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Osteocalcin as the recommended biopolymer for ^{14}C age dating of bone and $\delta^{13}C$ and $\delta^{15}N$ paleodietary reconstruction

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Abstract—Osteocalcin, a gamma-carboxyglutamic acid containing bone protein is tightly bound to the hydroxyapatite matrix of bone and is relatively more stable than the dominant collagen. Its distribution in nature is limited to vertebrates. Osteocalcin and collagen have been isolated from modern and fossil bone samples of different organisms in different depositional environments for analysis of their $\delta^{13}C$, $\delta^{15}N$, and ^{14}C content. We present evidence suggesting that osteocalcin is a more suitable protein fraction for obtaining accurate ^{14}C age estimates and/or $\delta^{13}C$ and $\delta^{15}N$ for paleodietary reconstruction from bone samples.

INTRODUCTION

THE EARLIEST DESCRIPTIONS of diet in prehistoric humans were based upon inferences from artifact assemblages or anecdotal accounts of midden constituents. Column sampling performed with proper statistical controls is adequate for measuring quantities of small, well-preserved, and evenly distributed food remains such as seed, shell, or fish bone (TREGANZA and COOKE, 1948; MEIGHAN, 1972). It is less accurate, however, at predicting quantities of larger, unevenly distributed components such as animal bone. Similar problems are encountered with faunal analyses where spotty distribution patterns are complicated by non-representation of bones due to diagenesis, butchering patterns, or scavenger activity. Plant remains pose unique problems. They infrequently are preserved in archaeological sites and when present, mostly represent uneaten foodstuffs.

In contrast to standard midden analyses, trace element and stable isotope analysis of human bone collagen provide a direct means for estimating the contribution of broad categories of food items to the diet. This is possible because the slight, but detectable differences in the isotopic composition of different food types are ultimately reflected in bone collagen (DENIRO and EPSTEIN, 1978, 1981; SCHOENINGER and DENIRO, 1984; CHISHOLM *et al.*, 1982; MINAGAWA *et al.*, 1986). The following sections discuss the basis for using stable carbon and nitrogen isotope ratios for dietary reconstruction.

Carbon isotope ratios

Carbon isotope ratios discriminate C_3 plants from C_4 and CAM (Crassulacean acid metabolism) plants and also differentiate marine from terrestrial organisms. Different classes of plants have different carbon isotope ratios, depending on the type of biochemical reaction they utilize to obtain their carbon. C_3 plants use a different enzymatic pathway to fix atmospheric carbon during photosynthesis than do C_4 plants. The enzyme responsible for the C_3 pathway discriminates $^{13}CO_2$ to a greater extent than that for C_4 plants (BENDER, 1968, 1971), resulting in lower $\delta^{13}C$ values ranging from -22 to -33 (mean -25), whereas those of C_4 plants fix carbon with a $\delta^{13}C$ of -9 to -16 (mean -12). CAM plants, which have the ability to use both C_3-like and C_4-like photosynthetic pathways, tend under natural conditions to resemble C_4 plants in their isotopic composition (BENDER *et al.*, 1973; OSMOND *et al.*, 1973). These differences have been exploited by many researchers to document the introduction of plants from one isotopic group into an environment where the natural vegetation consists of plants of another isotopic type. This has been particularly useful in documenting the progression of maize (a C_4 plant) agriculture in the New World (VAN DER MERWE and VOGEL, 1977; BENDER, 1968; DENIRO and EPSTEIN, 1981; VAN DER MERWE, 1982; SCHWARCZ *et al.*, 1985).

Carbon isotopes can also discriminate between marine and terrestrial organisms (TAUBER, 1981; CHISHOLM *et al.*, 1982, 1983). This is possible due to the different isotopic composition of the carbon source in each environment. Marine organisms use seawater bicarbonate, which is approximately 7 per mil more positive than atmospheric carbon dioxide. Thus, the mean $\delta^{13}C$ value of organic carbon in marine organisms is -18 compared to the mean $\delta^{13}C$ value of -25 for terrestrial C_3 plants. Unfortunately, marine $\delta^{13}C$ values may be mimicked in a terrestrial animal that feeds on a mixture of C_3 and C_4 land plants. Due to this complication, it is

sometimes impossible to assess the marine contribution to the diet in any human population that utilizes both marine foods and large quantities of maize or other C_4 plants, by use of the $^{13}C/^{12}C$ isotope method alone.

Nitrogen isotope ratios

Studies on animals raised on diets of known nitrogen composition demonstrate that the $\delta^{15}N$ of animal tissues is determined by the $\delta^{15}N$ of their diets (DENIRO and EPSTEIN, 1981). $\delta^{15}N$ values of bone collagen was shown to be about 3 per mil more positive than that of the diet. The nitrogen isotopic composition of diet ultimately depends on the composition of nitrogen compounds available for uptake by plants at the base of the food chain. Subsequently, it was shown that nitrogen isotope ratios distinguish between marine and terrestrial foods in the diet (SCHOENINGER *et al.*, 1983; SCHOENINGER and DENIRO, 1984). Animals utilizing marine food sources are more enriched in ^{15}N than animals feeding on land plant sources, because the nitrogen sources utilized during protein synthesis in the two systems have a different nitrogen isotope composition. Marine plants have $\delta^{15}N$ values which are about 8 per mil heavier than those of terrestrial plants. These differences are then reflected in the food chain, with organisms feeding directly on either phytoplankton or land plants showing about a 3 per mil enrichment in ^{15}N over the starting $^{15}N/^{14}N$ ratio at each trophic level (DENIRO and EPSTEIN, 1981; MINAGAWA *et al.*, 1986; SCHOENINGER and DENIRO, 1984; SCHOENINGER, 1985). This has the effect of magnifying the differences between marine and terrestrial foods, as humans tend to feed on marine organisms higher in the food chain (fish) than on land animals (herbivores).

