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Expression of the Dietary Isotope Signal in the Compound-specific $\delta^{13}C$ Values of Pig Bone Lipids and Amino Acids

M. R. HOWLAND,[a] L. T. CORR,[a] S. M. M. YOUNG,[b] V. JONES,[a] S. JIM,[a] N. J. VAN DER MERWE,[c] A. D. MITCHELL[d] AND **R. P. EVERSHED,[a]***

[a] *Organic Geochemistry Unit, Biogeochemistry Research Centre, School of Chemistry, University of Bristol, Bristol, UK*
[b] *Archaeometry Laboratories, Department of Anthropology, Harvard University, Cambridge, Massachusetts, USA*
[c] *Department of Archaeology, University of Cape Town, Private Bag, Rondebosch, Cape, South Africa*
[d] *Growth Biology Laboratory, Agricultural Research Service, United States Department of Agriculture, Beltsville, Maryland, USA*

ABSTRACT Pigs were raised on six isotopically controlled diets to examine the dietary macronutrients used in the synthesis of bulk bone biochemical components (apatite, collagen and lipids) and individual compounds (bone fatty acids, cholesterol and amino acids from collagen). $\delta^{13}C$ values of apatite and bulk bone lipids reflected those of the whole diet, with $\Delta^{13}C_{\text{apatite-whole diet}} = 10.2 \pm 1.3‰$ and $\Delta^{13}C_{\text{bone lipids-whole diet}} = -2.4 \pm 0.7‰$. A wide variation observed in the $\Delta^{13}C_{\text{collagen-whole diet}}$ values (0.5 to 6.1‰) was hypothesized to reflect the relative importance of (i) the direct incorporation of essential amino acids, and (ii) the balance between direct incorporation and *de novo* synthesis of non-essential amino acids. Linear regression ($n = 6$) was used to assess the relationship between the $\delta^{13}C$ values of whole diet and bulk bone components and individual compounds. Whole diet $\delta^{13}C$ values showed a strong correlation with those of bone cholesterol ($R^2 = 0.81$) and non-essential fatty acids ($0.97 \leq R^2 \leq 0.99$). Not surprisingly, bone linoleic acid $\delta^{13}C$ values correlated well with dietary linoleic acid ($R^2 = 0.95$). Mass balance calculations using the $\delta^{13}C$ values of single amino acids accurately predicted the $\delta^{13}C$ value of whole collagen. The $\delta^{13}C$ values of whole diet were well correlated with those of the non-essential amino acids, alanine ($R^2 = 0.85$) and glutamate ($R^2 = 0.96$) in collagen. The essential amino acids leucine ($\Delta^{13}C_{\text{collagen leu-diet leu}} = 0.5 \pm 1.2‰$) and phenylalanine ($\Delta^{13}C_{\text{collagen phe-diet phe}} = -0.6 \pm 0.6‰$) showed little isotopic fractionation between diet and bone collagen. Copyright © 2003 John Wiley & Sons, Ltd.

Key words: diet; bone; amino acids; fatty acids; cholesterol; collagen; apatite; GC/C/IRMS

Introduction

Stable isotope analysis ($\delta^{13}C$ and $\delta^{15}N$) of the components of fossil bone and the tissues of modern animals can be used to reconstruct diet since the isotopic signatures of the local environment are incorporated into plants and passed along the food chain (Gannes *et al.*, 1998; Hobson, 1999). Until now, the majority of archaeological studies have focused on the analysis of collagen and apatite extracted from teeth and bones. Studies have addressed the relative importance of C_3 versus C_4 plants (Vogel & van der Merwe, 1977), animal protein versus

* Correspondence to: Organic Geochemistry Unit, Biogeochemistry Research Centre, School of Chemistry, University of Bristol, Cantock's Close, Bristol BS8 1TS, UK. e-mail: r.p.evershed@bristol.ac.uk

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vegetable protein (Richards *et al.*, 2000), and marine versus terrestrial foods (Tauber, 1981) in past human diets. Interpretation of the data from isotopic analysis relies on knowledge of the factors controlling the assimilation, biosynthesis and turnover rate of the tissue or compound.

Insights gleaned from the analysis of tissue samples from animals raised on controlled diets has been invaluable to our understanding of the carbon isotopic relationship between diet and consumer tissues (DeNiro & Epstein, 1978, 1981; Ambrose & Norr, 1993; Tieszen & Fagre, 1993). Feeding experiments have shown that the carbon isotopic composition of apatite reflects of that of the whole diet (Ambrose & Norr, 1993; Tieszen & Fagre, 1993). However, the use of apatite as a palaeodietary indicator is often restricted to tooth enamel because bone apatite is more susceptible to diagenesis and contamination from the burial environment (Quade *et al.*, 1995). In contrast to apatite, the carbon isotopic signal expressed in collagen is governed by the extent to which the carbon contained in dietary protein, lipids and carbohydrate is pooled for its synthesis. Thus, if dietary protein is routed for the synthesis of collagen then its isotopic composition will substantially under-represent the non-protein macronutrients of the diet. Essential amino acids and non-essential amino acids, whose sole precursors are essential, comprise 21.5% of the carbon atoms in collagen, defining the minimum amount of routing from dietary protein to bone collagen (Table 1).

