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ON ISOTOPES AND OLD BONES*

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This review charts the developments and progress made in the application of stable light isotope tools to palaeodietary adaptations from the 1970s onwards. It begins with an outline of the main principles governing the distribution of stable light isotopes in foodwebs and the quality control issues specific to the calcified tissues used in these analyses, and then proceeds to describe the historical landmark studies that have marked major progress, either in their archaeological applications or in enhancing our understanding of the tools. They include the adoption of maize agriculture, marine-focused diets amongst coastal hunter–gatherers, trophic level amongst Glacial-period modern humans and Neanderthals, and the use of savannah resources by early hominins in Africa. Particular attention is given to the progress made in addressing the challenges that have arisen out of these studies, including issues related to the routing of dietary nutrients. I conclude with some firm, and some more speculative, pointers about where the field may be heading in the next decade or so.

KEYWORDS: STABLE LIGHT ISOTOPES, BONES, TEETH, COLLAGEN, APATITE, DIET, MAIZE, MARINE

INTRODUCTION

The application of stable light isotope ratio analysis to past human diets has by now reached a certain level of maturity. It is over 30 years since the first pioneering publications appeared reporting the application of stable carbon isotopes to the uptake of maize amongst prehistoric woodland Americans (Vogel and van der Merwe 1977; van der Merwe and Vogel 1978). These first elegant applications built on a series of discoveries related to carbon isotope pathways in plant photosynthesis (e.g., Smith and Epstein 1971), the observations and experience garnered by radiocarbon chemists (e.g., Berger *et al*. 1964; Tamers and Pearson 1965; Bender 1968; Longin 1971; Hassan and Ortner 1977), and then controlled diet experiments (DeNiro and Epstein 1978) and observations from free-ranging animals (Vogel 1978) that provided the essential information about transfer of dietary isotope composition to animals' tissues.

The distinct advantage of a stable isotope natural abundance approach for dietary studies is that it reflects the foods *actually* eaten by an individual, or a group of individuals, rather than a palimpsest of waste of uncertain duration that typically preserves only a tiny fraction of the original material and overlooks those organic remains with low survival rates, such as plant foods. In the North American case, the results were decidedly unexpected, and prompted a re-examination of the earlier archaeological evidence for the formation of complex societies, and the adoption and spread of maize agriculture. They also prompted a longstanding debate about *how much* maize was reflected in the collagen isotope values, and the broader debate around this issue still permeates isotope dietary studies.

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The main challenges are about what the isotopic composition of various human tissues really means in terms of quantifiable dietary components—whether there is over- or under-representation, how we deal with issues of equifinality and variability, and whether the measured isotopic values have remained intact over the passage of time. We need to understand how *post mortem* processes may impact on the primary dietary information. These problems were posed early on and, in spite of clear advances, a significant number of the challenges are still current today.

As part of *Archaeometry's* 50th anniversary year, we were asked to chart the course of our field over the past half century or so, paying particular attention to the contributions that have appeared in this journal. Because the fundamental developments of stable light isotope ecology have taken place within many disciplines, the pioneering studies are scattered across an extremely wide literature, from geochemistry (the original 'home' discipline), to plant and animal sciences, archaeology and general science. This journal has published pioneering studies on the application of stable light isotope ratio analysis to Classical marbles in the Mediterranean (e.g., Herz 1992), but contributions in isotope applications to palaeodiets have tended rather to be directed at the issues of preservation of calcified tissues. In particular, a special 2002 issue of *Archaeometry* was devoted to the Fourth Bone Diagenesis meeting. For the purposes of this review, I have concentrated on the most fruitful major dietary applications, and on charting the subsequent progress in addressing the major issues that have arisen out of these studies. As alluded above, they include the issues of interpretation of quantity (*how much*), routing of dietary nutrients (*how representive*), and diagenesis in different tissues (*how reliable* are the analyses of bone and enamel, organic and inorganic components).

Because of the breadth of the field, I confine the review to a few exemplary studies, including the adoption of maize agriculture, marine-focused diets amongst coastal hunter–gatherers, trophic level amongst Glacial-period modern humans and Neanderthals, and the use of savannah resources by early hominins in Africa. Finally, I provide some pointers to the directions in which the field is heading, including high-resolution life history applications. As a start, it is useful to consider the main principles of stable light isotopes in foodwebs, and issues of preservation and quality control, before we consider specific applications to human diets.

