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Preservation of key biomolecules in the fossil record: current knowledge and future challenges

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We have developed a model based on the analyses of modern and Pleistocene eggshells and mammalian bones which can be used to understand the preservation of amino acids and other important biomolecules such as DNA in fossil specimens. The model is based on the following series of diagenetic reactions and processes involving amino acids: the hydrolysis of proteins and the subsequent loss of hydrolysis products from the fossil matrix with increasing geologic age; the racemization of amino acids which produces totally racemized amino acids in 10^5–10^6 years in most environments on the Earth; the introduction of contaminants into the fossil that lowers the enantiomeric (D:L) ratios produced via racemization; and the condensation reactions between amino acids, as well as other compounds with primary amino groups, and sugars which yield humic acid-like polymers. This model was used to evaluate whether useful amino acid and DNA sequence information is preserved in a variety of human, amber-entombed insect and dinosaur specimens. Most skeletal remains of evolutionary interest with respect to the origin of modern humans are unlikely to preserve useful biomolecular information although those from high latitude sites may be an exception. Amber-entombed insects contain well-preserved unracemized amino acids, apparently because of the anhydrous nature of the amber matrix, and thus may contain DNA fragments which have retained meaningful genetic information. Dinosaur specimens contain mainly exogenous amino acids, although traces of endogenous amino acids may be present in some cases. Future ancient biomolecule research which takes advantage of new methologies involving, for example, humic acid cleaving reagents and microchip-based DNA–protein detection and sequencing, along with investigations of very slow biomolecule diagenetic reactions such as the racemization of isoleucine at the β-carbon, will lead to further enhancements of our understanding of biomolecule preservation in the fossil record.

Keywords: amino acids; racemization; DNA survival

1. INTRODUCTION

The retrieval of biomolecular information of evolutionary value from fossils is a coveted yet often elusive goal. Although both proteins and DNA are components of the tissues of all organisms, they undergo degradation over geological time. The time range over which meaningful protein and DNA sequence information is preserved depends on a variety of variables such as the environmental temperature, humidity and the type of matrix in which the molecules are contained.

Proteins hydrolyse under geochemical conditions by cleavage of an internal peptide bond, an internal aminolysis reaction at the N-terminal position, which yields a diketopiperazine (cyclic dipeptide), and hydrolysis at the C-terminal position (see Bada 1991; Collins *et al.*, this issue, and references therein). The latter two reactions increase in significance as internal hydrolysis fragments the original protein. In the carbonate matrix, proteins are nearly completely hydrolysed to smaller peptides and free amino acids in about 10^6 years on the ocean floor, and in around 10^5 years in surface environments. In bones, hydrolysis of the main protein component, collagen, is even more rapid and little intact collagen remains after only $1–3 \times 10^4$ years, except in bones in cool or dry depositional environments.

DNA is even more fragile than proteins and it has been suggested, based on aqueous solution studies, that DNA sequences containing meaningful genetic information should not be preserved in most geological environments for more than 10^4 years (see for example, Pääbo & Wilson 1991; Lindahl 1993, 1997; Poinar *et al.* 1996). Depurination, involving the hydrolysis of the deoxyribose–adenine or guanine bond, followed by rapid chain breakage, is thought to be the main reaction in the fragmentation of DNA in the geological environment. The release of the purines generates apurinic sites in the DNA chain. At the apurinic sites, the free aldehyde form of the 2′ deoxyribose group is involved in a β-elimination

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reaction which rapidly breaks the DNA backbone at the 3′-phosphodiester bond of the apurinic sugar.

Recently it has been found that examining both the extent of amino-acid racemization and the relative amino-acid composition provides a useful index for the general assessment of biomolecule preservation, especially genetic information, in fossil specimens (Bada *et al*. 1994; Poinar *et al*. 1996). Amino acids are far more abundant than DNA in hard tissues such as bone and shell that comprise the major portion of the fossil record. Amino-acid diagenetic reactions are influenced by many of the same variables that affect the hydrolysis of DNA. In addition, amino acid racemization follows a predictable pattern (D:L aspartic acid > D:L alanine > D:L valine) over time that can be used to evaluate the critical problem of endogeneity vs contamination (Bada *et al*. 1973; Bada 1985*a*,*b*, 1991). Compared to DNA, straightforward sensitive techniques are available for the routine direct analysis of amino acids, along with their enantiomers (e.g. D- and L- isomers), in fossil samples weighing only a few milligrams (for example, see Zhao & Bada 1995).

We have developed a general model, based on analyses of modern and Pleistocene eggshell and mammalian bone specimens, for amino-acid diagenesis and preservation in a biomineral matrix. We then use this model to understand the level of preservation of original biomolecular components in a series of fossil specimens. Our measurements concentrated on the total content of aspartic (Asp) and glutamic (Glu) acids, serine (Ser), glycine (Gly), alanine (Ala), valine (Val) and leucine (Leu) because this group is representative of the various functionalities of the protein amino acids. The enantiomeric ratios of aspartic acid, alanine, and valine were also determined, thereby covering the range of amino-acid racemization rates. Minimal sample manipulation is involved in this analytical procedure, thus avoiding the possible contamination problems associated with the purification steps required for the isolation of protein and macromolecular components. Although it is possible that amino acids in some minor components in a fossil specimen may be less susceptible to diagenetic alteration than others (Masters 1987), the analyses we have carried out provide an overall general assessment of amino-acid preservation.