Hare *et al.* (1991) investigated the factors that influence the isotopic composition of bone collagen by rearing pigs on controlled diets and measuring the isotopic composition of single amino acids purified from bone collagen. This pioneering experiment provided important insights into the metabolism of essential and non-essential amino acids. Significantly, they showed that the enrichment between whole diet and bone collagen $\delta^{13}C$ values could be explained by the enrichment observed in some of its constituent amino acids. Glycine, glutamate and aspartate constitute 16.9, 9.7 and 4.7% of the carbon atoms in collagen, respectively, and were shown to be enriched in ^{13}C with respect to whole diet by 8, 6 and 3‰, respectively.

Interestingly, the $\delta^{13}C$ values of proline and glutamate in collagen were shown to differ by 5.7‰. This finding was unexpected since proline is synthesized *de novo* from glutamate and the authors suggested that this disparity was a consequence of the direct routing of proline from the diet. Unsurprisingly, the essential amino acids, threonine and valine, displayed similar $\delta^{13}C$ values in the both diet and in bone collagen. O'Brien and co-workers recently used gas-chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) to determine the input of adult diet into the synthesis of non-essential amino acids in the eggs of the moth *Lepidoptera* (O'Brien *et al.*, 2002). The authors concluded that with time an increasing proportion of the adult diet contributed to the isotopic composition of the non-essential egg amino acids, whilst the essential amino acids recorded the $\delta^{13}C$ signal of the larval host plant. Interestingly, Tuross *et al.* (1988) and Hare *et al.* (1991) showed that a consistent pattern is observed in the $\delta^{13}C$ values of amino acids from modern and fossil bone collagen. They postulated that deviations from this pattern could potentially indicate dietary stress, contamination from exogenous substances or unusual dietary habits.

The development of compound-specific stable isotope analysis of individual lipids has stimulated research using both natural abundance and enriched tracers (Guo *et al.*, 1993; Binnert *et al.*, 1995) in a wide range of applications from studies of ancient and modern foodwebs (Pond *et al.*, 1995; Stott *et al.*, 1999), lipid metabolism (Rhee *et al.*, 1997), and food authentication (Woodbury *et al.*, 1995). Stott *et al.* (1997) determined

Table 1. Percentage contribution of each amino acid to the carbon of bone collagen. The residue composition per one thousand are shown in parentheses (Vaughan, 1975)

Non-essential	Atom %	Essential	Atom %
Glycine	16.9 (327)	Lysine	4.5 (29)
Proline	14.7 (113)	Leucine	3.6 (23)
4-Hydroxyproline	13.0 (100)	Phenylalanine	3.3 (14)
Glutamate	9.7 (65)	Valine	2.4 (19)
Alanine	9.3 (119)	Threonine	2.1 (20)
Arginine	7.9 (51)	Isoleucine	1.7 (11)
Aspartate	4.7 (45)	Methionine	1.1 (8)
Serine	2.2 (28)	Histidine	0.7 (5)
Tyrosine	1.1 (5)		
Hydroxylysine	1.0 (6)		
3-Hydroxyproline	0.2 (2)		

the $\delta^{13}C$ values of fatty acids and cholesterol to investigate the routing and biosynthesis of lipids in pigs reared on controlled diets. The $\delta^{13}C$ values of the bone lipids recorded the isotopic composition of the experimental diets. The non-essential fatty acids had $\delta^{13}C$ values that were on average 3.5‰ lower than whole diet. The similar $\delta^{13}C$ values of the essential fatty acid linoleic acid in diet and bone suggested direct incorporation of dietary linoleic acid without significant isotopic fractionation. Cholesterol has been demonstrated to be an indicator of the $\delta^{13}C$ value of short-term whole diet in the tissues of rats reared on controlled diets (Jim, 2000; Jim *et al.*, 2001). Cholesterol $\delta^{13}C$ values have been used in conjunction with bone collagen isotope ratios to distinguish between the dietary habits of coastal and inland archaeological human populations (Stott *et al.*, 1999).

Before applying a multi-proxy approach to the study of diet, it is important to determine the ways in which established biochemical and nutritional processes impact on the compound-specific $\delta^{13}C$ values of major biochemical classes. Such insights can only be gained through the analysis of animal tissues from controlled feeding experiments. Pigs have been used for this study and they are close metabolic analogues for humans, thus, results from this work will be of particular importance for human palaeodietary reconstruction. Hence, our approach was to raise pigs on controlled diets (Young, 2002) and to conduct carbon isotope analyses of bone bulk tissue and individual compounds, to investigate the dietary macronutrients utilized in the synthesis of bone tissues and to probe their individual dietary isotope signals at the molecular level.

The results included in this paper are a subset of a much larger study and are presented with the aims of:

(i) Testing the relationship between $\delta^{13}C$ values of bone collagen and its constituent amino acids. This is an essential step in assessing the use of single amino acids as complementary proxies for bulk collagen.
(ii) Investigating the relationships between tissue biochemical components and diet $\delta^{13}C$ values. This aspect of the work is an important first step in validating the use of such an approach before undertaking mathematical modelling, or applying compound-specific stable isotope analysis to palaeodietary or ecological research.

