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Compound-specific approach to the $\delta^{13}C$ analysis of cholesterol in fossil bones

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Abstract—$\delta^{13}C$ values for cholesterol isolated from fossil animals, namely those of whales dated at 9735 ± 160 yr and 75000 ± 15000 yr B.P., are reported. Gas chromatography (GC) showed that 12 and 15 μg of cholesterol per gram dry weight of bone were recovered from the bones of these two fossil animals, respectively. Conventional gas chromatography–mass spectrometry (GC–MS) confirmed the identity of the ancient cholesterol and that the peaks separated by GC were of high purity. Gas chromatography–combustion–isotope ratio-mass spectrometry (GC–C–IRMS) of cholesterol recovered from the fossil specimens yielded $\delta^{13}C$ values that plotted within the range expected for the bulk fat of marine mammals. Significantly, the isotopic depletion between the cholesterol and collagen was similar for both ancient and modern whale bones, indicating reliable preservation of the $\delta^{13}C$ signal between the ancient cholesterol and the protein constituents. The potential to derive $\delta^{13}C$ values from individual lipids, for which the carbon skeletons are completely unaffected by decay processes, significantly extends the scope of palaeodietary studies. © 1997 Elsevier Science Ltd

Key words—cholesterol in fossil bones, palaeodiet, $\delta^{13}C$ in Cretaceous sediments, GC–C–IRMS

INTRODUCTION

Stable isotopes and trace elements preserved in mineralized tissues have been used in recent years to study the dietary preferences of ancient individuals, populations and food webs (DeNiro and Epstein, 1978, 1981; Van der Merwe, 1982; Schoeninger, 1985). The collagenous, non-collagenous proteins and individual amino acids of archaeological and palaeontological bones and teeth have proved to be valuable sources of $\delta^{13}C$ and $\delta^{15}N$ information for palaeodietary reconstruction (DeNiro, 1987; Schwarcz, 1991). The most commonly used method of obtaining stable isotopic information in vertebrate organisms involves the analysis of collagen, the major structural protein of bones and teeth, which possesses a well-defined amino acid sequence which varies little with diet. $\delta^{13}C$ and $\delta^{15}N$ values measured on bone collagen have been used successfully to assess the dietary input of C_3 and C_4 plants, terrestrial vs. marine dietary dependence, and to assess the isotopic fractionation associated between different trophic levels.

Collagen comprises a complex mixture of several different amino acids, mainly glycine (33%), proline (12%), hydroxyproline (10%) with glutamate, aspartate, alanine, serine and valine in lower proportions, each having individual but variable $\delta^{13}C$ and $\delta^{15}N$ isotopic compositions (Tuross *et al.*, 1988). The $\delta^{13}C$ values of these amino acids have been commonly shown to vary by ca. 17‰ (Tuross *et al.*, 1988), and in extreme cases up to 25‰ (Van Klinken, 1989). The bulk isotopic composition of the collagen will therefore be a composite of the fractionation of carbon and nitrogen isotopes into these amino acids during collagen biosynthesis (Hare and Estep, 1983). Amino acids are differentially stable and, therefore, any preferential loss of amino acids during diagenesis will ultimately bias the overall isotopic signature of isolated collagen (Tuross *et al.*, 1988). The fidelity of the bulk collagen signal is therefore heavily reliant upon achieving isotopic fidelity of each of the individual constituent components. Moreover, the stable isotopic signal obtained from collagen and non-collagenous proteins is biased towards the proteinaceous constituents of the diet (Krueger and Sullivan, 1984).

Until recently, the lipid components of bones and teeth had not been studied in detail and, more significantly, had been completely neglected as a source of palaeodietary information. We have recently observed that significant amounts of lipids, particularly cholesterol and its diagenetic congeners, are recoverable from ancient human and animal bones (Evershed *et al.*, 1995). Cholesterol was frequently found to be the most abundant constituent of the bone lipid extracts, corresponding to either: (i) the remnants of either the original blood-borne lipid (in the case of vascular bones) or (ii) the fat component of bone marrow that would have been present at the time of death of the organism. In this

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paper, we report the use of GC–C–IRMS to determine $\delta^{13}C$ values of cholesterol, with the aim of developing ($\delta^{13}C$) cholesterol as a source of palaeodietary information for use in conjunction with $\delta^{13}C$ and $\delta^{15}N$ bone collagen and bioapatite data. The ability to derive isotopic information from an individual compound source, such as cholesterol preserved in ancient mineralized tissues would provide a method of deriving palaeodietary information relating to the fats and carbohydrate components of the diets of ancient organisms.

