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Stable carbon isotopic evidence for differences in the dietary origin of bone cholesterol, collagen and apatite: Implications for their use in palaeodietary reconstruction

SUSAN JIM,[1] STANLEY H. AMBROSE,[2] and RICHARD P. EVERSHED[1,*]

[1]Organic Geochemistry Unit, Biogeochemistry Research Centre, School of Chemistry, University of Bristol, Bristol BS8 1TS, UK
[2]Department of Anthropology, University of Illinois, Urbana-Champaign, Urbana, IL 61801, USA

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Abstract—Rats were raised on a variety of isotopically controlled diets comprising 20% C_3, C_4 or marine protein and C_3 and/or C_4 non-protein or energy (i.e. sucrose, starch and oil) macronutrients. Compound specific stable carbon isotope ($\delta^{13}C$) analysis was performed on the cholesterol isolated from the diet ($n = 7$) and bone ($n = 15$) of these animals and the values compared with bulk $\delta^{13}C$ measurements of bone collagen and apatite. The dietary signals reflected by these three bone biochemical components were investigated using linear regression analysis. $\delta^{13}C$ values of bone cholesterol were shown to reflect whole diet $\delta^{13}C$ values, collagen to reflect mainly dietary protein values and apatite to reflect whole diet values. Further correlations between dietary protein-to-energy spacings ($\Delta^{13}C_{prot\text{-}engy} = \delta^{13}C_{protein} - \delta^{13}C_{energy}$) and whole diet-to-bone component fractionations ($\Delta^{13}C_{bcomp\text{-}wdiet} = \delta^{13}C_{bone\ component} - \delta^{13}C_{whole\ diet}$) indicates that for hypothetical diets where protein $\delta^{13}C$ values are equal to energy values, fractionations between whole diet and bone biochemical fractions are –3.3‰ for cholesterol, +5.4‰ for collagen and +9.5‰ for apatite. Moreover, the narrow range of variation observed in apatite-to-cholesterol spacings ($\Delta^{13}C_{apat\text{-}bchol}$) suggests that cholesterol $\delta^{13}C$ values can potentially also be used as an independent test for the isotopic integrity of apatite $\delta^{13}C$ values. These insights into bone cholesterol, collagen and apatite dietary signals, diet-to-bone fractionations and bone component-to-bone component spacings provide the basis for more accurate interpretations of the dietary behaviour of archaeological populations and food webs when the $\delta^{13}C$ analysis of bone is employed.

1. INTRODUCTION

Stable carbon isotope ($^{13}C/^{12}C$) analysis has been widely used since the 1970s to reconstruct aspects of ancient human diets (van der Merwe and Vogel, 1978; Tauber, 1981; Boutton et al., 1984; Schwarcz et al., 1985; Johansen et al., 1986; Buikstra and Milner, 1991; Lubell et al., 1994; Katzenberg et al., 1995; Ambrose et al., 1997; Richards and Hedges, 1999). Such investigations have largely relied on the $\delta^{13}C$ analysis of collagen and apatite recovered from human archaeological skeletons. Evershed et al. (1995) first reported the preservation of cholesterol and its diagenetic congeners in ancient human and animal bone. Significant quantities of cholesterol (2–50 μg/g dry weight of bone) were isolated from bone samples of varying age (75,000 B.P. to 19th century) recovered from different geographical locations and burial environments. Although the preservation of cholesterol in the oldest sample studied, 75,000-yr-old fossil whale bone, can be attributed to its unusual burial environment in permafrost, Upper Palaeolithic (23,000–29,000 B.P.) and Mesolithic (7500 B.C.) samples from limestone and peat sediments also yielded appreciable quantities of cholesterol (2–27 μg/g dry weight of bone). This discovery led to the investigation into the potential of bone cholesterol as a new source of palaeodietary information (Stott and Evershed, 1996; Stott et al., 1997a). Preliminary research carried out on tissue samples from controlled animal feeding experiments has shown that the dietary information or *dietary signal* derived from cholesterol differs from that derived from collagen and apatite, suggesting that its analysis in conjunction with that of other bone components will give a more complete insight into palaeodiet (Stott et al., 1997b, 1999; Jim et al., 2001). Indeed, the use of bone cholesterol $\delta^{13}C$ measurements in a comparative study of the diets of English inland (Medieval; Abingdon Vineyard; Oxfordshire) and coastal (Saxon-Medieval; Barton-on-Humber; N. Lincolnshire) populations, allowed the detection of subtle dietary differences between the two populations, which were not detectable in the bulk $\delta^{13}C$ values of collagen alone (Stott et al., 1999).

Accurate palaeodietary reconstruction using stable carbon isotope analysis requires: (1) knowledge of the foodstuffs that were available for consumption, which can be gleaned from floral or faunal remains or from archaeological records; (2) knowledge of the $\delta^{13}C$ values of such foodstuffs; (3) accurate measurement of the biogenic $\delta^{13}C$ values preserved in ancient bone and food remains; and (4) an understanding of the isotopic relationship between dietary macronutrients and bone biochemical components. Where food remains are not preserved, $\delta^{13}C$ measurements should be made on modern plants and animals that were likely to have contributed to the diet and from the same locality as the population under study in order to estimate possible ranges of dietary $\delta^{13}C$ values. Although dietary interpretations of past populations have been gleaned using the $\delta^{13}C$ analysis of bone collagen and apatite, there has been much discussion over the accuracy and limits of such interpretations because the precise isotopic relationship between the diet and specific bone biochemical components is not fully understood. This relationship, termed the fractionation factor or difference between diet and bone component $\delta^{13}C$ values, denoted as $\Delta^{13}C_{bcomp\text{-}diet}$ ($=\delta^{13}C_{bone\ component} - \delta^{13}C_{diet}$), is complex and

* Author to whom correspondence should be addressed (r.p.evershed@bristol.ac.uk).

dependent on many factors, including the nutritional status and digestive physiology of the individual under study and the biosynthetic pathway and turnover rate of the bone biochemical component (Koch et al., 1994). In this paper, through the study of rats raised on isotopically controlled diets, we shall address the key question: what is the relationship between diet (both in terms of whole diet and different dietary macronutrients) and bone biochemical component $\delta^{13}C$ values? In other words, do all bone components reflect the diet as a whole, or are there biases for a particular bone component towards a specific dietary macronutrient? The stable isotopic relationship between diet and bone is primarily dependent on the effects of differing metabolic pathways and digestive adaptations (Schwarcz, 1991, 2000; Hedges and van Klinken, 2000), although age, sex, and environmental and nutritional stress may also have secondary effects (Heaton et al., 1986; Sealy et al., 1987; Ambrose, 1991). A thorough understanding of the factors that underlie this question will result in significant refinements to palaeodietary reconstruction using $\delta^{13}C$ analysis. Indeed, better insights into the fractionations that occur between diet and bone would not only lead to more accurate qualitative (e.g., clarifying protein, energy and lipid resources) and perhaps, quantitative dietary reconstruction, but may also allow more subtle differences in diet such as seasonal or migratory behaviour, or differences due to social status or disease to be investigated.

2. THE DIETARY SIGNALS OF BONE COLLAGEN, APATITE AND CHOLESTEROL

Interpretations of the dietary signal obtained from the $\delta^{13}C$ analysis of bone collagen and apatite have been much debated (DeNiro and Epstein, 1978, 1981; van der Merwe, 1982; Krueger and Sullivan, 1984; Chisholm, 1989; Lee-Thorp et al., 1989; Schwarcz, 1991, 2000). Central to the debate is the question of whether specific dietary macronutrients are routed to specific body tissues (e.g., dietary proteins to bone collagen), or whether the carbon from all dietary macronutrient pools is mixed or 'scrambled' before the synthesis of tissues in the body. If routing of dietary macronutrient carbon does occur, then the isotopic composition of bone collagen, for example, may reflect little of the isotopic compositions of dietary macronutrients other than protein. Important initial insights regarding the flux of dietary carbon into the body tissues of animals were gleaned from the feeding experiment performed by DeNiro and Epstein (1978). They raised a variety of different animals on monotonous diets (nematodes, insects, shrimps, snails and mice) and reported an average enrichment of $0.8 \pm 1.1‰$ ($\Delta^{13}C_{animal\text{-}diet} = \delta^{13}C_{animal} - \delta^{13}C_{diet}$) between the carbon isotopic composition of the whole animals ($\delta^{13}C_{animal}$) and that of their respective diets ($\delta^{13}C_{diet}$). The study also showed that where the determination of the $\delta^{13}C_{animal}$ value was impractical, i.e., for mice, the values of diet-to-tissue fractionations ($\Delta^{13}C_{tissue\text{-}diet}$) for different tissues and organs, such as bone collagen, hair and spleen, varied considerably. Moreover, the isotopic relationships among the dietary macronutrients, namely $\delta^{13}C_{total\ organic\ matter} > \delta^{13}C_{lipid}$, $\delta^{13}C_{carbohydrates} > \delta^{13}C_{lipid}$ and $\delta^{13}C_{protein} > \delta^{13}C_{lipid}$, were shown to be inherited by the tissues of the animals raised on them. These results, together with those from other controlled feeding experiments, led to the suggestion that carbon may in fact be routed from dietary macronutrient fractions (i.e., its proteins, carbohydrates and lipids) to corresponding consumer tissue biochemical fractions. This contrasted with the linear mixing or 'scrambling' model, which proposed that carbon from all dietary macronutrients is proportionately allocated to all consumer tissues.

