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Molecular Evolutionary Clock and the Neutral Theory

Motoo Kimura

National Institute of Genetics, Mishima, 411 Japan

Summary. From the standpoint of the neutral theory of molecular evolution, it is expected that a universally valid and exact molecular evolutionary clock would exist if, for a given molecule, the mutation rate for neutral alleles *per year* were exactly equal among all organisms at all times. Any deviation from the equality of neutral mutation rate per year makes the molecular clock less exact. Such deviation may be due to two causes: one is the change of the mutation rate per year (such as due to change of generation span), and the other is the alteration of the selective constraint of each molecule (due to change of internal molecular environment). A statistical method was developed to investigate the equality of evolutionary rates among lineages. This was used to analyze protein data to demonstrate that these two causes are actually at work in molecular evolution. It was emphasized that departures from exact clockwise progression of molecular evolution by no means invalidates the neutral theory. It was pointed out that experimental studies should be done to settle the issue of whether the mutation rate for nucleotide change is more constant per year or per generation among organisms whose generation spans are very different.

Key words: Protein evolution — Population genetics — Mutation rate

Introduction

One of the most remarkable features of molecular evolution is its clocklike progression, namely, for a given protein or sequence of DNA, the rate of amino acid or nucleotide substitution is roughly constant among diverse lineages as well as within lineages over time. This is known as the "molecular evolutionary clock," and its existence was first suggested by Zuckerkandl and Pauling (1965). Since then, this property has been used extensively to reconstruct phylogenies, particularly when fossil records are missing or incomplete. Recent studies of Osawa's group on the evolution of a wide range of organisms using 5S rRNA sequences (see Hori et al. 1985), and those of Sibley's group on bird phylogenies using DNA–DNA hybridization (Sibley and Ahlquist 1986) are particularly successful examples.

At the same time, there has been much dispute regarding the accuracy and the underlying mechanism of the "clock"; some authors have even doubted the validity of the rate constancy hypothesis. In retrospect, it is interesting to note that Zuckerkandl (1965) made a suggestion contradicting the rate constancy hypothesis, that a great many polypeptide molecules of "living fossils" might possibly differ very little from those of their ancestors of millions of years ago. If we accept the viewpoint that evolution at the molecular level is caused by accumulation of slightly advantageous mutations as suggested by Zuckerkandl and Pauling (1965), such a prediction might very well be valid.

On the other hand, from the standpoint of the neutral theory, I predicted (Kimura 1969) that "genes in 'living fossils' may be expected to have undergone as many base (and therefore amino acid) substitutions as corresponding genes (proteins) in more rapidly evolving species." Since then, ample evidence has accumulated supporting my prediction based on the neutral theory (see Kimura 1983).

The rate-constancy hypothesis or the concept of the molecular evolutionary clock has been particularly controversial when used to estimate the date

Offprint requests to: M. Kimura

of the human–ape divergence. Wilson and Sarich (1969) made a bold suggestion, based on molecular data and assuming clocklike regularity of molecular evolution, that the date of human–ape divergence is only 4 or 5 million years (Myr) ago. Since this differed so much from the then accepted date of at least 20 or 30 Myr (see for example, de Beer 1964, who placed the date about 30 Myr), it caused a great deal of controversy. It now appears that the molecular approach gives much more reliable information on this problem than the paleontological one (Pilbeam 1984), particularly if a "generation time effect" is taken into account.

Some authors (e.g., Wilson et al. 1977), while strongly supporting the validity of the molecular clock concept, do not consider the underlying mechanism to be important. I have a different view, and in this paper, I intend to discuss the accuracy of the molecular evolutionary clock from the standpoint of the neutral theory.

Population Genetics of Mutant Substitutions

For our discussion of the mechanism underlying the molecular evolutionary clock, we must make a clear distinction between mutation at the individual level and substitution of molecular mutants (such as different amino acids) at the population level.

The process of molecular evolution consists of a sequence of events in which rare mutants increase their frequencies and spread through the species, finally reaching the state of fixation (100% in frequency). A great majority of such mutants, including those having a small selective advantage, are lost from the population by chance within a small number of generations. Only a tiny fraction can spread through the whole population to reach fixation, for which a very long time is required.

In the case of selectively neutral mutants (i.e., selectively equivalent alleles), it takes on the average $4N_e$ generations until fixation (Kimura and Ohta 1969), where N_e is the effective size of the population; N_e is roughly equal to the number of breeding individuals in one generation.

Let us now consider the sequence of events by which mutant genes are substituted one after another in the population (species) in the course of time. We denote by k_g the rate of mutant substitution per generation. Since each substitution may take a very long time, the rate should be measured as a long term average. The rate of substitution thus defined is independent of how quickly individual mutants spread into the population. What matters is the average interval between consecutive fixations.

Consider a gene locus (or a corresponding protein), and let v be the mutation rate per generation for a particular class of mutants (such as selectively neutral amino acid changes). Further, let u be the probability of ultimate fixation of an individual mutant. This is the probability that an individual mutant which appeared in the population eventually spreads in the population, reaching 100% in frequency.

