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RADIOCARBON DATING OF BONE OSTEOCALCIN: ISOLATING AND CHARACTERIZING A NON-COLLAGEN PROTEIN

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ABSTRACT. Osteocalcin, a non-collagen bone-matrix protein, has been examined as a possible source of autochthonous ^{14}C data in fossil bones where collagen has been seriously degraded. Extraction procedures for osteocalcin yield a well-characterized product that can be clearly distinguished from collagen. The Gla content indicates that osteocalcin is present in the fossil bones at levels similar to the range present in modern bone. However, it appears to be extracted primarily as proteolytic polypeptide fragments rather than as an intact protein. Concordant ^{14}C determinations are obtained on osteocalcin and gelatin extracts from the same bone when the collagen is relatively well preserved. However, increasing discordances in the ^{14}C values of the osteocalcin and gelatin fractions are associated with reduced concentrations of the gelatin extract in the bone.

INTRODUCTION

Ajie *et al.* (1990) reported the first set of ^{14}C measurements on osteocalcin, a non-collagen protein, from a series of modern and fossil bones. The purpose of these measurements was to assess the accuracy of osteocalcin ^{14}C age determinations in bone samples that exhibited low or trace levels of collagen. Widely-used methods of chemical pretreatment can yield anomalous ^{14}C ages for fossil bone that has lost >95% of its original protein content (Long *et al.* 1989; Hedges & Law 1989; Stafford *et al.* 1987, 1990, 1991; Taylor 1992). Many bones of significant anthropological interest from most tropical and many temperate sites of Pleistocene age have typically been subjected to severe diagenesis, characterized by trace amounts of protein extracts that do not exhibit collagen-like amino-acid patterns (Taylor 1991).

We are concerned with the biochemical nature, and especially the purity, of the osteocalcin fraction on which ^{14}C determinations have been obtained. Thus, we have obtained amino acid composition, radioimmunoassay, electrophoresis and stable isotope data on the osteocalcin extracts.

OSTEOCALCIN

Osteocalcin is one of several non-collagenous proteins contained in bone. Also known in the biomedical literature as BGP or "Bone Gla protein," osteocalcin was discovered in the early 1970s during a search for the source of the calcium-binding, vitamin-K dependent amino acid, gamma-carboxyglutamic acid or Gla (Hauschka, Lian & Gallop 1975; Price *et al.* 1976; Hauschka 1977; Hauschka & Gallop 1977). It is a low molecular weight protein (5200–5900 daltons) with 46–50 amino-acid residues per molecule, which contains 2 or 3 (depending on species) residues of Gla per molecule. Human osteocalcin has 2 Gla residues (Poser *et al.* 1980). This amino acid is formed within the protein as a result of post-translational carboxylation of specific glutamic acid residues at specific sequence positions. Another protein containing Gla – the Matrix Gla Protein (MGP) – with a molecular weight of 9000–11,000 daltons has also been identified in bone. Hauschka *et al.* (1989) provide the most recent review of the literature on osteocalcin and MGP.

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Metabolic functions for osteocalcin may include local control of calcium deposition and removal, a regulatory role in mineral deposition and crystal growth, and possible mediation of the action of vitamin D. Osteocalcin content varies with the degree of bone mineralization within and among organisms; the more mineralized the bone, the higher the osteocalcin content. Because of this, it is most abundant in the midshaft portion of long bones. Mammalian bone generally contains from 1–2 mg of osteocalcin per gram of dry weight of bone. However, human bone contains significantly less; it averages about 0.28 mg of osteocalcin per gram of bone (Hauschka, Lian & Gallop 1978; Hauschka & Reid 1978; Hauschka *et al.* 1989). Averaged over the whole organism, mammalian osteocalcin comprises about 1% of the total bone protein and some 10–20% of the noncollagenous bone proteins (Price *et al.* 1976; Nishimoto & Price 1979; Hauschka *et al.* 1989).

The first published proposal to use osteocalcin for the dating of fossil bone was made by Hauschka (1980). Several properties of osteocalcin make it potentially a very useful protein for ^{14}C dating of fossil bones. First, it appears to bind tightly to hydroxyapatite, the major mineral component of bone. In this bound form, the protein should be well protected from biochemical degradation due to the buffering action of hydroxyapatite and the decreased accessibility to exogenous proteinases. Second, Gla has not been detected in a number of potential contaminants; Table 1 lists the materials examined by Hauschka (1977). Although several investigators continue to seek sources of osteocalcin in other organisms, we are not aware of any confirmed reports. If this limited distribution continues to be confirmed, major sources of diagenetic contamination would be minimized.

