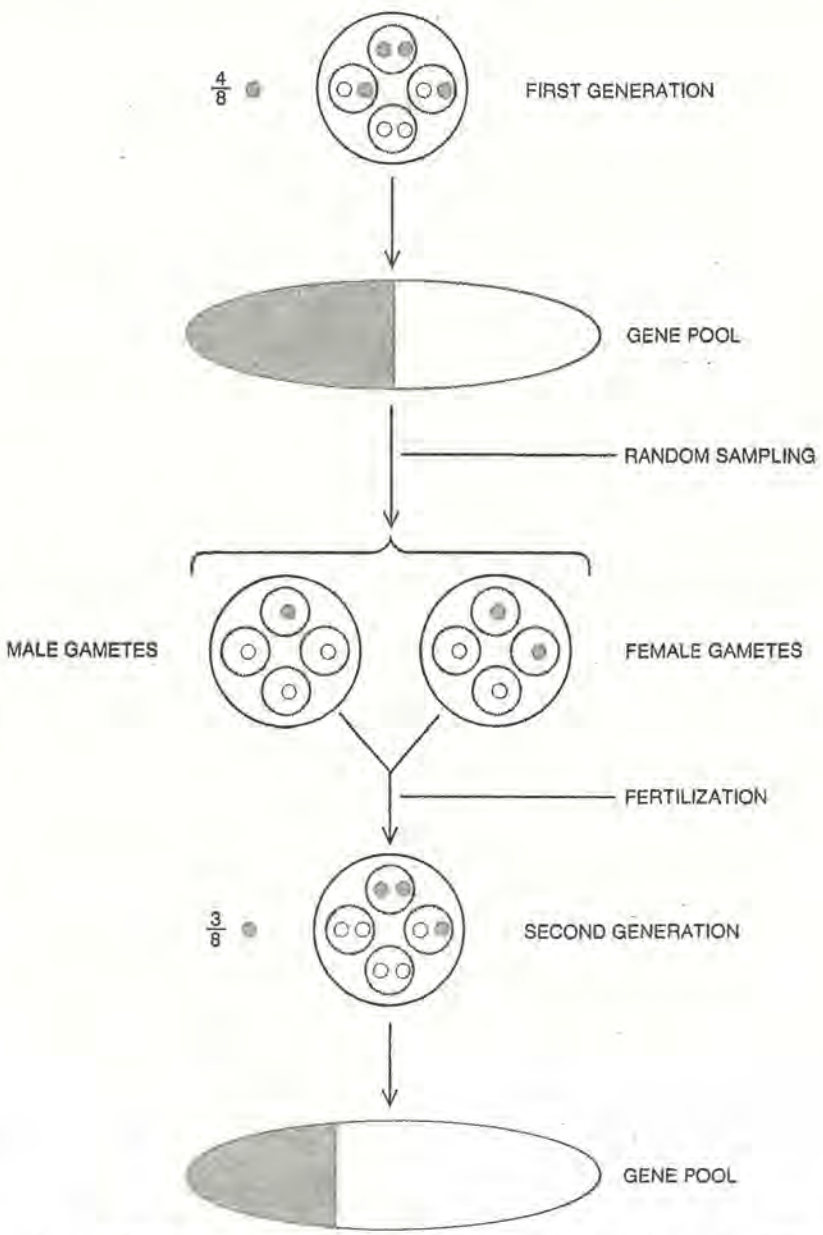
Molecular Evolution

Two major findings with regard to molecular evolution demonstrate particularly clearly that its patterns are quite different from those of phenotypic evolution and that the laws governing the two forms of evolution are different. One is the finding, alluded to earlier, that for each protein the rate of evolution in terms of amino acid substitutions per year is approximately constant and about the same in various lineages. The other is that molecules or parts of a molecule subjected to a relatively small degree of functional constraint evolve at a higher rate (in terms of mutant substitutions) than those subjected to stronger constraints do.

The constancy of the evolutionary rate is apparent in the molecule of hemoglobin, which in bony fishes and higher vertebrates is a tetramer (a molecule with four large subunits) consisting of two identical alpha chains and two identical beta chains. In mammals amino acids are substituted in the alpha chain, which has 141 amino acids, at the rate of roughly one substitution in seven million years. This corresponds to about one substitution in a billion years (or 10^{-9} substitution per year) per amino acid site. The rate does not appear to depend on such factors as generation time, living conditions and population size. The approximate constancy of the rate is evident when the number of amino acid differences between the alpha chains of various vertebrates is charted together with the phylogenetic tree showing the relations among the vertebrates and the times when they diverged from one another in evolution [*see illustration on pages 94 and 95*].

