from two specimens collected in the fall and two in late winter. The concentration of glycerol was calculated on the basis of total water content of the extracted muscles; that is, I assumed that glycerol was equally distributed in intra- and extracellular fluid spaces. Specimens of *H. versicolor* collected from terrestrial hibernation sites in late winter had full urinary bladders, and samples of their bladder fluid also contained approximately 0.3*M* glycerol (Fig. 2). Specimens collected in mid-May after migration from upland winter sites to breeding ponds had no glycerol and had also lost their tolerance to freezing. No glycerol was found in muscle extracts of *R. pipiens* or *R. septentrionalis*, so the correlation between glycerol content and frost tolerance was consistent both within and among species.

I had expected that winter survival of terrestrial frogs would involve frost avoidance through deep supercooling as is common among insects. I found instead an example of survival of a vertebrate after extensive freezing of body fluids. Complete freezing of extracellular water is consistent with my estimate of 35 percent of body fluid as ice (*12*). Frost tolerance is an important adaptation for survival of terrestrial frogs during winters of little snow when microclimate temperatures can easily fall below the supercooling point of these species. The occurrence of glycerol in association with frost tolerance suggests that this compound is a component of the cryoprotectant chemical system of these frogs.

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2. R. P. Hodge, *Amphibians and Reptiles in Alaska, the Yukon and Northwest Territories* (Alaska Northwest, Anchorage, 1976), pp. 18 and 55.
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4. T. Franke (personal communication) recorded soil-surface temperatures beneath leaf litter at a site of hibernating *H. versicolor* in Elm Creek Park Reserve, Hennepin County, Minnesota, through the winter of 1980–81 (model 1000 recording thermometer, Electric Auto-Lite Co., Toledo, Ohio). The minimum temperature was −7.2°C, and there were periods of 8 to 12 days when the temperature did not rise above −4°C. Frogs found at this site during February and March were at the same location as the sensing probe, that is, at the soil surface under leaf litter in the posture described by D. B. Ralin [*Comp. Biochem. Physiol.* **68**, 175 (1981)] for *H. versicolor* during desiccation.
5. Specimens of *R. sylvatica* were collected from upland forests in Itasca State Park, Clearwater County, Minnesota, in October 1979; *H. cruci-fer* from woods near Cedar Creek Natural History Area, Isanti County, Minnesota, in September 1980; *R. septentrionalis* from minnow traps in Itasca State Park in October 1980; *R. pipiens* from Rock Creek, Chisago County, Minnesota, in September 1980; and *H. versicolor* from upland woods in Elm Creek Park Reserve in October 1980 and in February and March 1981. All specimens collected during the fall were held in a refrigerator (4° to 8°C) for 20 to 30 days before testing, but the four specimens of *H. versicolor* collected during the winter were tested immediately; two of these frogs survived two cycles of freezing and thawing before being killed for extraction of muscles (*10*).
6. Individual specimens were placed in plastic containers with Styrofoam insulation below and above, with a copper-constantan (3-mil wires) thermocouple in contact with the mid-dorsal surface. Surface contact thermometry was used to measure supercooling points because invasive sensors usually bias results by providing artificial sites for nucleation of ice crystal growth inside the animals. The specimen holder was placed in a refrigerated bath (Forma Temp model 2025-2, Forma Scientific, Marietta, Ohio) with cooling rate controlled by manual adjustment of the thermostat. The thermocouple reference junction was held in distilled water and chipped ice at 0°C, and the thermoelectric voltage was used to measure and record specimen temperature (Omega 2809 digital thermometer, Omega Engineering, Stamford, Conn., and Gould 105 recorder, Gould, Inc., Cleveland) through the freezing cycle. A second thermocouple was used to monitor the bath temperature, which was lowered 0.6°C per hour until an exotherm occurred. Slow cooling was used to mimic rates of natural temperature change and to ensure thermal equilibrium between the specimen and bath before freezing. Temperatures of both the specimen and cooling bath were recorded to the nearest 0.1°C.
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8. The use of exotherms to estimate the amount of ice formed is similar to differential thermal analysis as described by M. J. Burke *et al.* [*Annu. Rev. Plant Physiol.* **27**, 507 (1976)]. The endothermic analysis of ice content is similar to that used by D. J. Murphy [*Physiol. Zool.* **52**, 219 (1979)].
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10. Frozen muscle was cut away from the hind legs, weighed, homogenized in cold 80 percent methanol (3 volumes per weight), and mixed with two volumes of chloroform from which the methanol-water phase was saved and centrifuged for 3 minutes at 11,600*g*. The supernatant was decanted and 5-μl samples were spotted on paper (Whatman No. 1 Chromatographic Paper) or cellulose thin-layer plates (Eastman 13255 Cellulose, Eastman Kodak Co., Rochester) together with mixed standards of glycerol-sorbitol, glycerol-glucose, and glycerol-fructose. After drying and development for 8 hours in either *n*-butanol:acetic acid:water = 12:3:5 or *n*-butanol:pyridine:water = 6:4:3, the chromatograms were dried and sprayed with periodate and iodide-starch as described by L. Somme [*Can. J. Zool.* **42**, 87 (1964)].
11. Technical details of high-performance liquid chromatography analysis (instrumentation, Waters Associates, Milford, Mass.) are described by D. L. Hendrix *et al.* [*J. Chromatogr.* **210**, 45 (1981)].
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13. I thank J. C. Underhill, J. L. Howitz, and especially T. Franke for their aid in collecting specimens. P. J. Regal, D. J. Merrell, and R. E. Lee provided useful reviews of my writing, and R. E. Lee and J. G. Baust provided high-performance liquid chromatography analyses.