TABLE 1. Materials in which Gla has not been found at concentrations >0.2 residues of Gla/1000 residues of Glu (Hauschka 1977)

Calcium-binding Vertebrate Proteins	
Thermolysin	Collagenase
Carp parvalbumin	Brain S-100 protein
Other Vertebrate Proteins	
Tubulin	Insulin
Hyaluronidase	Carbonic anhydrase
α-Lactalbumin	α-Amylase (hog pancreas)
α-Chymotrypsin	Semen
C3 complement	Erythrocyte membrane
Pepsin	Spectrin
Elastin	Saliva
Aldose reductase	Milk
Collagen	Procollagen (skin)
Plant Proteins	
Zein	Spinach, acetone powder
Bacterial Cell Cultures	
Bacilius subtilis	*Escherichia coli*
Pseudomonas fluorescens	*Aerobacter aerogenes*
Bacteroides melaninogenicus	
Reagents	
EDTA	Poly-L-glutamic acid

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Subsequent investigations have extended the usefulness of osteocalcin for geochronological and biogeochemical studies. One question was the degree to which the protein would be protected from chemical degradation on the scale of geologic time. Huq, Tseng and Chapman (1985) detected the osteocalcin antigen in two bones about 4 ka and 7 ka old. Ulrich *et al.* (1987) examined the osteocalcin content of six bovid bones recovered from geological contexts ranging in age from Miocene (*ca.* 13 Ma) to late Pleistocene (*ca.* 12 ka) and found that the osteocalcin antigen as well as its Gla residue remained detectable in all of the samples.

Extraction and purification procedures

Bone samples were first cleaned by ultrasonication in an ice-cold Tris-buffered protease inhibitor solution (TPIC) (Gundberg *et al.* 1984), then rinsed several times with ice-cold distilled water and freeze-dried. The dry bone was then powered in liquid nitrogen with a diamond mortar to a size less than 710 μ. The powder was then homogenized in ice-cold TPIC (1 gm/20 ml) for 30 min and allowed to settle. Decanting and rehomogenization with distilled water was continued until the supernatant was clear and colorless.

Two methods were examined to extract and purify an osteocalcin fraction: one using EDTA (Gundberg *et al.* 1984) and a modification of a method using formic acid (Poser *et al.* 1980). Since both methods appeared to produce a comparable product, and the formic acid method avoids the possibility of incomplete EDTA removal, we used the latter technique to extract the osteocalcin on which the initial ^{14}C and other analytical data were obtained.

The bone was demineralized in 20% formic acid (10 ml gm^{-1} of bone) followed by dialysis (Spectrapor 1 membrane tubing, mol. wt. cutoff 6000–8000) against deionized water for four days at 4°C with daily changes of water. (It has been experimentally determined that the highly charged osteocalcin molecule behaves hydrodynamically as a protein of 10,000 to 15,000 mol. wt. during dialysis extraction.) The content of the dialysis tubing was centrifuged and the supernatant reduced to a volume of 20 ml by freeze-drying. This was followed by gel filtration of the soluble extract on sephacryl S-200 in 6 M Guanidine HCl. Elution of osteocalcin was monitored by absorbance at 276 nm. The pooled osteocalcin fraction was freeze dried, dissolved in 0.07 M NH_4HCO_3 (1 mg ml^{-1}) and eluted from a DEAE ion exchange column with a 700 ml linear gradient of 0.07 M to 0.7 M NH_4HCO_3. Fractions of 5 ml were collected and the protein was monitored by A_{276}. The pooled osteocalcin fraction was reduced to a volume of 10 ml by freeze-drying, dialyzed against deionized water at 4°C for four days with daily changes of water, and subsequently freeze-dried to yield osteocalcin. We took appropriate precautions to avoid intersample contamination by using separate columns for modern and fossil samples, extensive column washing and isotopic and amino-acid analyses of blank column eluent buffers.

