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Indigeneity of organic matter in fossils: A test using stable isotope analysis of amino acid enantiomers in Quaternary mollusk shells

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ABSTRACT **Comparison of the $\delta^{13}C$ values of D and L enantiomers of individual amino acids was used to evaluate the presence of amino acid contaminants in Quaternary land snails. Measurements of $\delta^{13}C$ values of amino acid D and L enantiomers determined by combined gas chromatography, combustion, isotope-ratio mass spectrometry are reported. Conventional combustion techniques, following separation of aspartic acid and glutamic acid enantiomers by liquid chromatography, were also used to determine $\delta^{13}C$ as well as $\delta^{15}N$ values. Thoroughly cleaned samples ranging in age from 7000 to >100,000 yr B.P. are shown to have analytically identical $\delta^{13}C$ values for the D and L enantiomers of each amino acid, thus confirming that the amino acids are indigenous to the shells, even in Pleistocene samples. On the other hand, partially cleaned material shows divergence of isotopic values, thus indicating the presence of amino acid contaminants and emphasizing the importance of proper cleaning procedures. This approach provides a powerful method for assessing the indigeneity of amino acids in fossils.**

A variety of types of studies depend on the assumption that the organic constituents of fossils are indigenous. These include amino acid racemization/epimerization dating of marine (1), terrestrial (2, 3), and freshwater (4) samples, paleotemperature estimation from amino acid racemization rates (5), analysis of free amino acid concentrations for dating (6), taxonomic studies (7, 8), and reconstruction of paleodiets (9, 10) and paleoclimates (11–13) from the stable isotope composition of organic matter.

Indigeneity has previously been assessed by a variety of methods, no one of which has proved particularly reliable. Relative abundances of amino acids in fossils have been compared to modern samples of related species (14, 15). Such comparisons, however, are problematic because even in uncontaminated material, the proportions of the various amino acids will change over time as a result of more rapid breakdown of the less stable ones, such as serine, threonine, and aspartic acid. Amounts of amino acids may decrease by several orders of magnitude over thousands to millions of years (3, 15–17). The extent of racemization of amino acids has also been used to assess indigeneity, with the criterion being that very old samples should show equilibrium ratios of D/L amino acid enantiomers if they are uncontaminated (18). But racemization rates may be very reduced in certain molecular components (19, 20), especially where the amino acids have undergone condensation reactions with sugars (21). Microscopic examination has been used to show that the fossil matrix has been physically preserved (22, 23), but microstructure is not sensitive to either chemical preservation or the presence of contaminants (23). The similarity of the stable carbon and nitrogen isotope composition of bulk fossil organic matter to modern samples (24) and their dissimilarity to the sedimentary matrix (25) have also been used to indicate indigeneity of organic matter. As the amino acid composition changes over time due to diagenesis, however, the isotopic composition of the bulk organic matter may also change, regardless of contamination, due to the large differences in the isotopic composition of the individual amino acids (26). Carbon/nitrogen (C/N) ratios have been used to assess the presence of noncollagenous contaminants of collagen extracts from bone (24, 27). The fact that amino acids are common constituents of all sedimentary and aqueous systems poses a serious challenge to finding uncontaminated material. This challenge is particularly difficult for older fossils, where, with ensuing diagenesis, amino acid concentrations may begin to approach the natural background levels (nM/g) of the preserving environment.

Engel and Macko (28) introduced the idea of comparing the isotopic composition of D and L enantiomers of amino acids in order to determine the presence of contaminants. They showed that free amino acids retain their stable carbon and nitrogen isotopic integrity during racemization. It was hypothesized, therefore, that for samples in which there had been minimal contamination from the preserving environment, the stable isotope compositions of the D and L enantiomers of individual amino acid constituents should be approximately the same. On the other hand, if the amino acids are contaminated, the stable isotope composition of the D and L enantiomers will differ, except in the unlikely case where the contaminant has the same isotopic composition or the same D/L ratios as the indigenous amino acids. Subsequent analysis of D- and L-glutamic acid isolated by liquid chromatography (LC) from a Pleistocene sample of the marine bivalve *Mercenaria* revealed a depletion of several per mil in $\delta^{13}C$ and $\delta^{15}N$ values for the D enantiomer relative to the L enantiomer (29). Recently, the D enantiomer of aspartic acid was also found to be relatively depleted in the heavier isotopes, whereas no isotopic differences between the carbon isotopes of the neutral amino acid enantiomers were found (30). This result may indicate that the acidic amino acids are more readily contaminated. Alternatively, this difference may reflect the presence of glutamine and asparagine, which would be converted to glutamic acid and aspartic acid, respectively, during hydrolysis. The former amino acids would then essentially be contaminants of aspartic and glutamic acid (although of endogenous origin). At present no methods are available to determine whether asparagine or glutamine is present in proteins in these shells.