In a population consisting of N diploid individuals, if we assume that each mutant is represented only once at the moment of appearance, the rate of evolution per generation is

$$k_g = 2Nvu. \tag{1}$$

This formula is based on the consideration that in a population of N diploid individuals, 2Nv new mutants appear per generation, of which the fraction u eventually spread through the whole population. (For a haploid population, 2N in the above formula should be replaced by N.) As mentioned already, this rate is in the sense of a long-term average; to measure the evolutionary rate we must take a much longer time than required for individual mutant substitution.

If the mutant is selectively neutral, the probability of ultimate fixation is equal to its initial frequency, that is, $u = 1/(2N)$ in the diploid population, and therefore, from Eq. (1), we have

$$k_g = v. \tag{2}$$

In other words, for neutral alleles, the rate of evolution is equal to the mutation rate.

The above formulation of the evolutionary rate at the molecular level is implicit in my first paper on the neutral theory (Kimura 1968), but it was presented more explicitly in later writings (see Crow and Kimura 1970; Kimura and Ohta 1971). Note that for a haploid population, there are N genes so that we have $k_g = Nvu$ and $u = 1/N$. Therefore, we again obtain $k_g = v$, the same result as the diploid case. Note also that Eq. (2) is independent of the population size, and it holds even when the population size fluctuates from time to time. Furthermore, it is independent of the mode of reproduction; it is equally applicable to sexual and asexual organisms (Kimura and Ohta 1971). The formula also implies that the rate of evolution should be independent of the environment where organisms are placed, as long as the mutation rate remains the same.

Let g be the generation span measured in years, and let k_1 be the evolutionary rate per year so that $k_1 = k_g/g$. Then, from Eq. (2), we obtain, for the selectively neutral case, the following formula for the evolutionary rate per year:

$$k_1 = v_0/g. \tag{3}$$

In this formula, v_0 is the mutation rate per gener-

ation for selectively neutral alleles. (Subscript 0 in v_0 denotes that it refers to neutral alleles.)

Since the neutral theory assumes that a certain fraction, say f_0, of the mutations are selectively neutral, while the rest (i.e., $1 - f_0$) are sufficiently deleterious to be eliminated from the population, the above formula (3) can also be expressed as follows:

$$k_1 = f_0(v_T/g), \tag{4}$$

where v_T is the total mutation rate per generation so that $v_0 = f_0 v_T$.

Then, the existence of the molecular evolutionary clock means, if the neutral theory is valid, that v_T/g remains the same (i.e., constant) among diverse lineages and over time for a given gene (or a protein). Here, we assume that f_0 represents the level of selective constraint, and that f_0 is constant for a given molecule or a part of one molecule: generally speaking, f_0 is smaller for functionally more important molecules which are subject to stronger selective constraint.

The situation is much more complicated if mutant substitutions are caused exclusively by positive Darwinian selection. Let v_A be the mutation rate per generation for advantageous mutants, and let s be the selective advantage of an individual mutant over the preexisting allele. If we assume that s is small but $4N_e s$ is large so that $0 < s \ll 1$ and $\exp(-4N_e s) \ll 1$ for any of the advantageous mutants, then we have approximately $u = 2s(N_e/N)$ (Kimura 1964). Substituting this in Eq. (1), we obtain

$$k_g = 4N_e s v_A. \tag{5}$$

If s varies from mutation to mutation, then the mean selective advantage $\bar{s}$ (>0) may be substituted for s in the above equation. Furthermore, if we denote by f_A the fraction of advantageous mutants, so that $v_A = f_A v_T$, the equation which corresponds to (4) becomes as follows:

$$k_1 = 4N_e \bar{s} f_A v_T/g. \tag{6}$$

This means that the rate of evolution under natural selection depends on various factors, namely, the effective population size (N_e), the fraction of advantageous mutants (f_A) and their average selective advantage ($\bar{s}$), as well as the total mutation rate per year (v_T/g). Then, in order to explain the constancy of the evolutionary rate among diverse lineages, we must assume that the product of all these parameters remains constant among lineages. However, it is likely that these parameters tend to differ from species to species. Particularly, f_A should depend strongly on the environment where the species is placed, being high for a species offered a new ecologic opportunity but low for those kept long in a stable environment. It seems to me that a highly complicated and arbitrary set of assumptions must be invoked in order to explain the clocklike behavior of molecular evolution from the standpoint of the selectionists. Merely assuming that the amount of selection is equal among lines by no means leads to the constancy (equality) of the evolutionary rate, contrary to the statement of some authors. In fact, adaptive evolutionary change (due to mutant substitutions for which $4N_e s \gg 1$) should be characterized by marked differences in its rate among lineages over time, as evidenced by evolution at the phenotypic level revealed by paleontological studies (see, for example, Simpson 1944).