The current consensus, based upon the results of controlled animal feeding experiments, is that the ^{13}C content of bone apatite reflects that of the whole diet, i.e., proteins, carbohydrates and lipids (Ambrose and Norr, 1993; Tieszen and Fagre, 1993). Apatite carbonate is derived from blood bicarbonate and thus samples the total metabolic carbon pool and ultimately, the diet (Lee-Thorp et al., 1989; Schwarcz, 1991). In the metabolic steady state, virtually all ingested carbon atoms (>99%) leave the body as respired CO_2 from the lungs. Thus, respired CO_2 comprises the carbon from all dietary macronutrients in accordance to their proportions in the diet. Respired CO_2 is in isotopic equilibrium with blood bicarbonate. If blood bicarbonate is also in isotopic equilibrium with apatite carbonate, then the $\delta^{13}C$ value of bone apatite will reflect that of the diet as a whole (Schwarcz, 2000). Indeed, this model is supported by the results from the controlled feeding experiments carried out on rats and mice by Ambrose and Norr (1993) and Tieszen and Fagre (1993), respectively. Linear 1:1 correlations of whole diet and apatite $\delta^{13}C$ values were observed in both studies. Furthermore, $\delta^{13}C$ values of respired CO_2 from the latter study were shown to correlate linearly with both whole diet and apatite $\delta^{13}C$ values.

The results from these two feeding experiments have also demonstrated that the $\delta^{13}C$ value of bone collagen is biased towards that of dietary protein. Conventionally, the minimum amount of routing of dietary carbon to bone collagen is thought to be dependent on the relative proportions of essential amino acids (those that cannot be synthesised by the consumer and must therefore be absorbed directly from the diet) to non-essential amino acids comprising collagen. Essential amino acids and non-essential amino acids whose sole precursors are essential comprise 19.3% of the carbon atoms in collagen (Klepinger and Mintel, 1986; Schwarcz, 1991; Ambrose et al., 1997), defining the minimum amount of routing from dietary protein to bone collagen. In theory, the remaining amino acids can be obtained by *de novo* synthesis using carbon derived from non-protein sources, i.e., lipids and carbohydrates. However, a new tripartite division of amino acids (AAs) into indispensable (essential), dispensable (non-essential) and conditionally indispensable (Young and El-Khoury, 1995) suggests that protein routing should be much higher. Conditionally indispensable AAs include those: (1) with essential AA precursors, (2) whose synthesis is modulated by dietary supply, and (3) whose rates of synthesis are insufficient to meet increased demand under stress. It is undoubtedly more energetically efficient (in terms of the costs of *de novo* synthesis, deamination and transamination) to use dietary sources of non-essential AAs when available. If the conservation of energy is adaptive then the amount of routing of carbon from dietary protein to bone collagen should be much higher when the diet supplies an excess of each amino acid. A higher estimate of routing would be consistent with the results observed from the controlled feeding experiments performed by Ambrose and Norr (1993), and Tieszen and Fagre (1993).

Table 1. Rat dietary compositions and their macronutrient components. One percent of vitamins, 3.5% of minerals and 5% cellulose (wood and/or corn) were added to each diet.

Diet	Composition		Components and $\delta^{13}C$ values (%)			
Code	Protein	Energy[a]	Protein 20.0%	Sucrose 50.2%	Starch 15.5%	Oil 5.0%
D2A4	C_3	C_3	Milk casein (–24.5)	Beet (–24.2)	Rice (–26.4)	Cottonseed (–27.9)
D3G	C_3	C_4	Milk casein (–26.3)[b]	Cane (–11.0)[b]	Corn (–10.3)[b]	Corn (–14.9)[b]
D4H	C_4	C_4	Milk casein (–14.6)	Cane (–11.4)	Corn (–10.6)	Corn (–14.9)
D5I	C_4	C_3	Milk casein (–14.6)	Beet (–24.2)	Rice (–26.4)	Cottonseed (–27.9)
D6J2	Marine	C_3	Tuna fish (–17.8)	Beet (–24.2)	Rice (–26.4)	Cottonseed (–28.3)
D7K2	Marine	C_4	Tuna fish (–17.8)	Cane (–11.3)	Corn (–10.6)	Corn (–15.0)
D8L2	Marine	C_3/C_4(1:1)	Tuna fish (–17.8)	Beet/cane (–17.6)	Rice/corn (–18.3)	Cottonseed/corn (–21.6)

[a] Energy comprises sucrose, starch and oil components.
[b] From Ambrose and Norr (1993).

Cholesterol is the most abundant mammalian sterol and is essential for life. It is a major component of cell membranes and a precursor to the formation of bile acids, steroid hormones and vitamin D_3 in the body. Every cell in the human body contains cholesterol and has access to a large extracellular supply. Cholesterol is absorbed from the diet, and almost all cells (except red blood cells) are also able to synthesise their own cholesterol, although the most active sites are in the liver and small intestine (Myant, 1981; Benyon, 1998). Thus, cholesterol in the body is a balance between that which is absorbed from the diet and synthesised *de novo*, and that which is metabolised or excreted by the body.

An understanding of the dietary signal reflected by bone cholesterol can be gleaned when the pathways that affect its occurrence in bone are considered in more detail. Cholesterol is synthesised *de novo* from acetyl coenzyme A (acetyl-CoA), a common metabolite in the body that can be formed from the catabolism of dietary proteins, lipids and carbohydrates. Although the oxidation of ketogenic amino acids in the diet provides a source of acetyl-CoA, it is thought that cholesterol is synthesised from the acetyl-CoA derived predominantly from dietary lipids and carbohydrates (Sabine, 1977; Myant, 1981; Voet and Voet, 1995; Glew, 1997). Hence, the isotopic composition of the cholesterol that is synthesised *de novo* in the body is expected to be biased towards the $\delta^{13}C$ values of dietary lipids and carbohydrates, termed here collectively as the *energy* macronutrients of diet. However, dietary proteins that are not used for protein tissue synthesis are also used for energy metabolism, and can therefore become a substrate for cholesterol synthesis. For an omnivorous diet, carbohydrates are derived mainly from plant resources, whereas lipids and proteins are derived mainly from animal resources. Cholesterol in the diet originates only from animal resources (i.e., from meat and dairy products) and therefore its $\delta^{13}C$ value would be expected to reflect that of the dietary animal protein $\delta^{13}C$ value. Thus, it can be postulated that for a hypothetical omnivorous diet, where plant and animal resources have distinct $\delta^{13}C$ values, bone cholesterol $\delta^{13}C$ values would essentially reflect the isotopic composition of all dietary macronutrients (assuming that little or no fractionation occurs in the absorption of cholesterol from the diet). However, the extent to which dietary protein or energy $\delta^{13}C$ values are reflected by tissue cholesterol values, will depend not only on their relative proportions in the diet, but also on the relative importance of the processes of *de novo* synthesis vs. the direct absorption of cholesterol from the diet. For humans, it has been estimated that the amount of cholesterol that the body may synthesise in a day (typically 1.0–1.5 g) is at least twice that of the daily cholesterol intake for an average western diet (250–750 mg; Sabine, 1977). Approximately half of the dietary cholesterol will be absorbed by the intestine and the other half excreted, thus dietary cholesterol can be estimated to contribute to only 20% of total body cholesterol. Since the majority of tissue cholesterol is synthesised *de novo* in the body, bone or tissue cholesterol $\delta^{13}C$ values are thus expected to be biased towards that of the energy macronutrients of the diet, reflecting the protein and energy macronutrients in an approximate ratio of 1:4.

