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Amino Acid Racemization and the Preservation of Ancient DNA

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The extent of racemization of aspartic acid, alanine, and leucine provides criteria for assessing whether ancient tissue samples contain endogenous DNA. In samples in which the D/L ratio of aspartic acid exceeds 0.08, ancient DNA sequences could not be retrieved. Paleontological finds from which DNA sequences purportedly millions of years old have been reported show extensive racemization, and the amino acids present are mainly contaminates. An exception is the amino acids in some insects preserved in amber.

The invention of the polymerase chain reaction (*1*) has made it possible to determine DNA sequences from remnants of extinct species and past populations (*2, 3*). In addition, recent reports have claimed that DNA can be retrieved from paleontological finds that are millions of years old (*4*). However, because only a minority of ancient specimens contain amplifiable ancient DNA (*5*), false positives resulting from minute amounts of contaminating DNA pose a serious threat (*6, 7*). Although several ways to authenticate ancient DNA have been suggested (*2, 6, 8*), the field is in need of techniques that can indicate whether a particular ancient specimen may contain endogenous nucleic acids.

All amino acids used in proteins, with the exception of glycine (Gly), can exist in the form of two optical isomers, the D- and L-enantiomers, of which the L-enantiomer is used exclusively in protein biosynthesis. Once isolated from active metabolic processes, the L-amino acids undergo racemization to produce D-amino acids until eventually the L- and D-enantiomers of a particular amino acid are present in equal amounts. The rate at which racemization takes place differs for each amino acid and is dependent on the presence of water, the temperature, and the chelation of certain metal ions to proteins (*9*). Racemization is thus affected by some of the same factors that affect depurination of DNA, the major hydrolytic reaction responsible for the spontaneous degradation of nucleic acids (*10*). The racemization of aspartic acid (Asp), which has one of the fastest racemization rates, has an activation energy and rate constants over a wide temperature range (at neutral pH) that are similar to those for DNA depurination (*10, 11*). To test whether the extent of amino acid racemization is a useful indicator of the extent of DNA degradation in ancient specimens, we examined archaeological specimens from which DNA sequences have been retrieved (*12*). In order to ensure as far as possible that the samples used yield genuinely ancient DNA, we limited our analysis to nine cases that fulfill a number of criteria of authenticity (*2, 6, 8*) and we excluded human remains because of the inherent difficulty of recognizing contamination from contemporary humans (*2, 6, 8*). We also analyzed 17 samples, including some human samples, from which no ancient DNA sequences could be amplified.

No DNA sequences could be retrieved from samples in which the D/L Asp ratio was higher than 0.08 (Table 1), whereas all samples with D/L ratios below 0.08 yielded DNA sequences. Furthermore, there was a rough relation between the extent of Asp racemization and the length of the retrievable DNA sequences (Fig. 1). In samples in which the extent of Asp racemization was similar to that caused by the 6 N HCl hydrolysis procedure (D/L = 0.05), sequences between 140 and 340 base pairs (bp) could be amplified, whereas samples with greater amounts of racemization tended to yield only shorter DNA fragments.

No general correlation was observed between the age of the samples and the retrieval of DNA or the extent of racemization. However, of the nine samples that yielded DNA, seven stemmed from cold environments and four of them have been shown to contain smaller amounts of DNA damage than samples that do not yield amplifiable DNA sequences (*13*). On the basis of the racemization half-lives of Asp reported for bone in various climatic regimes (*9*), the finding that an Asp D/L ratio of about 0.1 is the limit for the retrieval of useful DNA sequences implies that the survival of DNA is limited to a few thousand years in warm regions such as Egypt and to roughly 10^5 years in cold regions. Such temporal limits for DNA retrieval are similar to those predicted from laboratory experiments (*10*). Aspects of amino acid preservation other than racemization do not show any correlation with DNA preservation (*14*).