To test the use of isotopic analysis of D and L enantiomers for detection of contaminants in fossil organic matter, we analyzed well-preserved samples of Holocene and Pleistocene land snails from the Negev Desert, Israel (Table 1). These samples were considered likely to be uncontaminated for several reasons: (*i*) the silty sediments in which they were preserved are quite low in organic matter; (*ii*) the D/L values

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Abbreviations: GC/C/IRMS, gas chromatography/combustion/isotope-ratio mass spectrometry; LC, liquid chromatography.

Table 1. Information on land snail samples used in analyses

Sample no.	Species*	Age, yr B.P.	A/I†	Sample type‡	Location§
Is-246	*T.s.*	7110 ± 270¶	0.23	Alluvial	166,073
Is-619	*T.s.*	9540 ± 220¶	0.32	RM	133,051
Is-555	*T.s.*	9700 ± 360¶	0.40	RM	131,065
Is-245	*S.a.*	100,000‖	0.51	Alluvial	166,073
Is-528	*T.s.*	>123,000‖**	0.63	RM	151,063

All samples are from the Negev Desert, Israel.
**T.s.*, *Trochoidea seetzeni*; *S.a.*, *Sphincterochila aharonii*.
†D-Alloisoleucine/L-isoleucine ratio.
‡RM, rodent burrow midden (see ref. 31).
§Local grid coordinates; map may be seen in ref. 31.
¶Radiocarbon age (mean ± SD), corrected for isotopic fractionation and age anomaly (32) but not calibrated.
‖Apparent U-Th age; A. Kaufman (Weizmann Institute of Science, Rehovot, Israel) and G.A.G., unpublished results.
**Minimum age, based on 2 SD error.

of all the amino acids show a regular increase with increasing radiocarbon age of the samples (33); and (*iii*) the carbon isotope composition of the bulk organic matter (which is composed mostly of amino acids and which relates to the presence or absence of ^{13}C-enriched C_4 plants) shows coherent geographic patterns over time, with more depleted values in the wetter, northern area and more enriched values in the drier south (11). For comparison, we also analyzed an aliquot of one of the samples that was not fully cleaned of secondary carbonates (calcite).

METHODS

The cleaning procedure for the shell samples (which are aragonite) consisted of sonication in distilled water, a mechanical cleaning under a stereo microscope (with a handheld Dremel tool fitted with various tips), and a brief dip in dilute HCl (see ref. 31 for details of procedures). For the partially cleaned aliquot of one sample, only the sonication and HCl dip procedures were used (no mechanical cleaning). Aliquots of the cleaned shells were hydrolyzed in 6 M HCl (24 hr, 100°C) and then desalted by cation-exchange (29). Amino acid concentrations were measured by HPLC (high-performance liquid chromatography) with post-column derivatization with *o*-phthaldialdehyde, and amino acid D/L values were measured by GC (gas chromatography, with N-P detector) after derivatization to trifluoroacetyl isopropyl ester amino acids (29).

Two methods were used for separation and analysis of D and L enantiomers: an LC procedure and a combined gas chromatography, combustion, isotope-ratio mass spectrometric (GC/C/IRMS) procedure. An LC procedure was used for separation of aspartic acid and glutamic acid, followed by HPLC separation of the enantiomers, conventional sealed-tube combustion, and measurement of $\delta^{13}C$ and $\delta^{15}N$ values with a VG Prism isotope-ratio mass spectrometer (details of the procedures are given in ref. 29). For the GC/C/IRMS analyses, amino acid enantiomers were derivatized as for GC measurements and analyzed directly for stable carbon isotope compositions with a Hewlett–Packard 5890 GC (with Chirasil-Val 50 m × 0.25 mm i.d. column) interfaced to a VG Prism isotope-ratio mass spectrometer via a combustion furnace and water trap. Details of this method, including calculation of the $\delta^{13}C$ values for the underivatized amino acid enantiomers from the derivative values, are reported elsewhere (30, 34). In most cases, the acidic amino acids (aspartic acid and glutamic acid) were separated by LC from the other amino acids and these two sets of amino acids were analyzed in separate GC/C/IRMS runs. In addition, for one sample, we investigated D and L enantiomer stable isotope compositions among different molecular weight components separated by ultrafiltration. This procedure made possible the assessment of whether diagenetic losses of particular fractions of the organic matter (e.g., free amino acids) would have any effect on the relative isotopic composition of the D and L enantiomers. An aliquot of the sample was dissolved in 6 M HCl and subjected to an ultrafiltration method (35) to recover several molecular weight fractions of free amino acids and peptides (<500 M_r, 500–3000 M_r, and an insoluble fraction that did not pass through a >10,000 M_r cut-off filter).