A programme of controlled feeding experiments using rats, carried out by Ambrose (unpublished data), has provided the opportunity to study in greater detail the effects of isotopically distinct diets on the $\delta^{13}C$ values of bone cholesterol, collagen and apatite. This extensive study includes animals that were raised on monotonous pure C_3 or pure C_4 end-member diets and those in which the isotopic composition of dietary proteins differed from that of the energy macronutrients, thus providing an opportunity to address questions concerning the precise origin of the dietary signals of bone cholesterol, collagen and apatite. Collagen and apatite $\delta^{13}C$ values from one of these diets (D3G in Table 1) have been previously reported in Ambrose and Norr (1993). The primary aim of this study was to investigate the carbon isotopic relationship between diet and bone cholesterol, i.e., to gain an understanding of the dietary signal reflected in bone cholesterol $\delta^{13}C$ values. The new insights into the metabolic behaviour of bone cholesterol will underpin its combined use with existing bone collagen and apatite techniques in the interpretation of ancient diets. These findings have important implications for how archaeological bone $\delta^{13}C$ values should be interpreted to gain more accurate insights into ancient diets.

3. MATERIALS AND METHODS

3.1. Sample Description

Holtzman albino rats were raised on a variety of purified and pelletized diets comprising 20.0% protein, 50.2% sucrose, 15.5% starch, 5.0% oil, 5% fibre, 3.5% minerals and 1% vitamins. One day after insemination, sperm-positive, 90-d-old female rats were placed on diets that their offspring would consume. Birth occurred 21 d after insemination and weaning occurred 21 to 23 d later. The sexes were separated

Table 2. Tissue sample codes, sex and pair of individual animals. Dietary compositions and sample groupings are also listed. M denotes male and F denotes female. First, second and third pair animals were sacrificed at 91, 131 and 171 d old, respectively.

	Sample			Dietary composition	
Group + no.	Code	Sex	Pair	Protein	Energy[a]
A2	C3	M	2	C_3	C_3
A3	C3	F	2	C_3	C_3
A4	C3P/C4	M	3	C_3	C_4
A5	C3P/C4	F	3	C_3	C_4
A6	C4	M	1	C_4	C_4
A7	C4	F	1	C_4	C_4
A8	C4	M	2	C_4	C_4
A9	C4P/C3	F	2	C_4	C_3
A10	C4P/C3	M	3	C_4	C_3
B1	MP/C3	F	1	Marine	C_3
B2	MP/C3	F	2	Marine	C_3
B3	MP/C3 + C4	F	1	Marine	C_3/C_4 (1:1)
B4	MP/C3 + C4	M	2	Marine	C_3/C_4 (1:1)
B5	MP/C4	M	1	Marine	C_4
B6	MP/C4	M	2	Marine	C_4

[a] Energy comprises sucrose, starch and oil components.

before sexual maturity. Normal room temperature (20°C) was maintained, and food and water were provided *ad libitum*. Male and female pairs were sacrificed at 91, 131 and 171 d after birth.

Fifteen rat forelimbs from 7 distinct diets were sampled for lipid analysis, in addition to the whole diets and dietary oils. Dietary compositions and $\delta^{13}C$ values of individual dietary macronutrients comprising the diets are shown in Table 1. A weighted mean $\delta^{13}C$ value for the energy macronutrients (i.e., sucrose, starch and oil) was calculated for each diet using their percentages in the diet, their percentages of digestible carbon and their individual $\delta^{13}C$ values. Table 2 lists all the animals that were studied. The 15 experimental animals can be separated into two groups: (1) group A ($n = 9$), whose individuals were raised on a diet comprising 20% terrestrial protein (milk casein), and 70% energy macronutrients, and (2) group B ($n = 6$), whose individuals were raised on a diet comprising 20% marine protein (tuna flesh) and 70% energy macronutrients.

3.2. Bulk $\delta^{13}C$ Analysis of Whole Diet, Dietary Macronutrients, Bone Collagen and Bone Apatite

Bone collagen and apatite extraction procedures and $\delta^{13}C$ measurements are summarized here and are described in detail in Ambrose and Norr (1993). Lipids were extracted from clean ground bone using petroleum ether. Collagen was extracted by demineralisation with 0.1 mol/L HCl, treated with 0.125 mol/L NaOH, solubilised at 95°C and freeze-dried. Whole diet, dietary macronutrients and bone collagen were combusted at >800°C with Cu, CuO and Ag foil in evacuated sealed quartz tubes. Bone apatite carbonate was prepared by deproteinisation with NaOCl, treated with 1 mol/L acetic acid to remove adsorbed carbonate, freeze-dried, and reacted under vacuum with 100% H_3PO_4 at 25°C. CO_2 was cryogenically distilled off-line, and analysed on dual inlet isotope ratio mass spectrometers at the Anthropology Department, University of Illinois (Nuclide 6-60 RMS) or the Illinois State Geological Survey (Finnegan MAT Delta E).

3.3. Chemicals and Precautions in Lipid Analysis

All solvents used were of HPLC grade and purchased from Rathburn chemicals. The internal standard *n*-tetratriacontane, cholesterol standard, sodium hydroxide pellets and derivatising agent *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% v/v trimethylchlorosilane were purchased from the Sigma Chemical Co. All glassware and ceramics employed were washed with Decon 90, dried in an oven and rinsed with chloroform:methanol (2:1 v/v) before use. Disposable rubber gloves were worn throughout the whole experimental procedure, from sample preparation to instrumental analyses, to prevent the contamination of samples with finger lipids. Extraction, filtration and saponification (neutral and acid) blanks were used to monitor and locate any contamination that may be introduced during the experimental procedure.

3.4. Preparation of Diet and Bone Samples for Lipid Extraction

Rat forelimbs were dissected into three different tissue types: bone (ulna and radius), skin, and flesh. Bones were then manually cleaned of adhering flesh, cartilage and tendons by scraping with a scalpel. Half a bone was used for the extraction procedure, typically the upper ulna. Rat bone and diet pellets were ground in a pestle and mortar before extraction and liquid nitrogen was employed to aid the bone crushing process. Ranges of rat sample weights are as follows: 0.03 to 0.13 g of powdered bone and 0.83 to 1.41 g of powdered diet pellets.

3.5. Extraction of Lipids From Diet and Bone Samples

Samples were transferred into large screw-capped vials and a known quantity of *n*-tetratriacontane (1 mg mL^{-1} in chloroform) was added as an internal standard. The samples were extracted with chloroform/methanol (2:1 v/v, 5–10 mL) by ultrasonication (3 × 1 h, Decon F5200b) where the supernatant was removed and replaced intermittently. The extract was then concentrated to ~5 mL under a gentle stream of nitrogen gas (5 psi) in an evaporation unit (TurboVap LV Zymarck Corporation, Hopkinton, MA 01748, USA) with the thermostatic bath set at 40°C. Suspended particulates were removed from the extract by centrifugation (1800 rpm, 20 min, MSE Mistral 1000) and filtration through a short pipette column packed with activated alumina. The total lipid extract was then transferred into preweighed sample vials and concentrated down to dryness under $N_{2(g)}$. The vials were re-weighed and stored at 4°C until required for analysis.

3.6. Saponification of Total Lipid Extracts

The total lipid extracts were subjected to hydrolysis. Aliquots of the total lipid extracts were transferred into screw-capped test tubes, blown down to dryness under $N_{2(g)}$ and hydrolysed with 0.5 mol/L methanolic NaOH (2 mL) at 70°C in a water bath for 1 h. After cooling, the mixture was extracted using hexane (3 × 2 mL) yielding the neutral cholesterol-containing fraction.

3.7. Derivatisation of Neutral Fractions

Neutral fractions were converted to their trimethylsilyl (TMS) derivatives using *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA)

Table 3. Whole diet (*w*diet), dietary protein (prot), energy (engy) and cholesterol (*d*chol) $\delta^{13}C$ values. Diet and average bone cholesterol concentrations are also shown.