As with carbon isotopes, overlap in $\delta^{15}N$ ratios of marine and land animals may be observed under certain conditions. Organisms inhabiting estuaries or coral reef environments are likely to have $\delta^{15}N$ values similar to those of terrestrial organisms, due to high nitrogen fixation rates (CAPONE and CARPENTER, 1982; SCHOENINGER and DENIRO, 1984). If food from these environments is incorporated into the diet, there will be an apparent under-representation of the marine components. Consumption of large quantities of $\delta^{15}N$-depleted legumes (VIRGINIA and DELWICHE, 1982) might also skew marine/terrestrial contrasts, although this has yet to be documented in human populations (SCHWARCZ *et al.*, 1985).

The combined application of carbon and nitrogen isotope ratios may resolve discrepancies resulting from the use of only one isotope (SCHOENINGER *et al.*, 1983). However, there are certain conditions when the application of both isotopic techniques will still be inconclusive. For example, combined $\delta^{13}C$ and $\delta^{15}N$ analyses sometimes do not permit a thorough dietary reconstruction of the native inhabitants of tropical island such as the Bahamas, where a diet consisting of C_4 agricultural products and marine resources are gathered from both coral reefs and the open ocean (KEEGAN and DENIRO, 1988). A similar situation is likely to be found in coastal Peru, where archaeological populations are known to have cultivated both maize and non-legumes while also exploiting marine resources. Under such conditions, resolution of dietary contributions becomes difficult.

Radiocarbon dating

Radiocarbon measurement is usually achieved by measuring the indigenous carbon extracted and purified from a suitable fraction of the material. This measurement is then used to derive the age of the material. Radiocarbon measurement for bones has conventionally been achieved by scintillation measurement of the β-disintegration of ^{14}C atoms in the mineral phase (usually hydroxyapatite) or the organic fraction (usually collagen). Each of these phases has been observed to have its associated problems for radiocarbon measurements (HEDGES and LAW, 1989). The exchange of carbon atoms of the mineral phase with ground water poses a serious problem in the use of hydroxyapatite. Contamination through secondary deposition and recrystallization of indigenous carbonate further complicates the use of hydroxyapatite. An indigenous organic fraction, such as collagen, which constitutes >90% of bone organic matter is considered to be more suitable for radiocarbon measurements of bone.

The development of the Accelerator Mass Spectroscopy (AMS) now makes it feasible to utilize milligram amounts of organic materials for ^{14}C dating and to refine existing, or develop new, pretreatment strategies for the ^{14}C dating of bone, including the direct dating of Holocene and Pleistocene hominid skeletal samples. This has been especially useful in dating bone fragments where only small quantities are available. In cases where the majority of the intact collagen remains, it is widely agreed that accurate ^{14}C age estimates can be obtained on this purified organic extract. However, accepted methods used to chemically pretreat bone samples can, in some cases, yield unreliable ^{14}C age

determinations when applied to fossil bones characterized by low to trace amounts of organic residues which do not exhibit a collagen-like amino acid pattern. The lack of effective pretreatment procedures for biochemically degraded bone limits the application of ^{14}C dating on bone samples from most tropical environments older than a few thousand years. These environments include regions from which valuable late Pleistocene hominid fossil bones derive. The use of collagen or any other fraction as a dating tool, therefore, should be approached with caution for the following reasons:

1) The sensitivity to contamination by modern carbon increases exponentially (1% contamination has 6,000 year effect for a bone 37,000 years old; HEDGES and LAW, 1989).

2) Carbon containing compounds from allochthonous environments may become physically and chemically mixed during diagenesis with carbon compounds indigenous to bone. Because the burial and fossilization environments represent a variety of different conditions, no patterns of interaction which occur are able to predict the origin of such exogenous contaminants.

Another bone protein, osteocalcin, which comprises 10–20% of the total non-collagenous protein of bone is tightly bound to the mineral phase and is present in bone of all vertebrate animals (HAUSCHKA, 1977; HAUSCHKA and GALLOP, 1977; HAUSCHKA *et al.*, 1982, 1983; GUNDBERG *et al.*, 1984). The properties of osteocalcin (see below) give it certain advantages over collagen in the study of fossil organic materials.

Osteocalcin is distinguished from collagen by its content of two or three (depending on species) residues of the vitamin K-dependent amino acid, gamma-carboxyglutamic acid (Gla). This amino acid is formed in the protein as a result of post-translational carboxylation of specific glutamic acid residues at specific sequence positions (GUNDBERG *et al.*, 1984). The number of Gla residues in human osteocalcin is reported to be two (POSER *et al.*, 1980). Some characteristic properties of osteocalcin relevant to the analysis of $\delta^{13}C$, $\delta^{15}N$ and ^{14}C in this study include:

1) It is a non-collagenous protein of low molecular weight (~6000 daltons) containing 46–50 amino acid residues per molecule. The amino acid sequence has been determined for more than a dozen vertebrate species (PRICE *et al.*, 1976; HAUSCHKA *et al.*, 1982) and has been reported to show strong conservation over hundreds of millions of years of divergent evolution (ULRICH *et al.*, 1987).

