Temporal Scaling of Molecular Evolution in Primates and Other Mammals[1,2]

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Molecular clocks are routinely tested for linearity using a relative rate test and routinely calibrated against the geological time scale using a single or average paleontologically determined time of divergence between living taxa. The relative rate test is a test of parallel rate equality, not a test of rate constancy. Temporal scaling provides a test of rates, where scaling coefficients of 1.0 (isochrony) represent stochastic rate constancy. The fossil record of primates and other mammals is now known in sufficient detail to provide several independent divergence times for major taxonomic groups. Molecular difference should scale negatively or isochronically (scaling coefficients < 1.0) with divergence time: where two or more divergence times are available, molecular difference appears to scale positively (scaling coefficient > 1.0). A minimum of four divergence times are required for adequate statistical power in testing the linear model: scaling is significantly nonlinear and positive in six of 11 published investigations meeting this criterion. All groups studied show some slowdown in rates of molecular change over Cenozoic time. The break from constant or increasing rates during the Mesozoic to decreasing rates during the Cenozoic appears to coincide with extraordinary diversification of placental mammals at the beginning of this era. High rates of selectively neutral molecular change may be concentrated in such discrete events of evolutionary diversification.

Introduction

Proteins and nucleic acids contain a wealth of information on the relationships of living plants and animals, complementing morphology in providing a full spectrum of knowledge of our extant flora and fauna. Fossils provide a more limited perspective on the form of extinct plants and animals. Fossils have value in expanding the known morphological diversity of life, but this is not their primary importance. Fossils are important because they provide, in outline if not detail, the independent historical framework required to make comparative morphology truly evolutionary. Similarly, fossils provide the historical background and temporal framework necessary for evolutionary interpretation in molecular biology.

Zuckerkandl and Pauling (1962, 1965) were among the first to relate molecular differences between living organisms to geological time. They noted that α-hemoglobin chains of humans and horses differ by 18 amino acid residues (Zuckerkandl and Pauling 1962). On the basis of radiometric dating of the fossil record as it was then known, humans and horses were estimated to have diverged ~100–160 Myr ago.

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Consequently it was reasonable to assume that each chain of α-hemoglobin averaged approximately nine evolutionarily effective changes/100–160 Myr ago, a rate equivalent to one amino acid replacement every 11–18 Myr ago. As befits a first attempt with limited data, Zuckerkandl and Pauling assumed average rates to be representative. Given two points and no additional information, a conservative assumption of linearity of intermediate values is axiomatic in all rate calculations.

Zuckerkandl and Pauling (1965) observed that there is a poor association between (1) number of amino acids inferred to have been replaced during the evolution of a given polypeptide chain and (2) functional change in the chain. Many replacements lead to relatively little functional change, whereas in other cases replacement of a single amino acid may lead to radical functional change. Consequently, it appears that functional change depends more on type than on number of amino acid replacements. Zuckerkandl and Pauling proposed that amino acid replacements proportional in number to elapsed evolutionary time cannot be ascribed to vital adaptive change. Amino acid replacements (or nucleotide substitutions) that occur at a constant rate are those that have little or no effect on the functional properties of a molecule. In other words, if most molecular change occurs at a constant rate, then we can assume that most molecular change is selectively neutral. This is the essence of the neutralist hypothesis of molecular evolution.

Zuckerkandl and Pauling's (1965, p. 148) quantitative model outlining the possible relationship of hemoglobin differences to geological time is illustrated graphically in figure 1. It incorporates a stochastic Poisson process to characterize observable change (n or d) but assumes from the beginning that total underlying change (m or d') is a linear function of time. The model is powerful in that a single point of evidence calibrating sequence difference in one pair of hemoglobins against geological time yields predicted times of divergence for all other hemoglobin pairs based solely on their sequence differences. For example, the model predicts that α-hemoglobin diverged from β- and γ-hemoglobin ~375 Myr ago, that β- and γ-hemoglobin diverged ~150 Myr ago, and that β- and δ-hemoglobin diverged ~25 Myr ago. In addition, times of divergence can be predicted for all organisms of known hemoglobin sequence.

A single divergence time for living taxa of known molecular difference is sufficient to calibrate the Zuckerkandl-Pauling model, but this datum by itself is not sufficient to test the appropriateness of a linear representation of molecular change over geological time. If we are willing to accept, with Zuckerkandl and Pauling, one point of calibration to the geological time scale based on a paleontologically constrained divergence of living taxa, then two, three, or four independent points of calibration should be equally acceptable. Multiple calibration points are required in inferring patterns of molecular change over geological time.

My point in reviewing the Zuckerkandl-Pauling model is to show how critically the neutralist hypothesis of molecular evolution depends on the idea of rate constancy—and to show that Zuckerkandl and Pauling assumed constant rates from the beginning. What evidence indicates that molecular change occurs at stochastically uniform rates over long periods of evolutionary time?

Notation, Analytical Methods, and Assumptions

Molecular difference d (or n following Zuckerkandl and Pauling 1965) can be quantified using whole-protein immunology (d_I), counts of amino acid difference (d_A), nucleic acid or DNA hybridization (d_D), and counts of nucleotide difference (d_N).