Diet	Composition		Diet $\delta^{13}C_{vPDB}$ values (?)				Cholesterol concentration (mg/g dry weight)	
Code	Protein	Energy[a]	*w*diet	prot	engy[a]	*d*chol	Diet	Bone
D2A4	C_3	C_3	–24.9	–24.5	–25.1	–27.6	0.04	0.42
D3G	C_3	C_4	–14.7[b]	–26.3[b]	–11.3[b]	–29.0	0.03	0.19
D4H	C_4	C_4	–12.2	–14.6	–11.7	–15.8	0.10	0.16
D5I	C_4	C_3	–22.3	–14.6	–25.1	–16.3	0.13	0.15
D6J2	Marine	C_3	–23.3	–17.8	–25.1	–30.6	0.22	0.32
D7K2	Marine	C_4	–12.9	–17.8	–11.6	–30.6	0.23	0.14
D8L2	Marine	C_3/C_4 (1:1)	–18.3	–17.8	–18.2	–30.7	0.20	0.18

[a] Energy comprises sucrose, starch and oil components.
[b] From Ambrose and Norr (1993).

containing 1% v/v trimethylchlorosilane. Twenty microlitres of BSTFA were added to each sample and the samples were placed on a heating block at 70°C (Multiblok Lab-Line) for ~1 h. Excess BSTFA was removed under a gentle stream of nitrogen gas and the trimethylsilylated extracts were diluted in an appropriate volume of hexane before analysis by high temperature-gas chromatography (HT-GC), high temperature-gas chromatography/mass spectrometry (HT-GC/MS) and gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS).

3.8. Gas Chromatography (HT-GC)

HT-GC analyses were carried out using 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). Samples were introduced either manually or by automated on-column injection (Hewlett Packard 7673 automatic sampler). The temperature of the oven was held isothermally at 50°C (2 min) and then increased to 350°C (20 min) at a rate of 10°C min^{-1}. Hydrogen was used as the carrier gas and flame ionisation detection (FID) was used to monitor the column effluent. Data were acquired and analysed using HP Chemstation software.

3.9. Gas Chromatography/Mass Spectrometry (HT-GC/MS)

HT-GC/MS analyses were performed using a Finnigan 4500 quadrupole mass spectrometer (source temperature, 280°C; electron voltage, 35 eV) interfaced to a Carlo Erba HRGC 5160 Mega series gas chromatograph. The GC was fitted with the same column as was used for HT-GC analyses and the same temperature program was employed. Hydrogen was used as the carrier gas. Data were acquired using an INCOS data system and processed using Interactive Chemical Information Software (ICIS) package. Peak assignments were made by comparison of mass spectra and retention times.

3.10. Gas Chromatography/Combustion/Isotope Ratio Mass Spectrometry (GC/C/IRMS)

GC/C/IRMS analyses were carried out using a Varian 3500 gas chromatograph coupled to a Finnigan MAT Delta-S isotope ratio mass spectrometer via a Finnigan MAT combustion interface (Pt/CuO) maintained at 850°C. Samples were introduced either manually or by automated on-column injection (Finnigan MAT A200S automatic sampler). The GC was fitted with a fused silica capillary column (50 m × 0.32 mm i.d.) coated with a dimethyl polysiloxane stationary phase (CP-SIL 5 CB, 0.25 μm film thickness). The temperature of the oven was held at 50°C (2 min) and then increased to 250°C at a rate of 10°C min^{-1}, then to 300°C (20 min) at 4°C min^{-1}. Helium was used as the carrier gas and the mass spectrometer source pressure was maintained at 9×10^{-5} Pa. Standardisation of runs was achieved using 6 aliquots of $CO_{2(g)}$ of known $\delta^{13}C$ value, which were injected directly into the ion source. Data were collected and processed using Finnigan MAT Isobase software. A C_{19} *n*-alkane of known $\delta^{13}C$ value was employed as a secondary standard which was measured every seventh run to monitor any fluctuations in instrumental measurements with time. The standard deviation of triplicate GC/C/IRMS analyses carried out on each sample was $\leq \pm 0.3$‰ and each analysis was checked for baseline line resolution of cholesterol and for the correct assignment of peaks. The cholesterol $\delta^{13}C$ values were corrected for the addition of the TMS group according to the procedure of Jones et al. (1991).

4. RESULTS AND DISCUSSION

4.1. Quantification and $\delta^{13}C$ Analysis of Cholesterol Present in the Diets

Quantification of total dietary cholesterol showed that cholesterol was present in the diets in varying concentrations, from 0.03 to 0.23 mg g^{-1} dry weight of diet (Table 3). Not surprisingly, the concentration of dietary cholesterol, which originates from the animal protein in the diet, was shown to reflect the type of protein present in the diets. Diets containing C_3 casein (D2A and D3G) exhibited the lowest cholesterol concentrations of 0.03 to 0.04 mg g^{-1} dry weight, diets containing C_4 casein (D4H and D5I) had intermediate cholesterol concentrations of 0.10 to 0.13 mg g^{-1} dry weight and diets containing tuna as the protein source (D6J2, D7K2 and D8L2) had the highest cholesterol concentrations of 0.20 to 0.23 mg g^{-1} dry weight. The diets were formulated using purified natural ingredients with the exception of C_4 casein. This casein was made from full cream milk powder imported from Kenya and purified at the food science pilot plant at the University of Illinois. Differences in the cholesterol content between the casein samples are due to the differential lipid extraction of the two proteins during processing.

The $\delta^{13}C$ values of dietary cholesterol ($\delta^{13}C_{d\text{chol}}$) were measured (Table 3) and for the terrestrial protein diets containing casein, $\delta^{13}C_{d\text{chol}}$ values resembled those of the protein ($\delta^{13}C_{\text{prot}}$). C_3 casein diets ($\delta^{13}C_{\text{prot}} = -24.5$‰), D2A4 and D3G, exhibited $\delta^{13}C_{d\text{chol}}$ values of -27.6 and -29.0‰, respectively. C_4 casein diets ($\delta^{13}C_{\text{prot}} = -14.6$‰), D4H and D5I, showed $\delta^{13}C_{d\text{chol}}$ values of -15.8 and -16.3‰, respectively. However, diets containing marine protein (tuna flesh; $\delta^{13}C_{\text{prot}} = -17.8$‰), D6J, D7K and D8L, exhibited $\delta^{13}C_{d\text{chol}}$ values of ~ -30.6‰. This extremely depleted and seemingly

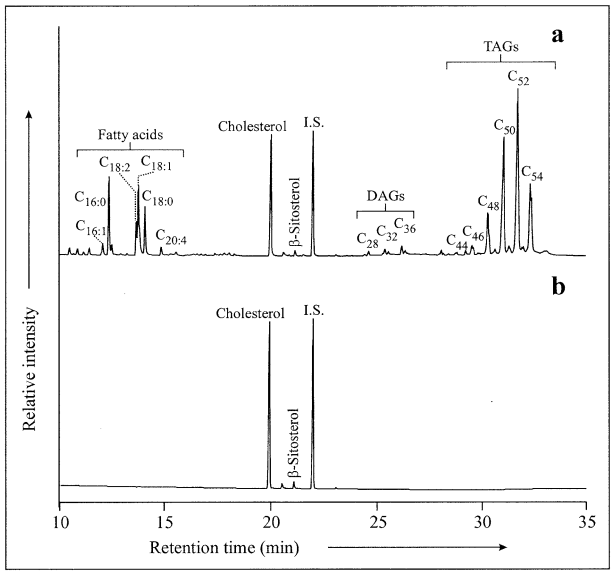

Fig. 1. Partial gas chromatograms of: (a) the total lipid extract, and (b) the neutral fraction of rat bone B6 (from diet MP/C4; I.S. = internal standard, *n*-tetratriacontane; DAGs = diacylglycerols; TAGs = triacylglycerols).

anomalous value was then tested by the analysis of additional tuna samples. Tuna flesh cholesterol was extracted from tinned and fresh samples, and demonstrated comparable $\delta^{13}C$ values of −26.2 and −27.8‰, respectively. Flesh cholesterol from mackerel and red mullet were also measured and gave $\delta^{13}C$ values of −27.4 and −21.9‰, respectively. It would seem that the range of cholesterol $\delta^{13}C$ values from marine fish may well extend beyond the ranges of what would generally be considered as marine from the bulk $\delta^{13}C$ analysis of fish flesh.

4.2. Bone Lipid Analysis

Typical distributions of bone total lipid extracts and neutral fractions are shown in Figure 1. Highly consistent distributions were shown for all the bone samples studied. Bone cholesterol concentrations were shown to range from 0.12 to 0.42 mg g^{-1} dry weight (Table 3) and were not a function of the dietary cholesterol concentration; the higher concentration of dietary cholesterol in the marine protein diets did not result in a higher concentration of bone cholesterol.