STABLE ISOTOPES AS DIETARY TRACERS

Target tissues

This review focuses on calcified tissues, since bones and teeth are by far the most common vertebrate tissues surviving into the archaeological record, although keratinous tissues such as hair and nails occasionally survive in more recent, special circumstances. The patterned isotope distributions described below are archived and analysed in bones and teeth, which are composite tissues made up of complex organic molecules and minerals. Collagen is the main protein in bone and dentine that provides the source for organic carbon ($^{13}C/^{12}C$),[1] nitrogen ($^{15}N/^{14}N$) and, to a lesser extent, oxygen ($^{18}O/^{16}O$), sulphur ($^{34}S/^{32}S$) and, most recently, hydrogen (D/H). It is composed of multiple helical peptide fibrils stippled with a fine, poorly crystalline

[1] By convention, stable isotope ratios are expressed in the δ notation, in parts per thousand (per mille or $^{o}/_{oo}$) relative to an international standard, as $\delta^{x}Z = (R_s/R_{ref} - 1) \times 1000$, where R is the isotope ratio ($^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, D/H or $^{34}S/^{32}S$). For carbon isotopes, the standard is the marine limestone PDB; oxygen and hydrogen isotopes may be expressed relative to PDB or to Standard Mean Ocean Water (SMOW), depending on the material being analysed; for nitrogen isotopes it is Ambient Inhalable Reservoir (AIR); and for sulphur isotopes it is the Canyon Diablo Triolite meteorite (CDT). Negative values denote that the sample has lower abundances of the heavier isotope than does the standard.

'cement' of mineral. Bone mineral and enamel are highly substituted biological calcium phosphate apatites, differing subtly in their chemistry and properties, from which $^{18}O/^{16}O$ can be determined from phosphate, and $^{13}C/^{12}C$ and $^{18}O/^{16}O$ from substituted carbonate. The timespan captured in these tissues differs. Since bone is a living tissue that turns over regularly within the life of an individual, isotope values reflect long-term averages that depend on the age of the individual. A recent study based on radiocarbon showed that turnover slows dramatically after full maturity is achieved (Hedges *et al.* 2007). In contrast, tooth enamel and dentine are incremental tissues that form during a limited, mostly juvenile, period of an individual's life, with the exception perhaps of the third molar. Consequently, isotope values reflect conditions at that time, although there is a little secondary dentine formation, and the nature of amelogenesis and primary and maturation mineralization in enamel means that time intervals are not discrete (see below).

Natural abundances of stable isotopes in foodwebs

The stable isotopes of an element differ slightly in their nuclear mass as a result of differences in the number of neutrons, leading to small but significant differences in their thermodynamic and kinetic properties (Sharp 2007). Molecules containing the higher-mass, rarer isotope tend to accumulate in the thermodynamically most stable component of a system—for instance, in the liquid rather than gaseous phase—or are slower to react in mass-sensitive kinetic reactions. In equilibrium, and incomplete or multidirectional physical and biochemical processes, the result is fractionation or partitioning. The principles governing physico-chemical fractionation are relatively well understood theoretically and empirically, and thus they provide a means of tracking the pathways of the 'life' elements through a complex series of chemical transformations.

The largest source of carbon isotope variability occurs in primary producers on land and in the oceans. In land plants, the two dominant photosynthetic pathways, C_3 and C_4 (after the number of carbon atoms fixed in the first product), differ in their net discrimination against ^{13}C during fixation of CO_2 (Smith and Epstein 1971; O'Leary 1981; Farquhar *et al.* 1989). In C_3 photosynthesis, strong discrimination against ^{13}C during CO_2 fixation by ribulose biphosphate carboxylase/oxygenase (RUBISCO) results in more negative $\delta^{13}C$ values in virtually all trees, woody shrubs, herbs and temperate or shade-loving grasses. Because plants following the C_4 pathway (tropical grasses and many sedges) concentrate CO_2 in bundle-sheath cells prior to release into the RUBISCO cycle, and as all of it is converted, fractionation is not expressed. C_4 photosynthesis is believed to be a relatively recent adaptation for lower pCO_2 and high solar radiation in the growing season (Ehleringer *et al.* 1997), so distribution of C_4 plants is confined to environments with such conditions. In C_3 plants $\delta^{13}C$ varies widely, from about −24 to −36‰ (global mean −26.5‰) depending on light intensity, temperature, humidity, moisture and recycling of CO_2 (O'Leary 1981; Farquhar *et al.* 1989; van der Merwe and Medina 1991). C_4 plant $\delta^{13}C$ (global mean −12.5‰) is less variable. Economically important C_3 cereals include wheat, barley, oats and rice, as well as all root staples such as potato, manioc and yam, while important C_4 plants include maize, sorghum, millet and cane sugar. In general, marine primary producers (e.g., phytoplankton, algae, diatoms and radiolaria) are enriched in ^{13}C compared to those in terrestrial C_3 ecosystems, because the source of carbon is mainly dissolved bicarbonate, which has relatively high $\delta^{13}C$ compared to atmospheric CO_2. The mean $\delta^{13}C$ is about −20‰ (Smith and Epstein 1971), but values vary.