2) It binds strongly to the hydroxyapatite (HAUSCHKA, 1977), the major mineral component of bone. In this bound form, the protein is very stable in part because of the buffering action of hydroxyapatite and partly due to the decreased accessibility to exogenous proteinases. Osteocalcin has been isolated from bovid bones 12,000 years and older (ULRICH *et al.*, 1987).

3) The protein is most abundant in bone (up to 2 mg/g), with lower amounts in dentin and cementum; enamel is devoid of osteocalcin (HAUSCHKA *et al.*, 1983, 1989). Osteocalcin has not been detected in any living groups other than vertebrates, and because of its content of Gla, it is strongly bound to the bone (ZYTKOVICZ and NELSESTUEN, 1976). Therefore, it is less likely that osteocalcin extracted from fossil bone will be contaminated by other protein or complex organic matter from the environment.

4) Osteocalcin can be isolated from bone free from high molecular weight proteins, humic and fulvic acids, polysaccharides, or other common soil contaminants (this study).

Dietary assessment ($\delta^{13}C$, $\delta^{15}N$) and ^{14}C measurements assume that no changes have occurred in the isotopic composition of the body carbon and nitrogen post-mortem, except by ^{14}C decay. However, post-mortem alterations that can change the isotopic configuration of the body carbon are often commonly observed in collagen, especially in buried bones (STAFFORD *et al.*, 1987). Post-mortem alteration (diagenesis) is most frequently induced by adverse environmental conditions resulting in degradation, modification, leaching, replacement, or contamination of collagen by similar or dissimilar exogenous organic materials. Depending on the extent of the diagenesis, bones are usually classified as "*well-preserved,*" "*moderately preserved,*" or "*poorly preserved.*" Well-preserved bone contains collagen having identical characteristics to those of modern fresh bone. In moderately preserved bone, the elemental C/N ratio of collagen may be different due to preferential loss of some amino acids. In poorly preserved bone, collagen is extensively degraded, and in extreme cases it is undetectable (by the absence of hydroxyproline in amino acid analysis).

Major diagenetic problems associated with collagen from poorly preserved bones are contamination and partial degradation leading to alteration of isotopic content. Introduction of contaminants in the form of exogenous free amino acids and proteins, humic and fulvic acids, and polysaccharides may occur through ground water or soil contact

and microbial action. These may react with the indigenous polypeptides of collagen, for example, through condensation reaction between sugar residues and amino groups, to provide exogenous contamination. Protein hydrolysis in acidic or alkaline aqueous environments and proteolytic attack resulting from bacterial action causes fragmentation and may allow the more soluble components to be leached out. For bone materials where diagenetic effects have significantly reduced and/or altered the collagen content, serious questions can be raised concerning the effectiveness of a preparative protocol for the isolation and purification of a meaningful indigenous organic fraction (HEDGES and LAW, 1989). To overcome these difficulties, we have examined the isotopic and biogeochemical characteristics of osteocalcin in a series of bone samples exhibiting wide variability in their residual collagen content.

PROCEDURES

Bone preparation procedures

Bone samples are cleaned by ultrasonication in an ice-cold tris buffered protease inhibitor cocktail (TPIC) (GUNDBERG *et al.*, 1984), containing the following: benzamidine, 5 mM; 6-aminocaproic acid, 10 mM; p-hydroxymercuribenzoic acid, 100 μM; phenylmethylsulfonyl fluoride, 30 μM; and tris HCl, 20 mM; pH 7.8. The bone samples are then rinsed several times with ice-cold distilled water and freeze-dried. The dry bone is powdered in liquid nitrogen with a diamond mortar to a size less than 710 μM. The powder is then homogenized in ice-cold TPIC (1 g/20 ml) for 30 minutes and allowed to settle. Decanting and re-homogenization with distilled water is continued until the supernatant is clear and colorless. In some cases, to remove contaminating pigments, the powder is allowed to settle in ice-cold 1 M HCl at 4°C for 20 minutes and then washed with distilled water to neutrality, and finally soaked in ice-cold 0.125 M NaOH at 4°C for 30 minutes; and again washed with distilled water to neutrality and lyophilized. These last procedures of alternate soaking in acid and base results in extractive loss and a reduced yield of osteocalcin.

Preparation of osteocalcin and collagen

Two methods are employed for the extraction and purification of osteocalcin. The first method is a modification of the EDTA procedure of GUNDBERG *et al.* (1984); the second is a modification of the formic acid procedure of POSER *et al.* (1980). As both methods produce the same high quality product and the second is considerably simpler, because it avoids problems of complete EDTA removal by dialysis, we recommend using it and describe it briefly below.

Demineralization of bone (typically 10–50 g) is accomplished in 20% formic acid (10 ml/g bone), followed by dialysis (Spectrapor 1 membrane tubing, molecular weight cutoff 6000–8000, Spectrum Medical Industries, Los Angeles, CA) against deionized water for four days at 4°C with daily changes of water. The content of the dialysis tubing is centrifuged and the supernatant reduced to a volume of 20 ml by freeze-drying. This is followed by gel filtration of the soluble extract on sephacryl S-200 in 6 M GuHCl. Elution of osteocalcin is monitored by absorbance at 276 nm. The pooled osteocalcin fraction is freeze-dried, dissolved in 0.07 M NH_4HCO_3 (1 mg/ml) and eluted from a DEAE ion exchange column with a 700 ml linear gradient of 0.7 M NH_4HCO_3 (GUNDBERG *et al.*, 1984). Fractions of 5 ml are collected, and protein is monitored by ultraviolet light adsorption at A_{276}. The pooled osteocalcin fraction is 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 lyophilized to yield the pure osteocalcin. Appropriate precautions to avoid intersample contamination include use of separate columns for modern and fossil samples, extensive column washing, and isotopic and amino acid analysis of blank column eluent buffers.