On the other hand, if evolutionary change is largely controlled by mutation pressure and random drift, as claimed by the neutralists, the constancy of evolutionary rate can be explained by assuming constancy of mutation rate per amino acid (or nucleotide) site among diverse lineages. This assumption has to be checked by future experiments.

Some Statistical Analyses of Protein Evolution

The evolutionary rate per year (i.e., k_g/g) is sometimes found to be inconstant, undermining the clocklike progression of molecular evolution. From the standpoint of the neutral theory, this can be explained as being due to two causes.

One is the change in the mutation rate per year (i.e., v_T/g), and the other is the alteration of the selective constraint (f_0) of each molecule. The former (change of v_T/g) is likely to be brought about by the change of the generation span (g), while the latter (change of f_0) is caused by the alteration of internal molecular environment such as gene duplication, or through change of interacting molecules, details of which are mostly unknown at present.

In this section, I shall present a few examples suggesting that these two causes are really at work in mammalian evolution. Before I go into data analysis, I shall develop a simple statistical method which will be useful for our analysis.

Let us assume a phylogenetic tree in which several branches diverge from a common ancestor A, as shown in Fig. 1. We denote by L the number of branches or lines involved ($L \geqq 3$). We consider a particular molecule, such as the α chain of hemoglobin, and let n_{aa} be the total number of amino acid sites compared between two lines. For simplicity, we assume that n_{aa} is constant for all pairs of comparisons. We denote by D_{ij} the observed number of amino acid differences between the i-th and j-th lines ($i = 1, 2, \ldots, L$; $j = 1, 2, \ldots, L$). Let X_i be the number of amino acid substitutions which have accumulated in the i-th line since divergence from the

common ancestor A. These L parameters (X_1, X_2, ..., X_L) may be estimated from L(L − 1)/2 observed numbers of amino acid differences by using the least square method as follows.

Let

$$S = \sum_{i=1}^{L} \sum_{\substack{j=1 \\ (j \neq i)}}^{L} (\tilde{D}_{ij} - X_i - X_j)^2 \qquad (7)$$

be the sum of squares of errors in fitting the observed numbers ($\tilde{D}_{ij}$) by the corresponding linear estimates ($X_i + X_j$). Note that the observed numbers here refer not to the raw data (D_{ij}) but rather to the corrected values ($\tilde{D}_{ij}$) in which multiple substitutions and back mutational changes are taken into account. In this paper, I use the well-known formula:

$$\tilde{D}_{ij} = -n_{aa}\log_e(1 - D_{ij}/n_{aa}). \qquad (7a)$$

[Note: for DNA sequence data, a different correction should be used (see Kimura 1980, 1981; see also Kimura 1983).]

Then, we determine X_i's so that S is minimized. Differentiating Eq. (7) with respect to X_i's and setting each of the resulting equations equal to zero, i.e., $\partial S/\partial X_i = 0$ ($i = 1, 2, \ldots, L$), we obtain

$$\hat{X}_i = \frac{1}{L-2}\left[\tilde{D}_{i\cdot} - \frac{\tilde{D}_{\cdot\cdot}}{2(L-1)}\right], \qquad (8)$$

where

$$\tilde{D}_{i\cdot} = \sum_{j}{}' \tilde{D}_{ij} \qquad (8a)$$

and

$$\tilde{D}_{\cdot\cdot} = \sum_{i=1}^{L} \tilde{D}_{i\cdot} \qquad (8b)$$

In these expressions, the hat (^) on X_i means that this is the least square estimate and the primed summation in Eq. (8a) indicates that the sum is over all j values excluding j = i. Furthermore, we assume that $\tilde{D}_{ij} = \tilde{D}_{ji}$. The large-sample standard error σ_i for $\hat{X}_i$ is given by the formula,

$$\sigma_i = \frac{1}{L-2} \cdot \sqrt{\left[\left(\frac{L-2}{L-1}\right)V(D_i) + \frac{1}{4(L-1)^2}\sum_{i=1}^{L} V(D_i)\right]}, \qquad (9)$$

where

$$V(D_i) = n_{aa}\sum_{j}{}'\left(\frac{d_{ij}}{1-d_{ij}}\right), \qquad (9a)$$

and

$$d_{ij} = D_{ij}/n_{aa}. \qquad (9b)$$

Table 1. The observed number of amino acid differences between hemoglobin α-chains of six mammals: $n_{aa} = 141$, L = 6 (data from Dayhoff 1978)

	Mouse	Rabbit	Dog	Horse	Bovine
Human	18	25	23	18	17
Mouse		27	25	24	19
Rabbit			28	25	25
Dog				27	28
Horse					18

Table 2. The number of amino acid differences between hemoglobin β-chains of six mammals: $n_{aa} = 146$, L = 6 (data from Dayhoff 1978)

	Mouse	Rabbit	Dog	Horse	Bovine
Human	27	14	15	25	25
Mouse		28	30	36	39
Rabbit			21	25	30
Dog				30	28
Horse					30

The standard error here is the probable error caused by sampling a restricted number, namely n_{aa}, of amino acid sites.

