Geochemical Evolution of Amino Acids in Dentine of Pleistocene Bears

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ABSTRACT A linear correlation was established between aspartic acid racemization ratio from cave bear dentine collagen and absolute dating. The high correlation coefficient obtained allowed age calculation through amino acid racemization. Aspartic acid and glutamic acid racemization kinetics have also been explored in dentine from a North American black bear (*Ursus americanus* Pallas). Three sample sets were prepared for kinetic heating experiments in nitrogen atmosphere: one water soaked, one with a water-saturated nitrogen atmosphere, and one without any moisture. It was possible to show that the presence of water is a factor controlling amino acid racemization rate. The aspartic acid in a heating experiment at 105°C shows an "apparent kinetics reversal" which can be explained by a progressive hydrolysis of amino acid chains (proteins and polypeptides). Because of the low potential of collagen preservation over long periods of time, the apparent kinetics reversal phenomenon will not affect the dating of old material where no traces of collagen remain. An apparent kinetics reversal was not observed in glutamic acid, which racemizates more slowly.

Amino acid racemization is well established as a dating method for mollusk shells; it has been applied to bones with generally less success,[1–12] and it has been less widely studied in teeth enamel[4] and dentine.[13–15] Extensive and very successful work has been done on aspartic acid racemization in modern human teeth for determination of age at death.[16,17]

In a great number of Spanish caves, abundant remains of fossil mammals appear. In many cases the fossils are associated with lithic artifacts of the Upper Paleolithic or ceramics that allow a precise dating. When the paleontological remains are associated with lithics of the Middle Paleolithic, the dating of the deposits is much more complicated. The difficulties notably increase when there are older sediments along with the lithic devices of the Lower Paleolithic and their dating becomes subjective. On the other hand, monospecific accumulations in the caves (bears, hyenas, lions, etc.) are very frequent, lacking faunal assemblages for biostratigraphical dating of the site.

Fossil bears are very common in the European paleontological record. Usually they appear as large monospecific bone and tooth accumulations without stratigraphical relationships. In some localities hominids are associated with the fossil bears.

Until now it has been possible to establish an amino chronological differentiation between Middle and Upper Pleistocene bear species (*Ursus deningeri* von Reichenau and *Ursus spelaeus* Rosenmüller-Heinroth),[18] and both Middle Pleistocene *U. deningeri* and Upper Pleistocene *U. spelaeus* aminozones have been established for the Iberian Peninsula. However, until now it has been impossible to transform racemization ratios into age calculations. To approach the aminochronological use of racemization ratios in bear dentine, we performed the present work on racemization-absolute dating correlation and experimental kinetics.

AMINO ACID RACEMIZATION AND AGE CALCULATION

In the application of amino acid racemization ratios for dating it is necessary to obtain "local mathematical models" based on calibration of amino acid racemization rates against well-established radioactive dating methods, such as ^{14}C and U/Th.[19]

It may be possible to use kinetic data for dating purposes because racemization follows a temperature-controlled first-order reversible kinetic model (FOK) and most of the paleontological localities are located in caves. Caves are ideal environments because perennial, saturated moisture is present in the fossil-bearing muddy sediments and a very stable thermal history can be expected, making it possible to ascertain their thermal history. In Spanish caves the air temperature change, during an annual cycle, was less than

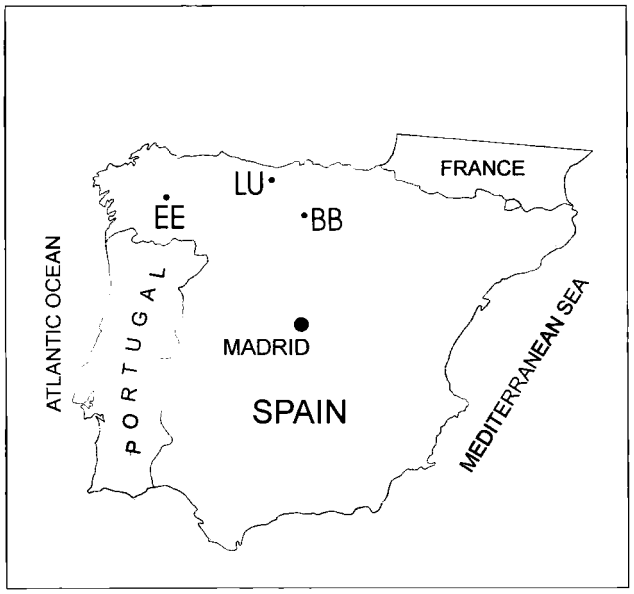

Fig. 1. Geographical situation of sampled localities.