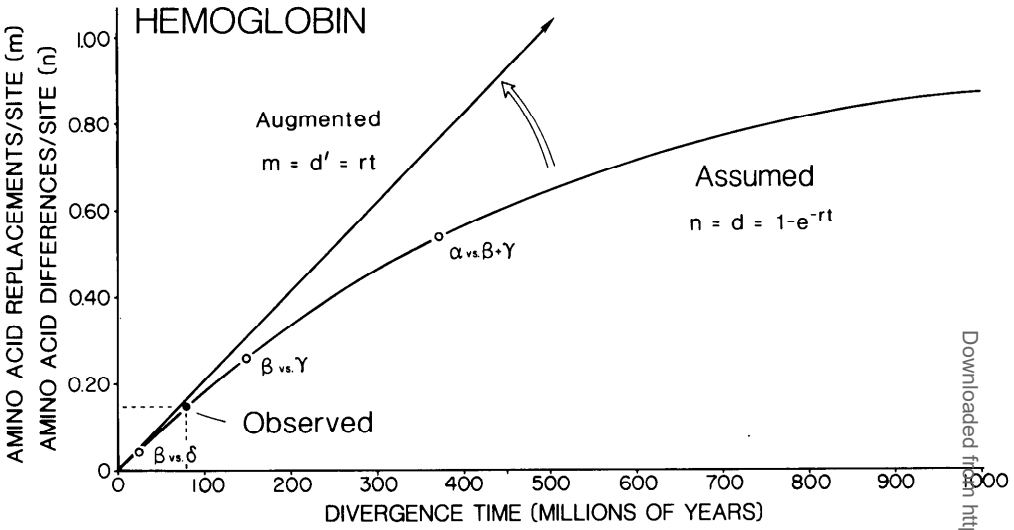


FIG. 1.—Zuckerkandl-Pauling model of amino acid sequence difference in human and other mammalian hemoglobin expressed as a function of geological time. Two forms of the model are shown here. The original expression describes observable sequence difference, n or d, as a concave-downward exponential curve asymptotic to 1.00 differences/site (cf. fig. 3 in Zuckerkandl and Pauling 1965); augmented linear expression describes amino acid replacements per site, m or d', as a function of divergence time, t, correcting for undetected replacements (Dickerson 1971). Direction of augmentation is shown by open arrow. The augmented sequence difference may exceed 1.00 replacements/site. Both expressions of the Zuckerkandl-Pauling model *assume* a constant rate of underlying amino acid replacement. The rate constant r = 0.00203 amino acid replacements/site/Myr is determined by substituting coordinates of one calibration point representing 0.15 differences/site (15%) and an 80-Myr time of divergence for humans versus nonprimate mammals. Sequence differences observed for α- vs. β- and γ-, β- vs. γ-, and β- vs. δ-hemoglobin chains (circles) yield predicted times for their evolutionary divergences. The average rate of change of molecular difference over the associated divergence interval is the slope of a tie line connecting any given point with the origin (instantaneous rate is given by the slope of the curve itself). Although a single observation (or the average for a group of observations) is sufficient to calibrate this model, multiple independent observations are required to test it.

These observed differences are routinely augmented in various ways to account for undetected replacements and substitutions, thereby yielding distance measures d' (or m following Dickerson 1971)—e.g., immunological distance d'_I, amino acid replacement d'_A, nucleic acid distance d'_D, and nucleotide substitution d'_N—corresponding to the differences listed above. The most common augmentation (including that of Zuckerkandl and Pauling and shown in fig. 1) portrays observed difference d as an exponential function of time, $d = 1 - e^{-rt}$, and distance d' as a linear function, $d' = rt$. Here r is a rate constant equal or proportional to the number of replacements or substitutions per site per unit divergence time t (in Myr). Variations of this Poisson process underlie most augmentation of observed difference d to yield molecular distance d'. Methods of augmentation are of less concern here than interpretation of augmented distances (d') as a linear or nonlinear function of time.

Temporal scaling refers to change in a dependent variable (e.g., d') over time. If we adapt the allometric power function widely used in morphological studies, molecular distance d' can be modeled as a simple function of divergence time: $d' = at^b$. A power function (as opposed to an exponential function) is appropriate here because it includes linearity as a special case (there are values, specifically $b = 1$, for which the first

derivative is a nonzero constant). Temporal scaling of molecular distance is *isochronic* when the exponent or scaling coefficient $b = 1.0$. This is the linear model of molecular evolution of Zuckerkandl and Pauling (1965) and later workers. Temporal scaling is *allochronic* when $b \neq 1.0$. By convention $b > 1.0$ is considered to represent *positive* scaling and $b < 1.0$ is considered to represent *negative* scaling. Logarithmic transformation of a power function yields a linear equation, $\log d' = \log a + b \log t$, facilitating solution for a and b. Log a and scaling coefficient b correspond to the constant and slope coefficient, respectively, in least-squares regression of dependent variable log d' on independent variable log t.

The null hypothesis of isochrony or stochastic constancy of molecular change over time—that is, the hypothesis that scaling coefficient $b = 1.0$—can be tested by calculating and comparing values of b based on empirical observation. Confidence limits for each empirically determined b are exactly equivalent to critical values necessary to test the null hypothesis: the null hypothesis is rejected when and only when 1.0 lies outside a 95% confidence interval for empirical b. Confidence intervals given here are calculated as estimated b plus or minus the product of its SE multiplied by critical values of a two-tailed Student's t-distribution for $\alpha = 0.05$ and $N^* - 2$ degrees of freedom (Sokal and Rohlf 1969, p. 435). N^* represents the total number of different values of the independent variable, as distinguished from total sample size N. Use of t for $\alpha = 0.05$ and $N^* - 2$ degrees of freedom makes the confidence intervals appropriately conservative in testing the hypothesis that $b = 1$.

Although a power function provides an explicit test of linearity, the behavior of power functions at the origin (first derivative or slope of zero) makes them inappropriate as models relating molecular distance to time. An exponential model (e.g., $d' = a[1 - e^{-rt}]$) is a better choice, and exponential models are likely to provide better interpolated prediction of unknown times of divergence based on molecular distances. Exponential models cannot be used to test for linearity because their first derivative is never a nonzero constant.

None of the analyses presented here includes error estimates for individual d or d' values; nor do any include error estimates for individual t values. Both variables, d or d' and t, are subject to complex observational and analytical error that is difficult to estimate and rarely reported. As in any study, incorporation of additional error would increase the uncertainty of all conclusions.

The known fossil record of mammals is assumed here to represent, in broad outline, the history of mammalian evolution. This outline is tested constantly, and future discoveries may alter it significantly; however, paleontological (and molecular) discoveries in recent decades have all been ones of refinement rather than broad-scale revision. Paleontological divergences considered here all represent times of diversification of morphologically distinctive higher taxonomic groups (orders, suborders, infraorders, or superfamilies) with an abundant fossil record in subepochs of first appearance. Broad relationships and timing of critical divergences are generally better known for higher taxonomic groups of mammals than they are for shorter-lived families, genera, and species.

Extinct species and higher taxa recognized morphologically in the geological past are assumed to represent distinct molecular genetic entities, just as extant species and higher taxa recognized morphologically represent distinct genetic entities in the living record. Consequently, times of morphological differentiation in mammalian history are assumed to coincide broadly with times of molecular genetic differentiation. One could argue that the genetic diversity of higher taxa (e.g., modern mammalian orders)

has resided cryptically in one or a few closely similar species of a long-existing genus or family (say, Cretaceous *Gypsonictops* or Leptictidae) for long intervals of geological time, but there is no evidence to suggest or support this. Postulating such radical decoupling of morphology and diversity from genetics would have far-reaching consequences precluding calibration of molecular evolution against the geological/evolutionary time scale and diminishing the importance of molecular evidence for understanding organismal evolution. The following discussion has little meaning if molecular evolution takes place independently of morphological evolution.

