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Radiocarbon, ^{13}C and ^{15}N analysis of fossil bone: Removal of humates with XAD-2 resin

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Abstract—Humic acids are the predominant source of error in the ^{14}C and stable isotope analysis of fossil bone organic matter. XAD-2 resin will quantitatively remove humates and give the highest yields of protein from bones with variable types of preservation. Decalcified bone, gelatin and base-leached residues can vary up to 5‰ for $\delta^{13}C$ and by 1‰ on $\delta^{15}N$ relative to XAD-treated fractions. Simultaneous analysis of ^{14}C age, $\delta^{13}C$ and $\delta^{15}N$ is recommended because each isotope value can be independently affected by the bone's diagenetic history. Radiocarbon analysis is the most sensitive and $\delta^{15}N$ is least sensitive for detecting exogenous organic matter. The uncertainty of analyses on the best pretreated protein is ±0.5‰ for both $\delta^{13}C$ and $\delta^{15}N$ and is larger than previous estimates. The accuracy for all isotope analyses will be better assessed by using individual amino acids instead of total collagenous residues. Inaccurate ^{14}C dates on severely degraded bone are an indication that this class of fossils may be unsuitable for any isotopic analysis.

INTRODUCTION

ACCURATE ISOTOPIC ANALYSES on fossil bones require that original isotopic ratios be preserved and that all exogenous carbon and nitrogen be removed. These qualifications are easily met for geologically young bone or bone that has been preserved in dry-cave or permafrost environments (Table 1). In these environments collagen diagenesis and humate contamination are usually minimal and accurate isotope determinations are obtained.

In contrast, the majority of bones that are used for radiocarbon dating and stable isotope analyses have undergone moderate to severe diagenesis and are often contaminated with substantial amounts of humates and other foreign organic matter (Table 1). As diagenesis proceeds, chemical properties of bone protein are lost and it becomes increasingly difficult to separate endogenous organic carbon from humates and other foreign organic matter. Time, temperature, and burial conditions contribute in a complex manner to alter bone organic matter; however, major categories of fossil bone are distinguishable by their physical-chemical characteristic's (Tables 1 and 2).

The two dominant sources of error in using bone protein for isotopic analyses are: (1) the preservation state of the collagen and (2) whether or not humates contaminate the fossil. The preservation of fossil bone protein determines the amino acid composition of the bone and therefore the stable isotope composition of the total organic fraction. As diagenesis proceeds, there are changes in the molar proportions of each amino acid (Table 2). Because each amino acid has a unique isotopic value (HARE and ESTEP, 1983), diagenesis of collagen will theoretically alter the isotope value of the resulting organic fraction. The second and predominant source of error is from humates, which cause significant errors in stable and radioisotope analyses. The effect of humate contamination depends upon their concentration in bone and on the ^{13}C, ^{14}C and ^{15}N composition of the humate phases.

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Previous research in bone pretreatment

The radiocarbon dating of naturally-burned-bone (ARNOLD and LIBBY, 1951; DE VRIES and BARENDSEN, 1954) and of artificially pyrolyzed bone (MAY, 1955; RAFTER, 1955) was so inaccurate that bone was not recommended for radiocarbon dating (LIBBY, 1952; DE VRIES 1954). It was subsequently realized that adsorbed humic material could contaminate the bone protein (MUNNICH, 1957). Methods to remove adsorbed organic matter (predominantly humates) from bone included the extraction of gelatin (SINEX and FARRIS, 1959; LONGIN, 1970, 1971) and the isolation of collagen (BERGER *et al.*, 1964). Gelatin was used because it was believed that humates were insoluble in the 90°C, water (pH 3) that was used to extract gelatin (SINEX and FARRIS, 1959; LONGIN, 1971; EVIN *et al.*, 1971). Conclusive proof that humates were the predominant contaminant in bone was given by DE VRIES (in VOGEL and WATERBOLK, 1963), BERGER and LIBBY (1966), and HAYNES (1967). To remove humates from collagen, BERGER and LIBBY (1966), HAYNES (1967), GROOTES (1968) and ARSLANOV and GROMOVA (1971) adopted for bone the method used to pretreat charcoal for ^{14}C dating (DE VRIES and BARENDSEN, 1954; OLSON and BROECKER, 1958; DE VRIES, 1958). The method is the extraction of decalcified bone with 0.1 to 0.5 *M* NaOH. The NaOH and gelatin-extraction techniques were combined by PROTSCH (1975) into the procedure commonly used today (BERGLUND *et al.*, 1976). The method comprises decalcification of the bone powder in 0.8 *N* HCl, leaching of the residue with 0.5% NaOH, followed by gelatin extraction.

More extensive pretreatment methods for collagen include cation exchange chromatography (HO *et al.*, 1969) and the combination of the NaOH, gelatin and chromatography approaches (PROTSCH, 1975; BERGER, 1975). Reverse phase chromatography methods for humate-removal include the use of XAD resins (STAFFORD *et al.*, 1982, 1987) and charcoal (GILLESPIE *et al.*, 1984). Complete separation of collagen from contaminates has used the isolation of collagen-specific amino acids, for example hydroxyproline and proline (WAND, 1981; STAFFORD *et al.*, 1982, 1987; GILLESPIE *et al.*, 1984).

Bone organic fractions that are used for stable isotope analysis are extracted by methods adopted from the radiocarbon literature. The most widely used procedures are those of DENIRO and EPSTEIN (1981), SCHOENINGER and DENIRO (1984), KRUEGER and SULLIVAN (1984) and those compared by CHISOLM *et al.* (1983). All pretreatments include the extraction of gelatin from HCl-decalcified bone; variations are in the details of decalcification, filtration and whether or not NaOH extraction precedes gelatin isolation.

XAD adsorption resins

The XAD resins are porous, non-polar to weakly-polar adsorbents that are well suited for isolating weakly or non-ionized aliphatic and aromatic molecules from aqueous solutions. The XAD-1, 2 and 4 polymeric adsorbents are macroreticular, non-ionic, hydrophobic,