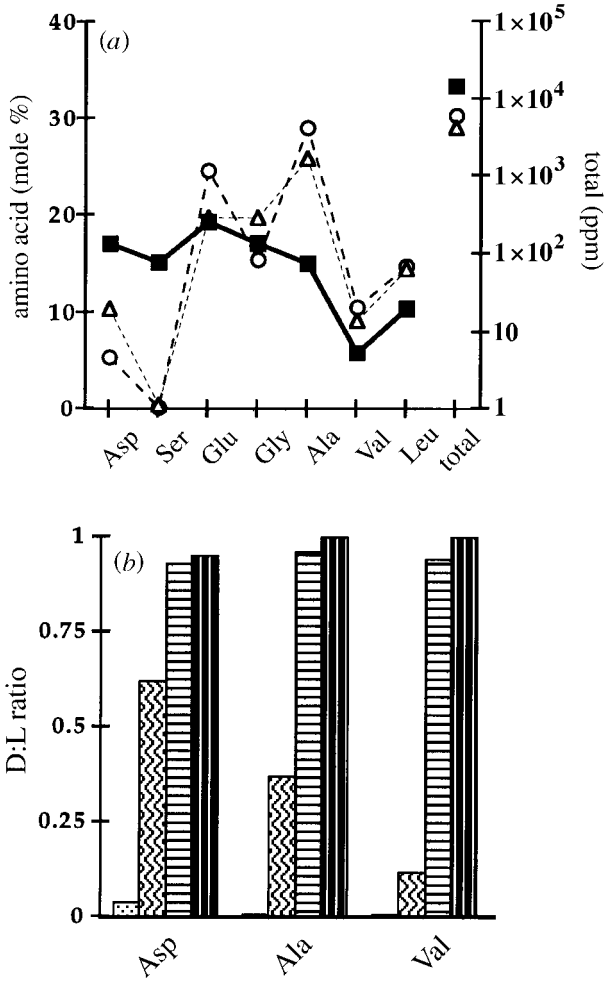

Figure 1. (*a*) The amino acid content (in ppm) and composition (expressed as mole % of the listed amino acids) in modern and Pleistocene ostrich eggshell. Solid squares, modern eggshell; open triangles, $2–4 \times 10^4$-year-old eggshell from Olduvai Gorge; open circles, $4–6 \times 10^5$-year-old eggshell from Olduvai Gorge. (*b*) The extent of racemization of aspartic acid, alanine and valine in modern, heated and Pleistocene ostrich eggshell. Columns (left to right): first, modern eggshell; second, heated modern landsnail (Goodfriend 1991); third, $2–4 \times 10^4$-year-old eggshell from Olduvai Gorge; fourth, $4–6 \times 10^5$-year-old eggshell from Olduvai Gorge. The D-amino acids present in the modern samples are from racemization which takes place during the acid hydrolysis step used in sample processing. The heated modern samples are included to show the expected pattern of racemization rates.

2. AMINO ACIDS IN MODERN AND PLEISTOCENE EGGSHELL AND BONE

Modern ungulate bone and ostrich eggshell samples were collected from surface remains on the Serengeti Plain around Olduvai Gorge, Tanzania. The Pleistocene mammalian bone and ostrich eggshell samples were from the Middle and Lower Naisuisu beds ($2–4 \times 10^4$ years old) and Upper Bed IV ($4–6 \times 10^5$ years old) at Olduvai Gorge; these samples were either generously supplied by the late Dr M. D. Leakey or collected under her supervision.

Sample pieces weighing about 100 mg were first manually cleaned to remove surface deposits, and then washed for a few minutes first with dilute HCl followed by rinsing with doubly distilled (2×) water; this surface etching procedure was repeated two to three times. After cleaning, the samples were dissolved in 2 × 6 M HCl and then hydrolysed for 24 h at 100 °C. After hydrolysis, the 6 M HCl was evaporated to dryness, the residue dissolved in 2 × water and the extract desalted using cation-exchange chromatography. The desalted extracts were then derivatized with *O*-phthaldialdehyde–N-acetyl-L-cysteine, and then analysed by HPLC using fluorescence detection (Zhao & Bada 1995). For amino-acid quantitation and accurate determination of enantiomeric ratios, a standard containing a racemic mixture of the selected amino acids was analysed at the same time as the samples.

The Pleistocene eggshell samples have retained 25–50% of the amino acids present in modern eggshell (figure 1*a*). The changes in the amino-acid composition of the fossil eggshells are characterized by a decrease in aspartic acid and serine, an increase in alanine, and only minimal changes in the other amino acids examined.

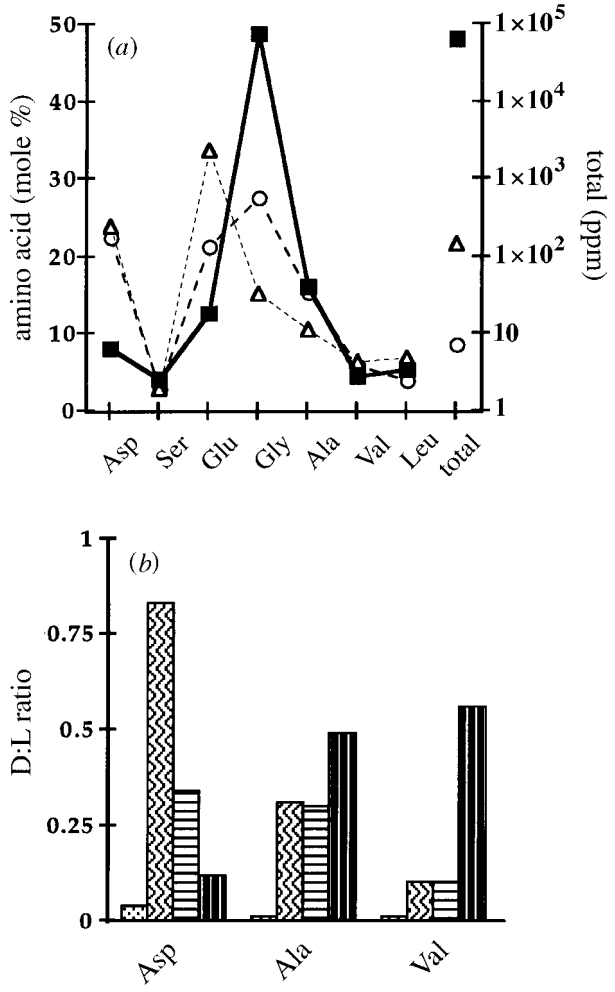

Figure 2. (*a*) The amino-acid content (in ppm) and composition (expressed as mole % of the listed amino acids) in modern and Pleistocene mammalian bone. Solid squares, modern bone; open triangles, $2–4 \times 10^4$-year-old bone from Olduvai Gorge; open circles, $4–6 \times 10^5$-year-old bone from Olduvai Gorge. (*b*) The extent of racemization of aspartic acid, alanine and valine in modern, heated and Pleistocene mammalian bone. Columns (left to right): first, modern bone; second, heated modern bone (Bada *et al*. 1973); third, $2–4 \times 10^4$-year-old bone from Olduvai Gorge; fourth, $4–6 \times 10^5$-year-old bone from Olduvai Gorge.