Analytical methods

Controlled animal feeding experiment

Female Poland China/Landracer crossbred pigs were reared in laboratory pens at the USDA Agricultural Research Service at Beltsville, Maryland by ADM. The pigs form part of a larger feeding experiment (Young, 2002). The six pigs embarked

Table 2. Composition and $\delta^{13}C$ values of experimental diets. Instrumental error: ±0.2‰

Diet components	$\delta^{13}C$ (‰)	% C*	% N*	Diet composition (%)					
				Diet 3	Diet 4	Diet 5	Diet 6	Diet 8	Diet 10
Ground maize	−12.2	39.3	1.3	10.3	30.4	50.0	69.2	—	74.1
Ground wheat	−27.4	40.1	1.6	71.8	50.7	30.0	9.9	82.9	—
Casein	−25.5	45.6	12.7	12.0	13.0	14.0	15.0	11.2	—
Menhaden fish meal	−18.7	41.5	9.9	—	—	—	—	—	23.0
Soybean oil	−33.5	78.3	0.1	2.0	2.0	2.0	2.0	2.0	2.0
Ca_2PO_4				2.5	2.5	2.5	2.5	2.5	—
$CaCO_3$	+0.4	11.8		0.5	0.5	0.5	0.5	0.5	—
NaCl				0.5	0.5	0.5	0.5	0.5	0.5
Selenium premix				0.05	0.05	0.05	0.05	0.05	0.05
Mineral premix				0.2	0.2	0.2	0.2	0.2	0.2
Vitamin premix				0.2	0.2	0.2	0.2	0.2	0.2
$\delta^{13}C$ whole diet (‰)				−24.7	−20.5	−18.0	−15.9	−25.7	−15.5

* % C and % N values were determined using a Carlo Erba CHN analyser.

on different controlled diets (Table 2) in early autumn 1997. The dietary histories of the pigs prior to this are unknown. The diets were formulated to contain 20% protein and to have a wide range in their $\delta^{13}C$ values. To achieve this goal diets were composed of combinations of casein, fish meal, ground maize, and ground wheat. These macronutrients were chosen for their characteristic stable isotope ratios. The casein was obtained from New Zealand, where cattle are fed predominantly C_3 plant fodder. Diets were mechanically ground in 250 kg batches to ensure homogeneity. Pigs weighed 10 kg at the start of the experiment and were raised on controlled diets until sacrifice when they weighed over 150 kg. They produced litters and were sacrificed in summer 1998. The adult pigs were sacrificed seven weeks after parturition. These animals were selected because they are nutritionally and physiologically comparable; they were raised on 20% protein diets and had produced litters.

After sacrifice, bone was cleaned of flesh by dermestid beetles and metarsal bone was used for all analyses.

Sample preparation

Apatite

Bone was powdered in a Spex mill (SPEX CentriPrep, New Jersey, USA). The resulting powder was lipid extracted with chloroform/methanol (2 : 1 v/v, 10 mL) by ultrasonication (4 × 30 min). The residue was treated with 4% NaOCl, rinsed with double distilled water, soaked in 0.1 M acetic acid for 24 h, rinsed again with double distilled water and freeze dried.

Collagen

Insoluble bone protein (collagen) was obtained as described by Sealy (1997). Bone was demineralized with 2% HCl, rinsed with distilled water, and soaked in 0.1 M NaOH overnight. Lipids were removed by soaking for 24 h in methanol/chloroform/water (10 : 5 : 4 v/v). After rinsing with distilled water the prepared collagens were freeze dried prior to analysis.

Cholesterol and fatty acids

All reagents and solvents used were, respectively, analytical and HPLC grade or better. Derivatizing agents were all from the same batch whenever possible. Analytical blanks were used to monitor contamination. Approximately 100 mg of bone was obtained by abrasion with a hand drill and transferred to screw-capped vials together with 50 µl of an internal standard, *n*-tetratricontane (1 mg/ml) (Stott *et al.*, 1999). After weighing, the samples were extracted into chloroform/methanol (2 : 1, v/v, 3 × 1 h, ultrasonication). An aliquot of each total lipid extract was transferred into screw-capped culture tubes, dried under N_2 and saponified with 2 ml 0.5 M methanolic NaOH (70°C 1 h). On cooling the neutral fraction was extracted into hexane (3 × 2 ml). The mixture was then acidified to pH 3 with 2 M HCl and the acid fraction extracted into hexane (3 × 2 ml). Neutral and acid fractions were stored in hexane at −18°C. Neutral fractions (cholesterol) and fatty acids were converted to their trimethylsilyl (TMS) ether and methyl ester derivatives (FAMES), respectively, as described in Stott *et al.* (1997).

Amino acids

After adding a known quantity of γ-amino butyric acid (0.1 M solution in 0.1 M HCl) as an internal standard, proteins (diets and collagens) were hydrolysed at 100 °C using 6 M HCl (500 : 1 v/w) in Young's tubes under vacuum for 24 h. HCl was removed under a gentle stream of N_2 at 40 °C. Due to the acid hydrolysis of proteins, glutamate and aspartate include contributions from the deamination of glutamine and asparagine, respectively. Amino acids were converted to their trifluoroacetyl-isopropyl (TFA/IP) esters in screw-capped tubes following the procedure of Silfer *et al.* (1991).