	Class I Modern	Class II Very Well to Well Preserved	Class III Moderately Well Preserved	Class IV Poorly Preserved	Class V Extremely Poorly Preserved
Whole bone % nitrogen	4.5-3.5	3.5-0.6	0.9-0.4	0.5-0.1	0.1 to <0.01
Characteristic amino acid composition.	Collagen	Collagenous	Collagenous	Collagen-Derived	Non-Collagen
Characteristic amino acids: Residues per 1000 nominal values — Hydroxyproline	90	90	90	30-80	0
Aspartic Acid	50	50	50	50-100	80-300
Glutamic Acid	70	70	70	70-130	70-260
Proline	120	120	120	100-180	30-90
Glycine	330	330	300-330	260-300	100-290
Alanine	105-110	120	120	100-120	70-120
Arginine	55	45	45	40-45	0-30
Physical characteristics of whole bone.	High compressive and tensile strength; spiral and conchoidal fracturing; waxy luster; dense impermeable mineral matrix.	Very well preserved if spiral and conchoidal fracturing exist; waxy luster is present throughout. Well preserved if interior of cortical bone becomes white and chalky with concomitant loss of conchoidal fracturing; exterior hard and waxy. As %N decreases, fracturing becomes uneven, perpendicular to bone axis; chalky luster progressively replaces waxy bone from interior toward exterior	Interior and exterior of bone are chalky; surface hardness decreases and porosity increases with decreasing %N. Uneven, hackly fractures perpendicular to bone's main axis.	Similar characteristics to Class III, with continued decrease in hardness, increase in porosity.	Soft, easily pulverized bone if no inorganic replacement occurs; low density. Hard consistence if mineralization by carbonates, Fe, Mn, or Si.
Percent of original bone morphology preserved and physical characteristics of collagenous bone-pseudomorph formed during decalcification.	100% Fine detail preserved	90-100% Fine detail preserved	70-100% Increasing loss of detail with decreasing %N	0-50% Amorphous insoluble residues predominate.	0-20% Amorphous residues.
Hot water extractable protein: "gelatin".	≥90 wt% of collagen; white acicular, crystalline lyophylizate.	>80 wt% of protein is soluble; modern appearance.	>50 wt% of protein; near-modern appearance.	20-50 wt% of protein extracted.	<10-20% of protein extracted Inorganic salts and humates predominate.
Amount and type of foreign-carbon contamination.	Zero to negligible	Zero to negligible; humates and CO_3 restricted to the outer few millimeters of bone surface.	Negligible to severe humate and CO_3 contamination, increasing with decreasing %N. Negligible to moderate Fe, Mn.	Moderate to severe; pervasive humate and CO_3 contamination; and moderate Fe, Mn, Al, Fe, U, and F substitution.	Carbonate usually >> humate contamination: secondary calcite, hematite, limonite, pyrite, pyrolusite, geothite, and gypsum.
Temporal and geologic conditions controlling preservation.	Recent	Recent to >100,000 yr. Artic (permafrost) burial, arid and hyper-arid caves, constant-temperature non-leached carbonate caves, reduced sediments (especially impermeable clays). Short-time burial in more porous, permeable sediments (>5000 yr).	Recent to >100,000 yr. Minimal to moderate water-leaching; cool to warm environments, fluctuating temperatures; most depositional environments and lithologies.	Recent to >100,000 yr. Predominantly >100 yr. Similar geologic conditions to III, except longer surface exposure before burial; greater oxidation of young fossils; greater geologic age for cooler, less leached, reducing environments.	Recent to >100,000; long-duration surface exposure or extensive oxidized-water leaching; porous, permeable sediments; soil zones, sand dunes, leached caves: carbonate-rich, humid to subtropical burial sites. Geologic conditions favorable for eventual mineralization.
Minimum chemical processing required.	Weak-HCl insoluble collagen or gelatin from decalcified bone. Individual amino acids are used for tracing biochemical processes and ecological pathways.	XAD-purification of hydrolyzed gelatin; individual amino acids for high-precision, multiple-dating of pivotal fossils.	XAD-purification of hydrolyzed gelatin or collagen; use of individual amino acids is encouraged as %N decreases and degree of humate contamination increases.	XAD-purification of hydrolyzates of hot-water-soluble or HCl insoluble residues; multiple dates on individual amino acids are highly recommended.	XAD-purified hydrolyzates of weak-acid insoluble residue; individual amino acids from weak-HCl soluble and insoluble fractions; sequential dating of 3-6 chemical fractions is mandatory. Fossils with <0.10% N may be irreversibly contaminated with young-age proteins and peptides, and humate-bound amino acids.
Probability of obtaining accurate isotopic measurements.	Extremely high	Very high	Very high to high	High to moderate	Low to not presently recommended for analysis.

TABLE 1. Classification of fossil bones based on physical and chemical properties. The order of importance, from greatest to least amino acid composition, whole-bone nitrogen content, chemical composition and % extractable gelatin, physical properties, humate contamination and depositional environment. Certain criteria immediately define specific classes; modern bones (I) have experienced no burial or environmental exposure and have ≤100‰ alanine; class V is defined by the absence of hydroxyproline, regardless of nitrogen content. Classes II and III have amino acid analyses that are identical to modern collagen, except alanine is proportionally increased; II and III are distinguished from IV by amino acid composition. Class II is separable into very well preserved bone with spiral fracturing and waxy luster; well preserved bone has hard, waxy exterior but develops a chalking interior. Class III fossils have a collagen amino acid composition, but have lost surficial and interior hardness, lack waxy luster and are chalky-textured throughout. Class IV specimens have a collagen-derived amino acid composition—all amino acids exist, but hydroxyproline and glycine are diminished and aspartic acid and glutamic acids are proportionally increased. Class V specimens are characterized by loss of hydroxyproline and often arginine, several fold increases in aspartic and glutamic acids, and decreased abundance of glycine and proline.

styrene-divinylbenzene copolymers that have surface areas of 100, 330 and 750 m^2 and pore dimensions of 200, 90, and 50 Angstroms, respectively (KUNIN, 1977). The XAD-7, 8, 9, and 11 resins are crosslinked polymethacrylate esters that are weakly polar and more hydrophylic than the styrene-divinylbenzene polymers (KUNIN, 1977). The methacrylates have very weak ion exchange capacities (AIKEN *et al.*, 1979). Rhom and Haas Amberlite XAD resins and their analogs are marketed under several trade names including: Waters Porapak, Bio-Rad Biobeads Sm-2, & 4, Foxboro Chromosorb, Sigma Chemical XAD, Supelco Supelpak and Sigma Chemical and Serva Chemical Companies' XAD.

Only XAD-1, 2 and 4 are suitable for isotope work because the resins are physically and chemically stable at extremes of pH, solvent polarity and temperatures to 250°C. The more hydrophylic resins, especially XAD-7, decompose at high pH (AIKEN *et al.*, 1979). These resins are supplied with or without chemical pretreatment that removes unreacted monomers (AIKEN *et al.*, 1979; THURMAN and MALCOM, 1981). The XAD resins have been used to extract dilute chemicals from environmental and physiological fluids, to concentrate humates from fresh and marine waters and in liquid chromatography where weakly polar compounds are separated from aqueous solution. Properties and applications of the resins are reviewed in Table 3.