4.3. Patterns Among the $\delta^{13}C$ Values of Diet and Bone Components

Table 4 presents the bone cholesterol, collagen and apatite $\delta^{13}C$ values. Two animals from each diet were studied, except for diet D4H (sample code C4) where three animals were studied. The smallest ranges of intra-pair variations in bone $\delta^{13}C$ values ($\Delta^{13}C_{pair}$) were observed for collagen, where $\Delta^{13}C_{pair} < 0.3$‰. For apatite, $\Delta^{13}C_{pair} < 0.6$‰, except for the C3 group where a variation of 1.0‰ was observed. More variable intra-pair differences are observed for cholesterol, where $\Delta^{13}C_{pair} = 0$ to 1.8‰. The reasons for the differences in ranges of variation in $\Delta^{13}C_{pair}$ values observed between the bone components are not clear, but are likely to have some metabolic significance. The standard deviations observed for triplicate measurements of cholesterol for each animal were all within analytical precision (±0.3‰), so intra-pair variance is probably not related to analytical error.

Bone (cholesterol, collagen and apatite) $\delta^{13}C$ values were compared to the diet values (whole diet, protein, energy and cholesterol) to investigate the isotopic relationship between diet and bone. Figure 2a shows the diet and bone $\delta^{13}C$ values plotted for each diet. Dietary cholesterol (*d*chol) $\delta^{13}C$ values are consistently more depleted than their corresponding dietary protein values and this depletion is also observed for bone cholesterol (*b*chol) $\delta^{13}C$ values when compared to collagen and apatite values. This finding is consistent with the relative depletion in ^{13}C that occurs during lipid biosynthesis with respect to other biochemical pathways (DeNiro and Epstein, 1977; Hayes, 1993). Apatite is the most enriched bone component for most of the diets except for the MP/C3 and C4P/C3 diets where apatite $\delta^{13}C$ values are comparable to collagen values. Figure

Table 4. Bone cholesterol, collagen and apatite $\delta^{13}C$ values. All standard deviations observed for triplicate cholesterol measurements were within the analytical precision of the instrumentation (±0.3‰). Precision of the collagen and apatite measurements = ±0.1‰.

Sample		Bone component $\delta^{13}C_{vPDB}$ values (?)		
Group + no.	Code	Cholesterol	Collagen	Apatite
A2	C3	–28.4	–20.0	–14.0
A3	C3	–28.0	–19.8	–15.0
A4	C3P/C4	–20.3	–17.0[a]	–5.9[a]
A5	C3P/C4	–19.3	–16.8[a]	–5.8[a]
A6	C4	–15.9	–7.9	–3.0
A7	C4	–16.4	–7.7	–3.2
A8	C4	–15.7	–8.0	–3.2
A9	C4P/C3	–23.0	–12.4	–13.2
A10	C4P/C3	–23.0	–12.1	–12.7
B1	MP/C3	–24.9	–14.5	–13.1
B2	MP/C3	–26.7	–14.4	–13.7
B3	MP/C3 + C4	–22.7	–12.3	–8.8
B4	MP/C3 + C4	–20.9	–12.1	–8.4
B5	MP/C4	–17.5	–9.7	–3.2
B6	MP/C4	–18.6	–9.6	–3.7

[a] From Ambrose and Norr (1993).

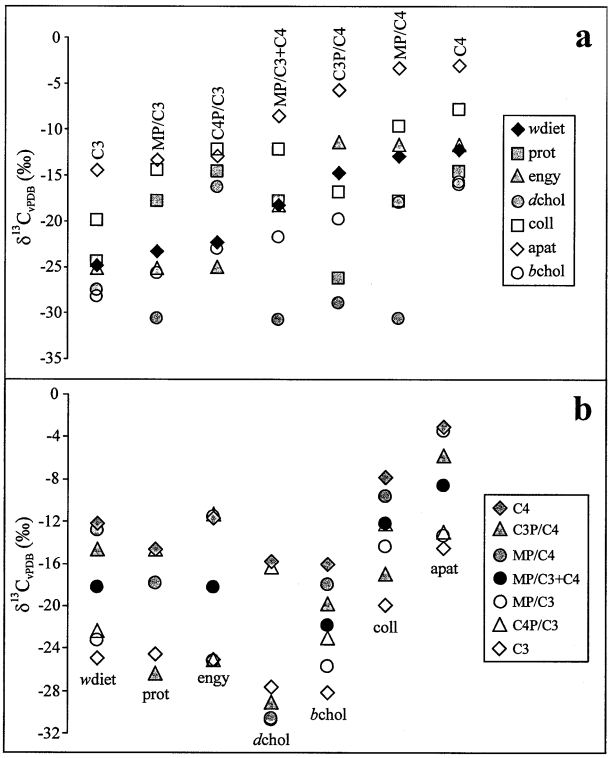

Fig. 2. δ^{13}C values from the controlled rat feeding experiment: (a) diet and bone δ^{13}C values for each diet, and (b) ranges of values observed for diet and bone δ^{13}C values. Each data point represents the mean value for the analyses of two animals, except for diet C_4, which represents data from three animals (*w*diet = whole diet; prot =protein; engy = energy; *d*chol = dietary cholesterol; *b*chol = bone cholesterol; coll = collagen and apat = apatite).

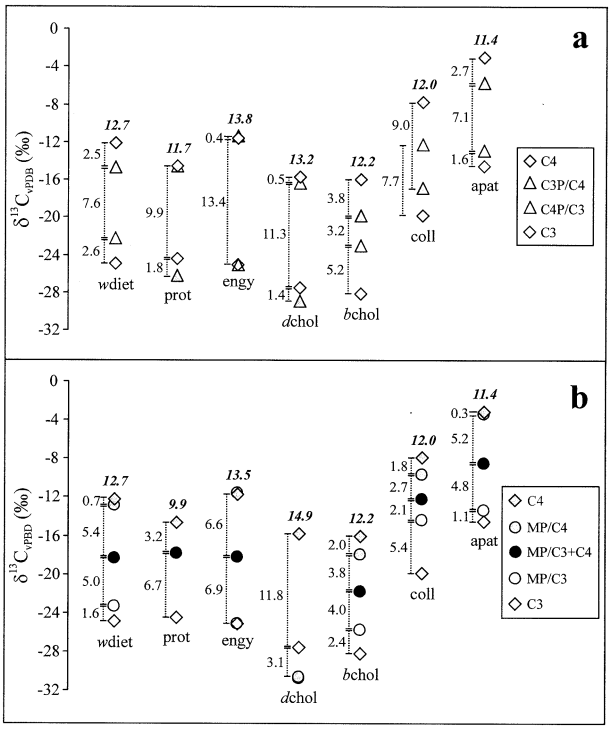

Fig. 3. Diet and bone δ^{13}C values for: (a) terrestrial protein, and (b) marine protein diets. Ranges of δ^{13}C values are shown in bold italics and variations in values between different diets are indicated (*w*diet = whole diet; prot = protein; engy = energy; *d*chol = dietary cholesterol; *b*chol = bone cholesterol; coll = collagen and apat = apatite).

2b presents the same data plotted with respect to whole diet, and each dietary macronutrient and bone biochemical component to show the ranges of their δ^{13}C values. The formulation of diets from essentially three isotopically distinct protein and energy sources resulted in whole diet δ^{13}C values that spanned the full natural range of variation, from a pure C_3 (−24.9‰) to a pure C_4 (−12.2‰) diet. The magnitude of the range for the whole diet values (12.7‰) is reflected in the ranges observed for all three bone components. Using average bone δ^{13}C values for each diet, cholesterol values varied from −28.2 to −16.0‰ (range = 12.2‰), collagen δ^{13}C values varied from −19.9 to −7.9‰ (range = 12.0‰) and apatite values varied from −14.5 to −3.1‰ (range = 11.4‰).