Because the racemization of Asp is faster than that of other amino acids (*9, 11*), the extent of racemization of Asp, among the amino acids analyzed here, should be the greatest, followed by alanine (Ala) and leucine (Leu), if all amino acids are of the same age. In contrast, a D/L ratio for Asp that is lower than that for Ala or Leu indicates contamination by more recent amino acids. For the samples from which ancient DNA sequences could be retrieved (Table 1), the extent of racemization of Asp was always greater than that for Ala and Leu, however, no authentic DNA sequences could be retrieved from samples in which the racemization of amino acids did not follow this pattern. Thus, amino acid racemization provides a way to identify the large majority of ancient samples that are not expected to yield any ancient DNA. The usefulness of this technique is enhanced by the fact that samples of only a few milligrams are sufficient for the analysis, and the results can be obtained in only a few days.

Ancient DNA sequences that are purportedly millions of years old have been reported from dinosaur bones, Miocene plant fossils, and amber inclusions (*4*). The D/L Asp ratio in the Utah dinosaur bone

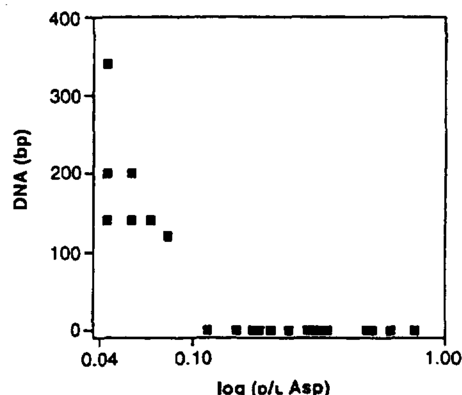

Fig. 1. Extent of Asp racemization plotted (as the logarithm of the D/L ratio of Asp) against the maximum length of DNA amplified (in base pairs).

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from which DNA sequences were reported (4) is 0.21, and is thus higher than for remains from which endogeneous DNA has been extracted (Table 2). Moreover, Ala is more racemized than Asp, an indication that the amino acids present in this specimen are a mixture of different ages. The poor preservation of the amino acids implies that no endogenous DNA should remain in this sample, which is in agreement with earlier results (7). The D/L Asp ratio of a *Tyrannosaurus rex* bone found in Montana (4) was 0.23, and Ala was more extensively racemized than Asp. Two dinosaur specimens from Antarctica, a cold depositional environment, also indicate substantial racemization as well as the presence of contaminating amino acids (Table 2). Thus, the prospects of retrieving DNA sequences from dinosaur fossils seem bleak.

Chloroplast DNA sequences have been reported from the approximately 17-million-year-old Clarkia deposit in Idaho (4). However, these results have been questioned (6–8). The amounts of Asp in the fossils are so low for several of the specimens that either the L or the D forms of Asp, or both, could not be accurately determined (Table 2) (15). However, both D- and L-Ala were detected, and in all cases, the D/L ratio for Ala was over 0.15, indicating extensive racemization. This result is consistent with the results of analyses of the preservation of lipids and other biopolymer (16) in Clarkia fossils, which indicate extensive microbial degradation.

The extent of racemization in some representative insects in amber and copal (Table 2) was close to what is caused by the hydrolysis procedure, an indication that little or no racemization has taken place. Because the amino acid concentration of the surrounding amber matrix is substantially

Table 2. Racemization of Asp, Ala, and Leu in paleontological samples from some of which DNA sequences have been reported. Samples for which the D/L ratios were difficult to determine because quantities of amino acids were too small are indicated as ND (20). The Utah and Montana dinosaur samples are identical to those from which DNA sequences have been reported (4) or in which the presence of DNA has been reported in the press. The Clarkia and amber specimens were similar to samples from which reported DNA sequences (4) stem. Ma, million years ago; ka, thousand years ago; ND, not determined. Gly/Asp ratios are given for bone samples only.