Table 2. Amino Acid Analyses of Fossil Bones that Vary in Geologic Age and Preservation

Sample	Cow Bone	Texas Bison	Texas Bison	Texas Bison	Texas Bison	Texas Bison	Dent Mammoth	Domebo Mammoth	Escapule Mammoth
Sample Number	TS82-40	77LLP-32	78LLP-6	78LLP-28	78LLP-27	78LLP-4	15-11	9-18	87NBS-142
Preservation Class	I	II	II	III	IV	IV	III	III	V
Geologic Age, Yr	Modern	CA. 700	CA. 500	5050	4900	10,700	11,000	11,000	11,000
Percent Nitrogen	4.5	3.0	2.3	0.95	0.35	0.09	0.83	0.69	0.03
Amino Acid (Residues °/₀₀)									
4-Hydroxyproline	90	97	94	95	66	30	91	84	0
Aspartic Acid	53	53	50	55	79	152	48	54	293
Threonine	19	16	16	16	17	11	19	23	29
Serine	35	32	31	28	28	18	36	37	0
Glutamic Acid	71	77	80	82	94	172	71	73	131
Proline	124	133	132	134	113	86	124	123	41
Glycine	322	293	296	291	287	267	327	322	279
Alanine	89	124	130	132	159	114	123	124	84
Valine	27	25	22	26	29	48	27	30	12
Methionine	7	6	6	5	3	10	5	7	0
Isoleucine	14	12	12	12	13	8	11	12	28
Leucine	32	28	28	27	28	27	27	33	29
Tyrosine	6	3	4	1	1	8	1	5	5
Phenylalanine	16	15	13	13	13	16	14	17	13
Histidine	5	4	6	8	2	0	2	4	0
Hydroxylysine	5	5	6	7	3	11	0	0	4
Lysine	28	26	27	26	26	23	28	29	51
Arginine	56	49	46	43	40	0	44	22	0
Total	999	998	999	1001	1001	1001	998	999	999

METHODS

Samples analysed

The fossil bones used in the experiment were bison (*Bison bison* and *B. antiquus*) that dated *ca.* 500 to 12,000 yr B.P. and three mammoths (*Mammuthus* sp.) that dated between 11,000 and 11,500 yr B.P. (HAYNES, 1984). The bison were from the Lubbock Lake Site, Texas and were collected *in situ* from alluviated valley sediments (STAFFORD, 1981). The mammoths were from three, 11,000 yr old Clovis-culture Paleoindian archaeological sites: the Domebo site, Oklahoma (LEONHARDY, 1966); the Dent site, Colorado (HAYNES, 1974); and the Escapule site, Arizona (HEMMINGS and HAYNES, 1969). Radiocarbon dates for the *Mammuthus* fossils are compiled in STAFFORD *et al.* (1987).

Table 3. Selected References on Properties and Uses of XAD Adsorption Resins

COMPARISON OF ADSORPTIVE PROPERTIES	Pietrzyk and Chu, 1977a; Thurman, Malcom and Aiken, 1978; Aiken, Thurman and Malcom, 1979.
DISTRIBUTION COEFFICIENTS FOR MODEL COMPOUNDS	Niederwieser, 1971; Burnham et al, 1972; Fritz & Willis, 1973; Grieser and Pietrzyk, 1973; Chu and Pietrzyk, 1974; Junk, et al, 1974; Pietrzyk and Chu, 1977a,b; Aiken, Thurman and Malcom, 1979; Dieterle, Faigle and Mory, 1979; Curtis, et al, 1981; Thurman and Malcom, 1981.
XAD RESIN CHEMICAL PROPERTIES AND APPLICATIONS	Grieser and Pietrzyk, 1973; Junk, et al, 1974; Kunin, 1977; Pietrzyk and Chu, 1977a; Aiken, Thurman and Malcom, 1979.
EXTRACTION OF ORGANIC COMPOUNDS FROM NATURAL WATERS	Riley & Taylor, 1969; Burnham et al, 1972; Junk, et al, 1974; Steelink, 1977; Mantoura and Riley, 1975; Kunin, 1977; Thurman and Malcom, 1979; Ho et al., 1983.
SEPARATION OF HUMATES FROM SOILS AND AQUEOUS SOLUTIONS	Riley & Taylor, 1969; Burnham et al, 1972; Junk, et al, 1974; Mantoura and Riley, 1975; Webb, 1975; Steelink, 1977; Aiken, Thurman and Malcom, 1979; MacCarthy, et al, 1979; Curtis et al, 1981; Sivaplan, 1981; Thutman and Malcom, 1981.
SEPARATION OF AMINO ACIDS, OLIGOPEPTIDES AND PURIFICATION OF PROTEINS	Zaika, et al, 1968; Zaika, 1969, 1970; Niederwieser, 1971; Holloway, 1973; Ho, et al., 1983.

Pretreatment of bone

The bones were broken into 1 cm sized fragments and repeatedly ultrasonicated in distilled water. The fragments were dried at 50°C., pulverized to <63 μm, then extracted with acetone and ethanol before decalcification with 4°C, 0.6 *N* distilled HCl. The acid-insoluble residue was concentrated by centrifugation and rinsed with distilled water. The acid-soluble fraction was filtered through 0.45 μm Millex-HV filters and rotary evaporated.

Gelatin extraction

The hot water soluble phase ("gelatin") was extracted by heating ≈ 200 mg of lyophilized, decalcified bone with 5 ml of H_2O (pH 3) for 24 hr at 90°C. Water-soluble and water-insoluble phases were separated by centrifugation and filtration before the supernatant was lyophylised.

Hydrolysis

Approximately 100 mg of either the demineralized bone powder or gelatin were hydrolysed with 10 ml of 6 *N* distilled HCl, for 24 hr at 110°C. Solids were removed by centrifugation before the filtered hydrolysate was treated by XAD chromatography.

XAD-2 chromatography to remove humates

Sigma Chemical Company 20 to 50 mesh XAD-2 resin was cleaned by repeatedly washing 500 g of resin in acetone and water. The aqueous suspension was alternately extracted three times for 30 min each with 80°C 3 *M* HCl and 3 *M* NaOH and then washed exhaustively with distilled water before soxhlet extraction for 24 hr each with methanol and acetone. After a final distilled-water-wash, the resin was stored in 1 *N* HCl.

The XAD-2 columns were prepared by filling 1 × 30 cm Bio-Rad Econo-columns with a 6 *N* HCl slurry of precleaned XAD-2 resin. Glass wool was packed on top of the resin and a 50 ml glass reservoir was attached with teflon tubing. The XAD-2 column is equilibrated with 3 bed volumes of 6 *N* HCl. The filtered hydrolysate is passed through the resin at 200 μl/min or at a rate sufficient to adsorb the fulvic acids in the upper one-third to one-half of the resin bed. The column is washed with two bed volumes of 6 *N* HCl, which is added to the purified hydrolysate. The eluate is oven dried or rotary evaporated to a viscous syrup.

Subsequent samples were pretreated with precleaned, analytical-grade Serva XAD-2 resin (100–200 μm), which was more efficient than coarse-size XAD. One to 3 ml of resin were poured into a 5 ml syringe, a 0.45 μm Millipore filter was added to the base and the resin was washed with 3 bed volumes of 6 *N* HCl. The protein hydrolysate was passed through the resin using the same procedure as for the 20–50 mesh XAD.