Relative Rate Test, Temporal Scaling, and Rate Constancy

The most widely cited test of rate uniformity in molecular evolution is the relative rate test developed by Sarich and Wilson (1967), but this is a *relative* test that does not address the critical question of how molecular change takes place over real evolutionary time. Molecular differences, however measured, must pass the following three tests to satisfy the Zuckerkandl-Pauling clock hypothesis:

1. Reciprocity Test

For any pair of taxa A and B, the molecular difference of A from B must be the same as that of B from A. This test is a test of the internal symmetry and consistency of molecular data.

2. Relative Rate Test

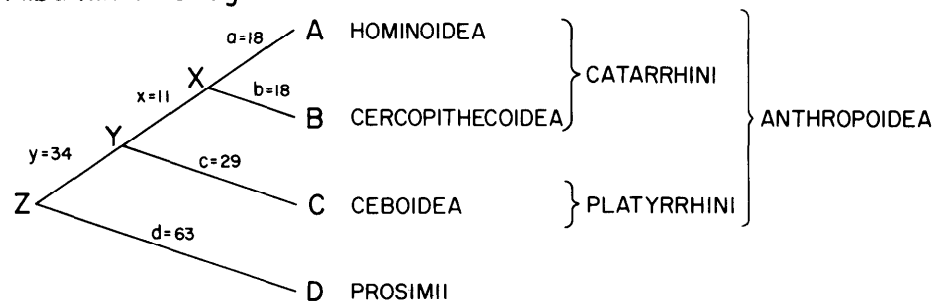
Taxa A and B must differ by the same amount from a more distantly related outgroup C. Expressed as a quotient of molecular difference and time, this test is a test of parallel rate equality. As shown in figure 2, the relative rate test is not a test of rate constancy. Slowing of molecular rates in all lineages of placental mammals subsequent to their divergence early in the Cenozoic would not be detected by this test.

3. Temporal-scaling Test

Molecular differences and independent historically or paleontologically determined times of divergence for species pairs A and A (origin or zero point), A and B, A and C, etc., must be mutually colinear (Gingerich 1985*a*). Given data that pass reciprocity and relative rate tests, these data must also pass a temporal-scaling test to demonstrate rate constancy or stochastic uniformity.

Fitch (1976) and Fitch and Langley (1976*a*, 1976*b*) were among the first to examine the colinearity of molecular differences and times of evolutionary divergence, showing that a straight line gives a reasonably good representation of the relationship between augmented nucleotide substitutions and divergence times in mammals (using divergence times provided by M. Goodman and L. Van Valen, personal communication). In another empirical study, serum albumin immunological distances calculated for selected pairs of mammalian carnivores and ungulates were compared with paleontologically constrained divergence times (fig. 3). Wilson et al. (1977) initially estimated the relationship between immunological distance (d'_I) and divergence time (t) in carnivores and ungulates to be $d'_I = 1.9t - 4$. Using the same data, Carlson et al. (1978) regressed divergence time on immunological distance, obtaining $t = 0.54d'_I$ (inverting, this implies that $d'_I = 1.85t$). A rigorous test of rate constancy was not attempted in either study.

As outlined above, linearity can be tested by fitting a power function to a set of observations and comparing an empirically determined exponent of the independent



Relative Rate Test:

$$\frac{a + x + c}{[t]} = \frac{b + x + c}{[t]} \Rightarrow \frac{a}{[t]} = \frac{b}{[t]} \quad \text{or} \quad a = b$$

$$\frac{(a+x) + y + d}{[t]} = \frac{c + y + d}{[t]} \Rightarrow \frac{a+x}{[t]} = \frac{b+x}{[t]} = \frac{c}{[t]} \quad \text{or} \quad a+x = b+x = c$$

FIG. 2.—The relative rate test as a test of parallel rate equality. The albumin phenogram illustrates the standard classification of primates used here. Living taxa A and B are descendants of hypothetical common ancestor X; living A, B, and C are descendants of hypothetical common ancestor Y; and living taxa A, B, C, and D are descendants of hypothetical common ancestor Z. The molecular difference between A and X, shown here in albumin immunological distance (d_i) units, is given by a (a = 18); distance between B and X is given by b (b = 18); distance between X and Y is given by x (x = 11), etc. All d_i values are taken from Sarich (1968, 1970). The relative rate test requires that molecular distances a + x + c = b + x + c and that (a + x) + y + d = (b + x) + y + d = c + y + d, as shown. Note that inclusion of time, t, in the denominator is unnecessary for this test and that substitution of any function of t, such as kt, t^k, or e^t would not change the result. In other words, the relative rate test is a test of parallel equality, not rate constancy. Discrepancies in relative rates may identify lineages evolving at unequal rates (precluding a single rate constant), but relative rates do not address the problem of how molecular differences or distances scale in real time.

variable with 1.00, the value necessary for linearity. In molecular evolutionary terms, the empirical exponent of divergence time (the temporal-scaling coefficient) is tested against the null hypothesis of linearity with a scaling coefficient of 1.00.

Fitch and Langley's (1976*a*) data, as presented, conform closely to linearity. The scaling coefficient for all 16 points is 1.03, with a 95% confidence interval ranging from 0.64 to 1.42. The breadth of this interval indicates that a wide range of nonlinear scaling models might also fit these data (the confidence interval is broad because the SE of the estimated scaling coefficient, 0.18, is large). Eliminating points 1–6 in Fitch and Langley's (1976*a*) data and their fig. 5 because they represent groups with poorly constrained divergence times and eliminating point 16 because it is outside the Cenozoic radiation of placental mammals, and then substituting divergence times of 25 Myr ago for points 7 and 8, 50 Myr ago for point 9, 55 Myr ago for points 10 and 11, and 65 Myr ago for points 12–15 (as justified below), we find that the scaling coefficient becomes 1.35 with a confidence interval ranging from 0.79 to 1.91. Linearity still cannot be ruled out, but there is some suggestion that nucleotide substitutions scale positively with divergence time (see Note added in proof).