The extraction of collagen in gelatin form is accomplished by the methods of DENIRO and EPSTEIN (1981), and SCHOENINGER and DENIRO (1984). Briefly, bone powder after TPIC washing is solubilized in 1 mM HCl for 10 hours at 90°C, filtered through a sintered filter, and lyophilized.

Isotope analysis

Aliquots of osteocalcin and collagen were combusted using the methods of MINAGAWA *et al.* (1984), and the resulting nitrogen and CO_2 analyzed by dual collecting isotope ratio mass spectrometry for their $\delta^{15}N$ and $\delta^{13}C$, respectively. ^{14}C analysis was performed using the accelerator mass spectrometer at DSIR-INS, Radiocarbon Laboratory, Gracefield, New Zealand, and conventional scintillation counting at Institute of Geophysics and Planetary Physics, University of California, Los Angeles. Atomic C/N ratios were calculated from the manometrically determined volumes of CO_2 and nitrogen gases produced by combustion of weighed protein samples at 875°C (MINAGAWA *et al.*, 1984; DENIRO, 1985).

Stable isotope ratios are reported in the δ per mil notation:

$$\delta^{13}C \text{ or } \delta^{15}N = [(R_{sample}/R_{standard}) - 1]1000 \quad (1)$$

where $R = {}^{13}C/{}^{12}C$ for $\delta^{13}C$ and $^{15}N/^{14}N$ for $\delta^{15}N$, respectively. Standard deviation for replicate analysis is $\pm 0.10‰$ for both $\delta^{13}C$ and $\delta^{15}N$, respectively. Standard deviation for osteocalcin analysis obtained by the two methods of extraction and purification (EDTA vs. formic acid) is less than ± 0.10 per mil for $\delta^{13}C$ and $\delta^{15}N$, respectively. ^{14}C dates are reported in years before present (BP) with a general precision of 1–1.5%.

Amino acid analysis

Samples for amino acid analysis were hydrolyzed under vacuum in a nitrogen atmosphere for 18 hours in 6N HCl at 110°C. Derivatization with phenylisothiocyanate was performed before HPLC separation and identification of amino acids. Amino acid contents are expressed in amino acid residues/1000.

Gamma-carboxyglutamic acid (Gla) in bone protein samples was determined by high resolution amino acid analysis after alkaline hydrolysis (2 M KOH, 22 hr, 108°C) as previously described (HAUSCHKA, 1977). A modified procedure involved hydrolysis of 30 mg bone powder samples in 0.3 ml 2 M KOH in 1.5 ml polypropylene

Table 1. GLA analysis

Sample	Residue GLA 1000 Residues GLU
HA-100	3.71
HA-101	2.99
HA-102	3.54
HA-103	5.26
HA-104	4.85
HA-105	4.91
HA-106	5.32
Bovine*	5.31
Human rib†	3.60

* Bovine samples reported in COLOMB *et al.* (unpublished result).
† HAUSCHKA (1977).

microfuge tubes, followed by perchloric acid ($HClO_4$) neutralization and analysis of the diluted hydrolyzate supernatants. Data are expressed as residues Gla/1000 residues Glu (glutamic acid). The Gla peak position in fossil bone samples was confirmed by spiking with an internal standard of authentic Gla.

Radioimmunoassay (RIA) of osteocalcin

A range of 30 to 200 mg samples of powdered bone were weighed into 1.5 ml microfuge tubes and extracted with 0.8 ml EPIC buffer (0.5 M ammonium EDTA, pH 6.1, containing proteinase inhibitors) by end-over-end mixing at 4°C for 18 hr (GUNDBERG *et al.*, 1984). A previously described non-equilibrium radioimmunoassay was utilized for osteocalcin quantitation (HAUSCHKA *et al.*, 1989), or the distantly related matrix Gla protein which also occurs in bone. Importantly, this assay detects only *intact* osteocalcin with high sensitivity (0.05 ng/tube) and specificity, but does not recognize proteolytic fragments of the native protein (GUNDBERG *et al.*, 1984; HAUSCHKA *et al.*, 1983). It exhibits equivalent crossreactivity with pure standards of human, monkey (*M. fascicularis*), and bovine osteocalcin which have a highly homologous 49 amino acid residue sequence (HAUSCHKA *et al.*, 1989). The primary antiserum was rabbit anti-bovine osteocalcin, used at a final dilution of 12,600-fold. Bone extracts were serially diluted in assay buffer and assayed over a wide range corresponding to 0.05–2000 μg bone/tube. The competing tracer was freshly prepared ^{125}I-monkey osteocalcin at 20,000 cpm/tube. Immuno-precipitation with goat anti-rabbit IgG second antibody was followed by centrifugation, washing of pellets, and gamma-counting. Data are expressed as the quotient B/B_0 as previously described (HAUSCHKA, 1977; GUNDBERG *et al.*, 1984). Monkey osteocalcin standard gave a midpoint $B/B_0 = 0.5$ at 0.24 ng/tube, with an interassay variation of 10%. All fossil human bone samples gave parallel dilution curves, indicating strong identity of the ancient antigen to the modern osteocalcin standard. Osteocalcin contents were calculated by equating the μg bone-equivalent extract/tube required to produce $B/B_0 = 0.5$ with the corresponding value for the osteocalcin standard.