Sample	Age	D/L Asp	D/L Ala	D/L Leu	Gly/Asp
	Dinosaurs				
Tyrannosaurus rex (Montana)	65 Ma	0.23	0.59	ND	1.18
Dinosaur bone (Utah)	65 Ma	0.21	0.91	ND	1.78
Hadrosaur (Antarctica)	65 Ma	0.16	0.16	ND	3.33
Ornithomimidea (Antarctica)	65 Ma	0.17	0.44	0.48	0.26
	Clarkia				
Modern leaf, Clarkia (Idaho)	0 Ma	0.05	0.02	0.01	
Clarkia leaf 1	17 Ma	ND	0.15	0.00	
Clarkia leaf 2	17 Ma	ND	0.29	0.00	
Clarkia leaf 3	17 Ma	ND	0.42	0.05	
Clarkia leaf 4	17 Ma	ND	0.32	0.55	
Clarkia sediment 1	17 Ma	ND	0.15	0.00	
Clarkia sediment 2	17 Ma	ND	0.14	0.00	
	Amber				
Hymenoptera (Dominican Republic)	0.1 ka	0.08	0.01	0.00	
Hymenoptera (Colombia)	3 ka	0.03	0.02	0.00	
Hymenoptera (New Zealand)	30 ka	0.03	0.00	0.00	
Hymenoptera (Dominican Republic)	35 Ma	0.01	0.01	0.00	
Diptera (Baltic Sea)	35 Ma	0.05	0.01	0.00	

Table 1. The extent of racemization of Asp, Ala, and Leu and DNA amplifiability for 26 archaeological and paleontological samples. DNA was extracted, amplified, and sequenced as described in the references. Briefly, for nonhuman samples 140 bp of the mitochondrial 16S ribosomal DNA (rDNA), or 120 bp of the mitochondrial 12S rDNA, were amplified, whereas for human samples primers for an 87-bp fragment of the mitochondrial control region were used. In all cases in which no DNA could be amplified, extractions were performed as in (18) and at least two attempts were made under conditions allowing amplification from single template molecules. For bone samples, Gly/Asp ratios are given.

Sample	Age (10^3 years ago)	D/L Asp	D/L Ala	D/L Leu	DNA (bp)	Gly/Asp	Reference
Equus sp. (California)	0.05	0.05	0.01	0.00	340	0.27	
Mylodon darwinii (Chile)	13	0.05	0.00	0.01	140	6.27	(5)
Mammuthus primigenius (Yuribei, Siberia)	9.7	0.05	0.00	0.00	200		(8)
Equus ferus (Siberia)	42	0.06	0.01	0.01	140	6.52	
M. primigenius (Khatanga, Siberia)	50	0.06	0.01	0.00	200		(8)
M. primigenius (Shandrin, Siberia)	35–40	0.06	0.01	0.00	200		(8)
E. hemionus (Alaska)	27	0.07	0.01	0.00	140	2.01	(18)
Mylodon darwinii (Chile)	13	0.07	0.04	0.00	140	10.20	(5)
Aptornis sp. (New Zealand)	3	0.08	0.01	0.00	120	1.95	(19)
Bos primigenius (Europe)	6.5	0.11	0.12	0.11	0	9.7	
E. ferus (Germany)	5.5	0.15	0.00	0.00	0	1.59	
Nothrotherium shastense (New Mexico)	13	0.17	0.01	0.00	0		
Papio cf. *cynocephalus* (Egypt)	2.3	0.18	0.02	0.00	0	18.50	
E. caballus (Chile)	20	0.20	0.25	0.00	0	3.10	
Human femur (Egypt)	4.5	0.21	0.02	0.00	0	0.10	
Megalonyx (Florida)	13	0.24	0.85	0.00	0	5.04	
Human femur (Egypt)	4.5	0.29	0.01	0.00	0	0.06	
Human femur (Egypt)	4.5	0.29	0.12	0.00	0	0.16	
Human femur (Egypt)	4.5	0.30	0.00	0.00	0	0.92	
Human femur (Egypt)	4.5	0.31	0.02	0.00	0	0.94	
Megalonyx sp. (Florida) (tooth)	13	0.33	0.44	0.23	0	0.40	
Glossotherium sp. (Cuba)	15	0.34	0.29	0.01	0	1.73	
Acratocnus odontrigonus (Puerto Rico)	15	0.49	0.61	0.15	0	0.413	
Scelidon chiliense (Peru)	15	0.51	0.81	0.15	0	0.61	
Eremotherium mirable (Peru)	13	0.60	0.27	0.14	0	0.53	
Megalocnus sp. (La Brea, California)	15	0.75	0.53	0.24	0	1.89	

lower than that of the insect tissue (*17*), the amino acids detected are likely to be endogenous. The surprising preservation of the amino acid stereochemistry in amber-entombed insects may be due to the anhydrous nature of the amber matrix. Because depurination of DNA would similarly be inhibited by anhydrous conditions, an amber matrix may provide conditions conducive to the long-term preservation of nucleic acids.