These changes are consistent with those observed in other fossil and heated ostrich eggshell samples (Brooks *et al*. 1990; Miller *et al*. 1991, 1992). The decrease in the aspartic acid content is likely caused by the rapid hydrolysis of peptide bonds containing this amino acid, and either the subsequent diffusive loss or decomposition of free aspartic acid (Bada 1991). The decrease in serine content is profound and is consistent with the known geochemical instability of this amino acid (Bada *et al*. 1978; Akiyama 1980). Alanine is a major product of serine decomposition and this reaction accounts for the increased alanine content of the fossil vs modern eggshell. The enantiomeric ratios (figure 1*b*) demonstrate that amino acids in the eggshell matrix are completely racemized in about 10^5 years in environments such as Olduvai Gorge, and that this racemic signal is retained in the eggshell for time periods of at least 6×10^5 years. (We also analysed ostrich eggshell from Lower Bed IV (age 8×10^5 years) at Olduvai Gorge, and the amino acids were also found to be racemic.) The presence of racemic amino acids in the Pleistocene eggshells confirms previous findings that biogenic carbonates such as eggshell are an excellent biomineral matrix for amino-acid preservation (Brooks *et al*. 1990; Miller *et al*. 1991, 1992).

In contrast to the eggshell results, the differences in composition of the Pleistocene bones in comparison to the modern specimens are more difficult to rationalize simply in terms of amino-acid diagenetic alteration reactions (figure 2*a*). Only a small fraction ($<0.2–0.3\%$) of the original amino acids remains in the Pleistocene samples compared to that in modern bones. Aspartic and glutamic acids are relatively enriched in the Pleistocene bones in comparison to modern bone, glycine and alanine are relatively decreased, while valine, leucine, and most notably serine, are nearly unchanged. The diagenetic alteration changes seen in eggshell, such as serine decomposition to yield alanine and loss of aspartic acid, are not found in the Pleistocene bones. The amino-acid compositional alterations of the Pleistocene bones are most likely explained by the following processes and reactions: leaching of collagen and its hydrolysis products from the bone matrix; selective preservation of minor non-collagenous bone protein components; introduction into the bone hydorxyapatite matrix of amino-acid-containing contaminants. especially aspartic and glutamic acids, from the surrounding environment (King & Bada 1979; Hare 1980; Bada 1985*a*; Masters 1987). Evidence that secondary contaminants are present is provided by the enantiomeric ratios (figure 2*b*), especially in the $4–6 \times 10^5$-year-old sample in which aspartic acid is the least racemized amino acid. The pattern of D:L ratios in the Pleistocene bones thus differs from that (e.g. D:L Asp > D:L Ala > D:L Val) predicted from the mechanism of amino-acid racemization and from laboratory studies of heated modern bone samples (Bada *et al*. 1973; Bada 1985*b*). The pattern that is observed is indicative of a mixture containing both endogenous and exogenous amino acids.

We conclude that bone is less likely than shell to retain its indigenous amino acids, and that the amino acids even in Pleistocene bones are a mixture of exogenous and endogenous components. In fact, based on the enantiomeric analyses of a large number of Pleistocene and Holocene age specimens (Bada *et al*. 1973, 1979; Bada 1985*a*), it appears that compared to shells, bones are such an open system with respect to the loss of original amino acids and the introduction of contaminants, that racemic amino acids in fossil bones are seldom observed. Tooth enamel seems to be a better matrix for preserving original amino acids than bone (Bada 1985*a*), although the number of investigations of this type of fossil material is fairly limited.

3. MODEL FOR DIAGENESIS AND PRESERVATION OF AMINO ACIDS IN FOSSILS

These results allow us to construct a general model for the preservation of amino acids in fossil specimens (figures 3 and 4). This model should be applicable to other biomolecules, such as DNA, whose diagenesis is influenced by some of the same variables and environmental factors as amino-acid diagenesis. Amino acids present in the biomineral matrix are initially contained in

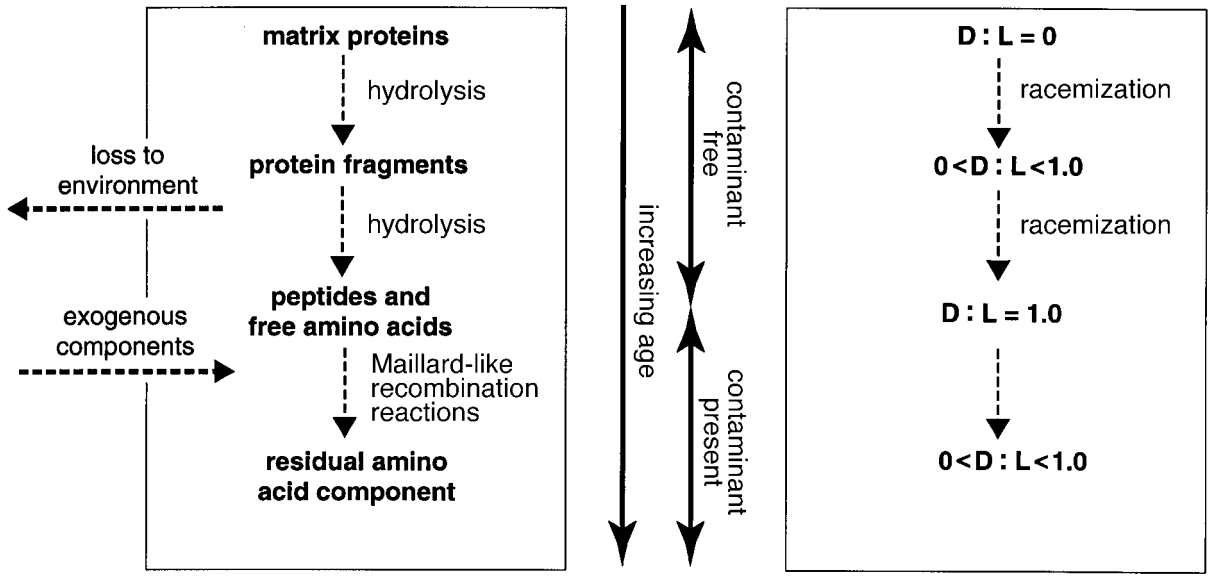

Figure 3. A model showing the diagenesis of the proteins and their constituent amino acids in the fossil matrix. As peptide bond hydrolysis takes place (left), the released amino acids can diffuse out of the matrix and are lost to the environment over geological time. Exogenous components can enter the fossil matrix and may react with remnant endogenous amino acids to form geopolymeric material similar to humic acids. The original L-amino acids present in the specimen undergo racemization (right), following a defined pattern of racemization rates (D:L Asp > D:L Ala > D:L Val). Racemization should be complete in < 1–5 million years in most environments on the Earth. The addition of contaminants would lower and distort the expected extent and pattern of racemization for the various amino acids.