An additional quantity which will be useful for investigating if the rates of evolution among lines are uniform is the statistic R. This is the ratio of the observed variance of X_i's to the theoretically expected variance under the assumption of uniformity, and if X_i's follow Poisson distribution, it can be shown that (L − 1)R follows the χ^2 (chi-square) distribution with L − 1 degrees of freedom. In terms of observed differences,

$$R = \frac{(L+1)V_D}{(L-1)\bar{D}}, \qquad (10)$$

where $\bar{D}$ and V_D are the mean and variance of $\tilde{D}_{ij}$ values (see p. 77 of Kimura 1983). Note that "the observed variance" here is not exactly identical to the variance of $\hat{X}_i$'s, since the latter does not contain the residual error variance (usually small) of least square estimation.

Let us now apply these methods to the analysis of actual data. Table 1 lists observed number of amino acid differences (D_{ij}'s) between six mammals (human, mouse, rabbit, dog, horse, and bovine) when their hemoglobin α-chains are compared. It is likely that these mammals diverged from each other late in the Mesozoic about 80 million years ago.

Noting that $n_{aa} = 141$ and L = 6, and applying Eq. (10) we obtain R = 1.26. With this R value, $\chi^2 = (L-1)R = 6.3$, and from a table of the χ^2 distribution with five degrees of freedom, we find

that deviation of R from unity is not statistically significant. So we cannot reject the hypothesis that the evolutionary rates among six mammals are the same with respect to α-hemoglobin. The estimated numbers of substitutions for these six lines are as follows:

Human: $\hat{X}_1 = 8.3 \pm 2.7$
Mouse: $\hat{X}_2 = 11.9 \pm 2.9$
Rabbit: $\hat{X}_3 = 16.9 \pm 3.1$
Dog: $\hat{X}_4 = 17.2 \pm 3.1$
Horse: $\hat{X}_5 = 11.6 \pm 2.8$
Bovine: $\hat{X}_6 = 10.1 \pm 2.8$

A similar analysis can be made for hemoglobin β with respect to the same set of animals, using data as listed in Table 2. In this case, noting $n_{aa} = 146$ and $L = 6$, we obtain $R = 3.1$. This gives $\chi^2 = 15.5$, and with five degrees of freedom, we find from a table of χ^2 that the deviation of R from unity is significant at about the 1% level. The number of amino acid substitutions for the six lines as estimated by using Eq. (8) turns out to be as follows:

Human: $\hat{X}_1 = 6.4 \pm 2.8$
Mouse: $\hat{X}_2 = 22.9 \pm 3.6$
Rabbit: $\hat{X}_3 = 9.9 \pm 3.0$
Dog: $\hat{X}_4 = 11.8 \pm 3.0$
Horse: $\hat{X}_5 = 18.4 \pm 3.7$
Bovine: $\hat{X}_6 = 20.3 \pm 3.5$

By inspection of these values, we immediately note that the human line accumulated much fewer amino acid substitutions, and the mouse line many more substitutions, as compared with the average mammals (i.e., about 15.0). If we remove these two extreme lines, and test the uniformity of the number of substitutions among the remaining four lines ($L = 4$), we obtain $R = 1.1$, a value expected under the assumption of uniformity. On the other hand, if we test the uniformity for a group consisting of mouse, human, and dog ($L = 3$), we obtain $R = 6.6$. These results are consistent with recent reports showing that the evolutionary rate in rodents is higher than the average mammal, while the rate of the human line is lower (Kikuno et al. 1985; Wu and Li 1985).

The difference in the results with respect to human and mouse lines for hemoglobulin α and β chains is likely to be due to statistical fluctuations, since the mutation rates and the levels of selective constraint appear to be the same for the α and β chains of these mammals. Therefore, it is desirable to repeat the statistical analysis after combining the data on the α and β chains, i.e., for $n_{aa} = 141 + 146 = 287$ and $L = 6$. This gives the following results:

Human: $\hat{X}_1 = 14.6 \pm 3.8$
Mouse: $\hat{X}_2 = 34.5 \pm 4.5$
Rabbit: $\hat{X}_3 = 26.9 \pm 4.3$
Dog: $\hat{X}_4 = 29.1 \pm 4.3$
Horse: $\hat{X}_5 = 30.0 \pm 4.4$
Bovine: $\hat{X}_6 = 30.2 \pm 4.4$

The R value turns out to be 1.9, giving $\chi^2 = 9.7$. With five degrees of freedom, the deviation of R from unity is significant at about the 5% level. In particular, it is clear that the human line definitely accumulated many fewer mutations than the mouse line.

The most likely cause of such differences in the evolutionary rate is the "generation time effect": If the mutation rates among organisms having widely different generation spans are roughly equal to each other when measured taking one generation as the unit, the mutation rate should be much higher, when measured per year, for an organism having a shorter generation span (and vice versa). Based on the neutralist principle that the rate of evolution is equal to the mutation rate for neutral alleles, the evolutionary rate of amino acid (or nucleotide) substitutions per year must be much higher in rodent than in man.