The alpha and beta chains have essentially the same structure, are about the same length and show roughly the same rate of evolutionary amino acid substitution. They arose through gene duplication some 450 million years ago and became differentiated as they accumulated mutations independently. If one compares the divergence between the alpha and the beta chain of man with the divergence between the alpha chain of the carp and the beta chain of man, it is evident that in both cases the alpha and beta chains differ from each other to roughly the same extent. Because the alpha chain of man and that of the carp differ from each other in about half of their amino acid sites this suggests that the alpha chains in two distinct lineages, one leading to the carp and the other to man, have accumulated mutations independently and at practically the same rate over a span of about 400 million years. Moreover, the rate of amino acid substitution observed in these comparisons is very similar to the rates observed in comparisons of the alpha chains in various mammals.

My assertion of constancy of the evo-



RANDOM CHANGES in gene frequency arise from random sampling of gametes (germ cells) in reproduction, as is shown here for a hypothetical population of four individuals (*gray circles*), each of which has two homologous genes (*solid color and open color*) inherited from the male and female parents. In the first generation the frequency of the "solid" allele is 4/8, and so the gene pool is 50 percent solid. Of the many gametes produced by a generation only a few are sampled, at random, in reproduction; here only one solid allele happens to be present in the four first-generation male gametes that engage in reproduction, so that the frequency of the solid allele changes to 3/8 in the second generation of individuals and hence in their gene pool.

lutionary rate at the molecular level has been criticized by, among others, Richard C. Lewontin of Harvard University, who called the asserted constancy "simply a confusion between an average and a constant" and "nothing but the law of large numbers." The remarks reveal a misunderstanding of the nature of molecular evolution. One is attempting here to compare intrinsic rates of evolution in different lineages. The death rates characteristic of man and of an insect do not become equal by merely being averaged over a long period of time or over a large number of individuals; there is no reason to expect two averages to converge on each other unless the intrinsic factors shaping them are the same. My point is that intrinsic evolutionary rates are essentially determined by the structure and function of molecules and not by environmental conditions.

Evolutionary rates are, to be sure, not precisely constant in the sense that a rate of radioactive decay is constant. My colleague Tomoko Ohta and I showed in 1971 that the variance (the squared standard deviation) of the evolutionary rate observed for hemoglobins and for the protein cytochrome *c* in different mammalian lines is about 1.5 to 2.5 times larger than the variance to be expected if it were due only to chance. Charles H. Langley and Walter M. Fitch did a more elaborate analysis at the University of Wisconsin School of Medicine, combining data for the alpha and beta hemoglobin chains, cytochrome *c* and fibrinopeptide *A;* they found a variation in rates of mutant substitution about 2.5 times larger than the expected variance due to chance fluctuations, and they took this as evidence against the neutral theory. Yet they also showed that when the estimated number of substitutions between diverging branches of a phylogenetic tree is plotted against the corresponding time of divergence, the points fall on a straight line, which suggests the substantial uniformity of the evolutionary rates. It seems to me to be wrong to emphasize local fluctuations as evidence against the neutral theory while neglecting to inquire into why the rate remains essentially constant.

Rates of Evolution

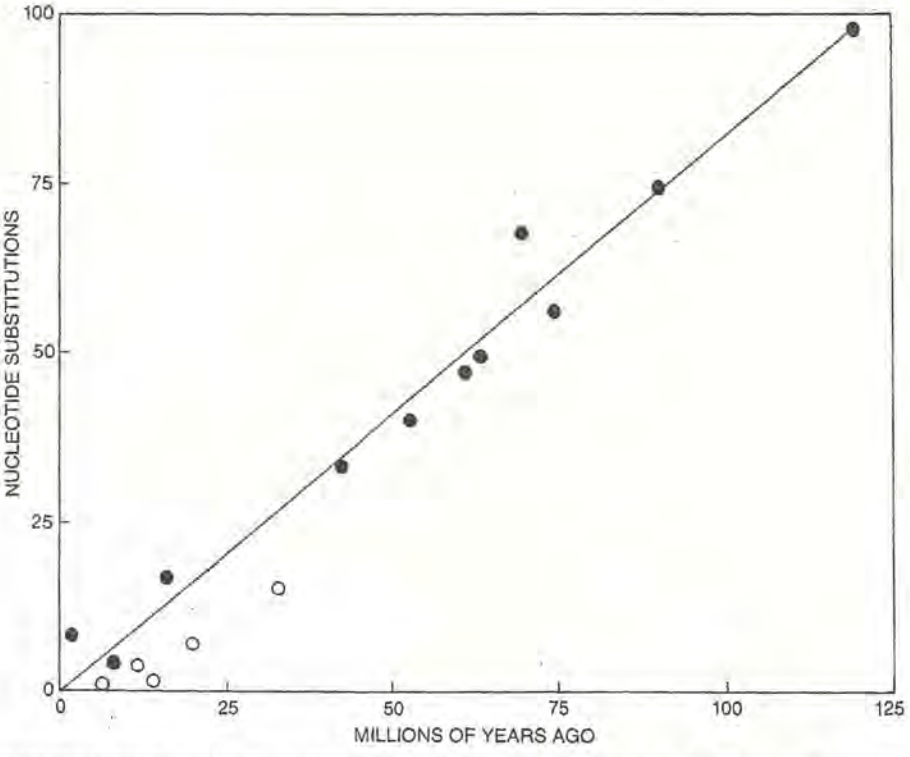
Turning now to the quantitative relations that determine rates of evolution, consider first the nucleotides constituting a genome: a single (haploid) set of chromosomes. For a human being the number of nucleotides is very large, on the order of 3.5 billion. Because the mutation rate per nucleotide site is low (perhaps 10^{-8} per generation, or one mutation per 100 million generations) one can assume that whenever a mutant appears it is at a new site. This assumption is called the "infinite site" model in population genetics.