Fulvic acids are desorbed from the resin by washing the column with water until the eluate pH is between 1 and 2. This step removes excess HCl but not fulvic acids, which are then eluted with 0.5 *M* NaOH, 1 *M* NH_4OH, acetone or methanol at room temperature. The base-eluted fulvic acids are acidified with HCl before drying. The XAD resin is used once and discarded because the resin retains traces of fulvic acids that cannot be desorbed.

Isolation of individual amino acids

Secondary (imino) amino acids were isolated for ^{14}C dating from the XAD-treated hydrolysate with a nitrosylation reaction that was followed by liquid chromatography (STAFFORD *et al.*, 1982). Primary amino acids were separated by a cation-exchange-procedure modified after MACKO *et al.* (1987). Amino acids for radiocarbon dating were isolated at the National Bureau of Standards.

Stable isotope analyses

Samples listed in Table 4 were analysed at the University of Arizona. Approximately 10 mg of sample were combusted in 8 mm O.D., Vycor tubes containing 1 g of CuO wire and 10 mg of silver foil.

Table 4. Isotopic Analysis of Various Treatments of Calbiochem Achilles-heel-tendon Collagen

Fraction Analysed	$\delta^{15}N$ ‰ (AIR)	$\delta^{13}C$ ‰ (PDB)
Collagen	+6.4	-12.8
Hydrolysed Collagen	+6.3	-12.8
XAD-2-treated Collagen Hydrolysates	+6.5	-12.5

Samples having both $\delta^{13}C$ and $\delta^{15}N$ determinations were analysed at the Geophysical Laboratory; ten mg of sample were combusted 1 hr at 900°C in evacuated, 6 mm O.D. quartz tubes that contain 1 g of CuO wire and 0.5 g of 20 to 30 mesh copper metal. After 1 hr of combustion, the furnace is cooled at 60°C/hr to room temperature, which converts quantitatively nitrogen oxides to N_2. Both methods are modifications of the STUMP and FRAZER (1973) and NORTHFELT *et al.*, (1981) techniques. Nitrogen and carbon isotope ratios are determined on N_2 and CO_2 gases separated by the procedure of MACKO (1981). Isotope values are reported as $\delta^{13}C$ and $\delta^{15}N$, which are calculated as:

$$\delta^{13}C = \left[\frac{^{13}C/^{12}C \text{ sample}}{^{13}C/^{12}C \text{ standard}} - 1\right] \times 1000‰ \text{ (PDB)}$$

$$\delta^{15}N = \left[\frac{^{15}N/^{14}N \text{ sample}}{^{15}N/^{14}N \text{ standard}} - 1\right] \times 1000‰ \text{ (AIR)}.$$

Measurement errors on standards are ±0.1‰ for $\delta^{13}C$ and ±0.2‰ for $\delta^{15}N$. The Peedee belemnite (PDB) carbonate is the standard for $\delta^{13}C$ measurements (CRAIG, 1953) and that for $\delta^{15}N$ is atmospheric nitrogen (AIR).

Radiocarbon measurement

Measurements of $^{14}C/^{13}C$ ratios were performed by tandem accelerator mass spectrometry (TAMS) at the University of Arizona. Sample carbon was converted to Fe-C targets with the procedure of JULL *et al.*, (1983). Details of the counting procedures are given in DONAHUE *et al.* (1983) and STAFFORD *et al.* (1987). Individual amino acids where converted to Fe-C targets by using the method of VERKOUTEREN *et al.* (1987).

RESULTS

The experiments provided data on the stable isotope composition and the radiocarbon ages of various fractions from fossil bone. The bison fossils were used for stable isotopic determinations, whereas the mammoth bones were used for both stable and radioisotope analyses.

Replicate gelatin extractions

The results in Table 5 are from bones with different amounts of humate contamination and different concentrations of protein. The experiment tested the LONGIN (1971) technique and its ability to separate gelatin from humates. As the weight percent of humates increased and collagen content decreased, the difference between replicate $\delta^{13}C$ analyses became greater. The variation in a single bone ranged from 0.5‰ for well preserved bone to 4.9‰ for the 12,000 yr old fossil. The percent of collagen remaining in the three bison bones was >90% in the 500 yr old fossil, 10% of modern in the 4900 yr old bone and less than 1% of modern for the 12,000 yr B.P. specimen.

XAD-2 isotope fractionation

XAD-2 resin was considered the best material to separate quantitatively the polar amino acids from the less polar hu-

Table 5. Comparison of $\delta^{13}C$ Values for Gelatin Extracted From Fossil Bison sp. by the Longin (1971) Method. Radiocarbon Data from Stafford (1981).

Lab No.	Sedimentary matrix	Geologic Age, yr B.P.	Skeletal Element	% modern Collagen	$\delta^{13}C$(PDB) ‰	Avg. $\delta^{13}C$ ‰	‰ range
77LLP-122	Cienega clay	ca 500	metatarsal(distal)	90	-9.1	-8.9	0.5
			metatarsal(proximal)		-8.6		
77LLP-5-1	Lacustrine clay	4910	tibia(distal)	10	-11.0		
			tibia midsection		-10.0		
			tibia midsection		-11.3	-11.1	2.2
			tibia(proximal)		-12.2		
77LLP-5-2	Lacustrine clay	4910	metatarsal(proximal)	1	-12.8	-11.8	2.1
			tibia(distal)		-10.7		
77LLP-17	Fluvial gravel	12,000	metacarpal(distal)		-18.0	-15.6	4.9
			metacarpal(proximal)		-13.1		

mates. To evaluate if XAD treatment affected isotope values, $\delta^{15}N$ and $\delta^{13}C$ were measured on collagen, hydrolyzed collagen and XAD-treated collagen hydrolysates from modern Calbiochem achilles heel tendon (Table 4). Among all three fractions, there was a 0.1‰ difference in $\delta^{15}N$ and an overall 0.3‰ variation in $\delta^{13}C$.

Sequential extraction of bone components

Because several pools of carbon exist in fossil bone, it is desirable to obtain $\delta^{13}C$ values on each protein-derived and humate fraction (Fig. 1). Sequential chemical extractions were made of the 4910 yr B.P. bison used in the previous experiment. The fossil had moderate amounts of both protein and humates and preliminary tests revealed that there was a 6‰ difference between protein and humate $\delta^{13}C$. In both the direct-hydrolysis and gelatin-hydrolysis methods, the $\delta^{13}C$ of fractions in each group became progressively more positive as purification proceeded. In both methods, the decalcified residue was 3‰ more negative than in the imino acid fractions. The value for imino gelatin-derived acids was −9.4‰ and −10.5‰ for direct-hydrolysis imino acids. Hydroxyproline $\delta^{13}C$ was −10.2 and proline $\delta^{13}C$ was −9.8‰ for the latter imino acids. Fractions significantly more negative than the preceding organic phases were fulvic acids, 6 *N* HCl insoluble residues, nitrosylated primary amino acids, and hot-water-insoluble residues.