Plant $\delta^{13}C$ values are reflected in the tissues of consumers. In the first controlled feeding experiment, DeNiro and Epstein (1978) showed that $\delta^{13}C$ of the whole animal is very similar to that of its diet (where it is possible to measure the whole organism), but there is partitioning

among tissues according to their chemistry and biosynthetic pathways. Thus isotopic differences, often expressed as Δ (difference) or ε (enrichment factor), vary between diet and particular tissues. The difference between diet and collagen $\delta^{13}C$ is generally about +5‰, as first observed by van der Merwe and Vogel (1978), based on their values for humans in a mono-isotopic C_3 biome. This offset is supported by many later studies of free-ranging herbivores (e.g., Sullivan and Krueger 1981; Lee-Thorp *et al.* 1989). Two well-controlled dietary experiments showed that the relationship is largely between dietary protein and collagen, because dietary amino acids are preferentially utilized for collagen tissue construction (Ambrose and Norr 1993; Tieszen and Fagre 1993) (see discussion below). A small trophic-level effect of about +1 to 2‰ is observed in subsequent steps, among omnivores and carnivores, and including humans.

Bone or enamel carbonate is formed in equilibrium with blood bicarbonate and its $\delta^{13}C$ is closely related; these values in turn are controlled by catabolic and respiratory processes (Krueger and Sullivan 1984; Passey *et al.* 2005b). The offset between dietary and bone carbonate $\delta^{13}C$ averages about +12‰ (Krueger and Sullivan 1984; Lee-Thorp *et al.* 1989); however, this varies according to body mass and dietary physiology. The controlled feeding studies for mice (DeNiro and Epstein 1978) and rats (Ambrose and Norr 1993) found Δ of <10‰; observations of many free-ranging herbivores suggest ~12‰, and analyses of horses gave 14‰ (Cerling and Harris 1999). More recently, results from controlled feeding studies of several small to large species suggested that offsets varied from +11 to +13.5‰ (Passey *et al.* 2005b). A likely cause of a large $\Delta_{diet\text{-}carb}$ is expiration of varying amounts of ^{13}C-depleted methane (Hedges and van Klinken 2000).

For nitrogen isotopes, variability in ecosystems reflects the balance between biological nitrogen fixation, complex recycling within the biosphere, and re-release of N_2 (Robinson 2001). Atmospheric N_2 is globally uniform in isotope composition, with a low $\delta^{15}N$ composition (0‰ by definition). On land, soils and plants are slightly higher in ^{15}N compared to atmospheric N_2 (Delwiche and Steyn 1970); soil and plant $\delta^{15}N$ is typically about +1–4‰ subject to variability caused by environmental aridity, leaching (with high precipitation), anoxia and salinity (Shearer *et al.* 1978; Heaton 1987; Handley and Raven 1992). A general 'rule of thumb' is that soil $\delta^{15}N$ is weakly inversely related to rainfall (Handley *et al.* 1999), but in practice the relationship that holds, although still variably, where mean annual rainfall is <400 mm (Heaton 1987). In the oceans, the most abundant form of nitrogen available to primary producers is recycled nitrate, with an average $\delta^{15}N$ value of about +5–6‰ in the productive upwelling centres along ocean margins (Liu and Kaplan 1989).

Nitrogen isotopes vary with trophic level, and a stepwise trophic shift of +2–6‰ in $\delta^{15}N$ from plants to herbivores, and from herbivores to carnivores, has been widely documented in marine and terrestrial foodwebs (DeNiro and Epstein 1981; Minigawa and Wada 1984; Schoeninger and DeNiro 1984; Sealy *et al.* 1987). In long marine foodchains, the effect is a stepwise enrichment in ^{15}N, resulting in distinct high $\delta^{15}N$ values in most marine foods and consumers (Minigawa and Wada 1984), compared to terrestrial foods (Schoeninger and DeNiro 1984). Freshwater ecosystems behave similarly to marine systems, so that freshwater foods also have high $\delta^{15}N$, although their $\delta^{13}C$ does not follow the same pattern as the marine system (Dufour *et al.* 1999). The trophic shift is probably the result of loss of ^{15}N-depleted excretion products (urea in the case of most animals; Ambrose 1991). However, as summarized by Hedges and Reynard (2007), there is considerable diet to tissue variability amongst species with different physiologies—we do not know what it is for humans, it may not be linear, and the effects of high- or low-protein diets are not well understood. Logically, if the process of urea loss/body ^{15}N-enrichment continues, values in animal tissues should become progressively