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Fossil Genes: Scarce as Hen's Teeth?

Kollar and Fisher (*1*) report that grafts of chick epithelium to mouse molar mesenchyme were induced to produce tooth enamel. Their experiments indicate that the loss of teeth in the class Aves should be attributed to factors other than the loss of genes for enamel production. We wish to draw attention to the profound implications of their results to modern evolutionary theory in ways not mentioned by them.

If we are to assume, as do Kollar and Fisher, that genes for enamel matrix proteins have no function in extant birds, and that such genes have remained unexpressed since toothed birds became extinct during the Cretaceous (*2*), we ask: what is the probability that any archaic gene could retain function after some 70 million years of unselected, random mutation?

Dormant DNA in living organisms is not frozen in time, remaining forever unchanged as a preserved fossil; instead it is transmitted from cell to cell and generation to generation via the same replicative machinery as any other DNA. Mutation is a slow but ineluctable part of this process that generates new alleles which must survive the pressure of negative selection to persist in time. Allelic variants of an unexpressed locus, however, are operationally neutral to the action of selection, and the accumulation of changes in wholly silent sites is expected to saturate after 100 million years (*3*).

According to conventional neutralist theory, proteins (the products of structural genes) evolve at a constant rate within the confines of selective constraints imposed by function (*4*). That is, realized amino acid substitutions are maximal when variants at a locus are independent of Darwinian fitness. Of the proteins surveyed thus far, fibrinopeptides appear to have evolved at the highest rate, with an estimated nine amino acid substitutions per amino acid site per 1 billion years (*4*). Kimura offers this particular example as one approaching selective neutrality; therefore, an equivalent expected rate for proteins of the resurrected bird-enamel locus is not unreasonably high. Using fibrinopeptides as a conservative baseline for near-neutral genes, we predict that the extant and putatively unexpressed bird-enamel locus would have experienced sufficient random mutations to effect substitutions at approximately 63 percent of the original amino acids in its protein product (*5*).

While it can be argued that numerous differences are known to exist within

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families of analogous proteins across taxa without loss of function in any, consider the likelihood of function in any if, say, more than half the amino acids were substituted randomly.