RESULTS AND DISCUSSION

Analysis for Gla shows that it is present in the fossil bones at levels similar to the range present in modern bone (Table 1). Eighty percent of total Gla in modern bone is associated with osteocalcin with the remainder occurring in matrix Gla protein and traces of vitamin K-dependent coagulation factors (HAUSCHKA *et al.*, 1989). RIA data, however, indicate that only a fraction of the normal osteocalcin is present (Table 2). Thus, a major fraction of the total measurable Gla is associated with non-immunoreactive fragments (polypeptides) of osteocalcin and matrix Gla protein which have been retained in the fossil materials. The overall RIA data of Table 2 do, however, demonstrate the presence of intact osteocalcin in the fossil bone samples. The fraction of the total original osteocalcin which remains intact, and thus detectable by RIA depends on the diagenetic conditions pertinent to each particular bone as long as mild laboratory extraction procedures are used. Further evidence that the extract isolated and identified as osteocalcin is truly osteocalcin and not collagen, or other high molecular weight proteins, is drawn from amino acid composition and from gel electrophoresis analyses. The amino acid composition of human fossil osteocalcin is similar to that of modern osteocalcin (Table 3), and collagen, or collagen peptide, contamination is precluded by the absence of hydroxyproline for the osteocalcin extract from the human samples. Additionally, to provide modern controls, Table 4 presents elemental and stable isotopes of carbon and nitrogen compositional data on collagen and osteocalcin extracts from a series of modern bones. Gel electrophoresis measurements performed on the fossil bone samples studied indicate the presence of only a low molecular weight protein in the size range of 6,000–17,000 daltons. The molecular size range obtained in this study by gel electrophoresis is similar for both modern and fossil osteocalcin and is also consistent with published results (TUROSS *et al.*, 1989; GUNDBERG *et al.*, 1984).

Unlike modern collagen, fossil and poorly preserved collagen shows mild to extreme variations in amino acid composition and elemental C/N ra-

Table 2. Comparison of intact osteocalcin in fossil bones to modern human, determined by radioimmunoassay

Sample	Intact osteocalcin (% of modern human)*
HA-100	3.3
HA-101	2.6
HA-102	5.2
HA-103	5.1
HA-104	17.4

* Modern human osteocalcin: 0.28 mg/g bone (HAUSCHKA, 1977).

Table 3. Amino acid residues/1000 of collagen and osteocalcin from bones that vary in age and state of preservation

	Collagen														
Sample no.	CGH-510	CGH-511	CGH-512	HA-100	HA-101	HA-102	HA-103	HA-104	HA-105	HA-106	HA-107	HA-108	HA-110	HA-111	HA-112
Amino acid															
OH-PRO	85	85	86	85	85	56	86	50	86	85	86	86	87	92	95
OH-LYS	2	2	2	2	2	1	2	1	2	2	2	2	1	2	2
ASP	48	48	48	48	48	66	47	55	47	48	47	48	42	45	47
GLU	73	73	73	73	73	91	74	83	74	73	73	73	73	76	77
SER	35	35	35	35	35	32	34	31	35	35	35	35	29	30	32
GLY	344	344	343	344	343	311	343	321	344	344	345	344	359	348	344
HIS	4	4	4	4	4	2	4	5	4	4	4	4	3	3	3
ARG	56	52	56	56	56	44	56	48	56	56	56	56	52	53	55
THR	19	19	18	18	18	14	18	17	22	23	23	22	15	15	15
ALA	111	111	110	111	111	141	112	130	106	105	106	106	113	115	117
PRO	118	118	118	118	118	101	119	121	120	120	121	120	125	126	122
TYR	3	3	3	3	3	5	3	3	2	2	2	2	2	2	2
VAL	24	24	24	24	24	28	24	20	23	23	23	23	25	26	27
MET	4	4	4	4	4	6	4	2	5	5	5	5	3	4	5
ILE	9	9	9	9	9	7	9	15	10	10	10	10	9	9	10
LEU	24	24	24	24	24	29	24	21	26	26	26	26	21	22	22
PHE	15	15	15	15	15	19	15	18	15	15	15	16	13	14	14
LYS	26	26	25	26	26	38	26	12	25	25	25	25	29	16	12
CYS	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

	Osteocalcin															
Sample no.	CGH-510	CGH-511	CGH-512	HA-100	HA-101	HA-102	HA-103	HA-104	HA-105	HA-106†	HA-107†	HA-108†	HA-110	HA-111	HA-112	*Modern human
Amino acid																
OH-PRO	0	0	0	0	0	0	0	0	0	21	21	19	0	0	0	0
OH-LYS	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ASP	102	102	102	102	101	102	103	102	100	120	121	122	104	104	103	102
GLU	102	102	104	103	101	100	102	103	104	81	82	80	147	150	151	143
SER	0	0	0	0	0	0	0	0	0	60	61	64	0	0	0	0
GLY	61	60	63	59	62	61	61	59	63	80	81	79	64	63	64	61
HIS	21	19	20	22	19	20	20	21	22	22	21	19	20	20	19	20
ARG	81	80	82	80	81	83	81	80	82	41	40	42	78	76	82	82
THR	0	0	0	0	0	0	0	0	0	41	40	40	0	0	0	0
ALA	60	62	61	61	63	62	62	61	60	101	103	101	80	86	80	61
PRO	143	140	141	142	142	139	140	139	143	80	81	80	137	149	137	143
TYR	102	100	101	101	102	100	102	103	103	60	63	61	104	96	104	102
VAL	60	63	63	61	61	62	60	62	61	41	40	42	45	47	43	61
MET	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ILE	19	22	20	20	21	21	21	20	19	63	61	61	21	23	20	20
LEU	101	101	103	102	100	105	103	102	102	62	60	62	107	102	104	102
PHE	41	41	40	40	43	41	39	40	40	0	0	0	43	42	43	41
LYS	0	0	0	0	0	0	0	0	0	19	21	21	0	0	0	0
CYS	40	40	39	42	42	40	43	43	41	41	40	40	40	39	41	41

* Composition of modern human osteocalcin (POSER *et al.*, 1980).