Instrumental methods

Continuous-flow IRMS (CF/IRMS)

CF/IRMS analysis of diets and collagen was conducted in triplicate at the University of

Cape Town using a Finnigan MAT 252 mass spectrometer coupled to a Carlo Erba NA1500 elemental analyser as described by Sealy (1997). The standard deviation of repeated measurements of a gelatin standard was 0.2‰. CF/IRMS analyses of total bone lipids was performed using a Carlo Erba NC2500 elemental analyser coupled to a Finnigan MAT DELTA^{plus} XL at the University of Bristol. The standard deviation of repeated measurement of a nylon standard was 0.3‰.

Dual inlet IRMS

Isotopic analysis of apatite was conducted at the NERC Isotope Geosciences Laboratory, Keyworth, UK as described by Yates *et al.* (2002) using a VG Optima dual inlet gas source mass spectrometer. The reproducibility of duplicate analyses was 0.07‰.

GC and GC/mass spectrometry (GC/MS)

GC and GC/MS analyses of cholesterol and fatty acids were performed as described by Stott & Evershed (1996) and Stott *et al.* (1997). GC and GC/MS analyses of amino acids were performed as described by Docherty *et al.* (2001).

GC/C/IRMS

GC/C/IRMS analyses of cholesterol and fatty acids were performed using a modification of the protocol described in Stott & Evershed (1996) and Stott *et al.* (1997). Co-injected standards of nondecane (cholesterol) and methyl esters of undecanoic, tricosanoic and heneicosanoic acids (Sigma-Aldrich products U-0250, T-9900 and H-3265) (fatty acids) of known isotopic composition were added to samples immediately prior to GC/C/IRMS analysis (Figure 1a and 1b). Carbon isotope values measured for FAMES and cholesterol TMS ethers in this research were corrected for the addition of derivatizing carbon (Rieley, 1994).

GC/C/IRMS analyses of amino acids were performed in triplicate (Silfer *et al.*, 1991; Docherty *et al.*, 2001). A reduction reactor was employed to remove nitrogen oxides which produce isobaric ions (Metges & Daenzer, 2000). Co-injected standards of TFA methyl esters of alanine, phenylalanine and lysine (Sigma-Aldrich products T3381,

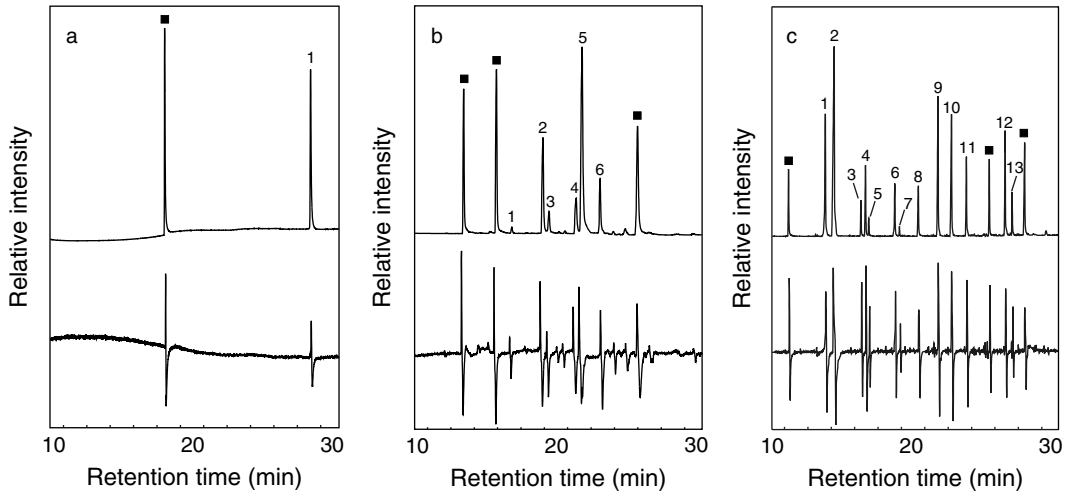

Figure 1. Partial GC/C/IRMS profiles of: (a) cholesterol from pig bone analysed as TMS ether; (b) fatty acids from pig bone derivatized to FAMES; and (c) amino acids from pig bone collagen derivatized to TFA/IP esters. The lower chromatogram represents the instantaneous ratio of the *m/z* 45/44 ions, whilst the upper chromatogram represents the *m/z* 44 ion current. Peak identities are: (a) 1, cholesterol; (b) 1, methyl tetradecanoate ($C_{14:0}$); 2, methyl hexadecanoate ($C_{16:0}$); 3, methyl hexadecenoate ($C_{16:1}$); 4, methyl octadecanoate ($C_{18:0}$); 5, methyl octadecenoate ($C_{18:1}$); 6, methyl octadecadienoate ($C_{18:2}$); and (c) 1, alanine; 2, glycine; 3, threonine; 4, serine; 5, valine; 6, leucine; 7, isoleucine; 8, γ-amino butyric acid (internal standard); 9, proline; 10, hydroxyproline; 11, aspartic acid; 12, glutamic acid; 13, phenylalanine; ■ represent co-injected standards.

T5006 and T4631) of known isotopic composition were added immediately prior to GC/C/IRMS analysis (Figure 1c). $\delta^{13}C$ values of amino acids were calculated from the $\delta^{13}C$ values of their TFA/IP derivatives using correction factors (Silfer *et al.*, 1991; Docherty *et al.*, 2001).