0.5°C,[20] and would therefore be negligible inside the fossil-bearing sediment. In fact, some authors[4] base their age calculation method on an approximate constant calculation from a previously dated (radioactive or paleomagnetic methods) locality. This constant is later used for age calculation of other paleontological localities.

Before any discussion, we need to consider amino acid and protein preservation in the sedimentary environment. In Mollusca, Crustacea (mainly Ostracoda), and Brachiopoda shells, there are intercrystalline and intracrystalline proteins. Intracrystalline proteins in calcite brachiopod shells rapidly evolve to free amino acids,[21] which usually show low racemization rates. However, not only rapid peptide bond decomposition but also a strong leaching of free amino acids from the shell occurs in a time span as short as from the beginning of the Postglacial (10,000 BP) to the present.[22] In teeth, the lack of a hard mineral structure in dentine is an added problem for protein preservation. In spite of there being three components of teeth (enamel, dentine, and cement), we prefer to sample dentine because of its higher protein content and because in cement a modern contamination from handling cannot be discounted. Dentine, as noticed in cub bear tooth, is the first component to mineralize; enamel and cement mineralize later.

MATERIALS AND METHODS

To obtain age calculation algorithms, we analyzed cave bear samples from three different localities (Fig. 1): 1) A bone sample from Eirós cave (EE) was ^{14}C (AMS) dated,[23] resulting in a date of 24,090 ± 440 BP. 2) In the *U. spelaeus*-bearing bed from La Lucia (LU) cave, some small stalactites appeared and a thin flowstone 2–3 cm thick sealed the bone-bearing bed. The two calcite samples were U/Th-dated and the obtained data are summarized in Table 1. 3) A combination of electron spin resonance (ESR) and U-series dating methods on the remains of the Sima de los Huesos (BB) bear gave a probable date of 320 ± 4 kyr.[24]

For kinetics analysis, we employed *Ursus americanus* teeth from a skull that was purchased in 1986 which is exhibited in the Historical and Mining Museum of the Madrid School of Mines. Because crushing was not possible, powder samples from canines and lower molar roots were obtained with an odontologist radial milling cutter; a slight heating was unavoidable. We avoided sampling the pulp cavity, where dry soft tissues "phantoms" still remained.

We selected five canines or third upper incisors from each locality because their conic crown shape effectively protects dentine from contamination. When possible, we chose perfect specimens of intermediate age, neither extremely old nor extremely young, and not having worn enamel, an open pulp cavity, an open apex root, or thin dentine walls.

Fifty mg of powdered dentine samples were obtained from the innermost part of the crown via drilling the tooth with a dental diamond drill. Powder from the outer part of the root, up to a limit of 1 mm deep, was rejected. Cementum layers were never sampled. This process produced an unavoidable slight sample heating. We drilled near the crown/root boundary avoiding black-colored root zones. Tooth powder was stored in small plastic boxes new from the factory.

For kinetic experiments, 100 mg of tooth powder and 2 g of quartz sand were placed in glass test tubes (135 mm long and 13 mm wide) with screw caps and Teflon septa. Ultraclean water (120 µl) was added and the screw caps were closed under nitrogen atmosphere. Previously, the quartz sand was deeply cleaned by oven baking at 600°C for 6 h. A blank was prepared to check for spurious amino acid contamination.

Glassware, Eppendorf plastic micro-test tubes, plastic micropipette tips, and Pasteur pipettes were new from the factory. All the water used in the analysis was Milli-Q quality from Millipore (Bedford, MA). Chemicals were Merck (Darmstadt, Germany), HPLC, or spectroscopy grade.

A very important analytical improvement was dialysis to eliminate free amino acids. With this process, we aimed to remove foreign amino acids and to obtain a homogeneous protein (from collagen) molecule. We employed a modification of the method proposed by Marzin.[25] The powder sample, 50 mg of dentine, was dissolved in 1 ml of 2N hydrochloric acid and sonicated. After the addition of 5 ml PBS buffer, the sample was dialyzed at 3,500 Da (Spectra/Por mnco 3500 membrane) for 20 h in a buffered solution with magnetic stirring at room temperature. The samples were then prepared according to the method of Goodfriend and Meyer:[26]

1) Hydrolysis was carried out in 6N hydrochloric acid in test tubes with Teflon-lined screw caps closed under nitrogen atmosphere in a heating block at 100°C for 20 h. Next, after 4 min in an Eppendorf centrifuge the supernatant was transferred, frozen in liquid nitrogen, and vacuum-dried in a plastic desiccator. Samples were redissolved with distilled water. In a further step water was evaporated under vacuum.