Let v represent the mutation rate per gamete per unit time (generation). Since each individual has two sets of chromosomes, the total number of new mutants introduced into a population of N individuals in each generation is $2Nv$. Now let u be the probability that a single mutant will ultimately reach fixation. Then, in a steady state in which the process of substitution goes on for a very long time, the rate k of mutant substitution per unit time is given by the equation $k = 2Nvu$. That is, $2Nv$ new mutants appear in each generation, of which the fraction u eventually reach fixation, and k represents the rate of evolution in terms of mutant substitutions. The equation can be applied not only to the genome as a whole but also, with good approximation, to a single gene consisting of several hundred nucleotides or to the protein encoded by a gene.

The probability u of ultimate fixation is a well-known quantity in population genetics. If the mutant is selectively neutral, u equals $1/(2N)$. The reason is that any one of the $2N$ genes in the population is as likely as any other to be fixed, and so the probability that the new mutant will be the lucky gene is $1/(2N)$. (This assumes that the process is being viewed over a very long period of time, since the average time for a neutral gene to sweep through the population is $4N_e$.) Substituting $1/(2N)$ for u in the equation for the rate of evolution ($k = 2Nvu$), one gets $k = v$. That is, the rate of evolution in terms of mutant substitutions in the population is simply equal to the rate of mutation per gamete, independent of what the population size may be.

This remarkable relation applies only for neutral alleles. If the mutant has a small selective advantage s, then u equals $2s$ with good approximation, and the equation for the rate of evolution becomes $k = 4Nsv$. That is, the rate of evolution for selectively advantageous genes depends on the size of the population, the selective advantage and the rate at which mutants having a given selective advantage arise in each generation. One should expect, in this case, that the rate of evolution would depend strongly on the environment, being high for a species offered new ecological opportunities but low for those kept in a stable environment. It is highly unlikely, I think, that the product Nsv should be the same in diverse vertebrate lineages, in some of which phenotypic evolution has been very rapid (as in the line leading to man) and in others of which phenotypic evolution has long since practically ceased (as in the line leading to the carp). And yet the observed rates of molecular evolution show remarkable constancy. It seems to me that this constancy is much more compatible with the expectation of the neutral theory, that is, with the equation $k = v$, than it is with the selectionist relation $k = 4Nsv$.

Even more striking than the constan-



NUCLEOTIDE SUBSTITUTIONS estimated from the total number of amino acid differences observed in seven proteins in 16 pairs of mammals are plotted against the time since members of each pair diverged. Except for lineages involving primates (*open circles*) the points fall close to a straight line, again suggesting approximate uniformity of a protein's molecular evolutionary rate. Data are from Walter M. Fitch of University of Wisconsin School of Medicine.