To characterize biochemically the formic acid-extracted osteocalcin fraction, amino-acid composition, radioimmunoassay and preliminary electrophoresis data were obtained on extracts from human and moa bone samples. Table 2 presents the Gla and osteocalcin content of five human bones, while Figure 1 illustrates dilution curves comparing radioimmunoassay data from monkey (*M. fascicularis*) (Hauschka, Carr & Biemann 1983), a modern human and a human fossil bone (HA-104) from the Haverty site (Haverty skeleton 4). HA-104 exhibited a dilution curve parallel to that of the modern bone control indicating strong identity of the ancient antigen to modern osteocalcin. The Gla content indicates that it is present in the fossil bones at levels similar to the range present in modern bone. However, the radioimmunoassay data, which detect only intact osteocalcin – not reacting to proteolytic fragments of the native protein – indicate that the fossil human bones retained only a fraction of the *in vivo* osteocalcin content of modern bone. The range

TABLE 2. Gla and Osteocalcin Content of Fossil Human Bone

Sample no.	Gla/Glu*	Osteocalcin content**
HA-100	3.71	3.3
HA-103	5.26	5.1
HA-101	2.99	2.6
HA-102	3.54	5.2
HA-104†	4.85	17.4

*Expressed as residues Gla/1000 residues Glu of alkaline-hydrolyzed whole bone powder; standard bovine Gla concentration = 5.31; modern human Gla concentration ranges from 2.5 to 8.5 depending on bone and location in body (Hauschka 1977 and unpublished data).

**Expressed as % of modern human where modern human osteocalcin content is set at 0.28 mg/g bone (Hauschka *et al.* 1989).

†See Fig. 1.

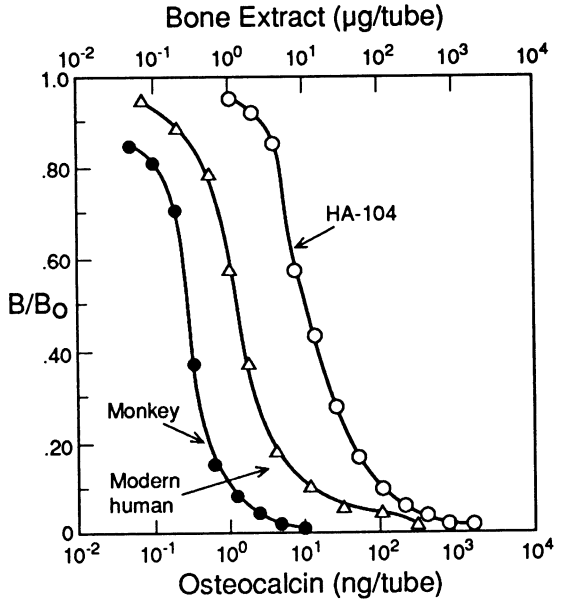

Fig. 1. Radioimmunoassay (RIA) of modern human bone (△), modern monkey (*M. fascicularis*) bone (●) and HA-104, fossil human bone (○) using non-equilibrium technique described in Gundberg *et al.* (1984). Primary antiserum was rabbit antibovine osteocalcin used at a final dilution of 12,600-fold. Extracts were serially diluted in assay buffer and assayed over a wide range corresponding to 0.05–2000 µg bone/tube (upper abscissa). Immunoprecipitation with goat antirabbit IgG second antibody was followed by centrifugation, washing of pellets and gamma counting. Data are expressed as the quotient B/B_o, where B is designated as the amount of ^{125}I-osteocalcin,specifically bound to antibody, and B_o is the value of B in the absence of added unlabeled osteocalcin. Monkey osteocalcin standard (lower abscissa) gave a midpoint $B/B_o = 0.5$ at 0.24 ng/tube, with an interassay variation of 10%.

in osteocalcin content exhibited by the fossil bones suggests that diagenetic conditions may, in part, control the amount of intact osteocalcin that can be extracted.

The amino-acid composition of the osteocalcin isolated from the fossil bones is clearly similar to modern human osteocalcin (Poser *et al.* 1980) and can be clearly distinguished from collagen. No hydroxyproline was found in the osteocalcin extracts of any of the human fossil bones examined. Further, preliminary data from electrophoresis indicate that the extraction procedures applied to fossil bone yield only a low molecular weight protein of 6000–15,000 daltons. This appears to exclude the presence of collagen proteins in the purified osteocalcin fraction.