Carbon isotope ratios are reported relative to the Pee Dee Belemnite limestone standard in parts per thousand (‰). Results are expressed as:

$$\delta^{13}C = [(R_{sample}/R_{standard}) - 1] \times 1000$$

where R_{sample} and $R_{standard}$ are the ratios of $^{13}C/^{12}C$ for the sample and the standard. Differences between isotope ratios are expressed as:

$$\Delta^{13}C_{A-B} = \delta^{13}C_A - \delta^{13}C_B$$

Results and discussion

Presented below are the results from the isotope analyses of the diet and bone tissue (collagen, apatite, bulk bone lipids, individual bone fatty acids and cholesterol and collagenous amino acids) of pigs (n = 6) raised on controlled diets. Bulk bone component $\delta^{13}C$ values are presented in Table 3 and these data support the findings of previous studies (Ambrose & Norr, 1993; Tieszen & Fagre, 1993), which are summarized below:

1) $\delta^{13}C$ values of apatite and bulk bone lipids reflected those of the whole diet with $\Delta^{13}C_{apatite\text{-}whole\ diet} = 10.2 \pm 1.3$‰ and $\Delta^{13}C_{bone\ lipids\text{-}whole\ diet} = -2.4 \pm 0.7$‰.
2) The isotopic spacing between collagen and whole diet ($\Delta^{13}C_{collagen\text{-}whole\ diet}$) ranged from 0.5 to 6.1‰.

The variability in the $\Delta^{13}C_{collagen\text{-}whole\ diet}$ values illustrates the complexity of tissue metabolism. The aim of the $\delta^{13}C$ measurements of individual lipids and amino acids presented below is to probe the factors controlling the isotopic composition of different bone biochemical fractions in relation to diet.

Cholesterol and fatty acids

The six most abundant fatty acids extracted from diet and bone and identified by GC and GC/MS were myristic acid ($C_{14:0}$), palmitic acid ($C_{16:0}$), palmitoleic acid ($C_{16:1}$), stearic acid ($C_{18:0}$), oleic acid ($C_{18:1}$) and the essential fatty acid linoleic acid ($C_{18:2}$ *cis* $\Delta^{9,12}$). The fatty acid distributions of the six diets varied due to the differing lipid compositions of maize, wheat, casein and fish meal, whereas consistent fatty acid distributions were shown in all pig bone samples where $C_{16:0} > C_{18:1} > C_{18:0} > C_{18:2} > C_{16:1} > C_{14:0}$. Thus, bone fatty acid composition was not related to dietary fatty acid compositions. Cholesterol, present in all diets, had an average concentration of 0.04 mg g^{-1} in the casein-containing diets and 0.13 mg g^{-1} in the fish meal-containing diet.

Bone fatty acid and cholesterol $\delta^{13}C$ values were ^{13}C-depleted by up to 3.4‰ with respect to whole diet values. This depletion in ^{13}C with respect to whole diet $\delta^{13}C$ values has previously

Table 3. Carbon isotopic compositions (‰) of whole biochemical fractions extracted from pig bone. R^2 values represent the correlation between whole diet and bone component $\delta^{13}C$ values

Bone component	Diet 3	Diet 4	Diet 5	Diet 6	Diet 8	Diet 10	Mean	1σ	R^2
$\delta^{13}C$ collagen	−20.6	−18.7	−16.9	−15.4	−19.6	−11.5			
$\delta^{13}C$ estimated collagen*	−18.4	−17.4	−14.7	−14.3	−19.2	−10.4			
$\delta^{13}C$ apatite	−14.2	−11.8	−9.0	−6.3	−13.6	−4.3			
$\delta^{13}C$ lipids	−26.2	−23.7	−20.8	−18.2	−27.5	−18.4			
$\Delta^{13}C_{collagen\text{-}whole\ diet}$	4.1	1.8	1.1	0.5	6.1	4.0	2.9	2.1	0.77
$\Delta^{13}C_{apatite\text{-}whole\ diet}$	10.5	8.7	9.0	9.6	12.1	11.2	10.2	1.3	0.91
$\Delta^{13}C_{lipids\text{-}whole\ diet}$	−1.5	−3.2	−2.8	−2.3	−1.8	−2.9	−2.4	0.7	0.98

* Estimated collagen values were calculated using single amino acid $\delta^{13}C$ values and their percentage contribution to collagen carbon (Table 1).

been observed in controlled feeding experiments using rats (Jim, 2000; Jim *et al.*, 2001) and results from a kinetic isotope effect occurring during the oxidation of pyruvate by pyruvate dehydrogenase to acetyl-CoA, the common precursor in the biosynthesis of all lipids (DeNiro & Epstein, 1977; Hayes, 1993). The general pattern observed for the $\delta^{13}C$ values of the non-essential fatty acids was: $\delta^{13}C_{C18:1} > \delta^{13}C_{C18:0} > \delta^{13}C_{C16:0} > \delta^{13}C_{C14:0} > \delta^{13}C_{C16:1}$ (Figure 2). Interestingly, an identical pattern of $\delta^{13}C$ values for these five fatty acids has previously been identified in redhead duck adipose fatty acids (Hammer *et al.*, 1998) and for $C_{16:0}$, $C_{18:0}$ and $C_{18:1}$ in the bones of pigs raised on controlled diets (Stott *et al.*, 1997). The factors controlling the relationship between the $\delta^{13}C$ values of fatty acids within a particular tissue are unknown, but they may involve isotopic fractionation during fatty acid desaturation or elongation (Rhee *et al.*, 1997).