higher with age. This is not observed, however, and it may be the case that isotope effects leading to trophic enrichment in ^{15}N are more marked in certain phases of maturation.

Biochemical processes induce minimal sulphur isotope fractionation in plants (Trust and Fry 1992) and in higher foodweb levels according to a single controlled feeding study (Richards *et al*. 2003b), so their distribution is largely governed by variations in underlying geology on land (ranging in $\delta^{34}S$ from −22 to +22‰), and the contrast with the uniform composition of the oceans ($\delta^{34}S = +20‰$). Krouse pioneered the application of sulphur isotopes to studies of location and human diets (e.g., Krouse *et al*. 1987), but earlier applications were limited by the large amounts of bone collagen required until improvements in continuous flow methods for sulphur isotopes emerged. Given the uniform oceanic composition, marine diets are detectable, but because of a strong sea-spray effect, marine-like $\delta^{34}S$ also reflects coastal or even island residence. Therefore $\delta^{34}S$ must be applied in combination with $\delta^{13}C$ and $\delta^{15}N$ (Richards *et al*. 2003b).

It is well known that the global distribution of hydrogen and oxygen isotopes is largely bound up with their behaviour in water (Dansgaard 1964). In animal tissues, however, analysis and interpretation of the two isotopes is separated because of the nature of the tissue archives. Studies of $\delta^{18}O$ in vertebrate bone and enamel mineral have a long history, with attention directed towards exploring $\delta^{18}O$ in apatite phosphate or carbonate as a palaeoclimate indicator (Longinelli 1984; Luz *et al*. 1984; Luz and Kolodny 1985). Dietary ecology is implicated, since water and oxygen in food contribute to body water $\delta^{18}O$, to a degree influenced by an animal's drinking habits and thermophysiology (Luz and Kolodny 1985; Bocherens *et al*. 1996; Kohn 1996; Sponheimer and Lee-Thorp 2001). Because hydrogen isotopes exchange rapidly and readily, studies have focused on non-exchangeable, tightly bound hydrogen in organic molecules. This work is in its infancy, in part related to the analytical difficulties. One recent study demonstrated that in addition to providing indications of ambient climate conditions, trophic-level effects are observed (Reynard and Hedges 2008). The implications of these variations for human diets have not yet been explored.

PRESERVATION AND QUALITY CONTROL

If we are to use stable isotopes as tracers in fossil or subfossil bones and teeth, we must be confident that the original isotope values have not been altered substantially *post mortem* and *post-burial*. All ancient calcified tissues are subject, inevitably, to some measure of alteration and, in fact, almost all bones and teeth disappear completely, relatively quickly, unless a narrow range of optimal conditions are met. The pathways of diagenesis in bone, dentine and enamel, and in the organic and inorganic components of these tissues, vary markedly because of their chemical and structural differences, while the main external influences remain those of moisture and pH, microbial attack, temperature and time (Hare 1980; Collins *et al*. 2002; Hedges 2002; Lee-Thorp 2002; Berna *et al*. 2004). Clearly, a close relationship exists between bone preservation (or the converse) and site formation processes (Weiner and Bar-Yosef 1990; Bell *et al*. 1996; Berna *et al*. 2004; Jans *et al*. 2004). In spite of the different pathways, the survival or destruction of the organic and inorganic components often appears to operate in concert, particularly for bone, which is a porous structure providing ready access to microbes and water, and where collagen and bioapatite provide some mutual protection (Hedges 2002).

Collagen

The stability and degradation of the major protein in bone and dentine, collagen, has been extensively studied because of its importance not only in isotopic but also radiocarbon and

racemization studies. On geological time-scales, collagen is short-lived compared to fossilized bioapatite, but it is a very robust biomolecule, and it has been repeatedly shown that measurable amounts of collagen can survive under optimal conditions for well over 100 000 years (Jones *et al*. 2001). Collagen denatures when hydrogen bonds are broken and thereafter fibrils dissolve away relatively quickly, explaining collagen's sensitivity to moisture, temperature and pH conditions. Collins *et al*. (2002) provided a synthesis of current understanding of the degradation of collagen and other biomolecules in the same special issue of *Archaeometry*. Non-collagenous proteins—in particular, osteocalcin—also survive in ancient bone but, disappointingly, their survival rate has proved to be poorer than that of collagen (Smith *et al*. 2005).