4.4. Diet to Bone δ^{13}C Relationships for Animals Raised on Terrestrial Protein Diets

Figure 3a presents the data from the terrestrial protein diets (C3, C4P/C3, C3P/C4 and C4). The isotopic relationship amongst the whole diet values, where $\delta^{13}C_{C4} > \delta^{13}C_{C4P/C3} > \delta^{13}C_{C3P/C4} > \delta^{13}C_{C3}$, is conserved in the δ^{13}C values of bone cholesterol and apatite. In fact, the isotopic relationship demonstrated by the apatite δ^{13}C values mirror those of the whole diet. Whole diet δ^{13}C values for C3P/C4 (−14.7‰) and C4P/C3 (−22.3‰) represent a difference from C4 (−12.2‰) and C3 (−24.9‰) diet end-member values of −2.5 and +2.6‰, respectively. These differences from end-member values are conserved in the apatite δ^{13}C values, where C3P/C4 (−5.8‰) and C4P/C3 (−12.9‰) animals show differences from C4 (−3.1‰) and C3 (−14.5‰) values of −2.7‰ and +1.6‰, respectively. Bone cholesterol δ^{13}C values also conserve the isotopic relationship amongst the whole diet values and do not reflect dietary cholesterol δ^{13}C values. This finding is consistent with previous work performed on the flesh cholesterol of these animals (Jim et al., 2001) and demonstrates that the majority of cholesterol in bone is synthesised *de novo*. In contrast to apatite and cholesterol δ^{13}C values, collagen values do not conserve the whole diet δ^{13}C pattern. A small difference of +2.5‰ between C4 and C3P/C4 whole diet δ^{13}C values resulted in a much larger difference of +9.0‰ in collagen values. Similarly, a small difference of +2.6‰ between C4P/C3 and C3 whole diet δ^{13}C values resulted in a large difference of +7.7‰ in collagen values. This clearly demonstrates the bias of collagen δ^{13}C values towards the δ^{13}C values of the protein in the diet.

4.5. Diet to Bone δ^{13}C Relationships for Animals Raised on Marine Protein Diets

Figure 3b shows the diet and bone δ^{13}C data for the pure C_3, pure C_4 and marine protein diets. Again, the isotopic relationship between the whole diet values, where $\delta^{13}C_{C4} > \delta^{13}C_{MP/C4} > \delta^{13}C_{MP/C3+C4} > \delta^{13}C_{MP/C3} > \delta^{13}C_{C3}$, is conserved for bone apatite and cholesterol values, as previously observed for

Table 5. R^2 values from linear correlations of diet and bone $\delta^{13}C$ values.

	R^2 values		
	Bone component		
Diet component	Cholesterol	Collagen	Apatite
Whole diet	0.92	0.48	0.99
Protein	0.16	0.72	0.04
Energy[a]	0.80	0.28	0.95
Dietary cholesterol	0.15	0.25	0.03

[a] Energy comprises sucrose, starch and oil components.

the terrestrial protein diets. This relationship is also conserved in collagen for the marine protein diets as these diets all have the same dietary protein $\delta^{13}C$ value ($\delta^{13}C_{prot} = -17.8‰$). Indeed, the bias of collagen $\delta^{13}C$ values towards protein $\delta^{13}C$ values can be clearly seen resulting in the marine collagen values plotting close to the C_4 end-member collagen value, mirroring the dietary protein isotopic relationship. The conservation of the whole diet pattern in bone cholesterol indicates that on these marine protein diets, the synthesis of cholesterol *de novo* is greater than the absorption of cholesterol from the diet (dietary cholesterol concentration ~0.21 mg g^{-1} dry weight). These marine protein diets contained twice as much cholesterol as the C_4 protein diets, but no bias towards dietary cholesterol $\delta^{13}C$ values is observed for bone cholesterol $\delta^{13}C$ values.

4.6. Correlations Between Diet and Bone $\delta^{13}C$ Values

The relationships between diet (whole diet, protein, energy and cholesterol) and bone (cholesterol, collagen and apatite) $\delta^{13}C$ values were investigated using linear regression. Table 5 summarises the R^2 values observed between each diet and bone component. Figure 4 shows the relationship between bone cholesterol and whole diet (Fig. 4a), collagen and dietary protein (Fig. 4b), and apatite and whole diet (Fig. 4c) $\delta^{13}C$ values. Collagen and apatite $\delta^{13}C$ values were shown to correlate best with protein ($R^2 = 0.72$; $p \leq 0.001$) and whole diet ($R^2 = 0.99$; $p \leq 0.001$) values, respectively (as was also demonstrated for the data presented in Ambrose and Norr, 1993), and these findings are consistent with the results from the controlled animal feeding study performed by Tieszen and Fagre (1993). Cholesterol was indeed found to correlate best with whole diet values ($R^2 = 0.92$; $p \leq 0.001$), as was previously hypothesised. The low value of R^2 between bone and dietary cholesterol ($R^2 = 0.15$; $p > 0.05$) indicates that the direct absorption of cholesterol from the diet was not the dominant pathway. Interestingly, the linear relationship between bone cholesterol vs. energy ($R^2 = 0.80$; $p \leq 0.001$) correlated better than the relationship between bone cholesterol vs. protein ($R^2 = 0.16$; $p > 0.05$), providing evidence for the hypothesis that cholesterol is synthesised *de novo* from acetyl-CoA derived predominantly from dietary lipids and carbohydrates.

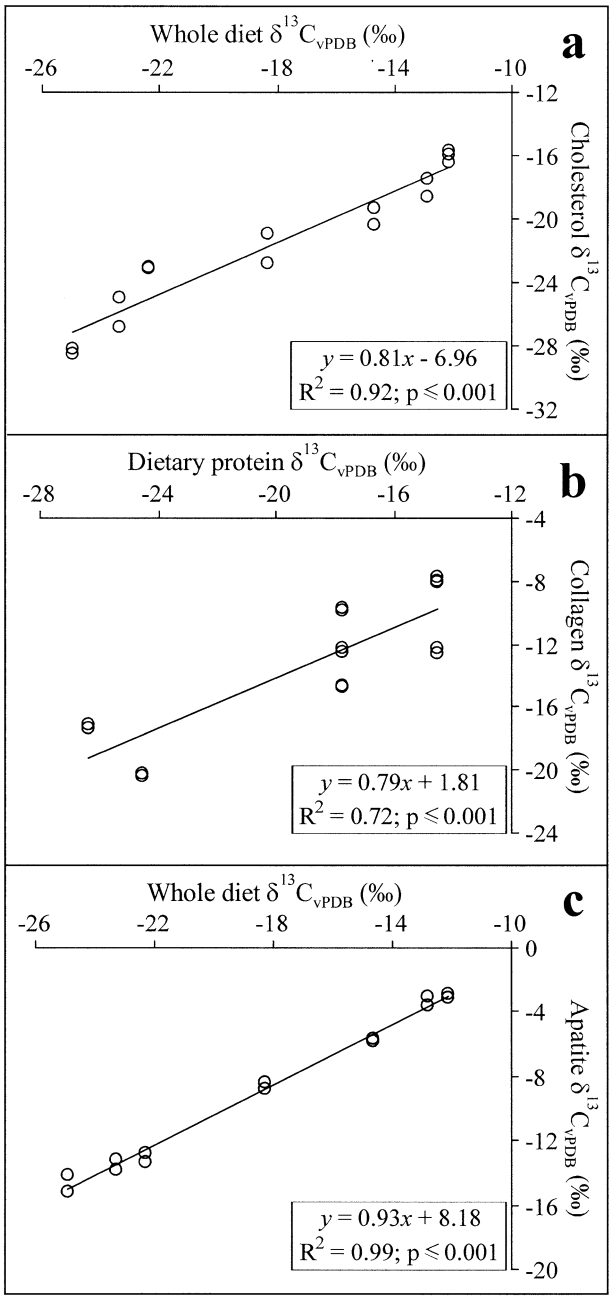

Fig. 4. Linear correlations for bone cholesterol, collagen and apatite $\delta^{13}C$ values with: (a) whole diet, (b) dietary protein, and (c) whole diet $\delta^{13}C$ values, respectively.

Table 6. Ranges and mean values of the fractionation between diet and bone δ^{13}C values ($\Delta^{13}C_{bcomp\text{-}dcomp}$; *b*comp = bone component; *d*comp = diet component).

	$\Delta^{13}C_{bcomp\text{-}dcomp}$ (?)					
	Bone component					
	Cholesterol		Collagen		Apatite	
Diet component	Range	Mean	Range	Mean	Range	Mean
Whole diet	5.0	–3.5	12.5	5.0	2.1	9.5
Protein	15.8	–2.7	7.3	5.8	19.2	10.3
Energy[a]	11.1	–3.5	18.7	4.9	7.0	9.4
Dietary cholesterol	19.9	3.7	17.1	12.2	24.4	16.7

[a] Energy comprises sucrose, starch and oil components.