Linear regression analysis was performed to investigate the origins of the dietary signals of bone fatty acids and cholesterol. A summary of the R^2 values observed between each diet and bone lipid is presented in Table 4. $\delta^{13}C$ values of all bone non-essential fatty acids correlated extremely well with whole diet values, ($0.98 \leq R^2 \leq 0.99$, Table 4). In addition, a good correlation between cholesterol and whole diet $\delta^{13}C$ values was observed ($R^2 = 0.81$). Non-essential fatty acids and cholesterol may be directly incorporated from the diet or synthesized *de novo* from acetyl-CoA, the common metabolite formed during the catabolism of dietary lipids, carbohydrates and protein. Thus, if *de novo* synthesis dominates over direct incorporation then the $\delta^{13}C$ values of the non-essential fatty

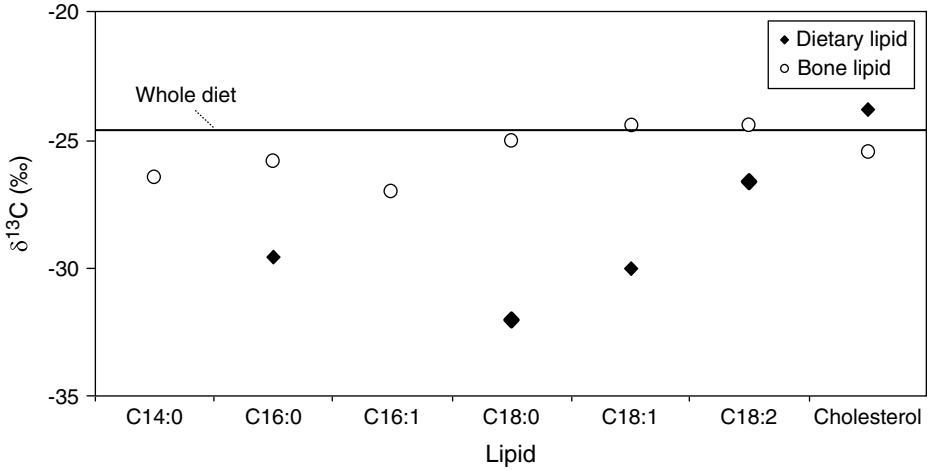

Figure 2. Dietary and bone cholesterol and fatty acid $\delta^{13}C$ values from diet 3.

Table 4. Regression statistics for whole diet and specific diet lipid (*x*) versus specific bone lipid (*y*) $\delta^{13}C$ values (m = gradient and c = *y*-intercept)

(n = 6)	Whole diet (*x*) vs. bone lipid (*y*)			Dietary lipid (*x*) vs. bone lipid (*y*)		
	R^2	m	c	R^2	m	c
$C_{14:0}$	1.00	1.01	−1.96	—	—	—
$C_{16:0}$	0.99	1.02	−0.85	0.87	1.30	12.72
$C_{16:1}$	0.97	0.80	−7.51	—	—	—
$C_{18:0}$	0.99	1.04	0.25	0.81	2.17	39.35
$C_{18:1}$	0.98	0.92	−1.65	0.87	0.96	2.77
$C_{18:2}$	0.81	0.93	−3.65	0.95	0.95	0.20
Cholesterol	0.81	0.44	−13.99	0.001	−0.01	−23.03

acids and cholesterol will reflect whole diet $\delta^{13}C$ values. Although cholesterol was present in all diets, the lower correlation with whole diet when compared to the non-essential fatty acids is probably not due to a higher degree of routing of dietary cholesterol as is indicated by the weak correlation between dietary and bone cholesterol $\delta^{13}C$ values ($R^2 = 0.001$).

Mammals lack the enzyme to form double bonds beyond the ninth carbon atom and therefore linoleic acid cannot be synthesized *de novo* and must be incorporated directly from the diet (Voet & Voet, 1995). Consequently, $\delta^{13}C$ values of bone linoleic acid are expected to reflect dietary values. The strong correlation shown between the diet and bone linoleic acid ($R^2 = 0.95$, Figure 3) was evidence for this direct incorporation. However, linoleic acid $\delta^{13}C$ values were more positive than dietary values by 1.3 ± 1.0‰ and this has previously been observed in a controlled rat feeding experiment (Jim *et al.*, in press). This finding was attributed to isotopic fractionation during the catabolism of excess dietary linoleic acid or in its conversion to other metabolites.