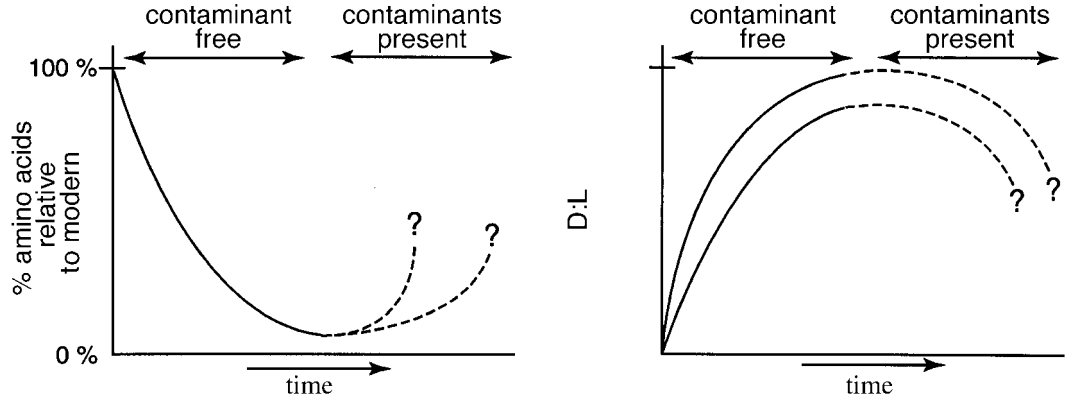

Figure 4. The effect of the various diagenetic reactions and processes shown in figure 3 on the amino-acid content (left) and the amino acid D:L ratios (right) of fossil specimens. In contamination-free samples, amino acids are steadily lost from the fossil matrix and the extent of racemization increases, eventually resulting in a racemic mixture. Contamination introduced into the fossil could result in an increase in the amino-acid content, and lower than expected D:L ratios. Well-preserved fossils would be expected to fall on the solid line, whereas those which are contaminated would fall on the dashed lines.

proteins, which, as they undergo hydrolysis, liberate free amino acids and small peptides. The half-life for peptide bond hydrolysis in the biomineral matrix in temperate environments is estimated to be in the order of 10^5 years or less (Bada 1991), varying somewhat with the type of biomineral. The free amino acids released by hydrolysis can be lost to the surrounding environment, or, as in the case of serine and possibly aspartic acid, undergo decomposition. As a result, the total amino-acid content of a fossil decreases with time. The carbonate matrix of shell retains original amino acids and their diagenetic products better than the hydroxyapatite matrix of bone. Secondary amino-acid contaminants may be introduced into the biomineral matrix from percolating groundwater, and during alteration and recrystallization of the inorganic components of the biomineral. The rate of introduction of contaminants is a function of the type of biomineral matrix. For example, compared to shell, bones are apparently more porous with respect to both the loss of original amino-acid components and the introduction of exogenous amino acids.

Racemization of the amino acids originally present in the biomineral matrix also takes place and the extent of racemization is a measure of overall biomolecule degradation and preservation. The relative rates of racemization of the various amino acids should follow a predictable sequence (e.g. D:L Asp > D:L Ala > D:L Val). If no contaminants have been introduced into the system, based on the racemization half-lives determined from known age fossils, all amino acids should be totally racemized in < 5–10 million years in cold depositional environments; in temperate regions a racemic mixture of

all amino acids would be attained in <1 million years (Bada 1985*a*, 1991). Contamination introduced into the fossil would be enriched in L-amino acids, and these exogenous amino acids would not exhibit the same relative proportions as those originally present in the biomineral matrix. Contamination would thus lower the enantiomeric ratios produced via racemization, and thus the relative extent of racemization of the various amino acids would not correspond to the pattern predicted by the racemization mechanism.

The extent of amino-acid racemization can be used to predict the survival of ancient DNA. Investigations have shown that when the D:L aspartic acid ratios are <0.1–0.15, and alanine is less racemized that aspartic acid, meaningful DNA sequence information is preserved (Poinar *et al*. 1996). This extent of aspartic-acid racemization, along the racemization rates estimated from fossils of known age (Bada 1985*a*, 1991), predicts that DNA should only be preserved for periods of only a few thousand years in temperate regions and no more than 10^5 years in colder, high latitude areas. This conclusion is in close agreement with the DNA survival times under geochemical conditions estimated from aqueous solution studies (Pääbo & Wilson 1991; Lindahl 1993, 1997).

Amino acids, the free amino group of peptides and proteins, and the bases in DNA with exocyclic primary amino groups (cytosine, adenine and guanine) may undergo Maillard-type condensation reactions with sugars, including the sugars at apurinic sites in DNA, and thus become incorporated into melanoidin or humic acid-like compounds (see Bada 1998, and references therein). Amino sugars present in the matrix (such as chitin), or introduced into a fossil specimen as contaminants, would also be components in this reaction because they contain both sugar and amine components. The Maillard reaction products include high molecular weight components (several thousand daltons), collectively known as melanoidins, which have characteristics similar to humic acids. An important aspect of these humic-acid like geopolymers is that they may be much more stable with respect to hydrolysis, especially when an aminoketose produced by the Amadori rearrangement is the major product. Thus, whereas proteins and DNA are generally rapidly hydrolysed into small peptides and short DNA fragments, when these biomolecules are captured and bound in Maillard reaction products they are probably much more resistant to hydrolysis, and could thus be preserved over longer geological time periods. Because the Maillard reaction could result in the incorporation of both original components and contaminants into the humic acid-like material, fossil specimens may contain endogenous and exogenous components in the form of these high molecular weight geopolymers.

4. AMINO ACID AND BIOMOLECULE PRESERVATION IN SOME REPRESENTATIVE FOSSIL SPECIMENS

(a) *Human remains*

Obtaining original biomolecular information from ancient human skeletal material is perhaps the 'Holy Grail' of ancient biomolecule research, and amino-acid preservation information plays a central role in these efforts. Given the small sample requirement and the rapid processing time (with a new 'one-pot' sublimation-based method this is only few hours (Glavin & Bada 1998)), amino-acid screening provides an easy method for evaluating the potential for DNA survival in fossil samples. Even if valuable human material is considered too precious for these preliminary analyses, associated faunal material can be used to make the initial assessment.

It is now recommended that before PCR-based DNA amplification is attempted on valuable human remains the extent of amino-acid racemization be evaluated in order to determine the prospect that the sample contains endogenous DNA (Poinar *et al*. 1996). This racemization screening approach was recently used prior to the successful amplification of DNA from the Neandertal-type skeleton discovered in the last century near Düsseldorf, Germany (Krings *et al*. 1997).