The intrinsic mutation rate for neutral alleles may also change in some lines due to change (usually loss) of selective constraint. One of the best examples suggesting this is the exceptionally rapid evolutionary change of insulin observed in the guinea pig (*Cavia*) and also in its relative the coypu or nutria (*Myocastor*). (These animals belong to the group of hystricognath rodents.)

Table 3 lists the number of amino acid substitutions among seven mammals including guinea pig and coypu. First, if we exclude the coypu, which is more closely related to the guinea pig than to the other five mammals (human, elephant, sheep, sperm whale, and rabbit), and apply the above statistical method, that is, Eqs. 8, 9, and 10 (noting $n_{aa} = 51$ and $L = 6$), we obtain

Human: $\hat{X}_1 = 1.2 \pm 1.7$
Elephant: $\hat{X}_2 = 1.1 \pm 1.6$
Sheep: $\hat{X}_3 = 1.7 \pm 1.7$
Sperm whale: $\hat{X}_4 = 1.7 \pm 1.7$
Rabbit: $\hat{X}_5 = 1.5 \pm 1.7$
Guinea pig: $\hat{X}_6 = 20.5 \pm 3.1$

and

$$R = 13.2.$$

This very high value of R clearly comes from the fact that the guinea pig line accumulated more than 10 times as many amino acid substitutions as the rest of the lines since divergence from the common ancestor. The R value becomes even higher if we

Table 3. The number of amino acid differences between insulin (A and B peptides) of seven mammals (data from Dayhoff 1978)

	Elephant	Sheep	Sperm whale	Rabbit	Guinea pig	Coypu
Human	2	4	3	1	18	18
Elephant		2	4	3	17	19
Sheep			2	4	18	20
Sperm whale				3	18	20
Rabbit					18	18
Guinea pig						15

Excluding insertions and deletions, 51 amino acid sites are compared ($n_{aa} = 51$)

select three lines, namely, human, elephant, and coypu lines. For this set of comparisons (L = 3), we obtain R = 18.4.

On the other hand, if we exclude from Table 3, guinea pig and coypu, and test the uniformity of substitution rates among the remaining five lines (human, elephant, sheep, sperm whale, and rabbit), we get R = 0.62, indicating strong uniformity. The average number of substitutions for these five lines turns out to be $\bar{X} = 1.44$.

From the data given in Table 3, we can also estimate the numbers of amino acid substitutions in various branches including guinea pig and coypu as illustrated in Fig. 2. It is interesting to note that the rates of amino acid substitutions in the two lines, one leading to guinea pig and the other leading to coypu from their nearest common ancestor B, are roughly equal to each other even if both have very high rates. Assuming that the ancestor A goes back to 80 million (8×10^7) years ago, the average rate of amino acid substitutions in the mammalian line, excluding guinea pig and coypu, turns out to be $1.44/(51 \times 8 \times 10^7)$ or 0.35×10^{-9}/aa/year (per amino acid site per year). On the other hand, the average rate for these hystricognath rodents since their descent from the ancestor A is 5.2×10^{-9}/aa/year. From the standpoint of the neutral theory, a possible explanation, first proposed by Kimura and Ohta (1974), is that the insulin of these hystricognath rodents lost its original selective constraint at an early stage of evolution so that most amino acid changes in that molecule became neutral, i.e., nonharmful (see also Jukes 1979). This is consistent with the observation that in the guinea pig, zinc is absent from the insulin-producing cells, coinciding with the loss of the usually invariant histidine B10 (for more detailed discussion and relevant references, see p. 114 of Kimura 1983).

It is concluded that due to loss of selective constraint, as evidenced by a drastic change in the molecular environment, mutations started to accumulate in guinea pig and coypu insulins at a very high rate. In this connection, investigation of the

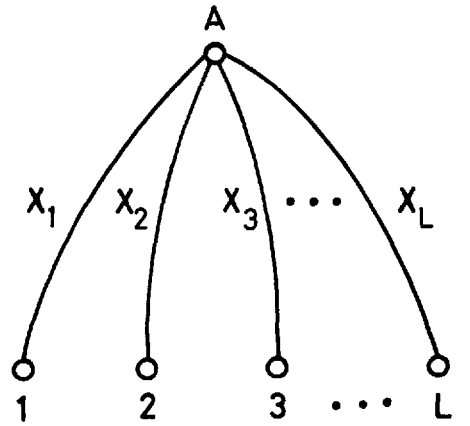

Fig. 1. A phylogenetic tree in which L lineages ($L \geq 3$) diverge from their common ancestor A. Letters X_1, X_2, etc. denote the number of amino acid substitutions accumulated in the individual lineages

evolutionary rates of the middle segment (C peptide) of proinsulin in mammals, including guinea pig, is of interest. The C peptide is the part which is removed when the active insulin (A and B peptides) is formed from its precursor, proinsulin. It is known that the C peptide generally has a much higher rate of amino acid substitution in evolution than insulin.