A second bison (78LLP-28; Table 2) from the same stratum as 77LLP-5 (Table 5) and a *ca.* 500 yr B.P. bison (78LLP-6; Table 2) were used for separation of individual amino acids (Fig. 2). The $\delta^{13}C$ values for the same amino acid at each geologic age are offset by the same amount (3‰), which is

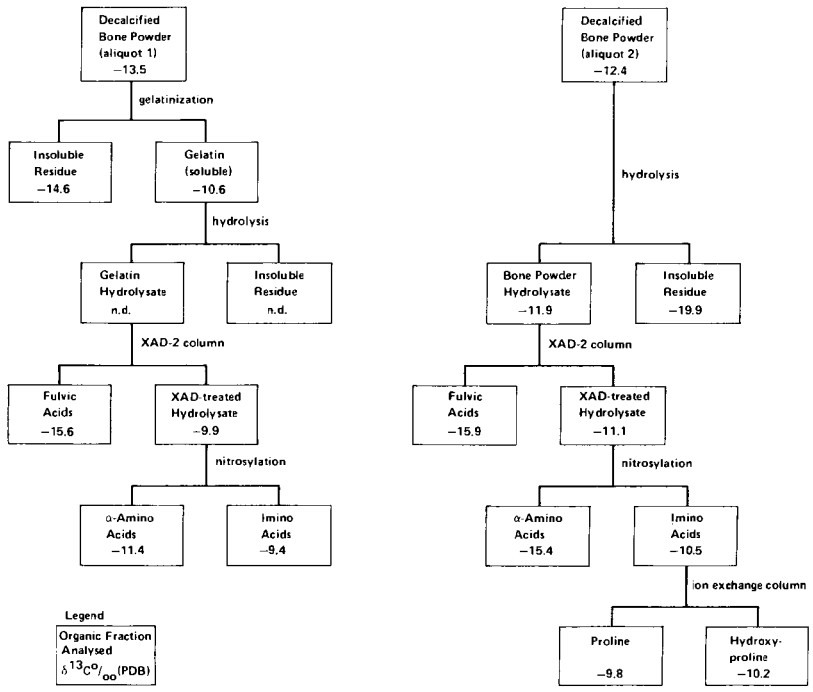

FIG. 1. Comparison of $\delta^{13}C$ values for the sequential purification of a 4900 yr old *Bison* sp. tibia by the direct hydrolysis and gelatin/hydrolysis methods. Bone powder was divided into two lots and decalcified with 0.6 *N* HCl at 4°C. In the direct method, the HCl insoluble residue was hydrolysed and the 6 *N* HCl hydrolysate was passed through an XAD-2 column. In the gelatin-hydrolysis procedure, gelatin was extracted from the HCl insoluble fraction and the gelatin was then hydrolysed and passed through the XAD-2 column. Imino acids were isolated by nitrosylation of the hydrolysates (STAFFORD *et al.*, 1982).

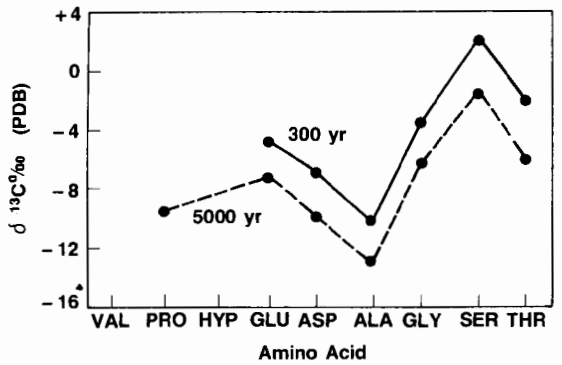

FIG. 2. Comparison of $\delta^{13}C$ values of individual amino acids from two *Bison bison* bones of differing geologic age. The fossils are from the same locality (Lubbock Lake site, Texas; STAFFORD, 1981).

the value obtained for multiple analyses of total-collagen at 4900 and 500 yr B.P. (Table 5; STAFFORD, unpublished data).

Mammoth fossils

Accurate radiocarbon measurements are essential for the age dating of fossil bone. It was important, therefore, to monitor the ^{14}C activities in protein and humate phases and to compare these experimentally-determined ages with the known-ages for each fossil. Radiocarbon ages and stable isotope compositions in Table 6 and Figs. 3, 4 and 5 were determined for three mammoths that were known to date between 11,000 and 11,500 yr B.P.

Domebo mammoth

Ten of the radiocarbon dates on organic fractions statistically agree with the known age of the elephant (Table 6). Fractions giving apparent radiocarbon ages younger than 11,000 years were fulvic acids (5000 yr) and the acid-soluble phase (9540 ± 480 yr B.P.).

The average $\delta^{15}N$ value (+12.5‰) for Domebo mammoth protein fractions was within 1‰ of that for fulvic acid extracts. The exceptions were primary and secondary amino acids prepared by nitrosylation. The most significant carbon isotope difference was between fulvic acids and bone organic matter. The average $\delta^{13}C$ of fulvic acid was −19‰ compared to the average of −11.5‰ for bone organic carbon. Other fractions isotopically more negative than bone organic carbon were the hot-water-insoluble phase (−17.5‰) and 6 *N* HCl insoluble carbon (−19.9‰).

Domebo mammoth bone was extracted a second time to evaluate how additional chemical treatments affected stable isotope values. The results, shown in Fig. 6, are that XAD and charcoal-purified hydrolyzates have $\delta^{13}C$ values of −10.3 to −11.7‰, which are 4 to 5‰ more positive than decalcified bone. The $\delta^{13}C$ values in the humate phases (between −17.3 and −21.3‰) are significantly more negative than bone organic matter. In contrast, the nitrogen isotope values of humates vary by ≤1‰ between the collagen and humate fractions. The $\delta^{13}C$ in gelatin is 3‰ more positive than in decalcified bone and is 1‰ more negative than XAD-treated hydrolyzed gelatin.

NaOH-extracted collagen is 3‰ more negative in $\delta^{13}C$ than untreated collagen. Gelatin was obtained from two base-extracted residues. The first gelatin fraction (from 0.5% NaOH leached collagen) was isotopically identical to the base-extracted residue (Fig. 6), whereas gelatin from 1% NaOH

Table 6. Radiocarbon and Stable Carbon and Nitrogen Isotope Analyses for Three Known-age Mammoths. AA- University of Arizona Accelerator ^{14}C Laboratory Number; GX- Geochron Laboratories. N.D.: Not Determined. Samples are Listed in Order of Their Pretreatment