Carlson et al.'s (1978) study yields an empirical exponent of immunological distance of 0.78 (the inverse would be 1.28), with a 95% confidence interval ranging from 0.64 to 0.92. Contrary to their claim, the relationship they present is significantly

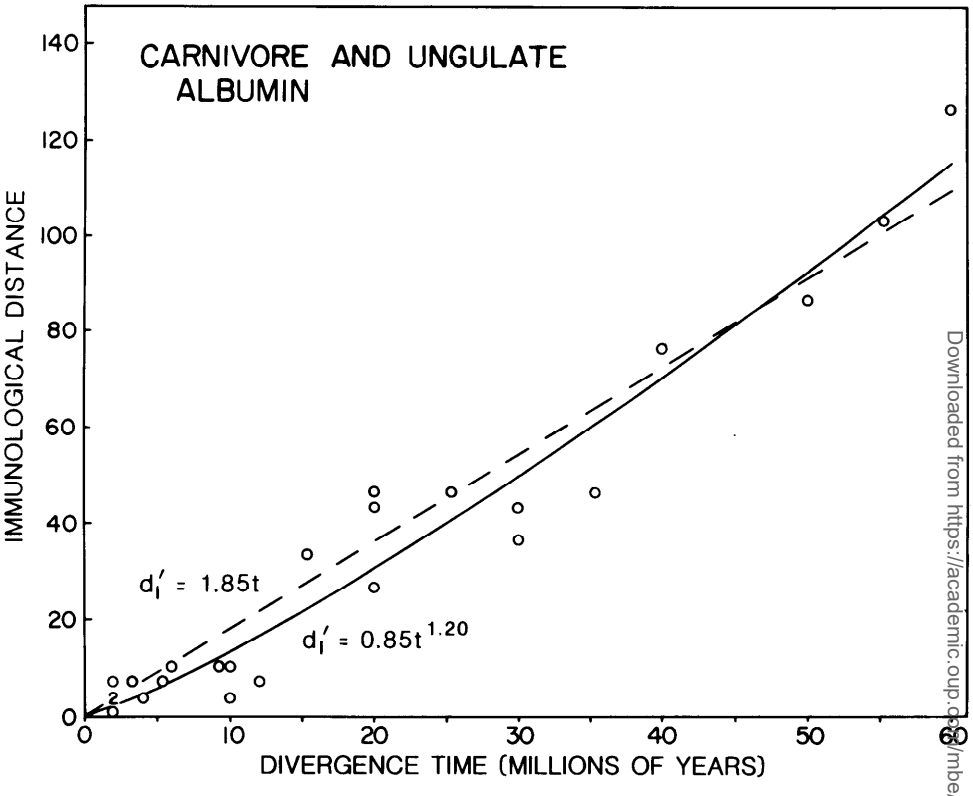


FIG. 3.—Temporal scaling of mammalian carnivore and ungulate albumin difference over Cenozoic time. The abscissa is divergence time (t); the ordinate is albumin difference in immunological distance (d_I') units. Data are from Wilson et al. (1977). A dashed line represents the linear null model of Carlson et al. (1978); the solid line represents the nonlinear fit to data based on the assumption that d_I' and t are both independent variables. The scaling coefficient of 1.20 is the slope of the principal axis of log d_I' and log t. The 95% confidence interval for this slope ranges from 1.01 to 1.44, excluding linearity (1.00). Regression of log d_I' on log t yields a scaling coefficient of 1.10 for d_I' as a function of t (also nonlinear, but not significantly so). The average rate of change at any point is given by the slope of a tie line to the origin (the instantaneous rate is given by the slope of the curve itself). Concave-upward curve implies deceleration of albumin evolution in Cenozoic carnivores and ungulates.

nonlinear. Expressing immunological distance as a function of divergence time, appropriate for temporal scaling, yields a scaling coefficient of 1.10 with a 95% confidence interval ranging from 0.91 to 1.29; and linearity, by this test, cannot be ruled out. Treating immunological distance and divergence time as independent variables, the principal axis of log d_I' versus log t has a slope of 1.20 with a 95% confidence interval of 1.01–1.44 (fig. 3). Both treatments of Fitch and Langley's mammal data and all three treatments of Wilson and Carlson's carnivore and ungulate data yield positive scaling of immunological distance and divergence time, although only two of the five treatments yield scaling coefficients that are statistically significantly greater than the 1.00 value required for linearity.

Temporal Scaling in Primate Evolution

Immunological distance (d_I') values for primate albumins published by Sarich (1968, 1970) have acceptable reciprocity, and, as shown in figure 2, they also pass the

relative rate test, demonstrating parallel rate equality. Primate immunological distances can be scaled against geological time using paleontologically constrained divergences. Primates have a reasonably dense and continuous fossil record that, by virtue of wide interest, is unusually well studied. The evolutionary history of primates, which is known in outline (e.g., Gingerich 1984), constrains divergence times of major groups within the order. The following four divergence times are sufficiently well established on the basis of fossils to be considered here:

1. Divergence of Primates from other orders of placental mammals as part of the Cretaceous-Tertiary (Mesozoic-Cenozoic) transition, a relatively short interval of geological time during which major reorganization of terrestrial faunas took place. Placental mammals exhibit little diversity before this transition, and none of the modern orders are recorded before the early Cenozoic. The placental-primate divergence at or just after the Cretaceous-Tertiary boundary is securely dated at 65 Myr ago.

2. Divergence of Prosimii-Anthropoidea as part of the Paleocene-Eocene transition, an interval of geological time ~60–50 Myr ago. Here I shall use 55 Myr ago as the best estimate. The divergence being dated is that between tarsiiform and lemuriform primates of modern aspect, which first appeared at or near the beginning of the Eocene (anthropoids appear to be derived from one or the other—which group is ancestral to Anthropoidea is unimportant here).

3. Divergence of Platyrrhini-Catarrhini as part of the Eocene-Oligocene transition, an interval of geological time ~40–35 Myr ago. Here I shall use 40 Myr ago as the best estimate of divergence time. Catarrhini and Platyrrhini are first known with certainty in the Oligocene of both Old and New Worlds.

4. Divergence of Cercopithecoidea-Hominoidea associated with the Oligocene-Miocene transition, an interval of geological time ~20–25 Myr ago. Here I shall use 25 Myr ago as the best estimate for this divergence. Hominoidea are known in the Oligocene, but Cercopithecoidea are not known before the early Miocene.

For purposes of analysis, Radinsky (1978) estimated the Prosimii-Anthropoidea, Platyrrhini-Catarrhini, and Cercopithecoidea-Hominoidea divergence times to be 53, 45, and 25 Myr ago, respectively. Szalay and Delson (1979) gave corresponding times of 55, 40, and 30 Myr ago. Pilbeam (1984) estimated the latter two divergences at 45 and 32 Myr ago. All are in close agreement. Use of greater divergence times for Platyrrhini-Catarrhini and Cercopithecoidea-Hominoidea, as suggested by Pilbeam, would change results presented here very little, systematically increasing rather than decreasing all scaling coefficients.