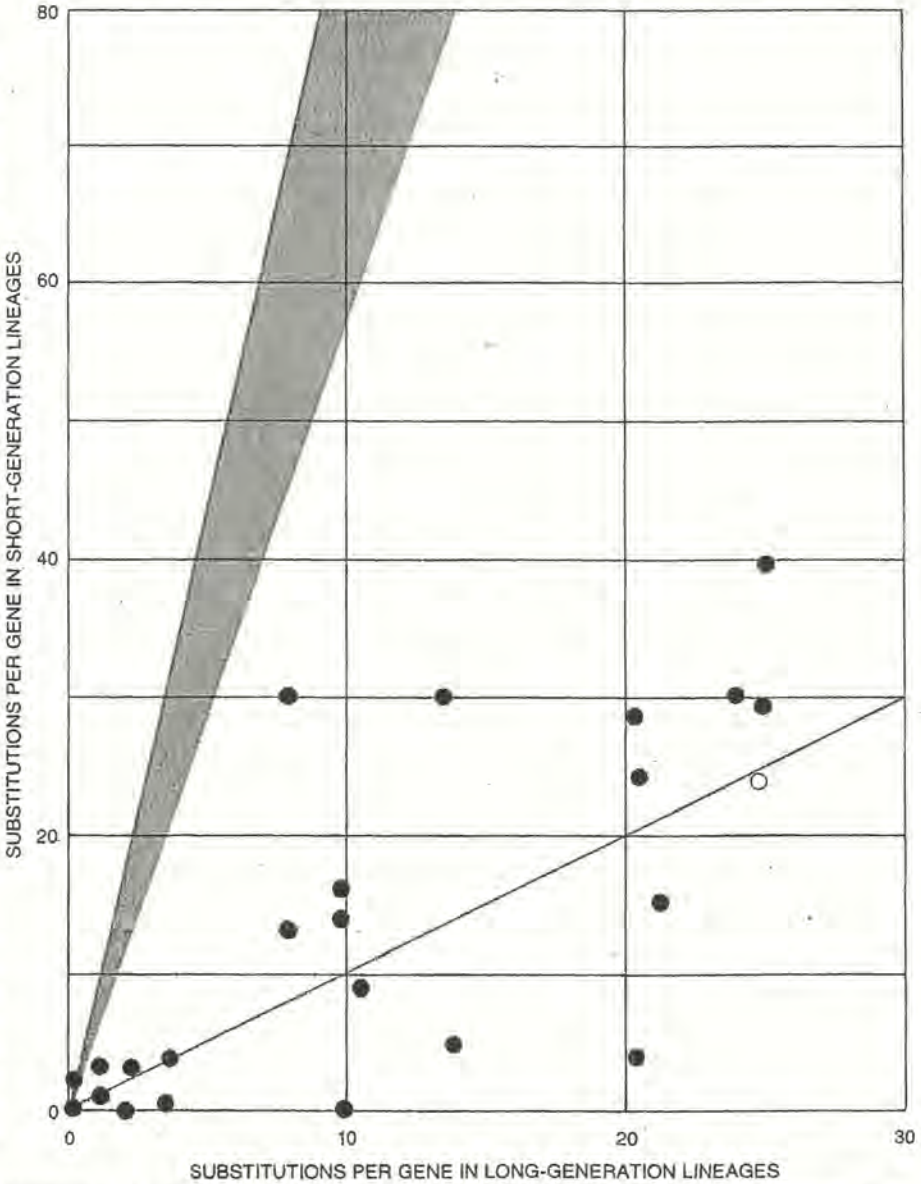
cy of the rate of evolution is the second major feature of molecular evolution: the weaker the functional constraint on a molecule or a part of a molecule, the higher the evolutionary rate of mutant substitutions. There are regions of DNA between genes, for example, and in the case of higher organisms even within genes, that do not participate in protein formation and must therefore be much less subject to natural selection; some recent research has indicated that nucleotide substitutions are particularly prevalent in such relatively unconstrained regions of DNA.

Functional Constraint

This relation between a relative lack of selective constraint and a relatively high rate of molecular evolution has been well established for certain proteins. Among the proteins so far investigated the highest evolutionary rate has been found in fibrinopeptides, which appear to have little function, if any, after they become separated from fibrinogen to yield fibrin, a protein that plays a role in blood clotting. The same effect is observed in the case of the C chain of the proinsulin molecule, a precursor of insulin. The C chain, which is cleaved from the precursor to form active insulin, evolves at a rate several times higher than that of the active molecule. The effect of constraint on the evolutionary rate has also been noted for different parts of the hemoglobin molecule. The precise structure of the surface of the molecule is presumably less significant than the structure of the pockets, in the interior of the molecule, that hold the iron-containing heme groups. Ohta and I have estimated that the regions of both the alpha and the beta chain that are at the surface of the protein evolve about 10 times faster than the regions forming the heme pocket.

The genetic code is based on groups of three nucleotides, with each triplet "codon" in a strand of RNA specifying a particular amino acid of the protein chain encoded by the RNA. For example, the codon *GUU* (the letters stand for particular nucleotide bases) specifies the amino acid valine. So does *GUC*, however; the genetic code is "degenerate," with most amino acids being designated by two or more synonyms, which typically differ only in the third position of the triplet. As a result a large fraction (perhaps 70 percent) of all random nucleotide substitutions at the third position are synonymous changes and do not lead to amino acid replacements. There is growing evidence that evolutionary nucleotide substitution goes on at a particularly high rate at the third position. Michael Grunstein of the University of California at Los Angeles and his colleagues compared the RNA sequences encoding the protein histone IV in two species of sea urchin. They found that although the protein has maintained a practically unchanged amino acid sequence for about a billion years, a number of synonymous nucleotide differences are found in the RNA sequences of the two species. On the basis of their data and paleontological evidence on the length of time since the species diverged, I have estimated that the rate of nucleotide substitution has been roughly 3.7×10^{-9} per year at the third position, a very high rate. What is remarkable is that there have been so many synonymous mutant substitutions in the histone-IV gene in spite of the very low rate of amino acid changes in the corresponding protein.