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12. External surfaces of bone sections (~1 mm) were removed, and samples were ground under liquid nitrogen in a Freezer/Mill 6700 bone grinder (Spex Industries, Edison, NJ). Then 0.01 to 0.5 g were hydrolyzed in doubly distilled 6 N HCl for 24 hours at 100°C. Glassware was cleaned by immersion in 10 M HCl for 2 weeks, then rinsed in doubly distilled water and baked at 250°C for 1 week. Soft tissue samples were briefly rinsed in 0.01 N HCl and hydrolyzed as above. Subsequently, samples were dried under vacuum over NaOH. Bone samples were redissolved in doubly distilled water and desalted with a cation exchanger (50W-X8) (Bio-Rad) as in J. L. Bada, *Earth Planet. Sci. Lett.* **15**, 223 (1972). Soft tissue samples were dissolved directly in 0.4 M sodium borate. Amino acids were derivatized with *O*-phthaldialdehyde/*N*-acetyl-L-cysteine (OPA/NAC) and analyzed by high-pressure liquid chromatography (HPLC) (Gilson, Middleton, WI) with fluorescent detection as described in M. Zaho and J. L. Bada, *J. Chromatogr. A* **690**, 55 (1995). Mock samples were analyzed in parallel with each series of samples, and background amino acid concentrations were subtracted from sample values. For quantitation and accurate determination of enantiomeric ratios, a standard containing a racemic mixture of the selected amino acids was analyzed on the same day as the samples. To determine the extent of racemization caused by the experimental procedure, we analyzed five bovine serum albumin (Pharmacia) samples; they were found to have a D/L ratio for Asp of 0.034 ± 0.0035. Variation due to sample processing was tested by the analysis of 10 samples from the same bone, yielding a D/L ratio of 0.056 ± 0.0036. We investigated the relative fluorescence of OPA-derivatized D and L forms of Asp, Ala, and Leu by analyzing samples with D/L ratios of 1. The observed ratios (0.867, 0.85, and 0.80, respectively) were used to adjust values determined for the samples.
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14. For example, the total amounts of the six amino acids analyzed [Asp, serine (Ser), Ala, Gly, Leu, and valine (Val)] do not correlate with the retrieval of DNA sequences (H. N. Poinar, M. Höss, J. L. Bada, S. Pääbo, data not shown). Furthermore, a ratio of Gly to Asp of 5.5 or larger can be used as a rough estimate of the preservation of collagen in bone [H. Elster, E. Gil-Av, S. Weiner, *J. Archeol. Sci.* **18**, 605 (1991)]. However, DNA could be extracted both from bones in which collagen was preserved according to this criterion and from some in which it was not.
15. Only minute amounts of amino acids were present in these samples, and these values were not significantly different from those in the surrounding sediment. As a result, the D/L ratios for Asp could not be determined in most cases. The presence of bacterial amino acid decomposition products, for example, β-alanine and γ-amino-*n*-butyric acid, in these specimens also suggests bacterial contamination (J. L. Bada, unpublished observations).
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20. The Utah and Montana dinosaur samples were too small to allow for the removal of surface material. Instead, they were rinsed several times with 0.01 N HCl and then with doubly distilled water before hydrolysis. The remaining dinosaur bones were processed like the other bones (*12*). Clarkia sediments containing fossil leaves were opened in a hood, photographed, and rinsed with 0.01 N HCl. Leaves were then scraped off into sterile vials, hydrolyzed, desalted, and analyzed by HPLC (*12*). Surrounding sediments were similarly analyzed. Amber samples were processed as in (*11*).

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