A wealth of amino-acid data on human remains already exists because of the extensive investigations that have been carried out on racemization dating. This information in turn can be used to predict which human skeletons may have retained potentially useful DNA sequence information. Although the German Neandertal skeleton had a D:L aspartic acid ratio (e. g. 0.12) consistent with DNA preservation, most others from this critical period of human evolution probably do not. For example, the Middle Eastern skeletons from the Tabun, Qafzeh and Skhul sites (Masters 1982), as well as other Pleistocene age skeletons France and Spain (Cooper *et al*. 1997), have D:L aspartic-acid ratios >0.3 indicating extensive biomolecule degradation. Based on the Olduvai Gorge results presented in figure 2, because of the warm environmental temperatures, skeletons from sub-Saharan Africa, such as those from Border Cave in Swaziland (D:L Asp *ca*. 0.5–0.6; J. L. Bada, unpublished data), should contain extensively racemized aspartic acid and are thus clearly unsuitable for ancient DNA analyses.

Most palaeoindian remains of interest with respect to the settling of the New World also do not show much promise with respect to the retrieval of meaningful DNA information. For example, skeletons from Southern California coastal sites, such as Del Mar Man and others which have ages in the 5–9×10^3 years range in general have D:L ratios >0.14 (Bada 1985*a*). Although no racemization measurements have yet been carried out, an interesting exception may be the 'Kennewick Man' from Washington state, dated at 7880 ± 160 years, which apparently has excellent collagen preservation (Taylor *et al*. 1998).

Other exceptions where original amino acids and DNA might be preserved over time periods longer than expected would be skeletons from frozen environments and cold high latitude sites. One of the best known examples is the 5×10^3-year-old 'Ice Man' found in an Alpine glacier (Handt *et al*. 1994; Macko *et al*., this issue). Another interesting possible site is the Upper Cave at Zhoukoudian, China. Preliminary analyses of one of the Upper Cave skeletons which as an AMS-based radiocarbon age of 3×10^4 years yielded a D:L aspartic acid ratio of 0.1 (J. L. Bada, unpublished data), in the range where DNA would still be predicted to be preserved.

(b) *Amber-entombed insects*

Analyses using HPLC with fluorescence detection have found that the amino-acid content of amber-entombed

insect tissue is much higher than that of the surrounding amber matrix (Bada *et al*. 1994). The amino-acid compositions and enantiomeric ratios of insect tissues obtained from the various age copal and amber specimens were found to be essentially identical to those of modern insects (Bada *et al*. 1994; Wang *et al*. 1995; Poinar *et al*. 1996). There appear to be no significant changes in the overall amino-acid composition of the insect tissue either during the initial encapsulation of insects in tree resins or over the long geological period the insects have been entombed in amber. Even though serine is one of the most unstable amino acids, it is still preserved in amber-entombed insect tissues. These results are in conflict with the recent report that protein remnants, and by implication amino acids, were not detectable by pyrolysis-gas chromatography/mass spectrometry (py-GC/MS) in insect specimens obtained from Dominican amber (Stankiewicz *et al*. 1998). However, the HPLC method is far more sensitive in comparison to py-GC/MS. Taken together, the results obtained by the two methods are best interpreted as indicating that some level of amino-acid preservation is present in amber-entombed insects although there may be significant alteration of both the original proteins and amino acids, possibly because of the formation of humic-acid like condensation products.

In amber-entombed insects, no significant amino-acid racemization could be detected in comparison to the modern specimens (Bada *et al*. 1994; Wang *et al*. 1995; Poinar *et al*. 1996). The half-life for aspartic-acid racemization for insects entombed in amber is estimated to be about 1×10^9 years. This half-life is 10^4 to 10^5 times longer than the aspartic-acid racemization half-lives measured in fossil bones and shells from temperate environments. The slow amino-acid racemization rate in insect tissues preserved in amber has been attributed to the anhydrous nature of the amber matrix (Bada *et al*. 1994).

The retardation of amino-acid decomposition and racemization in insects encased in amber suggests that the degradation of other biomolecules might also be inhibited. If DNA depurination is retarded by more than a factor of 10^4 to 10^5, as is aspartic-acid racemization in the amber insect inclusions, then DNA fragments containing many hundreds of base-pairs could be preserved over time-scales of 10^7 to 10^8 years. However, efforts to verify that DNA is indeed preserved for extraordinary periods of geological time in amber-entombed organisms have yielded conflicting reports and the issue remains unsettled (for example, see Cano *et al*. 1993; DeSalle *et al*. 1992; Lindahl 1993, 1997; Austin *et al*. 1997). From an amino-acid preservation perspective, DNA survival in amber-entombed insects, even for a period of 10^8 years, is not unreasonable.

(c) *Dinosaur specimens*

The survival of endogenous biomolecules in dinosaur specimens is also controversial. Based on the model shown in figures 3 and 4, the possibility that dinosaur remains contain original amino-acid constituents and DNA sequence information would seem highly unlikely. This inference has not deterred biomolecular and stable isotopic investigations of dinosaur material (for example, see Woodward *et al*. 1994; Muyzer *et al*. 1992; Ostrom *et al*. 1993; Schweitzer *et al*. 1997). However, these results are compromised by problems of endogeneity and contamination. An illustration is the report of Bocherens *et al*. (1988) that a 'collagen' fraction isolated from an *Anatosaurus* bone had a $\delta^{13}C$ value of $-15‰$, which implies that this dinosaur had a diet consisting mainly of C_4 plants. However, this carbon photosynthetic fixation pathway only developed roughly 15–20 Ma ago (Ehleringer *et al*. 1991), long after the extinction of the dinosaurs.

To further investigate biomolecule preservation in dinosaur specimens, we carried out amino-acid analyses of some late Cretaceous age dinosaur bones and eggshells (these were obtained from Dr Mark Goodwin of the Museum of Paleontology, University of California, Berkeley, CA). These samples were subjected to the same type of amino-acid analyses used in the investigations of the Pleistocene eggshell and bones specimens.

The dinosaur eggshells (data not shown) were found to contain roughly 0.1–1% of the amino acids present in modern ostrich eggshells. However, the expected compositional changes observed during diagenesis of Pleistocene eggshells (see figure 1) are not observed in the dinosaur eggshells in that serine was found to be one of the more abundant amino acids present. In addition, all of the detected amino acids have low D:L ratios, and alanine is more racemized than aspartic acid. The presence of relatively unracemized amino acids and abundant serine in our dinosaur eggshell samples indicates that the amino acids are exogenous contaminants which were added fairly recently based on the predominance of L-amino acids. Amino-acid analyses of other dinosaur eggshells from France have found D:L alanine and D:L valine ratios near 1, and the amount of serine was below detection, so original amino acids may be still preserved in some dinosaur eggshell specimens (Shimoyama *et al*. 1989).