Placental mammals did not begin to radiate until the late Cretaceous-Paleocene transition (~65 Myr ago), and the divergence of primates from other placental mammals cannot be pushed back past this point. Paleocene mammalian faunas known from Asia, Europe, North America, and South America are much more primitive than Eocene faunas, precluding any significant diversification of primates of modern aspect before the Paleocene-Eocene transition. The lower (younger) limit on time of divergence for Prosimii-Anthropoidea is based on the early Eocene appearance of Omomyidae and Adapidae, primates of modern aspect classified with Tarsiiformes and Lemuriformes, respectively, one or the other of which gave rise to Anthropoidea. Lower limits for divergence of Platyrrhini-Catarrhini and Cercopithecoidea-Hominoidea are based on appearance of both representatives of each pair in the Oligocene and early Miocene, respectively. Upper (older) limits are based on reasonably good worldwide coverage of mammalian faunas lacking primates of the grade in question. Nonspecialists rarely appreciate the progressive nature of evolutionary change over geological time. On the basis of the fossil record, there was nothing like an anthropoid

before the Eocene, nothing like a catarrhine before the Oligocene, and nothing like a cercopithecoid before the Miocene, a finding that provides upper limits on the times of divergence discussed here.

Divergence times of families and subfamilies within the order Primates are poorly constrained by the fossil record. To take one example, the great variation in times of divergence for Hominidae-Pongidae estimated by paleontologists and paleoanthropologists during the past 20 years provides ample evidence that this separation is poorly documented paleontologically. The timing of hominid-pongid divergence is still something to be estimated from molecular clocks—and not an independent datum contributing to their construction.

Temporal Scaling of Albumin Immunological Distance

The divergence times of 65, 55, 40, and 25 Myr ago discussed above can be used to test the colinearity of primate albumin immunological distances and divergence times. Sarich's (1968, 1970) albumin immunological distances for comparisons involving Eutheria-Primates, Prosimii-Anthropoidea, Platyrrhini-Catarrhini, and Cercopithecoidea-Hominoidea are plotted against corresponding divergence times in figure 4. Sarich (1970) suggested a linear relationship: $d'_I = 1.67t$. Linearity requires a temporal-scaling coefficient (exponent of t) equal to 1.00. The appropriateness of a scaling coefficient of 1.00, indeed the appropriateness of a linear model, can be tested empirically by examining a best-fit power function. Exponentiating both sides of a linear regression describing dependency of log d'_I on log t yields the empirical curve $d'_I = 0.33t^{1.43}$, where 1.43 is the scaling coefficient of interest.

Standard methods characterizing the slope of a linear regression yield a confidence interval for the scaling coefficient based on (1) the SE of the estimated slope and (2) Student's t-distribution with $N^* - 2$ degrees of freedom (where N^* corresponds to the number of independent divergence times). Student's t-distribution is very large for one degree of freedom, and, in practice, four or more independent divergence times are required to achieve adequate statistical power for any test of the linear model. In the example discussed here, the SE of the regression slope is 0.069, and t with two degrees of freedom is 4.303. Consequently the 95% confidence interval for the scaling coefficient is 1.43 ± 0.30, or 1.13–1.73. The value of 1.00 required for linearity is well outside this confidence interval.

An empirical scaling coefficient significantly greater than 1.00 indicates positive temporal scaling of primate albumin d'_I values during the Cenozoic: the curve fit to data in figure 4 is concave upward. Positive temporal scaling indicates that primate albumins evolved at decelerating rather than at constant or accelerating rates.

Temporal Scaling of Other Molecular Distance

Fifteen additional studies of molecular distance that separate major groups of primates as well as primates from other placental mammals, provide further evidence of slowing rates of molecular change in primate evolution. All sixteen studies exhibit positive temporal scaling of molecular difference and divergence time (table 1). Scaling coefficients range from 1.08 to 2.61, with a geometric mean of 1.60. They form a uniformly distributed histogram when plotted on a proportional or logarithmic axis (fig. 5). Statistically, six scaling coefficients are significantly different from 1.00 (zero on a logarithmic scale), five scaling coefficients based on four independent divergence times are not significantly different from 1.00, and five scaling coefficients based on two or three divergence times lack adequate statistical power for any real test.

The sixteen primate studies listed in table 1 indicate that molecular distance

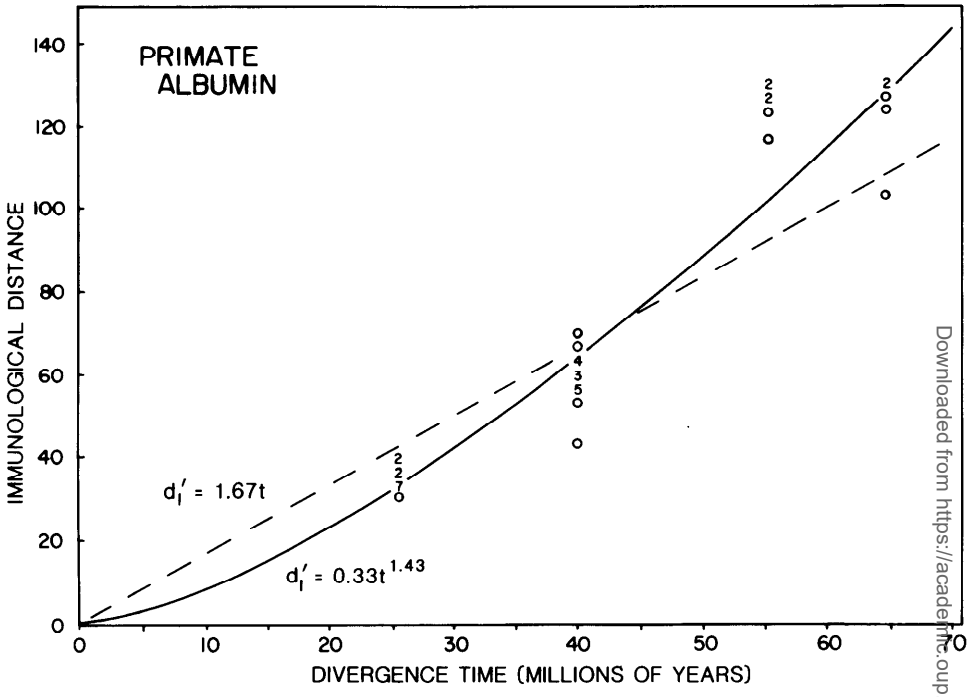


FIG. 4.—Temporal scaling of primate albumin difference over Cenozoic time. The abscissa is divergence time (t); the ordinate is albumin difference in immunological distance units (d_I'). Circles represent individual values, and integers represent multiple values falling at the same point. Nonlinear $d_I' = 0.33t^{1.43}$ (solid line) gives a better least-squares fit to data than does the linear model $d_I' = 1.67t$ (dashed line) proposed by Sarich (1970). Scaling coefficient 1.43 is significantly greater than 1.00. The average rate of change at any point is given by the slope of a tie line to the origin (the instantaneous rate is given by the slope of the curve itself), and a concave-upward curve implies deceleration of albumin evolution over Cenozoic time. The power-function representation of the nonlinear model shown here approximates the positive scaling portion of the more general sigmoid curve shown in fig. 6.

scales positively with divergence time over the course of the Cenozoic. The consistency of this pattern provides support for Goodman's repeated claim that molecular change has slowed during primate evolution (Goodman 1963, 1976; Goodman et al. 1983). Two treatments of Fitch and Langley's mammal data and three treatments of Wilson and Carlson's carnivore and ungulate data suggest that molecular change has probably slowed over the course of Cenozoic time in other placental orders as well.