These observations can be explained simply and consistently by the neutral theory. Suppose a certain fraction f_0 of all molecular mutants are selectively neutral and the rest are definitely deleterious. Then the mutation rate v for neutral alleles is equal to the total mutation rate v_T multiplied by f_0, so that the overall rate k of mutant substitution becomes equal to $v_T f_0$. Now assume that the probability that a mutational change is neutral (not harmful) depends strongly on functional constraints. The weaker the constraint is, the larger will be the probability f_0 that a random change is neutral, with the result that the rate of evolution k increases. The maximum evolutionary rate is attained when f_0 equals 1, that is, when all the mutations are neutral. In my opinion the high evolutionary rates observed at the third po-



MOLECULAR EVOLUTIONARY RATE in mammals that have a short generation time was compared with the rate in mammals having a long generation time by Allan C. Wilson and his colleagues at the University of California at Berkeley. Each point represents the ratio of the implied nucleotide substitutions in the two animals of a pair since the two diverged from a common ancestor; the open circle, for example, is for the beta chain of hemoglobin in the elephant (*abscissa*) and in the mouse (*ordinate*). If the rate of change per year were identical in both animals of a pair, the points would fall on a line (*solid color*). Actually the points are close to that absolute-time line and far from sector predicted for generation-time effect (*colored area*). Apparently molecular evolutionary rate is roughly constant per year, not per generation.

PROTEIN	EVOLUTIONARY RATE
FIBRINOPEPTIDES	9.0
PANCREATIC RIBONUCLEASE	3.3
HEMOGLOBIN CHAINS	1.4
MYOGLOBIN	1.3
ANIMAL LYSOZYME	1.0
INSULIN	.4
CYTOCHROME *c*	.3
HISTONE IV	.006

PROTEINS VARY WIDELY in their rate of evolution. Here the rate is given for several proteins in terms of the number of amino acid substitutions per amino acid site per billion years. The rate is particularly high for fibrinopeptides, which appear to have little function after they are cleaved from a precursor molecule to yield the active blood-clotting protein fibrin. Proteins such as fibrinopeptides are subject to less functional constraint, and evolve faster, than proteins whose precise shape is significant and hence subjects them to stronger constraint.

sition of the codon are rather near this limit.

The neutral theory, then, predicts that as functional constraint diminishes, the rate of evolution converges to the maximum value set by the total mutation rate. Confirmation of such a convergence, or plateauing, of molecular evolutionary rates by further studies would be strong evidence in support of the neutral theory. This interpretation of the molecular data will not make sense to selectionists. In their view molecules or parts of a molecule that are evolving rapidly in terms of mutant substitutions must have some important but as yet unknown function and must be undergoing rapid adaptive improvement by accumulating beneficial mutants. And they will see no reason to believe the upper limit of the evolutionary rate is related to the total mutation rate.

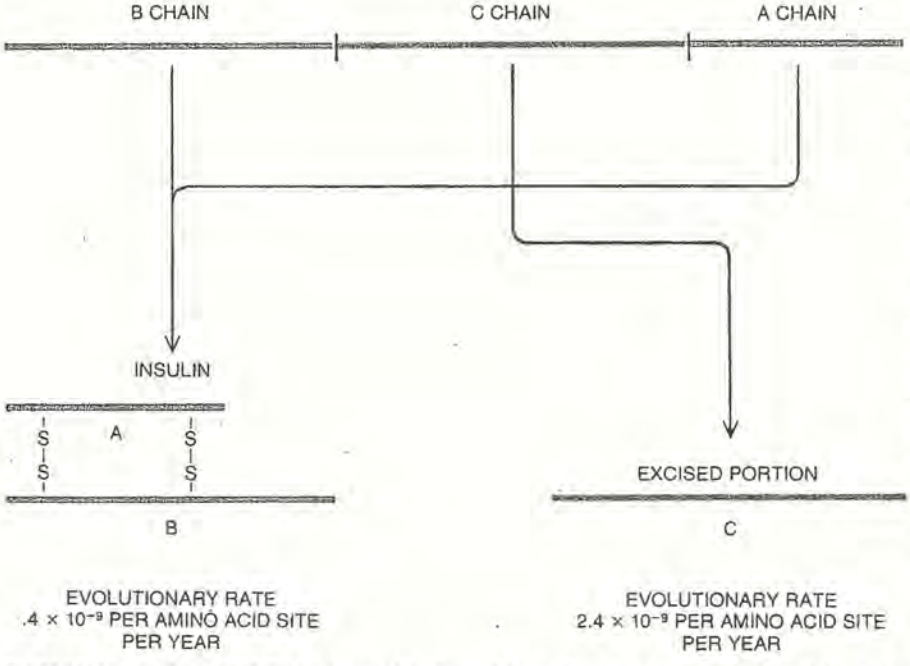
Polymorphism

Neutralists and selectionists also have diametrically opposed explanations for the mechanisms by which genetic variability is maintained within a species, particularly in the form of protein polymorphism: the coexistence in a species of two or more different forms of a protein. Neutralists maintain that polymorphisms are selectively neutral and are maintained in a population through mutational input and random extinction; in every generation a number of neutral mutants arise and in time either become fixed in the population or are lost, and in the process they contribute to genetic variability in the form of polymorphisms. In the neutral view polymorphism and molecular evolution are not two distinct phenomena; polymorphism is simply one phase of molecular evolution.