The dinosaur bones we analysed (figure 5*a*) have amino-acid contents similar to the Pleistocene bones, but their relative compositions resemble neither the modern nor the Pleistocene bones, nor can the compositional patterns be readily explained by invoking diagenetic alteration pathways. Surprisingly, the dinosaur bones were found to contain extensively racemized amino acids (figure 5*b*), suggesting that these amino acids are ancient. The *Tyrannosaurus* bone from Montana contains highly racemized aspartic acid and alanine (the amount of valine was too low to permit enantiomeric determination), and it has an amino-acid composition which somewhat resembles modern bone protein fractions in that glycine is the major amino acid. Why collagen remnants would be preserved in this dinosaur bone and not in the Olduvai Gorge samples shown in figure 2 is unclear, however. The *Tyrannosaurus* bone may contain trace amounts ($<10^{-2}$% of that present in modern bones) of endogenous amino-acid components, along with exogenous contaminants.

The *Edmontosaurus* bone is the most unusal dinosaur sample we analysed. Overall preservation of amino acids in this specimen is poor, with <0.1% of the amino acids present in modern bone being retained. Serine is the least abundant amino acid and the amino-acid composition is dominated by glutamic acid, valine and leucine, which account for 80% of the seven amino acids we investigated. There are no known bone proteins that have

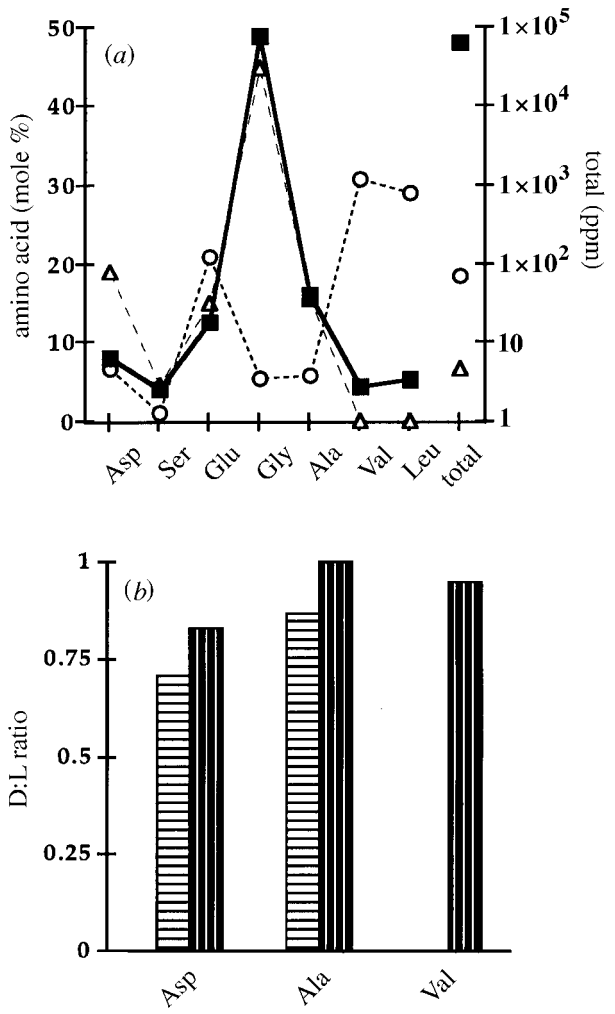

Figure 5. (*a*) The amino-acid content (in ppm) and composition (expressed as mole % of the listed amino acids) in dinosaur bones. Solid squares, modern mammalian bone; open triangles, *Tyrannosaurus* bone from Montana; open circles, *Edmontosaurus* bone from the north slope of Alaska. (*b*) The extent of racemization of aspartic acid, alanine and valine in dinosaur bone. Columns (left to right): first, *Tyrannosaurus* bone from Montana; second, *Edmontosaurus* bone from the north slope of Alaska.

amino-acid sequences dominated by these amino acids (Hall 1990). Peptide bonds of hydrophobic amino acids such as valine and leucine are very resistant to hydrolysis (Bada 1991), so it is possible that endogenous peptides enriched in these amino acids were retained in the *Edmontosaurus* bone, perhaps in humic acid-like condensation products (Bada 1998). Or this unusual mixture of amino acids may be simply derived from the selective accumulation and preservation of contaminants. The nearly racemic D:L ratios, especially the finding of nearly racemic valine, suggest that the amino acids in this dinosaur bone are very ancient, but the compositional data indicate that they may not be solely derived from bone proteins. Even though this specimen was recovered from a permafrost zone in Alaska, the early depositional environment was cool and temperate, with a temperature range of 2–8 °C (Clemens & Nelms 1993). At an average temperature of 5 °C, it is estimated that complete racemization of aspartic acid, alanine, and valine would take <10 million years. At subfreezing temperatures, racemization rates could be considerably slower because the system may be analogous to anhydrous conditions (Bada & McDonald 1995). In this case, complete racemization, especially of valine, may require more than 50 million years. Thus, the amino acids in the *Edmontosaurus* bone are at least several million years old and possibly even older, but they are possibly a mixture of both endogenous bone amino-acid components and ancient contaminants.

In summary, the dinosaur eggshells and bones we analysed contain only trace amounts, if any, of their original amino acids, along with exogenous components. Any endogenous amino acids that are present have undergone extensive diagenesis as evidenced by the presence of highly racemized amino acids. The general lack of unaltered, original amino-acid components and the presence of contaminants in these dinosaur samples implies that the possibility is remote that other biomolecular information such as nucleic acid and protein sequences would be preserved.

5. FUTURE POSSIBILITIES

Some of the lesser studied diagenetic reactions of some key biomolecules may be useful in providing additional ways of evaluating the level of original biomolecule survival under geochemical conditions. In addition, methodology development useful for investigations of biomolecules of evolutionary interest is in a stage of rapid advancement. Many of the new techniques have exceedingly low detection limits, in some cases allowing for detection at the single molecule level. The following are some examples.