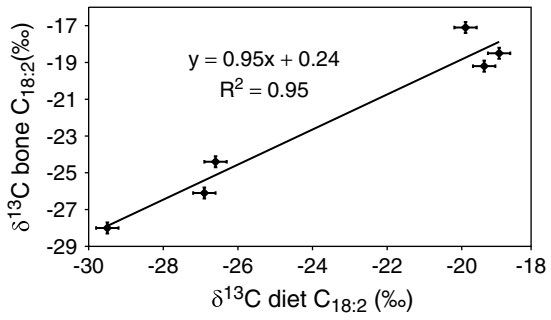

Figure 3. Linear correlation between dietary and bone $C_{18:2}$ fatty acid (◆) $\delta^{13}C$ values. Error bars encompass in the instrumental precision of ±0.3‰ for GC/C/IRMS and all replicate determinations were within this range.

Amino acids

An isotopic range of over 25‰ was observed for the 12 amino acids measured in the collagen of the pig raised on diet 3 (Figure 4), most likely as a consequence of their varied metabolic histories. The $\delta^{13}C$ values of the non-essential amino acids from the pig bone collagen were distributed across 10‰ (Figure 4), reflecting differences in their assimilation, transport and biosynthesis.

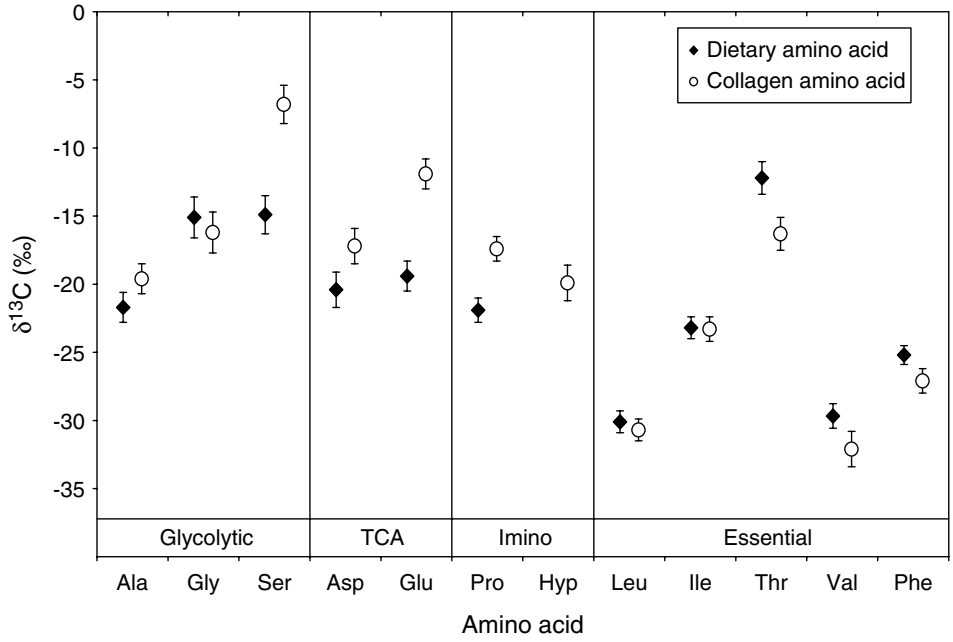

Figure 4. Dietary and bone collagen amino acid $\delta^{13}C$ values from diet 3. Error bars encompass the uncertainty associated with the use of correction factors for the determination of $\delta^{13}C$ values of derivatized amino acids (Docherty *et al.*, 2001).

This wide range contrasts markedly with the 5‰ range in the $\delta^{13}C$ values of the bone fatty acids and cholesterol (Figure 2), most likely because the latter share a common precursor, acetyl-CoA.

The 12 amino acids for which $\delta^{13}C$ values were obtained contribute 83.5% of the carbon atoms of collagen (Table 1). Mass balance calculations were used to estimate the $\delta^{13}C$ values of whole collagen using the $\delta^{13}C$ values of individual amino acids together with the amino acid composition of collagen shown in Table 1. The correlation between the calculated and measured bulk collagen $\delta^{13}C$ values was very strong ($R^2 = 0.96$). Estimated $\delta^{13}C$ values were on average 1.4 ± 0.6‰ more positive than the observed values. This systematic deviation may be because $\delta^{13}C$ values were not determined for arginine and lysine, which contribute 7.9 and 4.5% of the carbon to collagen, respectively. Previous work has shown that these amino acids are depleted in ^{13}C relative to bulk collagen and other amino acids (Hare *et al.*, 1991) which would largely account for the disparity between estimated and observed $\delta^{13}C$ values of collagen. Notwithstanding this, the strong correlation between the estimated and observed $\delta^{13}C$ values suggests that the compound-specific isotope values determined for the individual amino acids are meaningful and that there are no obvious systematic errors in the analytical approach.

Among the non-essential amino acids, collagen glycine was 8.4 ± 2.5‰ more enriched in ^{13}C than whole diet. Glycine is synthesized via several steps from 3-phosphoglycerate, an intermediate in the catabolism of dietary sugars. This synthetic pathway is a probable source of the reasonable correlation between bone collagen glycine and whole diet $\delta^{13}C$ values (Table 5, $R^2 = 0.69$). Figure 5 illustrates the strong correlation of the $\delta^{13}C$ values of collagen alanine and glutamate with the $\delta^{13}C$ value of whole diet. Alanine is formed by the action of alanine amino transferase on pyruvate. Pyruvate is the end product of glycolysis, so the $\delta^{13}C$ value of biosynthesized alanine is expected to be representative of dietary carbohydrate. Glutamate is a key amino acid in protein metabolism, donating its amino group in transamination reactions and therefore frequently interconverting its carbon skeleton with α-ketoglutarate, a tricarboxylic acid (TCA) cycle intermediate. The precursor for aspartate, oxaloacetate, is also a TCA cycle intermediate. Collagen glutamate $\delta^{13}C$ values were 6.5 ± 1.0‰ more positive than collagen aspartate $\delta^{13}C$ values. This consistent isotopic spacing may be attributable to isotopic fractionation by the enzymes that participate in the TCA cycle.