Selectionists maintain that polymorphisms are actively maintained by some form of "balancing selection," notable among which are heterotic selection, or "heterozygote advantage," and frequency-dependent selection. At one time the former was enthusiastically proposed as the main agent maintaining polymorphisms. There are instances in which individuals that are heterozygous for a particular gene (that carry a different allele of the gene on each of their two chromosomes) are fitter than individuals that are homozygous for either allele (that carry one or the other allele on both chromosomes). Selection will then tend to preserve both alleles in the population as a balanced polymorphism. In 1973, however, Roger D. Milkman of the University of Iowa found abundant polymorphisms in the bacterium *Escherichia coli,* which is a haploid organism: it has only one set of genes. Heterozygote advantage cannot explain such polymorphisms.

Nowadays many selectionists explain polymorphisms as being the result of frequency-dependent selection, in which the fitnesses of two alleles vary with their relative frequency. This was first proposed by the late Ken-Ichi Kojima of the University of Texas at Austin and his colleagues, who obtained results indicating marked frequency-dependent selection affecting the genes for the enzymes esterase-6 and alcohol dehydrogenase (ADH) in the fruit fly *Drosophila melanogaster;* Bryan Clarke of the University of Nottingham reported that he had confirmed Kojima's results in the case of ADH. On the other hand, experiments done by Tsuneyuki Yamazaki, now of Kyushu University, failed to show any such selection for esterase-5 alleles in *Drosophila pseudoobscura.* A group led by Terumi Mukai of Kyushu carried out extensive studies of selection for several enzymes in *D. melanogaster* and found no evidence for a difference in the fitness of variant forms of the enzymes. And recent careful, large-scale experiments by Mukai and Hiroshi Yoshimaru have failed to find any frequency-dependent selection for ADH in *D. melanogaster.*

If selection is not responsible for maintaining polymorphisms, what neutralist explanation is there for the fact that some proteins are more often polymorphic than others? Recently Richard K. Koehn of the State University of New York at Stony Brook and W. F.



C CHAIN OF PROINSULIN (*light color*) is cleaved from the precursor molecule and discarded, and the A and B chains (*dark color*) are joined by disulfide bridges (*S–S*) to form the active insulin molecule. In keeping with the C chain's relative freedom from functional constraint, it has been found to evolve at a much higher rate than the two chains of the active molecule.

Eanes, now of Harvard, and also Masatoshi Nei's group at the University of Texas at Houston, have shown that in various *Drosophila* species there is a significant correlation between the genetic variability (or polymorphism) of proteins and the weight of their molecular subunits. This is easy to explain according to the neutral theory because the larger the size of a subunit is, the higher its mutation rate should be. Harry Harris of the University of Pennsylvania and his colleagues could not find the same correlation when they investigated human polymorphisms, but they did find that single-subunit enzymes are more polymorphic than multiple-subunit ones, something that Eleftherios Zouros of Dalhousie University had earlier reported in *Drosophila*. One finding by Zouros and Harris fits the neutral theory particularly well: multiple-subunit enzymes that form hybrid molecules by combining with enzymes encoded by other genes have a clearly reduced level of polymorphism. The precise interaction among subunits required to form such enzymes would increase the degree of functional constraint and so reduce the possibility that a mutation will be harmless, or neutral.

Neutralists, in other words, consider molecular structure and function to be the major determinants of protein polymorphisms. Selectionists consider environmental conditions to be the major determinants. They have maintained that there should be a correlation between environmental variability and genetic variability. They predicted, for example, that organisms living at the bottom of the deep sea would generally be found to display little genetic variability because their environment is stable and homogeneous, whereas organisms living in the intertidal zone would display a great deal of genetic variability because their environment is a changeable one. The prediction was logical and plausible, but it failed: genetic variability has been found to be generally extremely high among organisms living at the bottom of the oceans and to be very low among organisms in the intertidal zone.

Models

In order to carry out quantitative studies based on population genetics one needs mathematical models for the mutational production of new alleles. The first such model was proposed in 1964 by James F. Crow of the University of Wisconsin and me. It is based on the fact that each gene consists of a large number of nucleotides, so that a practically infinite number of alleles can arise; the model therefore assumes that any new mutant arising represents a new allele rather than a preexisting one. The model predicts that variability within a species, in terms of the average heterozygosity H per gene, will be determined essentially by the product of the effective population size N_e and the mutation rate v per gene per generation, rather than by N_e and v separately. Specifically, H equals $4N_ev/(4N_ev+1)$. For example, if the mutation rate is 10^{-6} and the effective population size is 10^5, the average heterozygosity per gene will be about .286, that is, 28.6 percent of the individuals are heterozygous at each gene locus on the average. The larger either the population size or the mutation rate per gene per generation is, the closer the average heterozygosity will be to unity (or 100 percent).

The model assumes that alleles are identified at the level of the gene in terms of actual nucleotide substitutions. Most observations of variability depend, however, on the electrophoresis of proteins, which has much less resolving power and is far from revealing all nucleotide substitutions (or even all amino acid changes), so that the observed heterozygosity is less than the true amount. Even when the model is modified to take account of this problem, very large populations should, according to the neutral theory, display nearly 100 percent heterozygosity. When observations suggest otherwise, the theory is subject to criticism. For example, Francisco J. Ayala of the University of California at Davis has reported that in the neotropical fruit fly *Drosophila willistoni*, for which he estimates a very large effective population size of 10^9, he has found an observed heterozygosity of roughly 18 percent. He points out that even assuming a very low rate of neutral mutations per generation, 10^{-7}, the predicted heterozygosity is practically 100 percent.