In Table 4, the fraction of amino acid differences, i.e., D_{ij}/n_{aa} (D_{ij} = the observed number of differences; n_{aa} = the number of amino acid sites compared), is listed for 10 comparisons involving five mammals (guinea pig, rat, human, horse, and bovine). The C peptide consists of about 30 amino acid sites, but insertions or deletions are found in several comparisons, so I excluded these changes and counted only the number of amino acid substitutions. This means that n_{aa} may differ from comparison to comparison.

From these data, the number of amino acid substitutions *per sites*, namely $\hat{X}_i/n_{aa}$, can be estimated, and the following estimates have been obtained:

Guinea pig:	0.26 ± 0.11
Rat:	0.25 ± 0.10
Human:	0.13 ± 0.09
Horse:	0.16 ± 0.09
Bovine:	0.24 ± 0.11

These values show that the evolutionary rate of the guinea pig is not significantly higher than other mammals with respect to the C peptide of proinsulin. In fact, R = 0.66, obtained from this set of comparisons, strongly suggests that the rates of evolution of the C peptide are uniform among these five animals. The evolutionary rate per amino acid site per year turns out to be $k_{aa} = 3.3 \times 10^{-9}$ for guinea pig, and the average rate for these five animals is $\bar{k}_{aa} = 2.6 \times 10^{-9}$ (assuming 80 million years of evolution since the common ancestor).

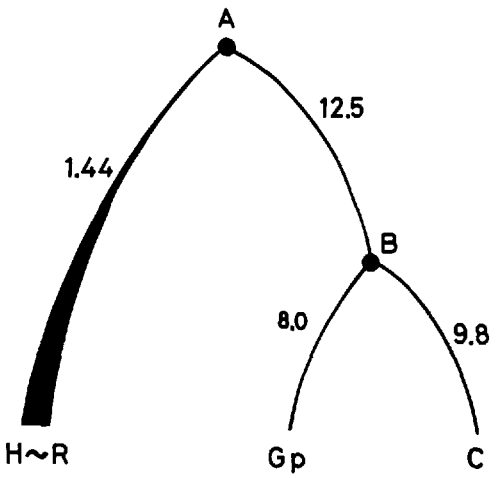

Fig. 2. A phylogenetic tree and the numbers of amino acid substitutions in various branches in the evolution of insulin of mammals including guinea pig (Gp) and coypu (C). In this figure, H ~ R denotes a group of five mammals consisting of human, elephant, sheep, sperm whale, and rabbit. The number of amino acid sites compared: $n_{aa} = 51$.

It is interesting to note that the remarkably high evolutionary rate, $k_{aa} = 5.2 \times 10^{-9}$, as found for insulin (A and B peptides) of guinea pig is rather near to $k_{aa} = 3.3 \times 10^{-9}$ obtained for the proinsulin C peptide of this animal. Note also that 5.2×10^{-9} is considerably lower than the corresponding amino acid substitution rate for pseudoglobin genes which is roughly, 10^{-8} or slightly higher (Li et al. 1981; Miyata and Yasunaga 1981). This means that insulin of guinea pig (and coypu) has not been totally liberated from selective constraint.

Using the numbers of amino acid substitutions given in Fig. 2, and assuming the uniformly high evolutionary rate of insulin in the hystricognath rodents since their descent from the common ancestor A (which we assume to be 80 million years ago), we can estimate the divergence point B for the guinea pig and the coypu. This turns out to be 33.3 million years ago. According to Romer (1968), the guinea pig group suddenly appeared in South America in the Oligocene (25–40 million years ago), so the above estimate is consistent with paleontological observations.

Discussion

As we have seen in the previous section, the generation time effect is one of the possible and (probably important) causes which make the molecular evolutionary clock less exact, reducing its foolproof reliability. However, the magnitude of this effect does not appear to be very large even among organisms whose generation spans are very different. In fact, in their attempt to test whether the generation span has any effect on the molecular evolutionary rate per year, Wilson et al. (1977) could not detect any such effect.

Table 4. The fraction (D_{ij}/n_{aa}) of amino acid differences between proinsulin C peptide of five mammals including guinea pig

	Rat	Human	Horse	Bovine
Guinea pig	11/29	9/29	10/29	11/26
Rat		10/31	12/31	9/26
Human			7/31	9/26
Horse				8/26

This table was constructed using data compiled in Dayhoff (1978)

However, recent works by Wu and Li (1985) and Kikuno et al. (1985) show that rodents (having a short generation span) evolve faster than human beings (which have a long generation span), particularly with respect to silent sites, suggesting a generation time effect. Also, Britten (1986) reports, based mainly on DNA–DNA hybridization data, that the evolutionary rate of DNA divergence of rodents is some five times higher than that of higher primates. He suggests that changes in the DNA repair mechanism are the primary cause of such difference. However, the possibility cannot be excluded, I think, that this represents the generation time effect.