(a) *Racemization of L-isoleucine and D-alloisoleucine at the β-carbon*

The racemization reactions at the β-carbon of L-isoleucine and its α-carbon epimerization product D-alloisoleucine, which yields D-isoleucine and L-alloisoleucine, are considerably slower than any other amino-acid racemization reactions (see Bada *et al*. (1986) for a discussion of these reactions and the structures for the various isoleucine stereoisomers). The racemization of L-isoleucine and D-alloisoleucine at the β-carbon has a half-life of roughly 10 million years in temperate environments, and perhaps as long as 100 million years at high latitude temperatures. Thus, the presence of D-isoleucine and L-alloisoleucine in fossil specimens would be an excellent indicator that some ancient components are preserved. Preliminary analyses of 3–4-million-year-old elephant enamel from Valdarno, Italy, indicate that D-isoleucine and L-alloisoleucine are present (M. Zhao and J. L. Bada, unpublished data). This result provides more evidence that tooth enamel is an excellent type of material for the long-term preservation of original biomolecules and their diagenetic products. Because of its widespread occurrence in the fossil record, tooth enamel clearly warrants further investigation.

The β-carbon isoleucine racemization products also appear to be present in the north slope of Alaska *Edmontosaurus* bone discussed above, which provides further evidence that at least some of the amino-acid components are very ancient. The problem still remains, however, of

determining whether the D-isoleucine and L-alloisoleucine in this late Cretaceous dinosaur bone are indeed ancient endogenous components or are ancient contaminants that have in turn undergone racemization.

(b) *Humic acid cleavage reagents*

The incorporation of endogenous biomolecules such as amino acids, peptides and DNA fragments into humic acid-like geopolymers which are preserved in the fossil matrix raises the intriguing possibility that some of the original molecules might somehow be retrieved. Extensive investigations of the *in vivo* non-enzymatic glycosylation of proteins and nucleic acids (generically known as the Maillard reaction) have been carried out because of the possible role of this reaction in ageing, in diseases such as cancer, and in the health complications associated with diabetes. It has been found that *in vivo* generated glycosylated proteins accumulate in long-lived tissues such as the eye lens nucleus (Monnier & Cerami 1981). Because of its possible deleterious effects to living organisms, efforts have been made to find ways of either mediating or reversing the Maillard reaction process. Of particular interest with respect to ancient biomolecule research is the use of reagents such as N-phenacylthiazolium bromide (PTB) which have been used to cleave Maillard or melanoidin products generated from the condensation of proteins with glucose (Vasan *et al*. 1996). After treatment with the cleaving reagent, the intact original protein (in this case, bovine serum albumin) was released. A similar approach may be useful in extracting endogenous biomolecules from fossil specimens. For example, the formation of humic acid-like condensation products in amber-entombed insects may be one of the reasons why efforts to replicate claims of successful DNA amplification in these interesting fossil specimens has proven to be so difficult, and the use of humic acid cleaving agents may help resolve this conflict. Some preliminary promising results in this area have in fact recently been obtained. Poinar *et al*. (1998) have shown that after treatment of a coprolite specimen with PTB, DNA with sequences characteristic of the source animal (in this case an extinct sloth) could be obtained whereas no DNA could be retrieved before treatment with the reagent. It is interesting to speculate that the use of humic acid cleaving agents on a variety of fossil specimens might result in the recovery of short intact peptides and nucleic acid sequences which previously were not accessible for study. This is obviously an exciting area for future ancient biomolecule research.

(c) *Microchip-based capillary electrophoresis separation and sequencing*

Direct analyses for the presence of protein–peptide and DNA sequences in fossil specimens could provide an additional means of evaluating the geochemical survival of portions of these key biomolecules in the fossil record. However, because in many cases only minute amounts of these biomolecules may be preserved (perhaps on the order of only a few thousand molecules or less), this has so far not been technically feasible. A relatively new technology which shows promise is microchip-based capillary electrophoresis (CE). The actual separation hardware, including buffer reservoirs and derivatization–reaction chambers, can be etched onto glass microchips with dimensions in the order of centimetres. Numerous channels can be etched onto the microchip which allows for the multiple separations and analyses to be performed in only a few minutes. The reagents, sample and solvents can be manipulated using electro-osmotic forces with no need for mechanical pumps or valves. Sensitive detection methods such as laser-induced fluorescence (LIF) or electrochemical detection can be used in a microchip CE system to achieve extremely low detection limits, sometimes in the range of a few molecules. An example for potential use in ancient biomolecule research is DNA separation–PCR amplification on a microchip (Woolley *et al*. 1996, 1997; Kopp *et al*. 1998). The separation of DNA fragments, followed by their amplification with PCR and the sequencing of the amplified sequences, has been completely achieved on a single microchip. A system such as this, for example, could be used to hunt for preserved ancient DNA directly in insect tissues obtained from amber, and thus perhaps help settle the controversy of whether DNA sequence information is indeed present in amber entombed insects. Similar protein separation–sequencing microchip CE-based systems are under development, and in the future these could be used to search for intact preserved peptides derived from specific proteins in a variety of fossils.

6. CONCLUSIONS

The geochemical reactions of amino acids have been thoroughly studied, and this information can be used to predict the level of survival of other important biomolecules such as DNA. Amino-acid analyses can be easily carried out and provide the bases of a routine method for evaluating the general level of biomolecule preservation in fossil specimens. The model we have developed based on the analyses of Pleistocene fossils predicts that original protein components should be hydrolysed to free amino acids which then diffuse out of the fossil matrix and are lost over time-scales of 10^5–10^6 years in most environments. Amino acids should also undergo complete racemization in <1–10 million years. Contaminants from the surrounding environment may be introduced into the fossil matrix. Condensation reactions involving both endogenous and exogenous molecules may result in the formation of humic acid-like components. The unravelling of this complex series of molecules, reactions and processes represents a major challenge in ancient biomolecule research. New techniques such as the use of humic acid cleaving reagents and microchip-based analyses will play a pivotal role in future research efforts.

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Discussion

R. P. Ambler (*University of Edinburgh, UK*). How dry is dry? Does one have any quantitative measure of the amount of water left in (say) amber or bacterial spores? One wants to know how many molecules of water there are left per amino acid residue or nucleic acid base.

J. L. Bada. I wish there was some way to measure the activity of water in the amber-entombed insect tissues. What we do know is that if you dry amino acids and DNA in a vacuum desiccator, heat the dry residues and measure both the racemization and depurination rates, they are several orders of magnitude slower than in aqueous solution. These observations have in turn been used to suggest that the lack of racemization in the amber insect tissues is because they are anhydrous. But, I agree with you that we really do not know how 'dry' they actually are.