† Moa osteocalcin composition is not completely known (HUQ *et al.*, 1990).

tios. Osteocalcin does not show such variations. The amino acid composition and elemental C/N ratios of osteocalcin from modern as well as from fossil (well-preserved, moderately preserved, and poorly preserved) bones are identical for the same species. Modern, unaltered collagen has an elemental C/N ratio averaging 3.2 for all vertebrates when bone collagen content >2%. Collagen from moderately to poorly preserved bones retaining their characteristic stable isotopic information has been shown to have elemental C/N ratios ranging from 2.9–3.6. Values outside this range suggest that the bone has

Table 4. Protein content, element C/N ratio, stable isotope ratio, ^{14}C age, and other biogeochemical data of collagen and osteocalcin

				Collagen					Osteocalcin							
Lab #	Species	OH-Pro[1]	Gly/Glu[2]	%wt[3]	C/N[4]	$\delta^{13}C$	$\delta^{15}N$	^{14}C age[5]	%wt[3]	C/N[4]	$\delta^{13}C$	$\delta^{15}N$	^{14}C age	$\Delta\delta^{13}C_{(O-C)}$*	$\Delta\delta^{15}N_{(C-O)}$*	$\Delta^{14}C$ age[5]
								Modern bone								
HA-3	dog	86	4.7	19.9	3.4	−14.5	+6.8		.37	4.3	−14.5	+6.9		0.0	0.1	
HA-4	dog	86	4.8	11.2	3.3	−15.0	+7.3		.38	4.3	−14.5	+7.5		0.5	0.2	
HA-14	deer	86	4.6	14.3	3.2	−20.5	+6.9		.26	4.4	−20.7	+7.0		0.2	0.1	
HA-15	deer	86	4.7	14.7	3.3	−19.8	+5.8		.30	4.0	−20.1	+5.8		0.3	0.0	
CGH-510	human	85	4.7	7.5	3.1	−20.9	+10.3		.24	4.1	−20.6	+10.4		0.3	0.1	
CGH-511	human	85	4.7	8.9	3.1	−17.0	+10.0		.20	4.2	−17.1	+10.3		0.1	0.3	
CGH-512	human	86	4.7	19.9	3.3	−19.6	+10.3		.25	4.2	−20.0	+10.3		0.4	0.0	
CGH-513	human	85	4.7	16.1	3.1	−17.4	+7.8		.26	4.2	−17.7	+7.9		0.3	0.1	
CGH-514	human	85	4.7	12.9	3.2	−18.6	+8.4		.25	4.2	−18.7	+8.5		0.1	0.1	
								Fossil bone								
HA-100	human (Egyptian)	85	4.7	11.9	3.1	−18.8	+12.1	3,845 ± 60†	.24	4.2	−19.0	+12.3	3,855 ± 93‡	0.2	0.2	10
HA-101	human (Egyptian)	85	4.7	4.4	3.1	−18.3	+12.4	3,630 ± 100†	.25	4.2	−18.5	+12.6	3,661 ± 100‡	0.2	0.2	31
HA-102	human (Egyptian)	56	3.4	<0.2	11.1	−17.1	+6.1	1,720 ± 550†	.25	4.2	−18.8	+12.6	3,260 ± 120‡	1.7	6.5	1,540
HA-103	human (Haverty)	86	4.6	11.6	3.1	−14.6	+17.8	7,259 ± 89‡	.25	4.3	−14.5	+17.8	9,090 ± 120‡	0.1	0.0	1,831
HA-104	human (Haverty)	50	3.8	<0.8	18.3	−24.9	+9.1	5,253 ± 94‡	.25	4.2	−26.9	+4.5	15,900 ± 250‡	2.0	−4.6	10,647
HA-105	moa	86	4.6	2.2	5.7	−22.0	+3.9	24,510 ± 250‡	.31	3.8	+22.1	+2.3	24,965 ± 220‡	0.1	−1.6	455
HA-106	moa	85	4.7	9.9	3.4	−22.8	−0.6	10,490 ± 88‡	.31	3.8	−22.9	−0.6	11,050 ± 110‡	0.1	0.0	560
HA-107	moa	86	4.7	11.0	3.4	−21.6	+1.1	15,000 ± 200‡	.31	3.8	−21.7	+1.2	15,275 ± 230‡	0.1	0.1	275
HA-108	moa	86	4.7	10.5	3.4	−22.8	+2.2	18,650 ± 250‡	.31	3.8	−22.9	+2.2	18,780 ± 260‡	0.1	0.1	130
HA-110	human (Haverty)	87	4.9	1.3	8.7	−15.6	+4.8	2,730 ± 90	.26	4.1	−16.6	+11.5	4,630 ± 260	1.0	−6.7	1,900
HA-111	human (Haverty)	92	4.6	1.2	9.9	−13.6	+15.1	3,870 ± 350	.25	4.2	−17.2	+14.1	12,600 ± 460	3.6	1.0	8,730
HA-112	human (Haverty)	95	4.5	0.9	17.9	−13.7	+10.2	4,710 ± 190	.25	4.2	−17.4	+16.6	11,960 ± 500	3.7	6.5	7,250

* O = osteocalcin; C = collagen.