Moreover, Sibley and his associates (Catzeflis et al. 1987) carried out DNA–DNA hybridization studies of some muroid rodents, and they came to the conclusion that the average evolutionary rate in these rodents is roughly 10 times as high as that of the hominoid primates. Note that even this much difference is not as large as the actual difference of the generation spans of these two animal groups; they must differ by a factor of at least 40 in the actual generation length.

Sibley and his associates have also discovered the generation time effect in birds (Sibley, personal communication). They found that birds that have longer generation times, i.e., whose first breeding age is higher (such as albatrosses that take 6–9 years until sexual maturity), tend to show less DNA divergence than those that mature more rapidly.

If the generation time effect is really important, particularly when organisms having widely different generation spans are compared, this means that, from the standpoint of the neutral theory, the nucleotide mutation rate is not quite constant per year but is higher for organisms having a shorter generation span (and vice versa). In other words, the number of mutational changes of nucleotides is dependent (albeit mildly) on the number of generations. Experimental studies bearing on this problem are much needed.

The second agent that interferes with the constancy of the evolutionary rate is an alteration of functional constraint of the molecule [leading to

change in f_0 in Eq. (4)]. This is exemplified by the guinea pig and coypu insulins (see previous section). This is probably the most dramatic example known on this topic. Another possible example is the rapid evolution of the opossum hemoglobin α studied by Stenzel (1974). In this case, the estimated rate of amino acid substitution is 1.7×10^{-9} per year, definitely higher than the standard value (1.2×10^{-9}) of this molecule in mammals. A change of selective constraint is suggested by the fact that in the opossum hemoglobin α, *Gln* occurs at position 58. This is the position which is almost invariably occupied by *His* (bound to heme) in the α hemoglobin of vertebrates. Note that the myoglobin sequence of the opossum is also known, but for this molecule, there is nothing unusual in its sequence (see p. 82 of Kimura 1983). Although we do not know at present what agents initiated such changes (mostly reduction) of functional constraint, it is possible that gene duplication is often responsible.

There may be other factors which also interfere with the accuracy of the molecular evolutionary clock, such as: (i) fixation of very slightly deleterious mutants during population bottlenecks (Ohta 1973, 1974, 1976), (ii) participation of compensatory neutral mutations in molecular evolution (Kimura 1985a,b), and (iii) fluctuation of the neutral space in the course of mutant substitutions (Kimura and Takahata, manuscript in preparation). Whether these factors are responsible for making the molecular clock less accurate to a significant extent remains to be seen.

Ohta and Kimura (1971) were probably the first to investigate statistically the variation of evolutionary rates among lines for a few molecules. They used the *F*-test to see if the observed variance is significantly higher than the variance expected by pure chance, and found that the *F* value is highly significant in β-type hemoglobins and cytochrome c, but not in hemoglobin α. For example, in cytochrome c, the ratio of the observed to expected variances turned out to be about 3.3.

This is consistent with the report of Jukes and Holmquist (1972) that rattlesnake cytochrome c has evolved three or four times as rapidly as turtle cytochrome c. In this case, however, the factor or factors that are responsible for such differences in evolutionary rates among lines is not clear.

It is often stated that, when the variance of evolutionary rates among lineages for a given protein (or a DNA region) is found to be significantly higher than the variance expected by pure chance, or when the estimated rates in different parts of a phylogenetic tree differ significantly, such an observation disproves the neutral theory.

Ayala (1986) analyzed the evolutionary rates of copper–zinc superoxide dismutase (SOD) among eight organisms, and he discovered that the estimated rates become progressively lower as more and more remote comparisons are made. This molecule (SOD) consists of about 150 amino acids, of which 55 are common to all sequences. Let us suppose that these 55 amino acids represent the "invariant sites," and fix our attention on the variable sites consisting of about 100 amino acids. If we use Ayala's (1986) Table IV, which lists the number of amino acid differences, and apply the method of the previous section to estimate the number of amino acid replacements accumulated in four mammalian lines (human, rat, horse, and cow) in the course of evolution from the common ancestor, we obtain the following estimates:

Human:	16.4 ± 5.2
Rat:	13.0 ± 5.0
Horse:	19.2 ± 5.4
Cow:	13.0 ± 4.9

We also obtian $R = 0.59$, suggesting a strong uniformity of the evolution of SOD among mammalian lineages. The average rate of evolution is $k_{aa} = 1.9 \times 10^{-9}$/aa/year, which is more than six times as high as the corresponding rate of cytochrome c (which Ayala mentions as a protein that exhibits more regular behavior over the whole geological span considered). Assuming this high rate, the number of amino acid substitutions that separate mammals and *Drosophila* should be about 2.3 per variable site. On the other hand, the corresponding observed number is about 0.6, and, even after corrections are made for hidden substitutions using Eq. (7a), the resulting figure is 0.92, which is only 40% of the value expected under the assumption of rate constancy. The discrepancy between the expected and observed values becomes much higher when we make a more remote comparison, such as human and yeast: the expected number of amino acid substitutions per site is 4.6, while the observed number (with a Poisson correction) is 1.2, which is only 26% of the expected value assuming rate constancy.