It seems that even when a large proportion of the original collagen molecules have disappeared, the isotopic composition remains intact. Uniform purification procedures are now used, all of which are modifications of the original Longin (1971) method. Chunks or powdered bone/dentine samples are demineralized in dilute HCl, nowadays at low temperature (5°C; see Richards and Hedges 1999a; Jones *et al*. 2001), followed by a gelatinization step, then filtration and freeze-drying, before small amounts are combusted and the CO_2 and N_2 introduced into a mass spectrometer. Standard deviations of replicate measurements are generally about ±0.1‰ for carbon and ±0.2‰ for nitrogen. Highly degraded collagen or humic contamination can produce altered stable isotope ratios but, again, standard protocols provide a straightforward, satisfactory quality control for collagen. Calculation of molar C:N ratios, and collagen yield and weight per cent C and N (Ambrose 1990), are routinely practised by the stable isotope community. The C:N measure in particular has proved to be extremely robust. The isotopic integrity of collagen, even in cases where little survives (~2%) was puzzling until it was shown that protein sequence and stable isotope information remain intact until a critical threshold of denaturation of fibrils and (high) loss is reached (Koon 2007).

Bioapatites

Although the minerals in bone and enamel are both biological apatites, they differ in ways that reflect their function, and also strongly influence diagenetic pathways. Bone apatite is highly substituted (including about 6% of CO_3^{2-}) with very low crystallinity,[2] so that it is a reactive material (Driessens *et al*. 1978; LeGeros 1991). Enamel apatite, on the other hand, has fewer substitutions (~3% CO_3^{2-}), higher crystallinity and density (LeGeros 1991) and higher-order prismatic structures (Boyde 1967). The organic matrix of mature enamel consists of very small amounts (<1%) of phosphoproteins and amelogenins, whereas the proportion of collagen remains high (~20–30%) in bone and dentine. The trend for bioapatites *post mortem* (where conditions are conducive to survival) is towards greater stability following processes of recrystallization and crystal growth (or Ostwald ripening). In bone, crystallinity indicators such as X-ray diffraction and Fourier transform infra-red show rapid increases after death, even in the absence of environmental promoters (Trueman *et al*. 2004), but changes in enamel are minimal even after very long periods (Lee-Thorp and van der Merwe 1987; Ayliffe *et al*. 1994). Recrystallization can introduce foreign ions into the crystal structures, but it is not inevitable that the original isotope composition is altered, as rearrangements and incorporation can be internal, drawn from surrounding fluid. In the case of enzymatically catalysed microbial attack (Blake *et al*. 1997; Sharp *et al*. 2000) combined with recrystallization, however, significant

[2] The term *crystallinity* denotes both size and perfection of crystals; in other words, poor crystallinity implies both internal distortion and small crystal size.

alteration of $\delta^{18}O$ was observed in bone phosphate, in spite of the belief that the strength of the P–O bond rendered it immune to diagenesis (Luz and Kolodny 1985). Enamel is not immune; Schoeninger *et al.* (2003) has shown that recrystallization to fluoroapatite can affect the isotopic composition of fossils from the tufa-rich Lake Turkana region. Over longer time-scales, ionic or isotopic exchange/diffusion processes may continue in both tissues, and precipitation of foreign minerals in cracks and pores includes pyrites, silicates and simple carbonates (Hassan and Ortner 1977).

The net result of these properties and observations is that bone apatite is vulnerable to the kinds of diagenesis that may frequently influence isotope composition, while enamel remains relatively immune. Most workers have responded by switching to enamel as sample material. Nevertheless, tooth enamel and bone are not equivalent in terms of the window reflected in an individual's life, as bone provides a broader perspective than that reflected in enamel. It has been argued that subfossil bone apatite can yield valuable information in many cases (Lee-Thorp and Sponheimer 2003), so we should not discard these opportunities too quickly.