Paradox of Positive Temporal Scaling

Positive temporal scaling with a slowdown in molecular evolution is paradoxical in light of principles affecting all rates of evolution. It is generally true that evolutionary rates measured over long intervals of geological time are lower than rates measured over short intervals (Gingerich 1983). In molecular terms, the number of undetectable amino acid replacements or nucleotide substitutions increases with increasing time of separation (because of multiple replacement or substitution at the same sites). Consequently, the number of detectable replacements or substitutions decreases as some function of total change, and rates calculated from this decreasing function are damped artificially over time in a manner analogous to that effected by the time averaging of rates of morphological evolution.

Table 1
Temporal Scaling of Molecular Change in Primate Evolution, Where Molecular Distance $d' = a$ (divergence time t)^b

Protein/Nucleic Acid	N	N^*	a	b	95% CI for b
Immunological distance (d'_I):					
Albumin (ID; Sarich 1968, 1970)	39	4	0.33	1.43	1.13–1.73
Transferrin (Sarich and Cronin 1976)	3	3	0.0037	1.54	...
Albumin + transferrin (Sarich and Cronin 1976)	30	4	0.83	1.42	0.96–1.90
Summed proteins (AD; Dene et al. 1976)	509	4	0.30	1.45	1.36–1.54
Amino acid sequence (d'_A; per 100 sites):					
Myoglobin (Romero-Herrera et al. 1973)	107	4	0.0006	1.37	1.07–1.67
Myoglobin (Romero-Herrera et al. 1978)	218	4	0.0014	1.12	0.82–1.42
Nucleic acid hybridization (d_D or d'_D):					
DNA (ΔTS [°C]; Kohne et al. 1972)	8	3	0.022	1.75	...
DNA (ΔTmR [°C]; Benveniste and Todaro 1976)	14	2	0.28	1.08	...
DNA (ΔTmR [°C]; O'Brien et al. 1985)	7	2	0.20	1.14	...
DNA (Δmode [°C]; C. Sibley, cited in Pilbeam 1983)	2	2	0.13	1.25	...
Nucleotide sequence (d'_N; per 100 sites):					
α-Hemoglobin (Goodman et al. 1983)	92	4	0.012	1.83	0.94–2.72
β-Hemoglobin (Goodman et al. 1983)	101	4	0.0029	2.20	1.55–2.85
Myoglobin (Goodman et al. 1983)	66	4	0.0016	2.30	1.70–2.90
Fibrinopeptide A and B (Goodman et al. 1983)	48	4	0.0004	2.61	1.64–3.58
Cytochrome c (Goodman et al. 1983)	24	4	0.0018	2.01	0.43–3.59
α-Lens crystalline A (Goodman et al. 1983)	19	4	0.0022	1.97	0.22–3.72

NOTE.—N = total number of taxa compared; N^* = number of independently determined divergence times; a = empirical constant; b = scaling coefficient; CI = confidence interval; ID = immunological distance; and AD = augmented distance.

Rates of molecular evolution for recent divergences are measured over shorter intervals of time than are rates for ancient divergences. Thus, even if underlying rates of molecular evolution based on total change per unit time (d') were constant, one would expect to see empirical rates based on observable change (d) speed up, with more recent divergences yielding progressively higher rates. Zuckerkandl and Pauling (1965) clearly recognized this in designing a model in which the average rate of change of observable sequence difference (slope of a tie line connecting any point with the origin) increases progressively for decreasing times of divergence (fig. 1). Conversely, rates of change of observable sequence difference decrease for increasing times of divergence as observable difference approaches the limit of 100% (representing change at all sites). Molecular difference should scale negatively with divergence time—but observable difference in Cenozoic primates scales positively.

Observable difference d, whatever the scaling coefficient, is always subject to the general constraint that change cannot exceed 100% (i.e., one replacement or substitution per site). Thus positive temporal scaling over shorter time intervals must eventually give way to negative scaling over longer intervals. Assuming that rates of molecular evolution maintain smoothness and continuity as they change, we expect that inflection from positive to negative scaling in the context of a fixed constraint on observable change might yield a sigmoid curve like that shown in figure 6.

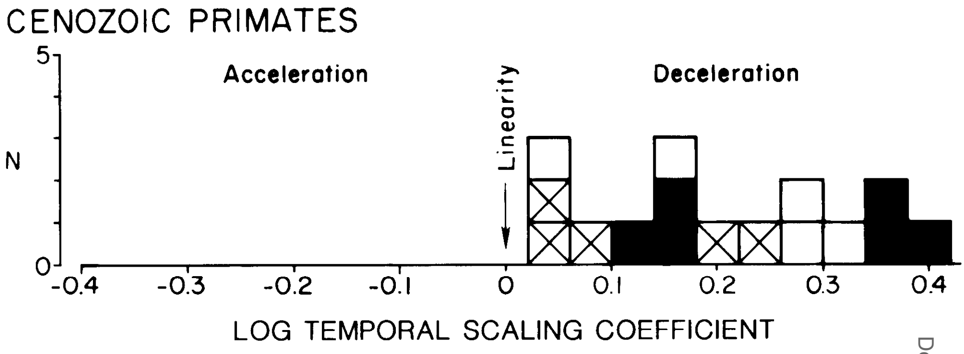


FIG. 5.—Histogram of temporal-scaling coefficients observed in 16 studies of molecular evolution in Cenozoic primates. Coefficients for scaling of immunological distances, amino acid replacements, nucleic acid hybridization, and nucleotide substitutions listed in table 1 range from 1.08 to 2.61 (0.03–0.42 on $\log_{10}$ scale). All scaling coefficients are positive (>1.00): six are significantly positive (shaded squares; 1.00 is outside 95% confidence interval for estimate), five are positive but not significantly so (unshaded squares; 1.00 is within 95% confidence interval), and five are positive but of unknown significance (cross-hatched squares; number of divergence times is less than four, providing inadequate statistical power for test).