There are two explanations for such discrepancies. The first is that the evolutionary rate has progressively increased along various lineages since their descent from the common ancestor that lived 1.2 billion years ago. The second explanation, which I think more plausible, is that the observed diminution of the rate in the backward direction is simply an artifact caused by the fact that it becomes progressively difficult to recover hidden substitutions by ordinary statistical means as more and more amino acid substitutions accumulate between the two sequences compared. This may be called the "apparent saturation effect." A similar case was reported by Perler et al. (1980) with respect to nu-

cleotide changes in silent sites in the evolution of preproinsulin gene.

Based on his analysis of SOD data, Ayala (1986) claims that the observed variance in evolutionary rate is much too large and thus inconsistent with the neutral theory. I do not think, however, that his claim is warranted unless the neutral mutation rate per year is shown to be equal among lineages over time and at the same time the molecular evolution of SOD has really accelerated.

Similarly, Gillespie (1984, 1986) criticizes the neutral theory on the ground that the observed value of R [such as computed by Eq. (10)] often turns out to be significantly higher than unity. For example, Ohta and Kimura (1971) analyzed three sets of data involving the α- and β-type hemoglobins and cytochrome c, and found that the observed variance is about three times higher on the average than expected by pure chance. Also, Langley and Fitch (1974) made a similar but more extensive analysis, and obtained results suggesting that the observed variance among lines is about 2.5 times as large as that expected from chance fluctuations.

Gillespie claims that such observations are incompatible with the neutral theory and that a model of evolution by natural selection, which he calls "episodic model," can fit the data better. His model is based on the idea that molecular evolution is episodic, with short bursts of rapid substitutions 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 natural selection.

More specifically, he considers a phylogeny as shown in Fig. 1 (which he calls a "star phylogeny"), and assumes that the number of episodes per lineage follows a Poisson distribution, and that the number of substitutions per episode also follows a certain probability distribution which is the same for all episodes in all lineages. He then states that this model has the remarkable property of predicting that the values of R are in a very restricted range, say from 1.0 to ~3.5, just as observed in the data.

I think it highly unrealistic to assume that the numbers of episodes in different lineages (which must experience different environments) follow the same probability distribution. Also, it is a moot point why natural selection acts in such a way that the number of substitutions per episode follows the same probability distribution for all episodes in all lineages. Here, natural selection is invoked arbitrarily to fit the results, while neglecting all the effects of the mutation rate, population size, selection coefficients, etc., as shown by Eq. (6). If, in reality, the relatively large variation (i.e., $R > 1$) in the number of amino acid substitutions among lineages is caused by some of the lineages having different intrinsic evolutionary rates, as discussed in the previous section, Gillespie's model breaks down completely. (For $R < 1$, his theory does not work anyway.)

From the standpoint of the neutral theory, the clocklike progression of molecular evolution can be explained by assuming that the rate of production of neutral mutations per year is nearly constant among related organisms for a given molecule over time. This means that, if the neutral theory is valid, the rate of evolution at the molecular level is directly proportional to the mutation rate. A dramatic example showing this is a very rapid evolutionary change observed in RNA viruses, which are known to have very high mutation rates: RNA viruses show evolutionary rates roughly a million times as high per year as that of DNA organisms.

Saitou and Nei (1986), who investigated the evolution and polymorphism of influenza A virus genes, found that the rate of nucleotide substitution is of the order of 10^{-3}/site/year for most genes studied. Since the mutation rate is estimated to be 0.01/site/year, and since this is much higher than the average substitution rate, the authors conclude that most influenza genes are subject to negative selection (i.e., $f_0 \ll 1$). An extremely high substitution rate and the clocklike progression of substitutions in this virus were also reported by Hayashida et al. (1985).

Similarly, Gojobori and Yokoyama (1985) estimated the evolutionary rates of both a retroviral oncogene (v-*mos* gene) and its cellular homolog and found that the former evolves nearly 0.8 million times faster than the latter. They pointed out that the rapid evolution of the former is caused by the correspondingly high mutation rate which is due to a lack of the proofreading enzymes that ensure accurate replication, coupled with the very high replication rates.

What is really remarkable in the evolution of these two types of RNA viruses is that not only do the nucleotide substitutions occur at extraordinarily high rates in clocklike fashion, but also synonymous substitutions (that do not cause amino acid changes) predominate over amino acid altering substitutions, indicating the typical pattern of neutral evolution.

Finally, for those readers who want to know more about the neutral theory of molecular evolution, I would recommend recent papers by Kimura (1986) and Jukes and Kimura (1984), and also, my book (Kimura 1983) on the subject, and the symposium volume by Ohta and Aoki (1985), which contains a number of relevant articles.

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