EXPERIMENTAL

Samples

Fossil whale bone fragments were taken from eroded permafrost deposits at Forlandsundet (Spitzbergen, Norway). Radiocarbon dating suggests an age of 9735 ± 160 yr for INSTAAR no. 3 DAU 7. INSTAAR no. M79-SB2 was dated, using chronostratigraphy, at 75000 ± 15000 yr B.P. Although the fragmentary fossils could not be identified as to a specific genus, they are thought to be representative of baleen whale species. Contemporary toothed whale rib bones (*Physeter catodon* and *Kogia simus*) were collected at autopsy, 24 h after the whales had beached (Tuross *et al.*, 1988).

Sample preparation and analysis

Following removal of exogenous lipids from the fossil whale bones by abrasion, the samples were crushed, weighed and extracted with chloroform/methanol (2:1 v/v, 2 × 30 min, ultrasonication). Prior to extraction, 5α(H)-cholestane (20 μg) was added as an internal standard. After centrifugation (1800 rpm, 30 min) the lipid extract was decanted to a vial, run through a short pipette column packed with silica and alumina, evaporated to dryness under a stream of dry nitrogen and stored at 4°C.

Contemporary whale bones were stripped of adipose fat and then extracted using the conditions as described above for the fossil bones. Lipid extracts were hydrolysed at 70°C (0.5 M NaOH in methanol, 1 h), acidified with HCl (pH 3) and extracted with hexane. Neutral lipids were separated from the acid fraction using amino-propyl (NH_2) solid phase extraction cartridges, conditioned with hexane, and then eluted with chloroform/2-propanol (2:1, v/v). Aliquots of total lipid, extracted from the fossil and modern whales, were derivatized as trimethylsilyl (TMS) derivatives [*N,O*-bis(trimethylsilyl)trifluoroacetamide BSTFA, 70°C; 1 hr] prior to gas chromatography (GC), gas chromatography–mass spectrometry (GC–MS) and gas chromatography–combustion–isotope ratio-mass spectrometry (GC–C–IRMS).

High-temperature GC was carried out on a Hewlett-Packard 5890 Series II gas chromatograph fitted with a fused-silica capillary column (15 m × 0.32 mm i.d.) coated with a dimethyl polysiloxane stationary phase (DB-1, 0.1 μm film thickness). Following an isothermal hold at 50°C (2 min) the temperature was increased to 350°C (20 min) at 10°C min^{-1}. GC–MS analyses were performed using a Finnigan 4500 quadrupole mass spectrometer (electron energy, 35 eV; filament current, 0.35 mA; source temperature, 280°C) interfaced to a Carlo Erba HRGC 5160 Mega series gas chromatograph. The column type and operating conditions are the same as those described above. Hydrogen was used as carrier gas.

The Finnigan MAT Delta-S GC–C–IRMS has been described previously by Hayes *et al.* (1990). Compound separation was performed on a Varian 3500 gas chromatograph fitted with a fused silica capillary column (50 m × 0.32 mm i.d.) coated with a dimethyl siloxane stationary phase (CP Sil-5-CB, 0.25 μm film thickness). The oven was programmed at 50°C (2 min) to 250°C at 10°C min^{-1}, then to 300°C (25 min) at 4°C min^{-1} using helium as a carrier gas. Individual organic compounds eluting from the GC capillary column are combusted to CO_2 and H_2O at 850°C in a cupric oxide-packed combustion furnace. Following the elimination of H_2O, the CO_2 is ionized in the ion source of the

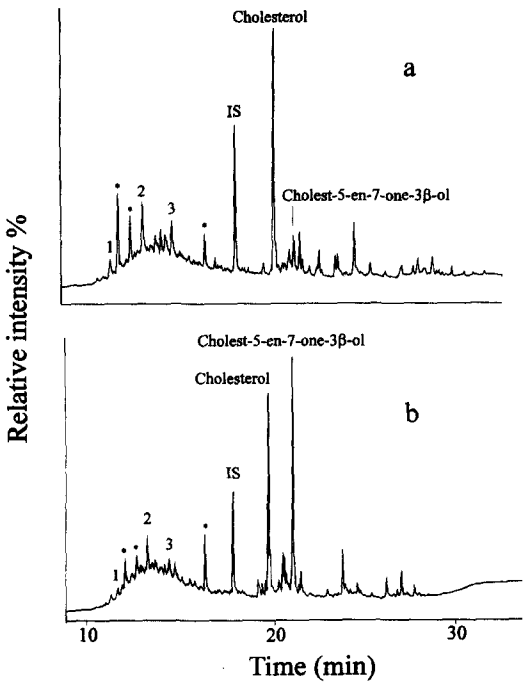

Fig. 1. Partial gas chromatograms of the trimethylsilylated total lipid extracts of fossil whale bone specimens: (a) INSTAAR no. 3 DAU7 (75000 ± 15000 yr B.P.); and (b) INSTAAR no. M79-SB2 (9735 ± 160 yr). Numbers 1–3 indicate myristic, palmitic and stearic fatty acids, respectively. IS = 5α(H)-cholestane internal standard; * = contamination from plastic packaging during storage.