4.7. Fractionation Between Diet and Bone Components ($\Delta^{13}C_{bcomp\text{-}dcomp}$)

One of the most important observations from the animal feeding experiments was the finding that the fractionation between diet and bone component δ^{13}C values is not necessarily constant (Ambrose and Norr, 1993; Tieszen and Fagre, 1993). Before this work, the modelling of collagen and apatite δ^{13}C values from field studies on herbivores and carnivores (Krueger and Sullivan, 1984; Lee-Thorp et al., 1989) showed that $\Delta^{13}C_{coll\text{-}wdiet} \sim 5‰$ and $\Delta^{13}C_{apat\text{-}wdiet} \sim 12‰$. The most detailed field estimate for herbivore $\Delta^{13}C_{apat\text{-}wdiet}$ is ~13.5‰ (Cerling and Harris, 1999). Variability in fractionation between herbivores and carnivores on diets in which dietary protein and energy have the same δ^{13}C values may be largely a function of the rates of methanogenesis (Metges et al., 1990). If so, then $\Delta^{13}C_{apat\text{-}wdiet}$ may also vary between ruminants and non-ruminants (e.g., equids vs. bovids). Although these fractionations hold true for the field animals studied, due to the more varied nature of human diets, in particular where dietary protein and energy δ^{13}C values differ significantly, human archaeological populations may exhibit $\Delta^{13}C_{bcomp\text{-}wdiet}$ fractionations that deviate substantially from those indicated above. Such deviations have been demonstrated in this study. Table 6 presents the ranges and mean values of the fractionation between diet and bone component δ^{13}C values ($\Delta^{13}C_{bcomp\text{-}dcomp}$). $\Delta^{13}C_{bcomp\text{-}dcomp}$ fractionations were shown to vary widely. Not surprisingly, from the correlations discussed previously, the narrowest ranges observed for bone cholesterol, collagen and apatite $\Delta^{13}C_{bcomp\text{-}dcomp}$ fractionations were found with respect to whole diet, protein and whole diet values, respectively, demonstrating a greater isotopic consistency with their inherited dietary signals. Indeed, the fractionation between whole diet and apatite values ($\Delta^{13}C_{apat\text{-}wdiet}$) was shown to vary by only 2.1‰ for all diets, demonstrating the integrity with which apatite inherits the whole diet δ^{13}C value. The fractionation between whole diet and bone cholesterol ($\Delta^{13}C_{bchol\text{-}wdiet}$), and dietary protein and collagen ($\Delta^{13}C_{coll\text{-}prot}$) δ^{13}C values exhibited larger ranges of variation of 5.0 and 7.3‰, respectively. These wider ranges indicate that the isotopic relationship between bone cholesterol and whole diet, and between collagen and dietary protein is more complex. In the case of bone cholesterol, complexities occur due to the balance between neosynthesis and routing of dietary cholesterol, and for collagen, it is clear that non-protein or energy carbon will also affect its δ^{13}C value.

4.8. Correlations Between $\Delta^{13}C_{bcomp\text{-}wdiet}$ Fractionations and $\Delta^{13}C_{dcomp\text{-}dcomp}$ Spacings

Greater insight into the sources of variation in diet-to-bone fractionations and the degree of routing from dietary macronutrients to bone biochemical fractions can be gained by plotting $\Delta^{13}C_{bcomp\text{-}wdiet}$ fractionations against: (1) the dietary protein-to-energy ($\Delta^{13}C_{prot\text{-}engy}$), and (2) the dietary protein-to-whole diet ($\Delta^{13}C_{prot\text{-}wdiet}$) spacings. Portraying isotopic relationships in this "double Δ" mode reveals the limitations of drawing conclusions about diet-to-bone relationships solely from the correlation coefficients (R^2) from linear regression analysis of diet and bone δ^{13}C values. We focus here on the gradients (*m*) and y-intercepts (*C*) of the regression equations in Figures 5 and 6 to interpret diet-to-bone relationships.

Figure 5 is a plot of $\Delta^{13}C_{bcomp\text{-}wdiet}$ fractionations against $\Delta^{13}C_{prot\text{-}engy}$ spacings. Significant differences in the gradients of these regression equations are observed, demonstrating the varying degrees on which $\Delta^{13}C_{bcomp\text{-}wdiet}$ fractionations depend on the difference between dietary protein and energy δ^{13}C values. The small gradients for $\Delta^{13}C_{apat\text{-}wdiet}$ ($m = 0.03$; $R^2 = 0.18$; $p > 0.05$) and $\Delta^{13}C_{bchol\text{-}wdiet}$ ($m = 0.17$; $R^2 = 0.74$; $p \leq 0.001$) indicate that these diet-bone fractionations

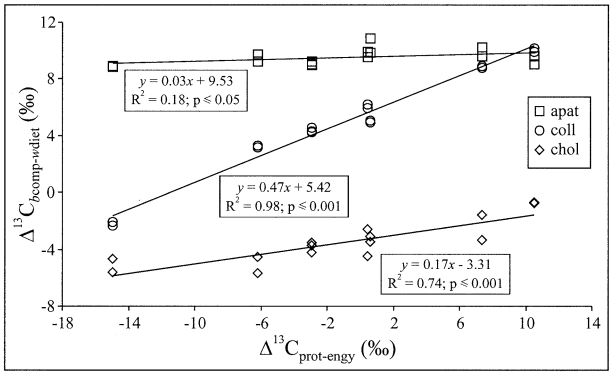

Fig. 5. Linear correlations between the dietary protein-to-energy spacing ($\Delta^{13}C_{prot\text{-}engy}$) and the bone component-to-whole diet fractionation ($\Delta^{13}C_{bcomp\text{-}wdiet}$).

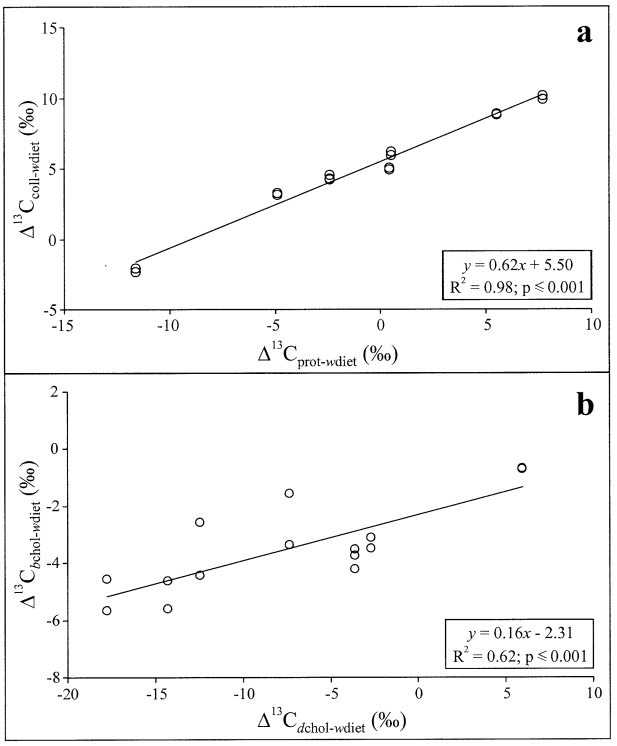

Fig. 6. Linear correlations between: (a) the dietary protein-to-whole diet spacing ($\Delta^{13}C_{prot\text{-}wdiet}$) and bone collagen-to-whole diet fractionation ($\Delta^{13}C_{coll\text{-}wdiet}$), and (b) the dietary cholesterol-to-whole diet ($\Delta^{13}C_{dchol\text{-}wdiet}$) spacing and bone cholesterol-to-whole diet $\delta^{13}C$ fractionation ($\Delta^{13}C_{bchol\text{-}wdiet}$).

are not significantly dependent on the $\Delta^{13}C_{prot\text{-}engy}$ spacing, whereas the steeper slope shown for $\Delta^{13}C_{coll\text{-}wdiet}$ ($m = 0.47$; $R^2 = 0.98$; $p \leq 0.001$) indicates a much greater reliance. These findings are consistent with the differences in the dietary signals observed for these bone biochemical components. Apatite and cholesterol $\delta^{13}C$ values reflect that of the whole diet and thus their fractionation with respect to whole diet values is not reliant on macronutrient isotopic differences. Collagen $\delta^{13}C$ values however, reflect that of the dietary protein and hence its fractionation from whole diet values is expected to be more heavily dependent on the isotopic difference between dietary protein and energy macronutrients. The y-intercepts of these regression equations show $\Delta^{13}C_{bcomp\text{-}wdiet}$ fractionations when $\Delta^{13}C_{prot\text{-}engy} = 0$ (i.e., when $\delta^{13}C_{prot} = \delta^{13}C_{engy}$). With respect to whole diet $\delta^{13}C$ values, cholesterol shows a depletion of –3.3‰ whereas collagen and apatite show enrichments of +5.4 and +9.5‰, respectively.