TABLE 1. Radiometric U/Th ages of two calcite samples from La Lucia locality

Sample	U (ppm)	Th (ppm)	U234/U238	Th230/U234	Th230/Th232	Nominal age (yr)	Corrected age (yr)
LU-1	0.06	0.01	2.45 ± 0.05	0.58 ± 0.01	22.660 ± 1.432	86,064 ± 2,492	
LU-1	0.09	0.06	2.42 ± 0.10	0.70 ± 0.02	7.935 ± 0.291	112,083 ± 6,152	77,231 ± 3,305
LU-3	0.04	—	2.63 ± 0.07	0.54 ± 0.01	—	76,424 ± 2,700	

LU-1: Samples from a 3-cm thick flowstone directly overlying bone bed.
LU-3: Sample was taken from a fallen stalactite included in the bone-bearing mud bed 5 cm thick.

2) The first derivatization step of the amino acids was esterification with 3M thionyl chloride in isopropanol. The vials, under nitrogen atmosphere, were left to react on the heating block at 100°C for just 1 h. The second derivatization step was N-trifluoroacetylation with trifluoroacetic acid anhydride. The vials were tightly closed and heated at 100°C for just 5 min on the heating block. Next, the samples were dissolved in 125 µl of n-hexane, shaken, concentrated to a final volume of 15–25 µl, and transferred to injection vials.

3) A volume of 0.2 µl of recent bear sample *U. americanus* and *U. spelaeus*) or 2 µl of old bear (*U. deningeri*) sample were injected into a Hewlett-Packard 5890 gas chromatograph. We used helium as the carrier gas, at a column head pressure of 6 psi, and a Chirasil-Val fused silica column from Chrompack. The detector was an NPD set at 300°C. Integration of the peak areas was carried out using HP's PEAK96 integration program that runs on a PC computer. The sensitivity limits of the method could be fixed according to the method-induced racemization (0.00–0.05, depending on the amino acid considered). As a laboratory routine, D/L-valine, D/L-alanine, D-*Allo*-isoleucine/L-isoleucine, D/L-proline, D/L-aspartic acid, L-hydroxyproline, D/L-phenylalanine, and D/L-glutamic acid peaks were identified.

RESULTS AND DISCUSSION

First, we want to state that the use of a previous dialysis process allowed a noteworthy accuracy in our analytical results that was not achieved in the former analyses carried out on nondialyzed samples. In the first sets of samples, we obtained racemization ratios of total (both free and bound) amino acids that were too erratic to be seriously considered. The effect of L-hydroxyproline peak was also important because in some analyses (typically those carried out with an old Chirasil Val column) it overlaps the L-asp peak and bizarre aspartic acid racemization ratio values were obtained. In our opinion, the use of dialysis and a newly purchased Chirasil Val column and NPD detector could be the key to success.

In spite of the advantages of the dialysis, we realized that there is a very strong time-related organic matter (collagen) loss. In Figure 2 we show total D+L aspartic acid peak areas adjusted for the sample weight and the injection volume to provide relative amounts. An excellent differentiation can be observed between the Middle Pleistocene (300-kyr-old) *Ursus deningeri* and the Upper Pleistocene (>110-kyr-old) *Ursus spelaeus* samples: there is a dramatic decrease in bound aspartic acid contents. *Ursus deningeri* areas are between 5×10^3 and 2.5×10^5, whereas *U. spelaeus* samples range between 7.5×10^5 and 10^7. In fact some *U. spelaeus* values are similar to those of modern *U. arctos* (brown bear <1 kyr old).

The next step was to establish a statistical relationship between the mean D/L aspartic acid racemization values and radiometric ages from the three radiometrically dated localities: Eirós (EE-*U. spelaeus*), La Lucia (LU-*U. spelaeus*), and Sima de los Huesos (BB-*U. deningeri*). A simple linear correlation model was used. The resulting equation is:

$$t = -64.92 + 1191(D/L\ Asp)$$

where t is time (in years) and D/L the mean aspartic acid racemization value. The correlation coefficient value is extremely high (r = 0.998) and significant ($P = 0.002$).