Table 1. Comparison between the $\delta^{13}C$ values of bone collagen and cholesterol of fossil and modern whales

Samples	$\delta^{13}C$ collagen (mean; n = 2)*	δ^{13} cholesterol (mean; n = 2)†	Spacing (collagen vs. cholesterol)
Fossil whale bone			
INSTAAR no. 3 7 DAU	−16.8	−25.7	8.9
INSTAAR no. M79-SB2	−17.1	−25.2	8.1
Modern whale bone			
Physeter catadon	−14.4	−21.5	7.1
Kogia simus	Not determined	−22.7	Not determined

*Values of $\delta^{13}C$ after Tuross *et al.* (1988).
†Values of $\delta^{13}C$ corrected for the derivatizing trimethylsilyl group.

mass spectrometer, whereupon carbon masses m/z 44, 45 and 46 are determined after resolution and amplification. Carbon isotopes are then measured relative to co-injected CO_2 standards and expressed as a delta notation relative to the PeeDee standard (*Belemnitella america*), $\delta^{13}C = [(R_{sample} - R_{standard})/R_{standard}] \times 1000$, where $R = {}^{13}C/{}^{12}C$ (per mil). All reported stable carbon isotope values of cholesterol are corrected for the addition of TMS derivatives (Jones *et al.*, 1991).

RESULTS AND DISCUSSION

GC and GC–MS analyses of fossil whale bone

GC–MS confirmed the presence of cholesterol [I] ($M^{+\cdot}$ 458, $[M\text{-}TMSOH]^+$ m/z 368), in the lipid extracts recovered from the two fossil animals [(Fig. 1(a) and (b)]. Comparison of the GC peak areas with those of the internal standard show similar amounts, 12 and 15 μg of cholesterol per gramme dry weight of bone respectively, were recovered from the bones of these two fossil animals. Cholest-5-en-3β-ol-7-one [II], a well-known oxidation product of cholesterol (Smith, 1981) was detected at concentrations of 2 and 22 μg per gramme dry weight of bone in the 10000-yr and 70000-yr-old whale bones, respectively. The presence of this and other degradation products of cholesterol may reflect the type of burial environment as well as give some indication of the subsequent diagenetic history of the bone (Evershed *et al.*, 1995). Since these degradation products possess intact carbon skeletons and are of the same biosynthetic origin to the precursor cholesterol, the potential exists to use their $\delta^{13}C$ values as an additional corroboratory source of ^{13}C information.