The extent of the dependence of collagen on dietary protein $\delta^{13}C$ values can be further investigated by plotting $\Delta^{13}C_{coll\text{-}wdiet}$ fractionations against $\Delta^{13}C_{prot\text{-}wdiet}$ spacings (Fig. 6a). A significant gradient ($m = 0.62$; $R^2 = 0.98$; $p \leq 0.001$) is observed for this relationship and can be interpreted as showing that 62% of the carbon atoms in collagen are derived from dietary protein. This estimate is much higher than 19.3%, which is the percentage expected due to the routing of essential amino acids from the diet, demonstrating that for these 20% protein diets, a substantial amount of non-essential or dispensable amino acids are also routed. Similarly, the extent of the routing of dietary cholesterol to bone cholesterol can be explored by plotting $\Delta^{13}C_{bchol\text{-}wdiet}$ fractionations against $\Delta^{13}C_{dchol\text{-}wdiet}$ spacings (Fig. 6b). The small gradient ($m = 0.16$; $R^2 = 0.62$; $p \leq 0.001$) observed for this relationship suggests that dietary cholesterol can account for 16% of the carbon in bone cholesterol. This estimate of routing is close to the predicted 20% discussed above.

Double Δ graphs can also provide insights into the systematic relationship of dietary macronutrient differences (i.e., $\Delta^{13}C_{prot\text{-}engy}$) to variations in both dietary component-to-bone component ($\Delta^{13}C_{bcomp\text{-}dcomp}$) fractionations and in bone component-to-bone component ($\Delta^{13}C_{bcomp\text{-}bcomp}$) spacings. Table 7 shows that the $\Delta^{13}C_{apat\text{-}bchol}$ spacing can be systematically changed from +9.8 to +14.9‰ (range = 5.1‰), the $\Delta^{13}C_{coll\text{-}bchol}$ spacing from +2.5 to +12.3‰ (range = 9.8‰) and the $\Delta^{13}C_{apat\text{-}coll}$ spacing from –0.8 to +11.2‰ (range = 12.0‰) as a function of the $\Delta^{13}C_{prot\text{-}engy}$ spacing. Bone cholesterol and apatite $\delta^{13}C$ values reflect the same dietary signal and thus a much smaller range is observed in the $\Delta^{13}C_{apat\text{-}bchol}$ spacings when compared to both the

Table 7. Dietary protein-to-energy ($\Delta^{13}C_{prot\text{-}engy}$) and bone component-to-bone component ($\Delta^{13}C_{bcomp\text{-}bcomp}$) spacings.

Sample	$\Delta^{13}C_{prot\text{-}engy}$ and $\Delta^{13}C_{bcomp\text{-}bcomp}$ spacings (?)			
Group + no.	prot-engy	apat-*b*chol	coll-*b*chol	apat-coll
A2	0.60	14.4	8.4	6.0
A3	0.60	13.0	8.2	4.8
A4	–14.96	14.4	3.3	11.2
A5	–14.96	13.6	2.5	11.0
A6	–2.92	13.0	8.1	4.9
A7	–2.92	13.2	8.7	4.4
A8	–2.92	12.5	7.7	4.8
A9	10.49	9.8	10.6	–0.8
A10	10.49	10.3	10.9	–0.6
B1	7.34	11.8	10.4	1.4
B2	7.34	13.0	12.3	0.7
B3	0.43	14.0	10.4	3.6
B4	0.43	12.5	8.8	3.7
B5	–6.19	14.3	7.7	6.6
B6	–6.19	14.9	9.0	5.9

$\Delta^{13}C_{coll\text{-}bchol}$ and $\Delta^{13}C_{apat\text{-}coll}$ spacings. The narrow range of variation in $\Delta^{13}C_{apat\text{-}bchol}$ may allow the use of bone cholesterol $\delta^{13}C$ values to evaluate the isotopic integrity of apatite $\delta^{13}C$ values. Carbonate in bone apatite is widely considered to be highly susceptible to diagenesis (Schoeninger and DeNiro, 1982; Nelson et al., 1986; Wang and Cerling, 1994; Koch et al., 1997). Even when bone collagen is preserved, and may be protecting carbonate from diagenesis, the isotopic integrity of apatite $\delta^{13}C$ values may be compromised (Koch et al., 1997). Thus, variability in $\Delta^{13}C_{apat\text{-}coll}$ could be interpreted as reflecting the diagenesis of apatite carbonate rather than any true dietary variation in whole diet and protein $\delta^{13}C$ values. Bone cholesterol is unlikely to be affected by diagenesis in the same way as carbonate as the bone matrix is not susceptible to the migration of soil lipids (Evershed et al., 1995). Hence, cholesterol $\delta^{13}C$ values could potentially provide an independent test of the diagenesis of apatite. On diets where $\Delta^{13}C_{prot\text{-}engy} = 0$, $\Delta^{13}C_{apat\text{-}bchol}$ should equal 12.8‰ (the difference between the y-intercepts of apatite and bone cholesterol in Fig. 5). On diets in which the $\Delta^{13}C_{prot\text{-}engy}$ is negative, e.g., on the far left hand side of Figure 5 where protein is C_3 and energy is C_4, $\Delta^{13}C_{coll\text{-}bchol}$ will be small (~+3‰) and both $\Delta^{13}C_{apat\text{-}bchol}$ and $\Delta^{13}C_{apat\text{-}coll}$ will be large (~+14 and +11‰, respectively; values from Table 7). Conversely, where $\Delta^{13}C_{prot\text{-}engy}$ is positive, e.g., on the far right hand side of Figure 5 where protein is C_4 and energy is C_3, $\Delta^{13}C_{coll\text{-}bchol}$ will be large (~+10.8‰) and both $\Delta^{13}C_{apat\text{-}bchol}$ and $\Delta^{13}C_{apat\text{-}coll}$ will be relatively small (~+10 and −0.7‰, respectively; values from Table 7). These findings will be fully evaluated in a future publication.

5. SUMMARY

The following is a summary of the most important insights gleaned from this study of rats raised on isotopically controlled diets:

1. The dietary signals demonstrated for bone collagen and apatite are consistent with the findings from Ambrose and Norr (1993) and Tieszen and Fagre (1993) where collagen $\delta^{13}C$ values reflect mainly that of dietary protein and apatite $\delta^{13}C$ values reflect that of the whole diet. This suggests that the combined measurement of bone collagen and apatite $\delta^{13}C$ values can provide different and complementary insights into diet.
2. Bone cholesterol $\delta^{13}C$ values were shown to reflect whole diet values demonstrating that cholesterol can play an analogous role to apatite in reconstructing whole diet $\delta^{13}C$ values.
3. Bone cholesterol $\delta^{13}C$ values can serve an important additional role in evaluating the isotopic integrity of apatite $\delta^{13}C$ values.

Thus, a more complete and reliable picture of ancient diet can only be gleaned from the analysis of all three bone biochemical components. The linear relationships between diet and bone $\delta^{13}C$ values revealed from this study may tentatively be used to estimate whole diet and dietary protein $\delta^{13}C$ values from archaeological bone values. However, it is clear that differences in the nutritional status and digestive physiologies of other animal species may result in different dietary signals to be reflected in bone cholesterol, collagen and apatite $\delta^{13}C$ values. Currently, it is not possible to accurately assess the relevance of these findings to other animal species or to humans and this highlights the need for further controlled feeding experiments (e.g., ruminants vs. monogastric animals) to be carried out. Differences in how ruminant and non-ruminant digestive physiologies can affect diet to tissue isotopic relationships have been evidenced in bone apatite (Krueger and Sullivan, 1984; Lee-Thorp et al., 1989) and in adipose tissue fatty acids (Dudd and Evershed, 1998). In addition, it is of particular importance to gain an insight into the relative rates of turnover of bone cholesterol, collagen and apatite since we do not expect archaeological populations to have subsisted on monotonous diets. Turnover rates have been estimated from the study of rats that were subjected to diet-switch experiments (Jim, 2000) and these results will be the subject of a further future publication. This study has demonstrated the potential of using bone cholesterol as a source of whole diet information. To date, cholesterol has been shown to be preserved in fossil bones from 75,000 B.P. (Evershed et al., 1995; Stott et al., 1997a) and longer timescales are currently being addressed. It is hoped that cholesterol $\delta^{13}C$ values will become a valuable tool in the assessment of Pleistocene human and faunal ecology.

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