RESULTS AND DISCUSSION

In general, the $\delta^{13}C$ and $\delta^{15}N$ values for the D and L enantiomers of individual amino acids in the total hydrolyzates of the fossil shells were found to differ by <1‰ (Table 2; Table 3, cleaned sample), indicating that the D enantiomers were derived via racemization of the L enantiomers, with no fractionation and with minimal contributions to either enantiomer from the preserving environments. For D- and L-alanine, the breakdown of other amino acids, such as serine or methionine (36, 37), may contribute to the total, and, thus, result in a deviation between the $\delta^{13}C$ values of the D and L enantiomers, such as that observed in the oldest samples (Is-528 and Is-245); this is essentially a form of contamination, although originating within the organic matrix.

In sample Is-619, differences of several per mil were observed for the same amino acid (e.g., aspartic acid) in different molecular weight fractions (Table 4). These differences may reflect fractionations resulting from natural diagenetic processes (e.g., hydrolysis, humification) or may in part result from the procedure used to isolate these molecular weight fractions from the shell. Nevertheless, the $\delta^{13}C$ values that could be determined for the D and L enantiomers of

Table 2. The C and N isotopic compositions (‰) of the D and L enantiomers of amino acids in total hydrolyzates of fossil land snail samples (*Trochoidea seetzeni*)

Amino acid	Is-246*		Is-528†‡		Is-619,§	Is-555,§
	$\delta^{13}C$¶	$\delta^{15}N$¶	$\delta^{13}C$	$\delta^{15}N$	$\delta^{13}C$	$\delta^{13}C$
D-Asp	−26.4	+2.9	−22.3	+20.0	−16.6	−9.5
L-Asp	−25.0	+3.6	−21.7	+19.7	−17.5	−10.9
D-Glu	−28.4	+3.4	−22.1	+22.2	ND	−11.5
L-Glu	−28.7	+4.2	−21.7	+22.1	ND	−12.6
Gly			−15.5 ± 0.4		−17.3	−7.4
D-Ala			−22.5 ± 0.4		ND	−27.0
L-Ala			−23.9 ± 0.5		−21.8	−27.9
D-Val			−30.5 ± 1.0		ND	ND
L-Val			−29.5 ± 0.1		−24.4	−21.0
D-Leu			−28.8 ± 1.7		ND	ND
L-Leu			−28.1 ± 0.1		−28.8	−31.5
D-aIle			−33.8		ND	ND
L-Ile			−30.4 ± 0.2		ND	−33.6

ND, not determined; either peak below detection or interference with coeluting peak.
*Sample analyzed by conventional combustion of enantiomers separated by LC.
†Asp and Glu analyzed by conventional combustion of enantiomers separated by LC; other amino acid enantiomers analyzed by GC/C/IRMS.
‡The errors shown for Is-528 are for analyses of preparations of separate sets of shells from this sample. The average analytical error for different GC/C/IRMS runs of the same preparation of a sample was 0.25‰.
§All amino acid enantiomers analyzed by GC/C/IRMS.
¶Stable carbon and nitrogen isotopic data are presented by the standard convention $\delta^{N}E = [R_{sample}/R_{standard} - 1]10^3$‰, where N is the heavier isotope of the element E and R is the abundance ratio of the heavy to light isotopes of the element. The reference standard for carbon is NBS-22 (−29.8‰) relative to Pee Dee belemnite carbonate (0.0‰). The standard for nitrogen is air ($\delta^{15}N$ = 0.0‰).

Table 3. Amino acid concentrations, D/L values, and $\delta^{13}C$ values for the fully cleaned and partially cleaned samples of Is-245

Amino acid	Fully cleaned			Partially cleaned		
	Conc., nM/g	D/L	$\delta^{13}C$*	Conc., nM/g	D/L	$\delta^{13}C$*
Glu	104.2	0.43		89.6	0.62	
D-			−21.3			−24.2
L-			−21.5			−16.4
Asp	89.0	0.69		75.0	0.57	
D-			−23.6			−25.1
L-			−23.8			−22.3
Ala	80.7	0.70		70.2	0.66	
D-			−22.6			−23.0
L-			−23.7			−27.0
Val	34.1	0.48		32.6	0.49	
D-			ND			ND
L-			−23.8			−23.7
Leu	50.1	0.51		54.0	ND	
D-			−27.8			−27.2
L-			−28.2			−30.6
aIle/Ile	24.7	0.51		24.4	0.47	
D-aIle			−26.6			ND
L-Ile			−27.5			−25.6
Gly	106.3	—	−10.8	84.7	—	−8.9

See text for explanation of cleaning procedures. ND, not determined.
*Measured by GC/C/IRMS.

individual amino acids in sample Is-619 were generally within 1‰ of each other in each molecular weight fraction.