† Conventional decay counting (Isotope Laboratory, UCLA).

‡ AMS direct counting (DSIR, New Zealand).

[1] Hydroxyproline composition in residues/1000.

[2] Glycine/glutamic acid ratios.

[3] Protein content expressed as weight percent.

[4] Elemental carbon/nitrogen ratios.

[5] Carbon 14 ages are given in years.

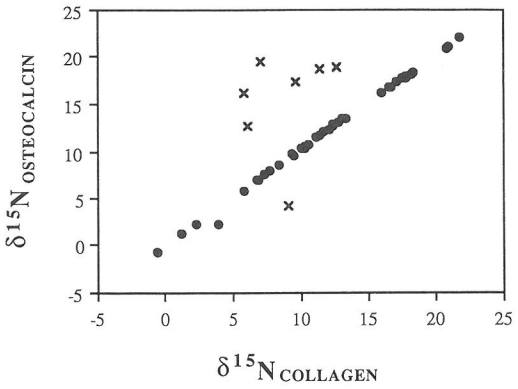

FIG. 1. Nitrogen isotope ratios of collagen vs. nitrogen isotope ratios of osteocalcin from 50 bone samples. Closed circles indicate bones with good collagen preservation. "x" indicates bones with poor collagen preservation.

undergone diagenetic modification, resulting in alteration of the collagen stable isotopic composition. Such bone materials have been shown to yield erroneous stable carbon and nitrogen isotope results (DENIRO, 1985). Most poorly preserved fossil bones fall into this category. Elemental C/N ratios of osteocalcin range from 3.6–4.4, depending on species. In individuals from the same species analyzed for this study, the elemental C/N ratio is constant irrespective of the state of preservation of the bone. The range of elemental C/N ratios in human bone osteocalcin is 4.18–4.33, yielding an average of 4.2.

Table 4 compares the protein content (expressed in weight percent), elemental carbon/nitrogen ratios, and stable isotope ratios of collagen and osteocalcin from a group of samples analyzed in this study. The samples selected cover the range from modern, well-preserved to poorly preserved fossil bones. It is apparent that whereas the protein content and elemental carbon/nitrogen ratios of collagen varied depending on the age and the preservation state of the bone, osteocalcin shows no such variations. Stable carbon and nitrogen isotope ratios of individual amino acids in collagen have been shown to be different from the bulk collagen. For example, glycine residues are often enriched in ^{13}C relative to the total collagen (TUROSS *et al.*, 1988). The divergence in stable carbon and nitrogen isotope ratios between osteocalcin and collagen values observed in some cases may reflect a perturbation in the amino acid speciation observed in the collagen. Mild perturbation may suggest moderate diagenetic effects resulting from partial elimination of the most soluble amino acid residues from the polypeptide chains, thereby inducing the fractionation observed in the stable carbon and nitrogen isotope ratios. Such mild perturbation may not affect the ^{14}C content as long as no introduction of exogenous materials has occurred. For samples HA-102, 104, and 112, where the collagen is almost completely depleted, the carbon and nitrogen isotope ratios of collagen show a large deviation from their ratios in osteocalcin. The large stable carbon and nitrogen isotope ratio shifts measured in these collagen samples show a pattern consistent with terrestrial soil contaminants (Figs. 1 and 2), where $\delta^{13}C$ and $\delta^{15}N$ shift towards values typical of leached products from C_3 plants. By contrast, however, the amino acid compositions, concentrations, and elemental C/N ratios of osteocalcin from these same samples are relatively consistent with those for modern samples.

Table 4 also reports other biogeochemical data, as well as ^{14}C data obtained from collagen and osteocalcin of fossil bone displaying various degrees of collagen preservation. Because of the generally small yields of organic extract, all but three of the ^{14}C analyses were obtained by the AMS technique. For samples HA-100, HA-101, HA-105, HA-106, HA-107, and HA-108, the ^{14}C ages of the collagen and osteocalcin fractions are essentially concordant. In those instances where the ^{14}C ages of the collagen and osteocalcin are discordant, the collagen is extremely poorly preserved (<2% of the original content), and the residual collagen has experienced degradation and differential loss of peptide fragments as reflected in the depressed OH-Pro and Gly/Glu values. These collagen ^{14}C ages are significantly younger than their osteocalcin values, suggesting contamination of the collagen components by younger carbon-containing substances.

Tables 3 and 4 provide stable isotopes of carbon

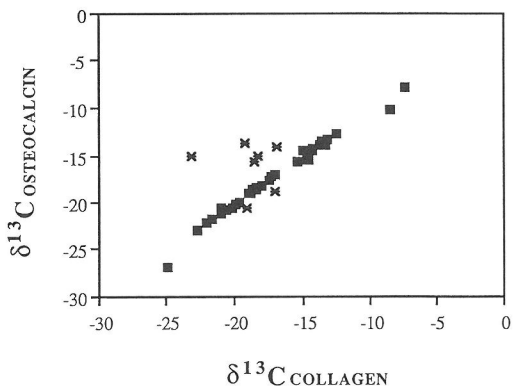

FIG. 2. Carbon isotope ratios of collagen vs. carbon isotope ratios of osteocalcin from 50 bone samples. Closed squares indicate bones with good collagen preservation. "x" indicates bones with poor collagen preservation.

and nitrogen, ^{14}C, and amino acid compositional data on collagen and osteocalcin extracts from a series of bones from the Haverty location of southern California. The discordance in the collagen/osteocalcin values for HA-104, HA-110, HA-111, and HA-112 (Haverty location, southern California) is particularly interesting, as the archaeological and physical anthropological context of skeletons from this location has been extensively studied and, on the basis of anatomical evidence (BROOKS *et al.*, 1991), it has been determined that the skeletons are genetically related and therefore they should have been buried at approximately the same historic period. However, previous ^{14}C ages on collagen cover a range at about 5000–10,500 yr BP (ENNIS *et al.*, 1986) for human skeletons from Haverty, whereas in this study a range of ages from 4630 to 15,900 were obtained from osteocalcin dating (Table 4). The significance of this is discussed in AJIE *et al.* (1990, 1991).