P. Finch (*Royal Holloway, University of London, UK*). Regarding the suggestion that Schiff base formation might 'stabilize' DNA after depurination is there any evidence? The expectation from organic and biochemistry is that Schiff base formation would accelerate DNA cleavage by phosphate elimination.

J. L. Bada. The Schiff base formed by the reaction of a primary amine and the sugar at a depurinated site would rapidly undergo the Amadori rearrangement. My suggestion is that the Amador product could inhibit the β-elimination reaction which breaks the phosphodiester bond and cleaves the DNA backbone.

D. R. Grocke (*University of Oxford, UK*). You discuss in your lecture some work you did on dinosaur bones from the North Slope, Alaska, and mention that the amino-acid results represent an exogenic signature. I have done some high molecular-weight isotope analyses on dinosaur material from the North Slope and found C_4 $\delta^{13}C$ signatures and a possible trophic structure on the basis of $\delta^{15}N$ values. Are these values representative of the exogenic signature or are they original signals? I state this in light of the work by Bocherens *et al.* (1994) who also record C_4 $\delta^{13}C$ values in Late Cretaceous dinosaur bones and plant material. In addition, Hasegawa (1997) and Grocke (1998) suggested that the $\delta^{13}C$ values of fossil terrestrial-derived organic matter reflect fluctuations in atmospheric CO_2, and are thus ultimately controlled by changes in the oceanic $\delta^{13}C$ reservoir. (Reference material: Bocherens *et al.* 1994; Hasegawa 1997; Grocke 1998.)

J. L. Bada. As I discuss in my paper, dinosaur specimens with $\delta^{13}C$ signatures characteristic of C_4 plants indicate contaminants are present because the C_4 photosynthetic pathway did not appear until about 15–20 million years ago (Ehleringer *et al.* 1991). In fact, the presence of the distinctive C_4 isotopic signal indicates that the exogenous components in these dinosaur specimens are less than 15–20 million years old. One problem I see with the North Slope samples is that C_4 plants probably have never grown or been abundant in this high latitude environment, so where did the C_4 $\delta^{13}C$ signature come from? It seems unreasonable that the $\delta^{13}C$ signature of atmospheric CO_2 could have been so enriched in ^{13}C during Cretaceous times that this is the reason for the dinosaur heavy carbon isotopic signatures. I base this comment on the $\delta^{13}C$ values of Cretaceous age amber which is only a few ‰ heavier in ^{13}C than modern-day tree resins (Bada & Stolp 1995). The dinosaur specimens from the North Slope of Alaska truly seem to be unusual in many ways and should be investigated further.

G. Eglinton (*University of Bristol, UK*). Could you get a more quantitative evaluation of the actual amino-acid content of the insect tissue in amber compared to the living insect? If the preservation is as good as you imply from the lack of racemization, then presumably the amino-acid yield in the amber-entombed insect issue should be similar to those of living insects.

J. L. Bada. Because the amber-entombed insect tissue samples are so small (on the order of tenths of a milligram), they are very difficult to accurately weigh. We have however made crude attempts at this, and find that in the insect tissues from amber the total content of Asp + Glu + Gly + Ala + Val was about 0.1–1% of the tissue weight (Wang *et al.* 1995). In dried modern insect tissues, the total content of these amino acids is 15–20%. Thus, in amber-entombed insects, there is an apparent loss of $>90\%$ of the original amino acids. However, the overall amino-acid composition of the amber-entombed insects is generally similar to that of modern insects. Even though the amino-acid content is lower, the composition is essentially unaltered in the amber-entombed insects compared to the living ones.

G. Eglinton (*University of Bristol, UK*). Hydrolysis with water is only part of the possible damage to both amino acids and DNA in the amber insects. Oxygen would be expected to diffuse into the amber inclusions quite easily and hence reduce the yields of amino acids through oxidation reactions. DNA should also be damaged by these reactions and this could be why there have been difficulties in recovering DNA from the amber insects.

J. L. Bada. I agree that hydrolysis is only part of the story. The loss of amino acids we see in amber-entombed insects may in fact be due to oxidation. Oxidation damage of DNA may inhibit PCR amplification and this could be part of the problem in obtaining ancient DNA from insects encased in amber. As Lindahl has noted, perhaps some other amino-acid reactions such as the oxidation of methionine could be used to assess the extent of oxidation damage (Lindahl 1996). Unfortunately, analyses for methionine and its oxidation products are a lot more difficult than amino-acid racemization measurements.

S. A. Macko (*University of Virginia, USA*). As you know, Peggy Ostrom (Ostrom *et al.* 1990, 1993) has shown that on the organic material isolated from well-preserved Cretaceous reptile fossils, there exist amino acids and the material shows ^{15}N signals consistent with a trophic structure. Simple modern or ancient bacterial contamination would not maintain this order. Further, a criterion that Mike Engel and I established (Engel & Macko 1986) for assessing contamination of amino acids in fossils uses

the fact that the stable isotopes of the amino-acid stereoisomers should be identical in uncontaminated materials. Perhaps that assessment could be used in your amino-acid extracts from the fossils. Additionally, we have observed that on fossils that are simply 'cleaned', the penetration by low molecular-weight contaminants can alter the ^{15}N and ^{13}C signal. For the Cretaceous fossils, we determined that the isolation of a high molecular-weight material (>5000 Da) was necessary and it was on this material that we made the isotope assessments. Perhaps this isolation procedure is the reason for differences between our observations.

J. L. Bada. I am aware of the Ostrom reports. What strikes me about these results is that the pattern of D:L ratios of the various amino acids is not consistent with that expected from racemization. For example, alanine was found to be more highly racemized than aspartic acid. This suggests to me that these are not original amino acids. Compared to the nearly racemic amino acids we found in the *Edmontosaurus* bone, the amino acids in the bones analysed by Ostrom *et al*. (1990, 1993) appear to be fairly recent in origin, based on their extent of racemization.

Another problem has to do with carbon and nitrogen isotopic signals reported by Ostrom *et al*. (1990). One of the distinctive features of the modern systems is that the marine environment is enriched in both ^{13}C and ^{15}N in comparison to terrestrial and freshwater environments. All of the Cretaceous aquatic fauna analysed by Ostrom *et al*. have similar carbon and nitrogen isotopic signatures, suggesting that there were no isotopic differences between marine and freshwater Cretaceous ecosystems like there is today. I am not sure why this should be the case. Again, I feel the problem is that these dinosaur bones contain a mixture of endogenous and exogenous components which have scrambled isotopic signatures.

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