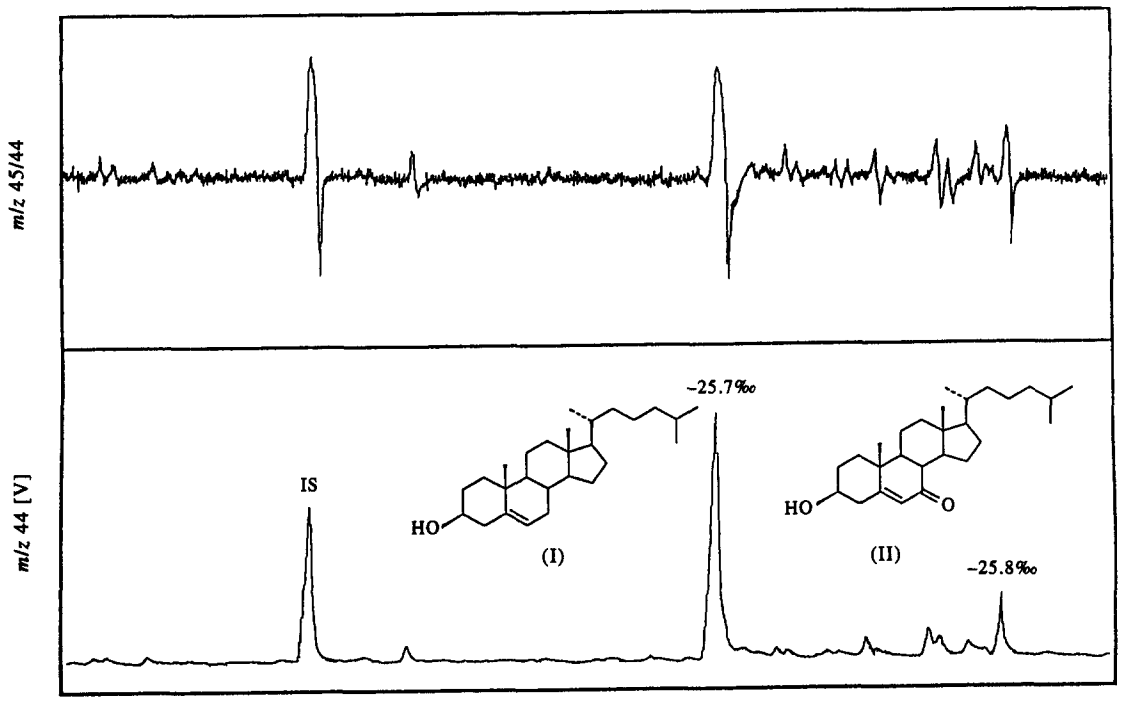

Fig. 2. Partial GC–C–IRMS profile of individual lipids in the 10000-yr-old fossil whale bone sample (INSTAAR no. 3 DAU7). The upper chromatogram represents the instantaneous ratio of the m/z 45/44 ions, while the lower chromatogram represents the m/z 44 ion current.

Gas chromatography–combustion–isotope ratio mass spectrometry

The partial GC–C–IRMS chromatogram of cholesterol and cholest-5-en-3β-ol-7-one from specimen INSTAAR no. 3-DAU7 (estimated age 10000 yr) is shown in Fig. 2. The baseline resolution which is essential for valid isotopic measurement, was achieved by use of a high efficiency (50 m) column and slower temperature programming (compare Figs 1 and 2). The $\delta^{13}C$ values of the cholesterol recovered from the modern and fossil whales are presented in Table 1. The values obtained exhibit the depletion previously reported for lipids of marine mammals (Tiezen *et al.*, 1983; Tuross *et al.*, 1988). Isotopic analysis of collagen and individual amino acids isolated from INSTAAR no. M79-SB2 and *Physeter catadon* had previously been performed using off-line combustion IRMS (Tuross *et al.*, 1988). A greater enrichment of ^{13}C is seen in the proteins compared with cholesterol, largely reflecting the isotopic fractionation occurring during the metabolic cycling of the amino acids (Hare *et al.*, 1991) together with the enhanced isotopic fractionation associated with lipid biosynthesis (DeNiro and Epstein, 1977). The measured collagen to cholesterol $\delta^{13}C$ spacings (Table 1) show a depletion of approximately 7.1‰ for the contemporary whales and 8.5‰ for the two fossil specimens. The small, yet probably significant differences (−2.6 to −3.4‰) in the $\delta^{13}C$ values of the collagen and cholesterol from the modern and ancient whale bones indicate that differences existed in the isotopic content of their diets, which must ultimately reflect the isotopic composition of the palaeoenvironmental primary carbon pool. The differences in the $\delta^{13}C$ isotopic signals of the cholesterol of the two fossil whales compared with the modern whales, are consistent with a trophic level effect existing between the diets of baleen and toothed whales (herbivory vs. carnivory).

An example of using the $\delta^{13}C$ value of cholesterol congeners as an additional source of ^{13}C information is shown in Fig. 2. The close similarity between the $\delta^{13}C$ values of the cholesterol and the 7-keto compound confirms their common biosynthetic origin. This approach will also be used with other cholesterol diagenetic products, e.g. stanols (Evershed *et al.*, 1995).

CONCLUSIONS

Reported herein are then first $\delta^{13}C$ values measured for individual lipids, specifically cholesterol, from fossil animal bones. Since the carbon skeleton of the cholesterol molecule is known to have been preserved intact, the fidelity of its isotopic signal is assured. The isotopic depletion between the cholesterol and collagen was similar for both ancient and modern whales, indicating a faithful preservation of the $\delta^{13}C$ signal between the lipid and protein constituents of the fossil bones. The ability to measure $\delta^{13}C$ values of individual lipid moieties preserved in mineralized tissues, provides a method of investigating carbon sources and cycling on a much shorter time-scale biologically, when compared with the slower turnover rates of carbon measured in bone collagen. In addition, the flux of carbon into the lipid (cholesterol) during biosynthesis will directly record the original isotopic composition of the carbohydrates and fats in the diet (similar to bioapatite but a potentially more reliable source of ^{13}C data on diagenetic grounds). The ^{13}C content of the ancient cholesterol, therefore provides an additional source of palaeodietary information that cannot be derived from stable isotope measurements of collagen, nor, more importantly, from any other palaeodietery indicator.

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