To examine the relationship between the stable isotope data of the osteocalcin and gelatin fractions, aliquots were combusted using the methods of Minagawa, Winter and Kaplan (1984), and the resulting CO_2 and nitrogen were analyzed for $\delta^{13}C$ (Fig. 2) and $\delta^{15}N$ values (Fig. 3). Shifts in stable

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isotope values appear to correlate with decreased gelatin concentrations at or below 1% of modern bone values. The changes in stable isotope values may correlate with partial elimination of the most soluble amino-acid residues and peptide fragments derived from the poly-peptide chains of these samples (Tuross, Fogel & Hare 1988), or perhaps reflect terrestrial soil contamination (Ajie *et al.* 1991).

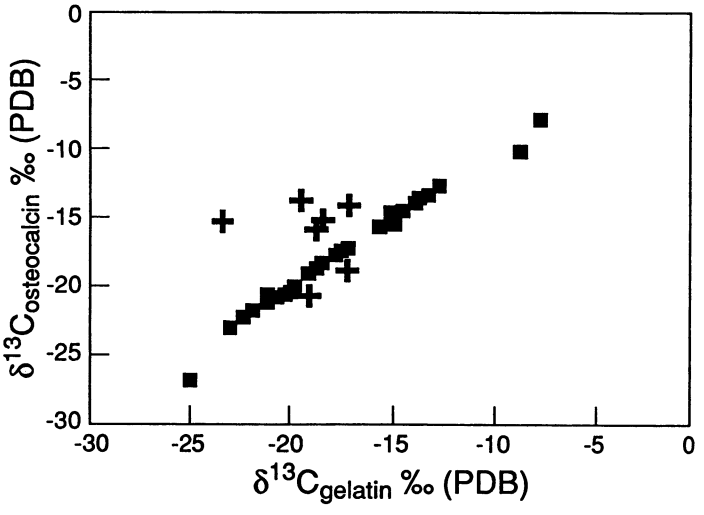

Fig. 2. Stable carbon isotope ratios of gelatin *vs.* osteocalcin from 50 modern and fossil bone samples. "+" indicates a bone with gelatin content of <1%.

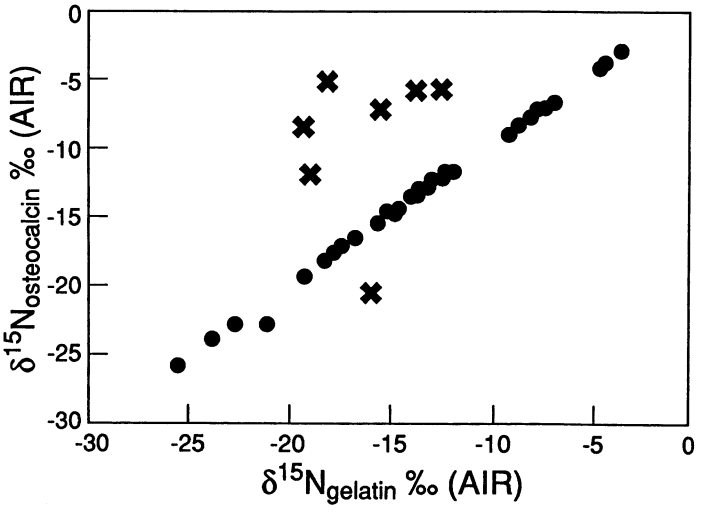

Fig. 3. Nitrogen isotope ratios of gelatin *vs.* osteocalcin from 50 modern and fossil bone samples. "x" indicates a bone with gelatin content of <1%.

Radiocarbon determinations on osteocalcin

To provide modern controls, Table 3 presents elemental, stable isotope, and amino-acid compositional data on gelatin and osteocalcin extracts from the series of modern human bones. Gelatin was extracted using methods outlined in DeNiro and Epstein (1981) and Schoeninger and DeNiro (1984). This involves washing in TPIC, solubilizing in 1 mM HCl for 10 h at 90°C, filtering and lyophilizing. As expected, the elemental carbon/nitrogen (C/N), hydroxyproline (Hyp), glycine/

glutamic (Gly/Glu) acid and glycine/aspartic acid (Gly/Asp) ratios reflect the presence of intact collagen. While the gelatin (collagen) concentration moderately varied in these samples, the osteocalcin concentration does not vary significantly.