We decided to adopt the more convenient and easier to handle linear model because, according to many authors,[27,28] for lower racemization ratios, between 0.00 and 0.40 (as in our case), a linear racemization behavior can be assumed. This linear D/L Asp ratio against radiometric age relationship cannot be considered an exception, since a similar behavior has been published for amino acid racemization in wool textiles.[29]

The radiometric ages and mean aspartic acid racemization values appear in an XY plot (Fig. 3); the origin ordinate value (0.05) is the induced aspartic acid racemization ratio measured in modern *U. americanus* dentine collagen,[18] which supports the correctness of the obtained correlation algorithm. We can conclude that there is good consistency between all of them. The age of *U. spelaeus* from La Lucia cave, deduced from U/Th dating of bear-bearing-bed-related speleothems, was 77 kyr. According to the average aspartic acid racemization ratio, it could be slightly older, approaching 90 kyr.

It was necessary to verify the supposed one-way sense of the racemization process because some authors[30,31] described an "apparent kinetic reversal" in the >1,000 Da peptide fraction in bivalve shells (*Ostrea* sp.). The "racemization kinetics" must be taken, at least, as the result of two

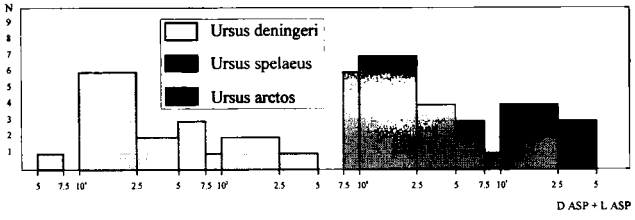

Fig. 2. Histogram of D+L aspartic acid peak areas (sample weight and GC analysis volume injection corrected).

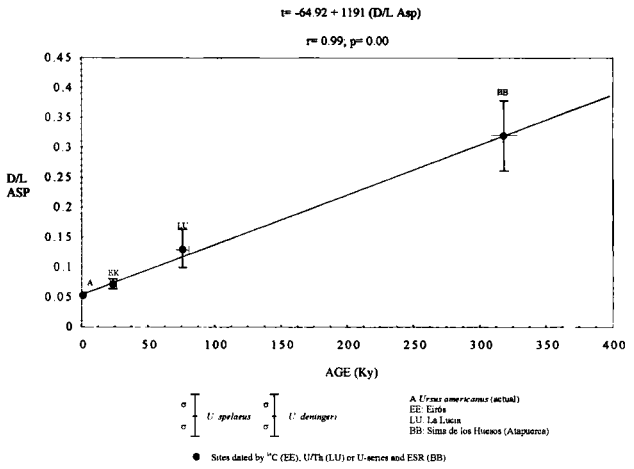

Fig. 3. XY plot of average D/L asp ratios and radiometric age of the three sampled localities.

different processes: reversible (racemization) or irreversible (hydrolysis) affecting proteins and peptides. The existence of a peptide hydrolysis resistance in the peptide bonds where hydrophobic amino acids are linked to aspartic acid has been reported, and since most of the racemization takes place at the terminal position of the peptide chains, an apparent racemization reversal can be produced. In our kinetic experiments (Fig. 4), this effect was evidenced by taking into account that the obtained k_L value for an adjusted model:

$$2k_L t = C + \ln \frac{1 + D/L}{1 - D/L}$$

corresponds to a first-order reversible kinetics model (FOK) where $k_D/k_L = 1$ and D/L is the racemization ratio. The value obtained was $k_L = 0.0042\ h^{-1}$ for aspartic acid in samples from the first 576 h of experiment (excluding the "kinetics reversal" interval).

Racemization rates in other amino acids (e.g., glutamic acid) are lower than in aspartic acid. The calculated k_L for glutamic acid is $k_L = 0.00045\ h^{-1}$ for the 1,704-h experiment.

Two additional kinetic experiments were carried out, heating the dentine samples at 65°C and 85°C for 1,272 h, in test tubes with quartz sand in an oven (full moisture conditions); the apparent rate constant k_L values are summarized in Table 2.

From the plot of ln k_L vs. T^{-1} (K^{-1}) following the Arrhenius relationships, we deduced that the activation energy for aspartic acid is 17.88 kcal/mol ($R^2 = 0.998$) and for glutamic acid 28.54 kcal/mol ($R^2 = 0.996$). No "kinetics reversal" was observed for glutamic acid at any heating temperature, nor for aspartic acid at 65°C and 85°C.In any case, the best method for amino acid racemization dating is to analyze a set of different amino acids making it possible, through similarity analysis, to determine apparent kinetics reversal and to correct it.