It is particularly interesting to note that for samples HA-104, HA-110, HA-111, and HA-112, there are large differences in $\delta^{13}C$ and $\delta^{15}N$, pointing to possible source contamination. Sample HA-104 yielded a $\delta^{13}C$ (−26.9 per mil) different from all other osteocalcin $\delta^{13}C$ values in this series and may represent an experimentally contaminated sample. Unfortunately, insufficient sample was available to repeat the analysis.

Ancient Egyptian chronology

Ancient Egyptian human remains are among the best preserved material available for radiocarbon dating. This is partly due to the process of mummification of dead humans in ancient Egypt and partly to the geographical location of burial.

Three bone samples (designated HA-100, HA-101, and HA-102) were part of the collection obtained from the British museum and generously donated by Dr. Rainer Berger, Radiocarbon Laboratory at UCLA. Collagen and osteocalcin were extracted from these samples and measured for their radiocarbon content. Because these bones derive from the IVth dynasty in Egyptian chronology, they should be about 3500 years old. Based on the concentration of the collagen from these samples, HA-102 is observed to have undergone a more severe diagenesis compared to the other two samples. Based on this observation, any anomaly in the radiocarbon ages is expected to be more prominent in this sample. Amino acid contents and elemental analyses for samples HA-100 and HA-101 are essentially similar to modern bone. The collagen and osteocalcin ^{14}C ages for HA-100 and HA-101 are essentially concordant, and also agree with the historical ages. However, in HA-102 (where the collagen is almost depleted but osteocalcin could be extracted), the ^{14}C ages are disconcordant, with osteocalcin reflecting the presumed historical age, and the collagen measured by BERGER (1970) showing a much younger age (Table 4).

Moa bones

This study also presents the biogeochemical characteristics, including ^{14}C dates, from bones of the extinct moa birds. Moa is a name popularly applied to the gigantic struthious bird belonging to the Ratitae group. They are among the largest flightless birds that once inhabited New Zealand. Living members of this family include the ostrich of Africa and Arabia (Struthionidae); the rhea of South America (Rheidae) and the emu of Australia (Dromacus). The extinct prototypes are the *Æpyornis* of Madagascar; the *Dromornis* of Australia and the moa of New Zealand (*Pachyornis elephantopus*). Fossilized moa bones have been excavated from numerous locations in New Zealand, and some of these bones are associated with sediment/soil ranging from near-Recent to Pliocene ages. Based on ^{14}C measured dates, it is widely believed that these birds underwent a process of extinction some time after New Zealand was settled by Polynesians (~700 yrs BP) and before it was settled by Europeans (~220 yrs BP). Because of the interest in Moa bones, several laboratories have undertaken numerous ^{14}C measurements, especially at the Department of Scientific and Industrial Research (DSIR), New Zealand. Part of a well-preserved bone sample collection from the Canterbury Museum, Christchurch, New Zealand, used at DSIR was made available for this study by Dr. John Hulston, and these bones are designated HA-105–HA-108. As these bone samples had already been analyzed for their ^{14}C content at the DSIR facility by β-counting using collagen (DSIR, unpubl. results), a comparative analysis of osteocalcin ^{14}C content was undertaken. Both collagen and osteocalcin were isolated from these bones, and the measured data indicate that the bones were well preserved, as evidenced by the C/N ratios of the collagen, OHP of the collagen, and concentration of collagen. Amino acid compositions from these extracts were also obtained. Although the amino acid composition of moa osteocalcin is not well documented, efforts are underway to determine this composition. A partial amino acid sequence for moa bone osteocalcin has been obtained (HUQ *et al.*, 1990). Interestingly, the presence of hydroxyproline

in the moa bone osteocalcin was observed in this study (Table 3).

The result of the isotope analyses are presented in Table 4. All the collagen ^{14}C dates were obtained from the study at DSIR, whereas the osteocalcin ^{14}C dates were obtained in this study. Both sets of results are concordant, the greatest variation being ±300 years. The ^{14}C age agreement between osteocalcin and collagen provides evidence for the promising utility of osteocalcin for biogeochemical studies.

SUMMARY

In this report, we have described the isolation of osteocalcin and collagen from bones of different organisms, different depositional environments, different degrees of preservation, and varying ages. In our comparative study, we find osteocalcin to be superior to collagen for stable carbon and nitrogen isotope studies and ^{14}C dating, especially for fossil and poorly preserved bones where collagen can give false results. Our observations demonstrate that when bone material is identifiable and the mineral phases are still present but collagen has largely disappeared, osteocalcin is more likely to be present and can be isolated and purified free of contaminant. The purified osteocalcin is observed to be present in two states: *viz.* intact protein and proteolytic polypeptides fragments. Based on the findings of this study, the use of osteocalcin is recommended for anthropogenic/paleoenvironmental reconstruction, where accurate chemical and isotopic information is desired from bones that have undergone extensive diagenesis.

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