A sigmoid curve approximating amino acid sequence difference (d_r) for mammalian myoglobin (fig. 6) illustrates two interesting points about myoglobin evolution. First, molecular change in myoglobin reaches a limit or plateau at ~16% observable amino acid sequence difference (0.16 changes/site). This plateau of variability is well below the maximum possible limit of 100%. Some 84% of amino acid residues, presumably those at sites of functional importance, are invariable, remaining unchanged in mammalian evolution. Second, divergence of anthropoid from prosimian primates at 55 Myr ago yields a constant rate *r* of 0.0027 differences/site/Myr divergence time, calculated according to the Zuckerkandl-Pauling model. A logistic model constructed to approximate the same data yields an intrinsic rate *r* of 0.15 differences/site/Myr, which is more than 50 times the rate derived from the Zuckerkandl-Pauling model. Choice of models is critically important in estimating rates of molecular evolution.

A logistic curve is but one of many smooth sigmoid curves, and it is employed here for illustrative purposes based on familiarity and ready interpretation of parameters. Other sigmoid functions may prove to be more appropriate: in particular, continuous functions involving an abrupt change of rate at the Mesozoic-Cenozoic boundary deserve consideration. Whatever function is employed, the paradox of positive temporal scaling in the context of a fixed limit on observable change is resolved by noting that positive scaling is always necessarily combined with negative scaling to remain within limits of observable difference.

Discussion

The idea that molecular clocks keep time in a uniform way has been challenged repeatedly by paleoprimatologists (e.g., Read and Lestrel 1970; Uzzell and Pilbeam 1971; Simons 1976; Radinsky 1978) because uniform clocks consistently yield divergence times for old divergences that are too old and divergence times for young divergences that are too young by comparison with the primate fossil record. Temporal scaling of molecular difference against divergence time is the only test of the molecular clock hypothesis, and temporal scaling yields coefficients that consistently exceed the value of 1.00 necessary for either colinearity of time and change or stochastic uniformity of evolutionary rates.

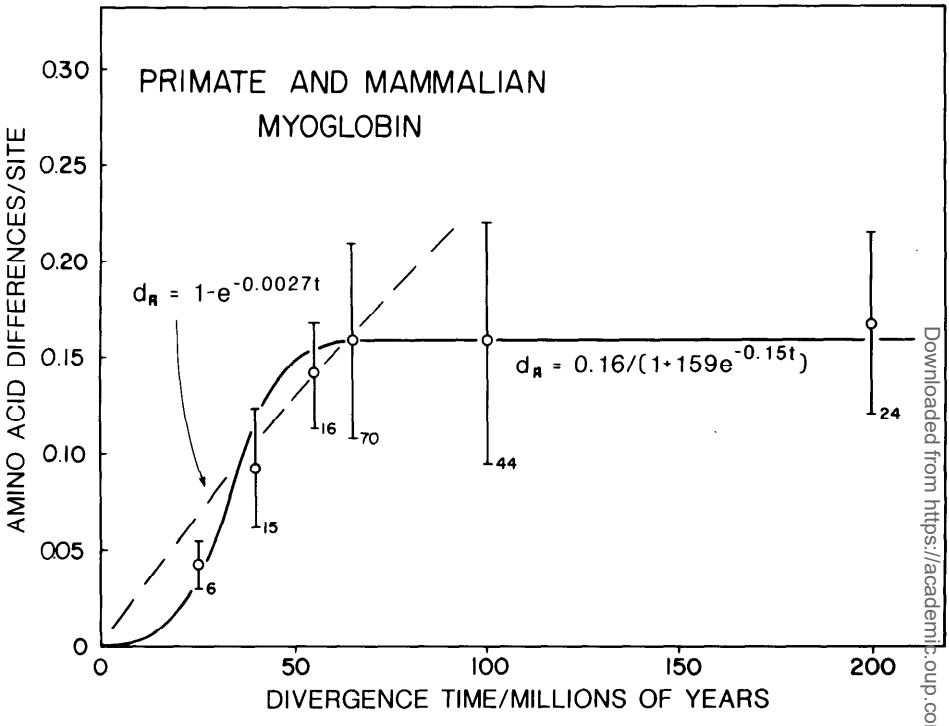


FIG. 6.—Empirical relationship of observed myoglobin sequence difference (d_A) and divergence time (t) in primates and other mammals. The distribution of means (circles) approximates a sigmoid curve (solid line). Vertical bars enclose 95% of variance (mean ± 2 SD), and subscripts denote sample size. Data are from Romero-Herrera et al. (1973) and Dickerson and Geis (1983). The dashed line represents the Zuckerkandl-Pauling model calibrated with 55-Myr-ago divergence time to yield maximum rate of 0.0027 differences/site/Myr. A sigmoid curve fit to the same data involves an intrinsic rate of ~0.15 differences/site/Myr, exceeding that estimated from the Zuckerkandl-Pauling model by a factor of more than 50. The sigmoid here is a logistic curve, fit by eye, based on an observed plateau at 0.16 differences/site, with arbitrary constant a = 159 making $d_A \approx 0$ when t = 0. The plateau in the observed sequence difference at 16% indicates that a limited fraction of amino acids in mammalian myoglobin are free to vary. The average rate of observable change at any point is given by the slope of a tie line to the origin (the slope of the curve itself is an instantaneous rate of observable change). Empirical distribution implies a change from increasing rates for Mesozoic divergences to decreasing rates for Cenozoic divergences. This transition from increasing to decreasing rates of observable change appears to coincide with the extraordinary diversification of placental mammals at the beginning of the Cenozoic. If so, the 65-Myr-ago break in the slope of the empirical curve may have been much sharper than that shown by this or any other logistic model.

It is worth considering how divergence times might be manipulated to achieve colinearity with total molecular difference. Lower limits on divergence times are set by documented appearance in the fossil record of representatives of both clades being compared, and thus lower limits are based on positive evidence offering little opportunity for alternative interpretation. The lower limit of the youngest divergence time employed here is based on the bilophodont molar of a fossil cercopithecoid (reported by Pilbeam and Walker [1968]) from Napak, an east African site yielding fossil hominoids and dated at 19–19.5 Myr ago (Walker and Pickford 1983). Conceivably, cercopithecoids and hominoids diverged as recently as 20 Myr ago. Assuming that albumin immunological distances evolved at a constant rate, a 20-Myr-ago divergence combined with an average d'_I of 34.6 separating Cercopithecoidea and Hominoidea yields $d'_I = 1.73t$. Given this relationship, one would predict the divergence of Anthropoidea

and Prosimii (average d'_I = 125.3) to have occurred at 72.5 Myr ago, some 7 Myr before the placental radiation leading to modern ordinal diversity in mammals and nearly 20 Myr before any primate of modern aspect related to either Anthropoidea or Prosimii is known in the fossil record.