There are at least two ways to deal with this apparent inconsistency. First of all, it is possible that the effective population size of *D. willistoni* has not been as large as 10^9 even if the apparent present size of the population is enormous. One can show mathematically that the genetic variability due to neutral alleles can be greatly reduced by a population "bottleneck" from time to time, after which it takes millions of generations for the variability to build up again to the theoretical level characteristic of a very large population maintained constantly over a long period. In this sense such neotropical species as *D. willistoni* may still show the effects of the bottleneck imposed by the last continental glaciation, between some 30,000 and 10,000 years ago. In addition the local extinction of colonies of a species, which may be fairly frequent, must reduce the effective population size.

A second possibility is that, as Ohta first proposed in 1973, the majority of "neutral" alleles may not actually be strictly neutral but rather may be very slightly deleterious. Adopting Ohta's idea, but retaining room for truly neutral mutations also, I have considered a model in which the selection coefficient s' against the mutant follows a particular distribution (the gamma distribution) [*see illustration on page 104*]. The mutation rate for variants whose negative selection (s') value is smaller than the reciprocal of two times the population size, or $1/(2N)$, can be considered the effectively neutral mutation rate v_e. It can be shown that this effectively neutral mutation rate decreases as the popu-

L. PICTUS MESSENGER RNA	GA **U**	AAC	AUC	CAA	GG **A**	AU **A**	AC **U**	AAA	CCG	GC **A**	AUC
S. PURPURATUS MESSENGER RNA	GA **C**	AAC	AUC	CAA	GG **U**	AU **C**	AC **G**	?	?	GC **U**	AUC
HISTONE IV AMINO ACID SEQUENCE IN BOTH SPECIES	Asp	Asn	Ile	Gln	Gly	Ile	Thr	Lys	Pro	Ala	Ile
	24	25	26	27	28	29	30	31	32	33	34

NUCLEOTIDE SEQUENCES of the messenger RNA encoding the protein histone IV in two sea-urchin species, *Lytechinus pictus* and *Strongylocentrotus purpuratus*, were compared by Michael Grunstein of the University of California at Los Angeles. There are four RNA nucleotides (*A, G, U* and *C*); three-nucleotide codons specify the various amino acids that constitute a protein. Most amino acids are specified by two or more synonymous codons, which usually differ only at their third position. In this short stretch of RNA coding for amino acid sites 24 through 34 of histone IV there are five synonymous differences (*color*) in third-position nucleotides. That is, there has been a high rate of nucleotide substitution at the unconstrained third position, leaving the amino acid sequences unaffected.

lation increases; in the case illustrated the rate is proportional to 1 divided by the square root of the population size. In this model the level of heterozygosity increases only slowly as the population increases. Moreover, given a realistic assumption about generation time, the rate of evolution in terms of mutant substitutions would be roughly constant per year for various lineages if the mutation rate per generation is constant. Note that although this explanation invokes natural selection, it is quite different from the selectionist explanation.