Lab Number	Sample Description	Radiocarbon Date YR BP	$\delta^{15}N$ ‰ (AIR)	$\delta^{13}C$ ‰ (PDB)
		Domebo Mammoth		
Direct Hydrolysis Method				
AA-824	0.6N HCl insoluble residue	10,820±270	+12.9	-11.5
AA-802A	0.6N HCl soluble residue from bone powder	9540±480	+8.0	-21.7
AA-802B	6N HCl insoluble residue from 0.6N HCl insoluble collagen	10,280±560	+8.6	-19.9
AA-825	XAD-purified, hydrolyzed, 0.6N HCl insoluble collagen	11,480±450	+12.0	-11.0
AA-811	Amino acids from XAD-purified 0.6N HCl insoluble residue	10,860±450	+23.6	-13.3
AA-808	Acetone/ether soluble α-amino acids from nitrosylated XAD-purified, hydrolyzed 0.6N HCl insoluble residue	11,280±530	+35.3	-10.1
AA-812	Fulvic acids from hydrolyzed 0.6N HCl insoluble residue; NH_4OH eluted and acidified	4910±320	+13.3	-19.7
Gelatin/Hydrolysis Method				
AA-814	0.6N HCl insoluble residue	10,690±640	+12.3	-12.7
AA-803	Unpurified gelatin	10,350±410	+12.0	-11.4
AA-805	XAD-purified, hydrolyzed gelatin	10,810±420	+12.1	-9.8
AA-810	Amino acids from XAD purified gelatin hydrolyate	10,120±450	N.D.	N.D.
AA-807	Acetone/ether soluble α-amino derived from XAD-purified hydrolyzed gelatin	11,330±470	N.D.	N.D.
AA-819	Fulvic acids from hydrolyzed gelatin: acetone elution	5130±290	+13.2	-18.4
83-37	Hot-water insoluble residue after gelatinization	N.D.	+13.5	-17.5
		Dent Mammoth		
AA-830	0.6N HCl insoluble residue from bone powder	8250±520	+7.2	-13.0
AA-831	Unpurified gelatin	9240±350	+6.9	-14.0
AA-832	XAD-purified, hydrolyzed 0.6N HCl insoluble residue	10,590±500	+6.8	-13.3
AA-833	XAD-purified, hydrolyzed gelatin	10,950±480	+6.9	-12.9
		Escapule Mammoth		
AA-834	0.6N HCl insoluble residue from bone powder	8500±470	+5.1	-24.9
AA-2653	0.6N HCl insoluble residue from bone powder	8460±270	N.D.	N.D.
AA-835	Unpurified gelatin	5210±270	+5.8	-17.4
AA-836	XAD-purified, hydrolyzed 0.6N HCl insoluble residue	4610±280	+8.5	-17.2
AA-2655	XAD-purified, hydrolyzed 0.6N HCl insoluble residue	4750±370	N.D.	N.D.
AA-2656	Aspartic Acid	3320±590	N.D.	N.D.
AA-2657	Glutamic Acid	3350±350	N.D.	N.D.
AA-2658	Serine	4070±490	N.D.	N.D.
AA-2660	Combined peaks of aspartic & glutamic acids, serine, threonine	2270±360	N.D.	N.D.
AA-2661	Glycine	4540±710	N.D.	N.D.
GX-11261	Epoxy preservative coating mammoth bone	3680±210	-2.0	-24.2

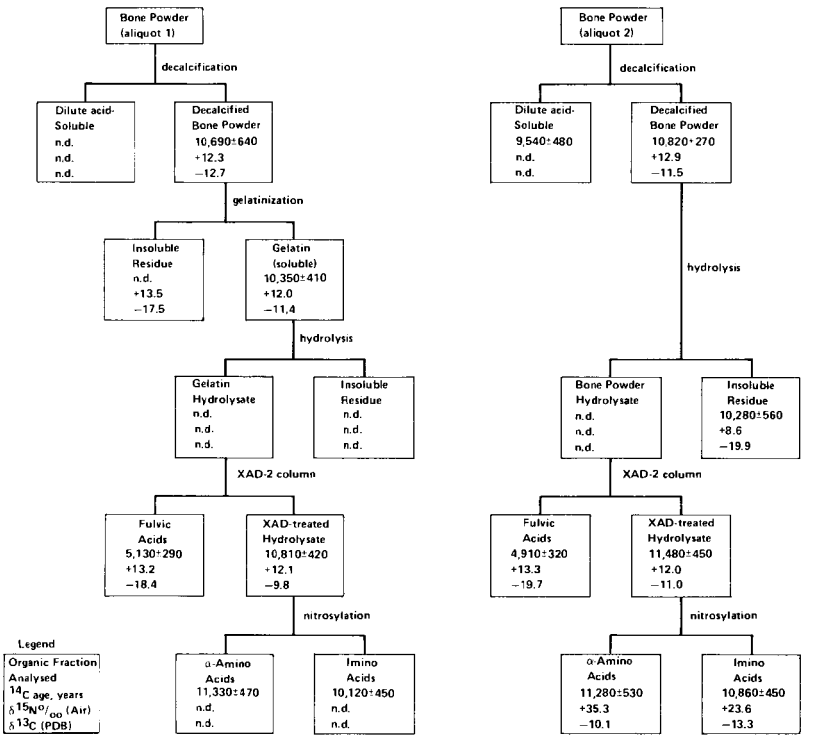

FIG. 3. Radiocarbon ages, $\delta^{13}C$ and $\delta^{15}N$ values for the Domebo mammoth, an 11,000 to 11,500 yr old mammoth associated with Clovis-culture artifacts, Oklahoma. See Table 4.

leached collagen had a $\delta^{13}C$ of −12.8‰. Weak-HCl extracted residue yielded 60% of its weight as hot-H_2O-extractable protein (gelatin) whereas the same weak-acid-insoluble residue, after NaOH extraction, gave 1% hot H_2O extractable protein.

Dent mammoth

The Dent mammoth fractions dated older as the sample became progressively more purified (Table 6; Fig. 4). The ages ranged from 8250 ± 520 yr on weak-acid insoluble collagen to 10,950 ± 480 yr on XAD-purified gelatin. The $\delta^{15}N$ values of all four fractions were within 0.2‰ of their average of +7‰. There was no significant difference in ^{13}C values between acid insoluble collagen and that purified with XAD-2 resin. Gelatin $\delta^{13}C$ was 1‰ more negative than collagen from which it was extracted, but became 1‰ more positive after it was hydrolysed and purified with XAD resin.

Escapule mammoth

The Escapule mammoth radiocarbon ages became younger as sample pretreatment increased (Table 6; Fig. 5). The oldest

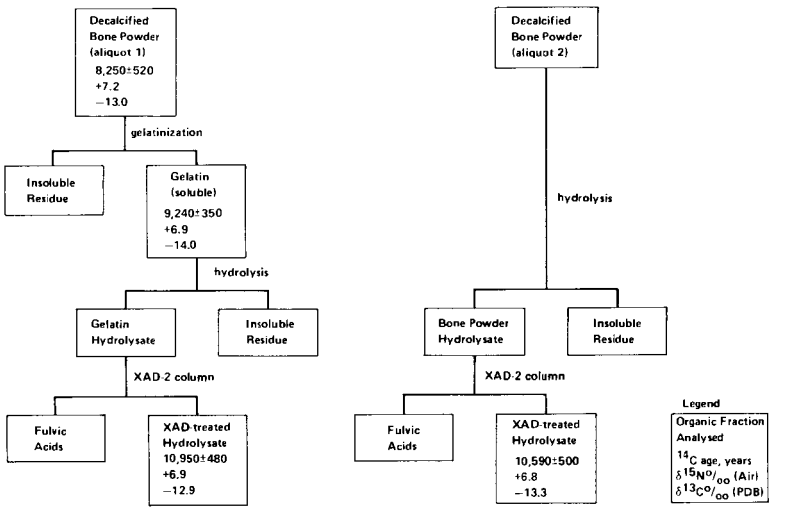

FIG. 4. Radiocarbon ages, $\delta^{13}C$ and $\delta^{15}N$ values for the Dent mammoth, Colorado.