In the absence of selective constraints, it is most unlikely that a silent gene could still function, or even be recognized, after more than 70 million years if the rates of protein evolution advocated by neutralist theorists are remotely reasonable. If Kollar and Fisher have uncovered a genuine phenomenon, then the thesis of Kimura and his colleagues requires serious revision. However, in view of the vast body of data upon which molecular evolutionary theory is based (*4*), unequivocal evidence is required to resolve the critical question: did chick cells actually make enamel matrix protein? Until the admittedly difficult task of characterizing the protein as nonmurine is performed, and assurances offered indicating that the genes responsible for the protein are indeed "quiescent," the burden of proof weighs heavily on Kollar and Fisher.

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1. E. J. Kollar and C. Fisher, *Science* **207**, 993 (1980).
2. Of at least 12 avian genera in the Cretaceous, perhaps two had toothed representatives. That geologic period extended from 135 to 70 million years before present. Precisely when toothed birds became extinct is unknown [see W. E. Swinton, in *Biology and Comparative Physiology of Birds*, A. J. Marshall, Ed. (Academic Press, New York, 1960); P. D. Gingerich, *Condor* **74**, 471 (1972)].
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5. Adjusting the evolutionary rate of the "nearly neutral" fibrinopeptides from nine amino acids per amino acid site per billion years, down to 70 million years becomes 0.07 its value, that is, 0.63 amino acid substitution per amino acid site for the putative bird-enamel locus.
6. We thank R. E. L. Black, M. A. Byrd, S. F. Hoegerman, and especially K. E. Weber for discussions.

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Grant and Wiseman (*1*) draw attention to the far-reaching implications of our report (*2*), and they question whether quiescent genes can maintain function. They assume that genes for enamel protein are present, and ask how a gene could retain function after 70 million years of unselected, random mutation. Grant and Wiseman impose hypothetical conditions on this complex problem and conclude that it is unlikely that unexpressed genes could function or even be recognized if mutations had occurred at the rate they propose.

Central to their argument is the contention that enamel genes have not been subjected to selection pressure. This supposition is open to question. Many structural, nonenzymatic proteins (for example, collagen and keratins) are families of proteins (*3*) that serve various functions and contribute to manifold phenotypic expression. For example, keratin filaments are related to cytoskeletal elements as well as to keratin complexes in the stratum corneum of skin. Selection is a complex interaction impinging on many phenotypic traits. Thus, a family of proteins may be subject to selective pressure even if one aspect of their function is lost as a consequence of developmental alteration. Little is gained from speculating about loss of function or about the extent of alteration in enamel protein because how these molecules function is not known. However, there is no evidence that they act enzymatically; in fact, they seem to function as a matrix. If so they could tolerate substantial amino acid substitutions before function is severely diminished.

For their argument, Grant and Wiseman chose fibrinopeptides as an example of proteins that mutate rapidly. But they overlook the significant observation (*4*) that enamel protein derived from fish to mammals cross-reacts immunologically. Immunological cross-reactivity suggests (i) that homologies exist in these molecules throughout the vertebrates; (ii) that there is probably less than a 40 percent difference between the proteins; and, thus, (iii) that the proteins are highly conserved. If Grant and Wiseman based their calculations on the mutation rates of more highly conserved proteins such as cytochrome c, collagen, or the histone H_4, their calculations would not have supported their argument. In addition, since enamel protein functions, the analogy with functionless fibrinopeptides is inappropriate. Very little is known about the molecular composition of enamel protein, or whether this is a group or family of proteins.

Relatedness of protein families, ever-increasing complexity of gene loci, and the importance of developmental controls of gene expression (*5*) all argue for a more sophisticated view of genetics, evolutionary theory, and developmental analysis. We believe that the validity of our findings can best be assessed by the presentation of experimental data, rather than by hypothetical arguments. We look forward to new experimental data that bear on this question.

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