Upper limits for divergence times are based on negative evidence: the absence of fossils representing one or both of the primate clades being compared. Thus, upper limits are less rigidly constrained than lower limits. However, placental mammals are so little differentiated in the Mesozoic and constitute such a minuscule component of known world Cretaceous vertebrate faunas (Lillegraven et al. 1979) that one cannot reasonably imagine Primates, let alone Prosimii and Anthropoidea, to have existed before the end of the Mesozoic. Even if one assumes that hominoids and cercopithecoids diverged at 20 Myr ago, there is no way to superimpose linear scaling of albumin evolution on the primate fossil record. The same logic holds as well for other measures of molecular difference. Positive temporal scaling and a slowdown in rate appear to be integral components of molecular evolution in this well-studied order, and positive scaling is likely to be the rule in other orders as well.

The first appearances of new primate grades, like those of new grades in other mammalian orders, are clustered in geological time, coinciding with Cenozoic epoch boundaries (late Cretaceous-Paleocene, Paleocene-Eocene, Eocene-Oligocene, Oligocene-Miocene, Miocene-Pliocene, and Pliocene-Pleistocene). Most epoch boundaries are marked by major climatic cooling-warming events affecting faunas on a worldwide scale. Worldwide cooling with associated continental endemism may be responsible for production of new diversity. Subsequent warming is clearly associated with faunal cosmopolitanism. The early Cenozoic fossil record for Africa, a likely equatorial center of origin for many primate groups, is poorly known; but profound faunal change during times of warming and cosmopolitan dispersal suggests that African and other equatorial faunas are being sampled in Asia, North America, and Europe at the beginning of the Paleocene, Eocene, and possibly Oligocene. To take one example, the first appearance of primates of modern aspect (primates of tarsier/lemur prosimian grade) in the early Eocene of North America is interpreted to reflect late Paleocene evolutionary events in Africa or South Asia (Gingerich 1986). This pattern of episodic dispersal of new primate and other mammalian grades provides additional justification for selecting 65-, 55-, and 40-Myr-ago divergence times for major groups of living primates.

The phyletic history of mammals is not a uniform series of many evenly or randomly spaced dichotomies but involves a much smaller number of major multichotomies in which the component dichotomies are closely packed (and probably, in many cases, unresolvable) in time. Brief intervals of diversification are separated by much longer intervals of relative faunal stability. One important consequence of this is that small differences in the large number of amino acid replacements or nucleotide substitutions distinguishing orders of mammals cannot be taken as evidence of interordinal relationships. Within primates, a few molecular similarities among many differences are probably not reliable evidence of the genealogy of distantly related suborders or infraorders.

The pattern of temporal scaling of molecular difference is important when predicting times of divergence for living primates with a poor fossil record. Ape-human divergence times predicted using empirical nonlinear scaling coefficients are approximately twice as great as those predicted using linear models of molecular change (Gingerich 1985*b*). Of greater significance, scaling molecular difference against geo-

logical time helps to delimit the role of selectively neutral change in molecular evolution. On the basis of the plateau in sequence difference for mammalian myoglobin (d_A in fig. 6), the average number of variable sites is only 16% of the total (84% are invariable). Rates of change within the variable 16% were sufficient to homogenize sequence differences, and, given limited mammalian diversity during the Mesozoic, we cannot resolve myoglobin rates (or most other rates) for the first 135 Myr of mammalian evolution. Comparable plateau levels for observable nucleotide substitutions (d_N, estimated from scaling of nonaugmented values in Goodman et al. 1983) are 22% in primate myoglobin, 22% in α-hemoglobin, 24% in β-hemoglobin, 20% in fibrinopeptide A and B, 7% in cytochrome c, and 7% in α-lens crystalline A.

Steepening of the curve of sequence difference versus time (fig. 6) suggests that the rate of myoglobin evolution increased markedly at 65 Myr ago, coinciding with the time of radiation of placental mammals. Interpretation of this increase in rate depends on how the curve is modeled. The logistic shown in figure 6 implies that rates increased slowly (concave-downward portion of curve) to reach a maximum of ~0.15 differences/site/Myr and then decreased (concave-upward portion of curve). Downward concavity implies a speedup in rates of molecular evolution, and subsequent upward concavity implies a slowdown. Assuming that the maximum rate of molecular evolution following placental diversification at 65 Myr ago reflects neutral change, then subsequent slowdown is likely to represent natural selection.

Rates of myoglobin evolution, like those of other proteins, have slowed following the diversification of placentals. To the extent that amino acid replacements and nucleotide substitutions change in proportion to elapsed evolutionary time, as Zuckerkandl and Pauling postulated, this change can be attributed to neutral evolution. However, there is little evidence that molecular change occurs at constant rates, and there is thus little basis for assuming that most molecular change is selectively neutral.

Note added in proof.—I am indebted to A. Templeton for the two following nonparametric tests of isochrony of molecular change. Nonparametric methods are appropriate when molecular distance (d') and time (t) are both random variables with unknown distribution functions and t is a stochastic predictor of d'. The null hypothesis of isochrony requires that the average rate of change of d' per unit t (the slope of a fit line to the origin) is independent of t itself.

Equation 8.2.20 of Puri and Sen (1985) yields a distribution-free test statistic:

$$L_n = (\sum a_i b_i)^2 (n - 1)/\sum a_i^2 b_i^2,$$

where $a_i = [\{\text{Rank } (d'_i/t_i)\}/(n + 1)] - 0.5$ and $b_i = [\{\text{Rank } (t_i)\}/(n + 1)] - 0.5$. Test statistic L_n approaches a χ^2 distribution with one degree of freedom as n approaches infinity.

Nonparametric tests often have greater statistical power than parametric approximations when underlying distributions are poorly defined. The data in Fitch and Langley's study discussed above (incorporating their points 7–15) yield an L_n of ~6.1, suggesting that the null hypothesis of isochrony be rejected with an error probability of ~0.02.

Approaching the problem in a different way, Spearman's rank correlation of d'_i/t_i and t_i yields $r_s = 0.875$, suggesting that the null hypothesis of isochrony (no correlation) be rejected with an error probability of ~0.002. Similarly, nonparametric tests are likely to increase rather than decrease the significance of all departures from isochrony documented in table 1.

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