The utility of stable isotopes for determining the indigeneity of amino acids in fossils is seen in a comparison of the $\delta^{13}C$ values determined for amino acid enantiomers from a thoroughly cleaned set of snail shells and a partially cleaned set of shells, all from the same sample (Table 3). The $\delta^{13}C$ values for the D and L enantiomers of individual amino acids in the fully cleaned sample were close to identical, as in the other fully cleaned samples analyzed (Table 2), whereas the $\delta^{13}C$ values for the D and L enantiomers of individual amino acids in the partially cleaned sample differed by several per mil, indicating a contribution from one or more sources exogenous to the shell. For both the D and L enantiomers of the partially cleaned shells, the $\delta^{13}C$ values deviate from those of the fully cleaned shells, indicating that the contaminating amino acids contain both D and L enantiomers. The concentrations of the various amino acids did not differ significantly between the two sets. Some differences were found in D/L values, but the pattern is not consistent: most amino acids are more racemized in the fully cleaned sample (aspartic acid, alanine, isoleucine), whereas glutamic acid is more racemized in the partially cleaned sample, and valine shows about the same value in both. Thus, the pattern is not a simple one of contamination only by younger or less racemized amino acids; rather, different amino acids show different patterns. These results confirm the initial hypothesis that in pristine fossil material, the D and L enantiomers of amino acids show similar isotopic composition. Furthermore, comparison of the isotopic values of enantiomers clearly shows the presence of contaminants in material that is not fully cleaned of secondary carbonates, even though this contamination does not show up as a significant deviation in the amino acid composition.

Table 4. $\delta^{13}C$ values* of amino acids in various molecular weight fractions of Is-619

Amino acid	Total hydrolyzate	<500	500–3000	Insoluble
D-Asp	−16.6	−15.8	−13.1	−12.4
L-Asp	−17.5	−16.5	−12.4	−13.8
D-Glu	ND	−15.7	−15.7	ND
L-Glu	ND	−17.6	−16.2	ND
D-Ala	ND	ND	−22.1	−21.4
L-Ala	−21.8	−22.3	−23.2	−20.3
D-Val	ND	ND	ND	ND
L-Val	−24.4	−24.2	−27.5	−26.2
Gly	−17.3	−16.3	−18.0	−19.1
D-Leu	ND	ND	ND	ND
L-Leu	−28.8	−26.9	−26.8	−27.8
D-aIle	ND	ND	ND	ND
L-Ile	ND	ND	−30.7	−32.4

ND, not determined.
*Measured by GC/C/IRMS.

CONCLUSIONS

All investigations of biochemical constituents of fossils must begin with an appraisal of their indigeneity. Even well-preserved fossils may not be entirely closed systems and the extent to which components have undergone exchange with the preserving matrix is often unclear. For fossil materials in which sufficient antiquity or thermal history has resulted in partial amino acid racemization, analysis of the stable isotope compositions of the D and L enantiomers of individual amino acids provides a powerful tool for establishing the indigeneity of these compounds. A number of problems are amenable to investigation by this methodology. Radiocarbon dating of organic matter in old or poorly preserved bones has long been problematic due to contamination by exogenous material; various methods of evaluating sample purity and processing bone for removal of contaminants have been used (38). Analysis of the stable isotope composition of amino acid enantiomers in bone would provide a useful check on indigeneity of amino acid fractions and could also be used to develop and improve procedures for processing bones for radiocarbon dating as well as for stable isotope analysis for paleodietary reconstruction (9, 10). The method would be particularly useful for validation of paleodietary work on ancient bone samples, such as Cretaceous dinosaurs (39, 40), in which the preservation of original organic material is more questionable. Mollusk shells have been widely used for amino acid racemization/epimerization dating of Late and Middle Pleistocene coastal marine deposits (1). Inconsistencies with other dating methods, such as U-Th, however, have brought into question the reliability of the racemization methods (41). Although contamination is not the only problem to which racemization methods are subject, evaluation of the indigeneity of the amino acids with stable isotope analysis of the enantiomers would provide a check on this aspect of the problem.

Whereas $\delta^{13}C$ values of amino acid enantiomers can now be measured relatively easily by GC/C/IRMS, the methodology for $\delta^{15}N$ values is still presently dependent on labor-intensive LC separations and subsequent conventional IRMS analyses. An additional GC/C/IRMS method for stable nitrogen isotope analyses, similar to that described herein for carbon, awaits development (42).

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