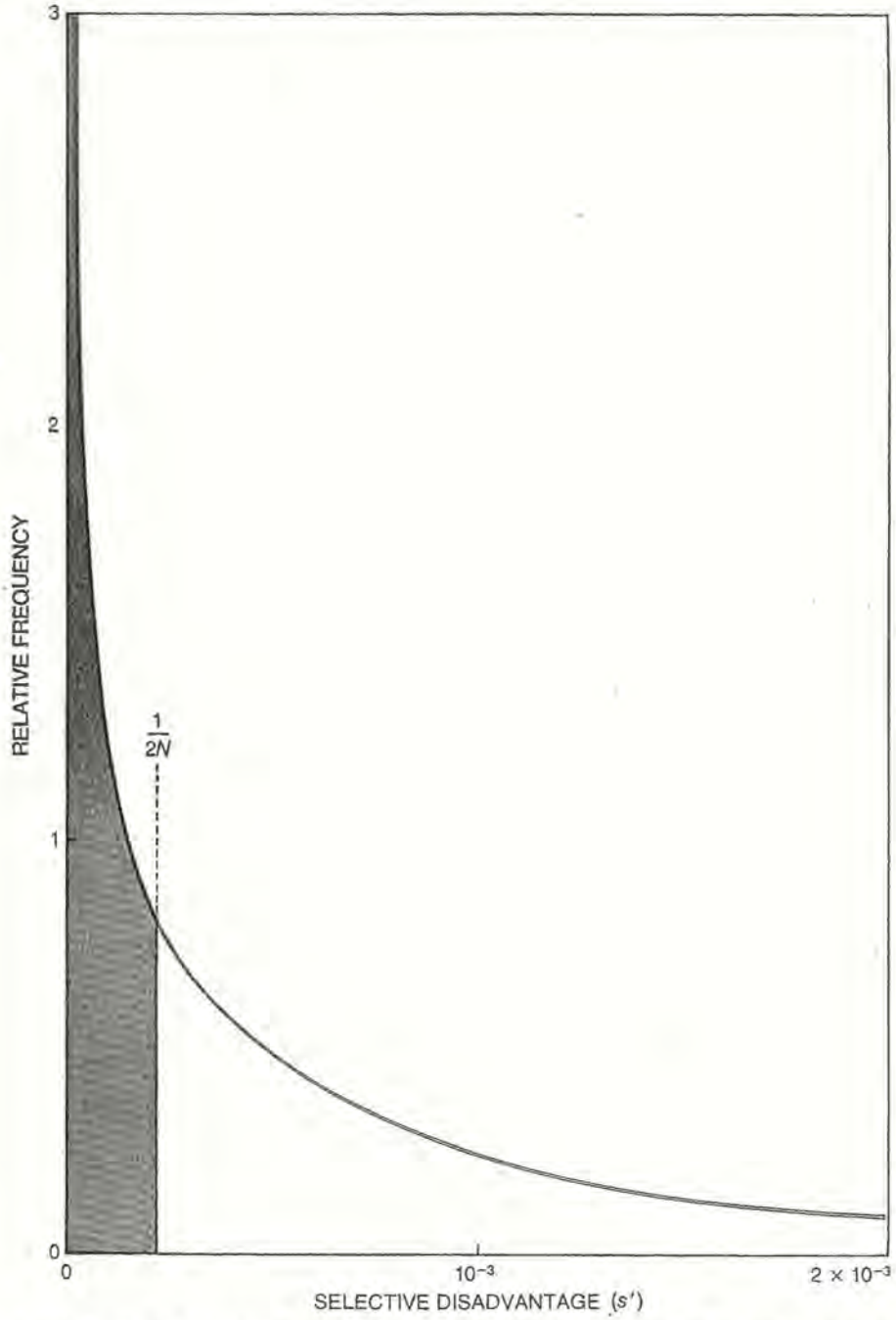
A Quantitative Approach

The neutral theory of molecular evolution and polymorphism that I have developed in collaboration with my colleagues Ohta and Takeo Maruyama is distinguished from most selectionist approaches—in particular from the approach of ecological genetics—in that it aims at a quantitative description of molecular evolution, which we attempt to achieve by manipulating diffusion equations. It is a venture in what might be called molecular population genetics. Nei and his associates at Houston have contributed greatly to this effort, in particular by connecting theoretical predictions with actual observations. They have shown, for example, that the variance of heterozygosity for particular enzymes within a species can be predicted fairly accurately by the neutral theory on the basis of observations of mean heterozygosity.

Because our theory is quantitative it is testable and therefore much more susceptible to refutation when it is wrong than are selectionist theories, which can invoke special kinds of selection to fit special circumstances and which usually fail to make quantitative predictions. To test the neutral theory, however, it is necessary to estimate such quantities as mutation rates, selection coefficients, population sizes and migration rates. Many evolutionary biologists maintain that such population-genetic quantities can never be accurately determined and that consequently any theory dependent on them is a futile exercise. I, on the other hand, believe these quantities must be investigated and measured if the mechanisms of evolution are to be understood. Surely astronomers and cosmologists cannot eschew theories set forth in terms of astronomical quantities simply because such quantities are hard to estimate accurately.

Darwinian selection acts mainly on phenotypes shaped by the activity of many genes. Environmental conditions surely play a decisive role in determining what phenotypes are selected for; Darwinian, or positive, selection cares little how those phenotypes are determined by genotypes. The laws governing molecular evolution are clearly different from those governing phenotypic evolution. Even if Darwin's principle of natural selection prevails in determining evolution at the phenotypic level, down at the level of the internal structure of the genetic material a great deal of evolutionary change is propelled by random drift. Although this random process is slow and insignificant in the time frame of man's ephemeral existence, over geologic time it makes for change on an enormous scale.

People have told me, directly and indirectly, that the neutral theory is not important biologically because neutral genes are not involved in adaptation. My own view is that what is important is to find the truth, and that if the neutral theory is a valid investigative hypothesis, then to establish the theory, test it against the data and defend it is a worthwhile scientific enterprise.



THEORETICAL MODEL assumes that most "neutral" alleles are actually slightly deleterious and therefore subject to a small coefficient of negative selection s'. The model shows the frequency distribution (*color curve*) of the value of s' for mutations at various sites in a gene. Mutations whose selective disadvantage is less than 1 divided by two times the population size N are effectively neutral. The area under the curve (*gray*) that is occupied by such mutants will decrease as the population increases. As the effectively neutral fraction of the mutants thus decreases, and as more mutants are consequently exposed to negative selection, the rate of molecular evolution will be reduced. In this model level of heterozygosity (a measure of variability) increases slowly with population size, bringing predictions of neutral theory into line with observation.