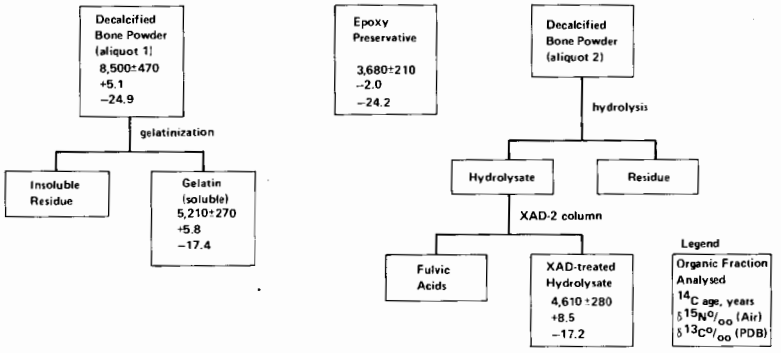

FIG. 5. Radiocarbon ages, ^{13}C and ^{15}N values for the Escapule mammoth, Arizona.

dates were on the weak-acid insoluble residue and the youngest dates were on individual amino acids, which ranged between 2270 and 4070 yr B.P. As sample treatment proceeded, the $\delta^{15}N$ values changed from +5.1 to +8.5‰ and the $\delta^{13}C$ values shifted by +7.7‰, from −24.9 to −17.2‰, respectively. Epoxy adhering to the bone had an apparent radiocarbon age of 3680 ± 210 yr, a $\delta^{15}N$ of −2.0‰ and a $\delta^{13}C$ of −24.2‰. Amino acid analysis of the epoxy gave no detectable amino acids at levels of picomoles amino acid/mg of epoxy.

DISCUSSION

The principal factors influencing the accuracy of isotope analyses on fossil bone are: (1) the molecular integrity of the bone protein and (2) the effectiveness of methods to remove humates, which have variable isotopic compositions and weight-percent-presence in bone. The uncertainty in analyses on 12,000 yr old bison gelatin (Table 5) may be due to both humate contamination and to the degraded condition of the bone's organic matter. The diagenesis of bone collagen affects the molecular weight and amino acid composition of peptides that are later extracted for analysis. Because the amino acids present in collagen have different isotopic compositions (HARE and ESTEP, 1983; Fig. 2), any variation in amino acid abundances would theoretically result in changes in $\delta^{13}C$ and $\delta^{15}N$ values.

An additional source of isotopic variation is from the chemical methods used to extract and purify collagen-derived organic matter. Replicate decalcifications for bison and mammoth bones had $\delta^{15}N$ ranges of 1‰ and $\delta^{13}C$ ranges of 4‰. The variation is probably due to humates that remain after decalcification and remain to contaminate succeeding fractions.

The replicate gelatin extractions from bison (Table 5) and the Domebo mammoth (Table 6) are an indication that extraction with hot water does not totally remove humates, which are partially soluble in weakly acidic solutions. Replicate extractions of gelatin differ by 0.6 to 2‰ for $\delta^{13}C$ and by up to 1.2‰ for $\delta^{15}N$. These data contradict conclusions that humates (fulvic acids) are insoluble during the gelatinization step (BERGLUND *et al.*, 1976; CHISOLM *et al.*, 1983). The molecular size of the humates may be an important factor in determining their solubility during decalcification and gelatinization. Geologically-young fulvic acids may have smaller molecular weights and greater solubility during gelatin extraction than older, more polymerized humic fractions that have higher molecular weights. The radiocarbon dates from the Domebo mammoth series support an hypothesis that

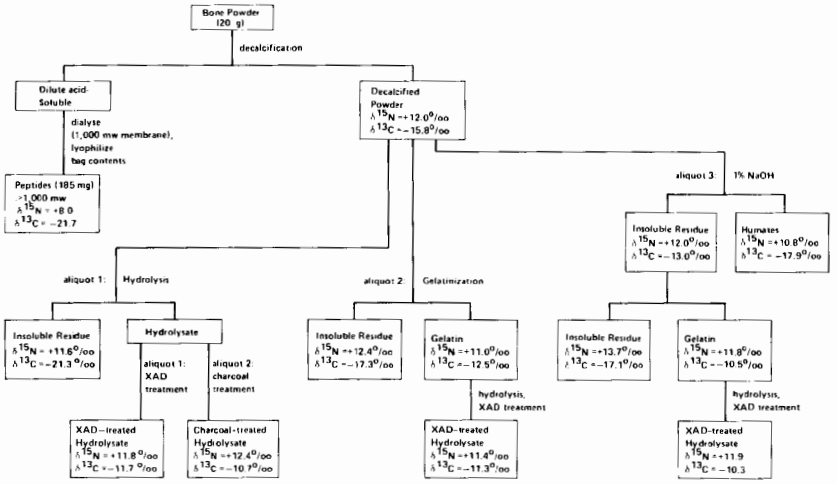

FIG. 6. Sequential extraction of Domebo Mammoth bone and chemical treatment of fractions to remove humic and fulvic acid contamination.

some higher-molecular-weight humates are contemporaneous with the fossil, and these humates may be the degradation products of endogenous bone amino acids and sugars. Although humate contamination may sometimes go undetected by ^{14}C analysis, the humic fractions will frequently alter the stable isotope ratios of bone collagen. The more negative $\delta^{13}C$ values for humates from the Domebo mammoth are an indication of humate contamination that went undetected by ^{14}C dating.

The XAD-2 treatment of protein and gelatin hydrolysates is considered the most effective method to remove humic contaminants. The resin introduces no nitrogen isotope fractionation and less than 0.1‰ change in $\delta^{13}C$. Gelatin extraction, especially for well-preserved bones, is recommended because this step excludes rootlets, lowers the amount of humates solubilized and provides a solution less contaminated with inorganic salts than if decalcified bone were hydrolyzed directly. Direct-hydrolysis and XAD-treatment of decalcified bone are recommended whenever sample weights are small and particularly when the fossil is severely degraded. Diagenetically altered collagen may contain <10% of its weight that is soluble in hot, acidic water. Direct hydrolysis eliminates the 10 to 90% mass-losses that occur during hot water extraction. A precaution in using the XAD method is that the hydrolyzates must be passed through the resin in at least 1 *N* HCl and preferably 6 *N* HCl; lower HCl molarities result in the preferential retention and loss of long-chain and aromatic amino acids (ZAIKA, 1970). Although it is feasible to pass soluble gelatin through XAD resin, this procedure is strongly discouraged because substantial amounts of gelatin would be adsorbed by the macroporous resin. In addition, the solutions would not be acidic enough to effect total protonation and adsorption of the fulvic acids. The XAD purification of hydrolyzates has two advantages over base desorption of amino acids from ion exchange resin (HO *et al.*, 1969). XAD-2 resin is physically and chemically more inert than ion exchange resins and secondly, NH_4OH will desorb simultaneously amino acids and weakly ionized fulvic acids that are bound to the cation exchange resin.