TABLE 3. Biogeochemical data on gelatin (collagen) and osteocalcin fractions of modern human bone

	Gelatin							Osteocalcin			
Sample no.	Wt%	C/N*	$\delta^{13}C$** (‰)	$\delta^{15}N$ (‰)	Hyp† (R/1000)	Gly/‡ Glu	Gly/§ Asp	Wt%	C/N*	$\delta^{13}C$** (‰)	$\delta^{15}N$ (‰)
CGH-512	19.9	3.3	−19.6	+10.3	86	4.7	7.1	.25	4.2	−20.0	+10.3
CGH-513	16.1	3.1	−17.4	+ 7.8	85	4.7	7.1	.26	4.2	−17.7	+ 7.9
CGH-514	12.9	3.2	−18.6	+ 8.4	85	4.7	7.2	.25	4.2	−18.7	+ 8.5
CGH-511	8.9	3.1	−17.0	+10.0	85	4.7	7.2	.20	4.2	−17.1	+10.3
CGH-510	7.5	3.1	−20.9	+10.3	85	4.7	7.2	.24	4.1	−20.6	+10.4

*Carbon/nitrogen ratio
**$\delta^{13}C$ PDB; $\delta^{15}N$ AIR
†Hydroxyproline composition in residues/1000
‡Glycine/glutamic acid ratio
§Glycine/aspartic acid ratio

A previous report presented ^{14}C and compositional data obtained on a series of fossil bones (Ajie *et al.* 1990: Table 2). In these samples, although gelatin content ranged from about 12% to <0.2%, osteocalcin concentration varied relatively little. What variation we observed was associated with differences between human and moa bone; there was virtually no difference in osteocalcin content measured in human bone even though the ages ranged from about 3 ka to almost 25 ka. In our view, these data supported the view that hydroxyapatite protects the osteocalcin from major diagenesis.

To examine variability in gelatin and osteocalcin ^{14}C values in a suite of fossil bones of assumed similar age but with significant variability in preservation of the gelatin/collagen fraction, we analyzed human skeletons from the Haverty (Angeles Mesa) site, Los Angeles County, California. A recent summary of analytical evidence concluded that the Haverty skeletons were interred over a relatively short time interval between 4 and 5 ^{14}C ka BP. This conclusion was, in part, based on ^{14}C values previously obtained on three of the Haverty skeletons (Brooks *et al.* 1991).

Table 4 lists the ^{14}C data obtained on gelatin and osteocalcin fractions obtained on 6 bones from 5 Haverty skeletons. For Haverty skeleton 1, both the gelatin and osteocalcin values are consistent with previous ^{14}C values obtained on this skeleton. However, as the percentage yield of the gelatin extract decreases and the C/N ratio progressively diverges from modern bone in other Haverty skeletons, there appears to be a trend for the discordance between the gelatin and osteocalcin ^{14}C values to increase. This suggests the possibility of a progressive contamination of the collagen in these samples. There is also a divergence from the pattern exhibited in modern bone in the stable isotope values of the gelatin and osteocalcin fractions. This is especially the case with regard to Haverty skeleton 4 (HA-104). In view of the fact that the osteocalcin ^{14}C age on Haverty skeleton 4 represents an age in excess of 12 ka, *i.e.*, potentially of pre-Clovis age, while other associated geological, archaeological and ^{14}C data indicate a much younger age, we will continue our studies with regard to the isotopic integrity of the gelatin and osteocalcin extracts obtained from all of the Haverty human skeletal samples, including HA-104.

TABLE 4. Comparison between gelatin and osteocalcin ^{14}C data on organic extracts from human bone samples from Haverty (Angeles Mesa) site, Los Angeles

	Gelatin								Osteocalcin					
Haverty skeleton	Wt%	C/N	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	Hyp (R/1000)	Gly/ Glu	Gly/ Asp	^{14}C age (yr BP)	Wt%	C/N	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	^{14}C age (yr BP)	$^{14}C_{ost}-{}^{14}C_{gel}$ (yr)
1**	12.9	3.3	--	--	--		--	5260 ± 520†	0.23	4.1	--	--	5540 ± 230	280
3*	11.6	3.1	−14.6	+17.8	86	4.6	7.3	7260 ± 90	0.25	4.3	−14.5	+17.8	9090 ± 120	1830
2**	1.3	8.7	−15.6	+ 4.8	87	4.9	8.5	2730 ± 90	0.22	4.2	−16.6	+11.5	4630 ± 260	1900
5**	1.2	9.9	−13.6	+15.1	92	4.5	7.7	3870 ± 350	0.22	4.3	−17.2	+14.1	12,600 ± 460	8730
5**	0.9	17.9	−13.7	+10.2	95	4.5	7.3	4710 ± 190	0.24	4.1	−17.4	+16.6	11,960 ± 500	7250
4*	0.8	18.3	−24.9	+ 9.1	50	3.9	5.8	5250 ± 90	0.25	4.2	−26.9	+ 4.5	15,900 ± 250	10,650