The kinetic experiment has shown the influence of moisture, also observed in bones,[32] in the racemization rate of dentine amino acids. Taking into account only the samples

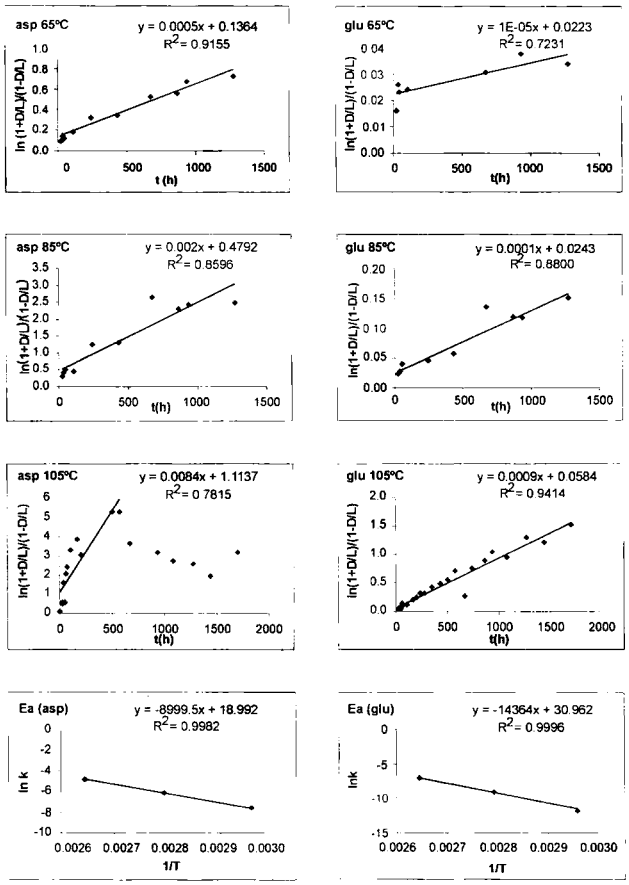

Fig. 4. XY plot of $2k_L t = C + \ln (1 + D/L)/(1 - D/L)$ for aspartic and glutamic acids from the 65°C, 85°C, and 105°C kinetic experiments carried out on actual *Ursus americanus* Pallas dentine. XY plot of ln k_L against 1/T (K^{-1}) for the estimation of the activation energies for aspartic and glutamic acids.

that were heated up to 100 h, k_L values obtained range from $k_L = 0.0008\ h^{-1}$ for the dry samples to $k_L = 0.0170\ h^{-1}$ (20 times greater) for the samples heated in the stove (full moisture conditions). The value obtained for the heating block samples (moisture-saturated atmosphere) is an intermediate one: $k_L = 0.0070\ h^{-1}$, due to the formation of a condensation water ring in the upper part of the test tube.

Some isolated samples were prepared by adding $CaCO_3$ instead of quartz sand but no relevant differences were obtained.

CONCLUSION

A linear aspartic acid racemization trend was found when average racemization ratio was regressed against ra-

TABLE 2. k_L values for aspartic and glutamic acids obtained from the kinetic experiments

Temperature (°C)	Time (hours)	k_L Asp (h^{-1})	k_L Glu (h^{-1})
65	1272	$2.5 \cdot 10^{-4}$	$5.0 \cdot 10^{-6}$
85	1272	$1.0 \cdot 10^{-3}$	$5.0 \cdot 10^{-5}$
105	1704	—	$4.5 \cdot 10^{-4}$
105	576	$4.2 \cdot 10^{-3}$	—

diometric ages of each locality. This means that the obtained mathematical equation can be confidently used for numeric age calculation. However, it is necessary to consider that local taphonomical conditions could play a very important role, either in intersample variation or in intrasample racemization ratio variation.

Kinetic experiments have demonstrated the development of an "apparent kinetics reversal" in aspartic acid, but it is not observed in glutamic acid. This problem in aspartic acid would not have a marked influence on dating since a theoretical 0.99 racemization ratio was reached before this apparent kinetics reversal appeared. However, in our experience, in very old teeth where high aspartic acid racemization ratios can be expected, no significant amounts of collagen remain.

The presence of water seems to be crucial for the amino acid racemization process, and a continued lack of moisture in sediments (in glacial or desert environments) could cause the process to stop temporarily. According to known Quaternary paleoclimatological evolution in Spanish caves, a continuous wet sediment environment could be inferred.

Results from kinetic experiments and paleontological sample analyses need to be considered in further kinetic experiments under different heating conditions.

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