The moderate correlations between the $\delta^{13}C$ values of the non-essential amino acids, proline and serine and whole diet (Table 5) may reflect a combination of assimilation from diet and *de novo* synthesis. The essential amino acids, leucine and phenylalanine, showed little isotopic fractionation between diet and bone collagen (Figure 6), i.e. $\Delta^{13}C_{\text{collagen phe-diet phe}} = -0.6 \pm 0.6$‰ and $\Delta^{13}C_{\text{collagen leu-diet leu}} = 0.5 \pm 1.2$‰. The correlations between $\delta^{13}C$ values for dietary and bone collagen valine, threonine and isoleucine (the other essential amino acids measured in this study) were weaker than those observed for

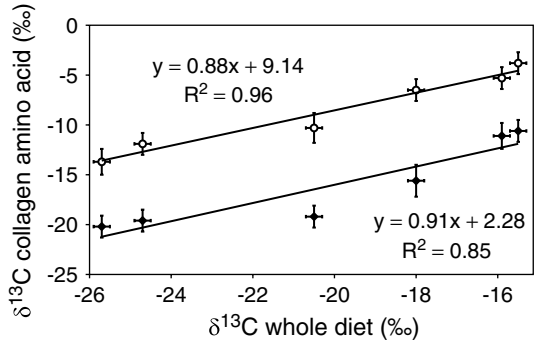

Figure 5. Linear correlations between whole diet, and bone collagen glutamate (○) and alanine (◆) $\delta^{13}C$ values. See caption of Figure 4 for explanation of error bars.

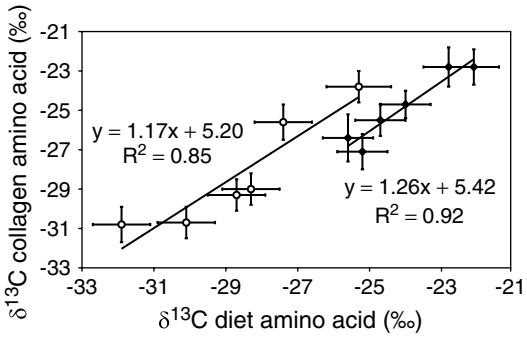

Figure 6. Linear correlations between dietary and bone collagen leucine (○) and phenylalanine (◆) $\delta^{13}C$ values. See caption of Figure 4 for explanation of error bars.

Table 5. Regression statistics for whole diet and dietary amino acid (x) vs. collagen amino acid (y) $\delta^{13}C$ values (m = gradient and c = y-intercept)

	(n = 6)	Whole diet (x) vs. collagen amino acid (y)			Dietary amino acid (x) vs. collagen amino acid (y)		
		R^2	m	c	R^2	m	c
Non-essential	Ala	0.85	0.91	2.28	0.47	0.86	−1.23
	Gly	0.69	0.82	4.80	0.34	0.59	−5.22
	Ser	0.59	0.86	11.17	0.79	1.16	6.80
	Pro	0.68	0.45	−7.89	0.53	0.53	−7.33
	Hyp	0.37	0.53	−7.14	—	—	—
	Asp	0.88	0.79	0.68	0.85	1.43	11.82
	Glu	0.96	0.88	9.14	0.87	0.99	6.28
Essential	Thr				0.26	−0.83	25.55
	Val				0.64	1.32	6.56
	Leu				0.85	1.17	5.20
	Ile				0.42	1.25	4.89
	Phe				0.93	1.25	5.41

leucine and phenylalanine (Table 5). The reasons for this are unclear and need to be explored further.

In summary, the wide variation observed in the $\Delta^{13}C_{\text{collagen-whole diet}}$ values can be explained by considering the relative importance of (i) the direct incorporation of essential amino acids, and (ii) the balance between direct incorporation and *de novo* synthesis of non-essential amino acids.

Conclusions

This study represents the most comprehensive investigation of the $\delta^{13}C$ values of individual fatty acids and amino acids from the long-term controlled feeding of large mammals performed to date. By using naturally labelled stable isotopic tracers at the bulk and compound specific level, this investigation has begun to assess the various metabolic processes governing the isotopic composition of individual compounds. Our principal finding are summarized as follows:

1) Bone cholesterol and non-essential fatty acid $\delta^{13}C$ values correlated well with whole diet.
2) Bone linoleic acid $\delta^{13}C$ values correlated well with dietary linoleic acid.
3) Mass balance calculations using the $\delta^{13}C$ values of single amino acids accurately predicted the $\delta^{13}C$ value of whole collagen.
4) The $\delta^{13}C$ values of the non-essential amino acids, alanine and glutamate, from bone collagen correlated well with whole diet.
5) The essential amino acids leucine and phenylalanine showed little isotopic fractionation between diet and bone collagen.

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