*Graphitization and AMS ^{14}C measurements at DSIR, New Zealand

**Graphitization at UCR Radiocarbon Laboratory and AMS ^{14}C measurements at AMS Laboratory, UC/LLNL, Livermore, California

†Previous decay counting ^{14}C analysis on Haverty skeleton 1: 5350 ± 150 (UCR-1349A: total acid soluble fraction) and 5280 ± 180 (UCR-1349D: total acid insoluble fraction after gelatin conversion with base soluble fraction removed)

APPLICATION TO DATING OF MACHU PICCHU HUMAN SKELETONS

Excavations in 1911 at the site of Machu Picchu (13°09′S, 72°32′W) in the Southern Andes of Peru revealed a series of graves containing human skeletons and associated grave goods. The skeletal and artifact assemblage from these excavations were subsequently housed at Yale University. Although the artifacts from Machu Picchu represent only Inca and early Colonial period materials, Eaton (1916) suggested that the site also contained a pre-Inca occupation. This interpretation has been disputed by later investigators.

Radiocarbon determination on bone collagen from five Machu Picchu human skeletons from the Yale collection yielded ages ranging from 640 to 2050 BP (Berger *et al.* 1988). We have obtained AMS ^{14}C ages on gelatin and osteocalcin fractions from these five skeletons (Table 5). The collagen appears to be well preserved as evidenced by the weight percent and C/N ratios of the extractable gelatin. Our ^{14}C data and particularly the concordant gelatin/osteocalcin values indicate that these skeletons are not earlier than the Inca period. This conclusion is entirely consistent with the current views of specialists in Andean studies concerning the dating of Machu Picchu materials (Christopher B. Donnan, personal communication, 1991). Because of the statistical ranges associated with the dates and the post-16th century de Vries effects, the skeletons could be much younger.

TABLE 5. Radiocarbon Determinations on Bone Samples from Machu Picchu, Peru

Bone (Yale)	^{14}C age (yr BP) Collagen*	Gelatin**	Osteocalcin**
3175	855 ± 365 (UCLA-2702B)	340 ± 150 (CAMS-822)	310 ± 150 (CAMS-821)
3211	1485 ± 185 (UCLA-2702D)	230 ± 130 (CAMS-816)	230 ± 120 (CAMS-815)
3212	---	320 ± 150 (CAMS-818)	350 ± 100 (CAMS-817)
3239	850 ± 325 (UCLA-2702F)	240 ± 120 (CAMS-820)	350 ± 90 (CAMS-819)
3248	640 ± 180 (UCLA-2702G)	420 ± 130 (CAMS-824)	300 ± 100 (CAMS-823)

*Berger *et al.* (1988) identifies the organic extract as collagen.

**This report; AMS analysis at Center for Acclerator Mass Spectrometry, University of California/Lawrence Livermore National Laboratory AMS Laboratory (J. S. Vogel, personal communication). Estimated $\delta^{13}C = -20‰$.

CONCLUSIONS

Osteocalcin is a non-collagen protein that holds promise for obtaining reliable ^{14}C age estimates on bone in which collagen concentrations have been seriously depleted. Using bones with a wide range of collagen preservation, we have obtained ^{14}C measurements on gelatin and osteocalcin extracts and have measured other indices of the biogeochemical status of the extracted fractions. In bones of presumed similar age, serious discordance in ^{14}C ages of gelatin and osteocalcin fractions appear to be accompanied by a decrease in gelatin content in the bone and anomalies in the stable isotope data.

Amino-acid composition, radioimmunoassay and electrophoresis studies of osteocalcin fractions indicate that the osteocalcin extraction procedures exclude collagen. The Gla content indicates that Gla is present in the fossil bones at levels similar to modern bone. However, the extractable osteocalcin appears primarily as proteolytic polypeptide fragments, rather than intact protein. The range in osteocalcin content in the fossil bones suggests that diagenetic conditions may control the amount of intact osteocalcin that can be extracted and its degree of fragmentation.

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