The XAD resins can also be used for concentrating and purifying fulvic acids for their isotope analysis. Fulvic acids can be isolated from several liters of dilute solution and the fulvic acids then eluted in a few hundred microliters of solution. Isotopic analyses on pure fulvic acid fractions are valuable for determining the temporal and geochemical origin of humates; the use of XAD as a chromatographic resin for humates (CURTIS *et al.*, 1981) enables these separations of specific fulvic acid phases.

The use of NaOH-leaching of collagen followed by gelatin extraction is an alternative procedure to XAD but a method that is less quantitative and results in significant sample losses. The technique is applicable only to fossils that give good yields of gelatin.

Further purification of bone organic matter includes the isolation of imino acids (hydroxyproline and proline) and primary amino acids. The nitrosylation reaction used to isolate imino acids is an inexpensive, efficient method to recover imino acids for radiocarbon dating; however, the data from the Domebo mammoth (Fig. 3, Table 6) and the bison (Fig. 1) are an indication that there are 10 to 20‰ errors in $\delta^{15}N$ and up to 5‰ errors in $\delta^{13}C$.

The series of stable isotope and radiocarbon results on known-age mammoths (Table 6) are an indication that no one isotope can be used to monitor sample purity. In the Domebo mammoth example, accurate radiocarbon dates were obtained despite unequivocal evidence that young-age fulvic acids were present. The Dent mammoth fractions dated older as humates were removed; however, there was no significant change in $\delta^{15}N$ and only 1‰ change in $\delta^{13}C$. Young-age humates were being removed as pretreatment progressed, but humate removal was detectable only by ^{14}C analyses. For the Escapule mammoth, the stable isotope data could be used to argue that young-age epoxy preservative was being removed and that the XAD-treated fraction was therefore the most accurate; however, the XAD fraction is the least accurate, as indicated by ^{14}C dates on individual amino acids. Whereas ^{15}N and ^{13}C-depleted epoxy apparently is being removed from the Escapule bone, the ^{14}C ages were still substantially younger than the bone's 11,000 yr age. The geologically-young ages of individual amino acids are strong evidence that the Escapule mammoth's original protein has been totally or largely replaced and contaminated with more recent organic matter. If replacement is a major characteristic of extremely poorly preserved bones, the prospects are very poor for using these specimens for any isotope analysis. All combinations of isotope ratios are possible for fractions in fossil bones and it is probable that contamination may exist despite apparently accurate values for some stable and radioisotopes. Had the bone collagen from the Domebo mammoth been 8 to 10‰ lighter in $\delta^{13}C$, the demonstrated presence of humates would have been impossible to establish.

CONCLUSIONS

The predominant contaminants affecting radiocarbon dates and stable isotope analyses in fossil bones are humic and fulvic acids. Hot-water extraction does not totally eliminate humates, a percentage of which are soluble in hot, acidic water. Untreated-gelatin is usable only from permafrost-preserved bones that have excellent preservation. NaOH extraction of gelatin is recommended only for well-preserved bones because in less well preserved fossils, humate removal is less reproducible and sample yields decrease dramatically. Humates are separable from fossil bone protein if collagen and gelatin hydrolyzates are pretreated with XAD-2, an effective humate adsorbent. The XAD-2 and -4 resins are recommended because they are chemically inert, they introduce minimal isotope fractionation, yields of amino acids are high and humates can be desorbed for their own analysis.

The uncertainty of stable isotope analyses on bone protein is much greater than is normally cited. Although carbon and nitrogen isotope analyses on modern collagen standards vary by ±0.1 and ±0.2‰ respectively, experimental uncertainty for both $\delta^{13}C$ and $\delta^{15}N$ is ±0.5‰ for fossil collagen. This uncertainty is due to combined errors from pretreatment methods, combustion, and diagenetically-induced changes in amino acid composition.

Humate contamination in bone is not always detectable by either radiocarbon or stable isotope assays, and the absence

of contamination in one isotope does not preclude contamination by another. Humates can have ^{14}C, ^{13}C and ^{15}N compositions that can be fortuitously identical to or different from those in bone collagen. The effect of any contaminant, especially humates, on bone analyses depends upon the absolute isotope abundance in the contaminant and the contaminant's weight-percent abundance in the analysed fraction. Contamination can not be quantified before a bone is pretreated, therefore fossil specimens should undergo a predetermined purification that comprises specific pretreatment steps. The minimum pretreatment should be the XAD-2 purification of either hydrolyzed gelatin or hydrolyzed decalcified bone. Isolation of individual amino acids will become essential as diagenetic alteration becomes greater. Simultaneous analysis of ^{14}C, ^{13}C and ^{15}N is highly recommended because a series of analyses will be more useful than single-isotope determinations. The least sensitive isotope for monitoring bone purification is ^{15}N, whereas ^{14}C is the most sensitive. Certain situations exist in which ^{13}C will detect contaminants that have the same ^{14}C age as the bone but are of exogenous geochemical origin.

Isotopic analysis of individual amino acids compensates for chemical heterogeneity of protein in bones from classes I–IV. Limits to the technique are indicated by accelerator ^{14}C dates an individual amino acids from severely degraded, Class V bone. Geologically-young ^{14}C ages on 11,000 yr old bone are evidence that very-poorly-preserved-bones are contaminated at the molecular level. Understanding what classes of bone can and cannot be analyzed will necessitate the exclusive analysis of specific amino acids throughout a study.

The ^{14}C content of humic and fulvic acids should be frequently assayed. XAD resins expedite the isolation of fulvic acids from liters of dilute solution, thus making routine the isotope analysis of these dilute phases. Chromatographic methods exist for the further resolution of humates into their molecular classes and these techniques should be applied not only to bone humates but to humates extracted from soils and sediments.

The accuracy of collagen isotope results can be dramatically improved with the methods suggested in this paper; however, these methods should not be considered the final solution to sample purification. The effects of organic contamination and collagen diagenesis on isotope analyses will not be totally understood and controlled until individual amino acids are isolated routinely and not until the geochemical cycling of amino acids is known. The use of individual amino acids will eliminate virtually all glue contamination, would lower the probability of non-collagen amino acid contamination from exogenous proteins and would eliminate much of the molecular and isotopic heterogeneity that results from protein diagenesis.

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