There is less agreement on protocols for detecting meaningful alteration of apatite, for eliminating contaminants and for establishing quality controls than is the case for collagen. This is because of uncertainty about the pathways and effects of diagenesis on isotopic composition and how best to gauge them, and because pretreatment protocols designed to eliminate contaminants can also introduce artefacts. It has been observed that many of the standard indicators of diagenesis do not correlate with one another (Hedges 2002) and, furthermore, they may indicate little about isotope alteration (Trueman *et al.* 2008). For instance, expected $\delta^{18}O$ distinctions between human groups in Mexican sites held, even though crystallinity was clearly altered (Stuart-Williams *et al.* 1996).

Unlike collagen, testing for reliability of apatite isotopic composition requires tests that rely on intrinsic natural isotopic variability. One approach is to establish a comparative $\delta^{13}C$ scale from animals that are known C_3 and C_4 feeders to mark the endpoints, against which unknowns can be compared (as first set out in Lee-Thorp and van der Merwe 1987). This works well, but there are limitations—it only applies in regions with distinct C_3 and C_4 floras, and there can be difficulties in assigning appropriate diets for long-extinct animals. Nevertheless, where it has been applied, the results have shown a remarkable robusticity in enamel $\delta^{13}C$ (e.g., Cerling *et al.* 1997). In making such present/past comparisons, we need to take into account that $\delta^{13}C$ of atmospheric CO_2, on which plants depend, has changed from about −6.5‰ in the pre-industrial era to −8‰ today as a result of fossil fuel burning (Friedli *et al.* 1986). At the same time, however, the fossil effect has not yet had a measurable effect on marine $\delta^{13}C$ values, and this difference could complicate studies of marine versus non-marine human diets.

Assessment of the reliability of $\delta^{18}O$ values is more intractable, because of the inherent variability of the system. One approach is to rely on the predictable variability within a faunal assemblage (Bocherens *et al.* 1996; Sponheimer and Lee-Thorp 2001); for instance, hippopotamus $\delta^{18}O$ is consistently lower than that of other animals in African faunal assemblages (Bocherens *et al.* 1996). A more universal test is to establish that predicted intra-annual $\delta^{18}O$ holds for high-resolution analyses of tooth crowns, following the approach established by Balasse (2003). Another is the comparison of $\delta^{18}O$ from the carbonate and phosphate ions, since the isotopic offset is known (Bryant *et al.* 1996; Iacumin *et al.* 1996), although there is some internal and inter-species variability (Martin *et al.* 2008).

Most standard purification procedures first eliminate the organic component of the powdered sample by means of weak NaOCl or H_2O_2, followed by etching in a weak, often buffered, acetic acid solution. The rationale is that the acid first attacks the more reactive phases comprising

the simple carbonate contaminants and more soluble apatite, whether biogenic or diagenetic. The dilute acetic acid protocol originally developed by Harold Krueger has remained in use with modifications (Sullivan and Krueger 1981; Lee-Thorp and van der Merwe 1987; Krueger 1991; Koch *et al*. 1997). Both steps in the protocol can induce chemical and isotopic artefacts, so the duration of the reactions must be limited, especially where the material is reactive. For instance, drilling produces very small particles that are significantly more reactive, and prolonged immersion can induce recrystallization. Our laboratory currently uses these protocols for very limited periods (30 and 5–10 min, respectively) in order to avoid dissolution and recrystallization. It should be pointed out that these weak acid protocols have limitations where material has been converted to highly stable fluoroapatite; in these cases the altered material—which may occur in patches—must be avoided.

MAIZE AGRICULTURE

The first application of $\delta^{13}C$ in human bone collagen from sites in North America was carefully chosen to represent the relatively simple case of importation of an isotopically distinct C_4 crop, maize, into a mono-isotopic C_3 environment (Vogel and van der Merwe 1977; van der Merwe and Vogel 1978). These authors found that the bone collagen of individuals in Northeastern American sites showed no isotope shift consistent with consumption of C_4 maize until about AD 1000, and thereafter bone collagen $\delta^{13}C$ increased sharply, reaching levels that suggested very high maize consumption, over 60%, by AD 1500 (Fig. 1). Subsequently, others have augmented these studies in similar or related areas where maize was an introduced crop, and produced similar results (e.g., Buikstra and Milner 1991).

It was the apparent *absence* of maize prior to AD 1000 that was most surprising, because there were strong indications that subsistence and social patterns were changing in the Early Woodland groups prior to this time. Was it possible that collagen $\delta^{13}C$ was under-estimating maize consumption prior to AD 1000? And were the proportions of C_4 carbon in collagen (50–60%) represented in the Late Woodland populations, as shown in several studies, consistent with the osteological evidence for the development of severe dietary deficiencies in some populations (Larson 1995)?

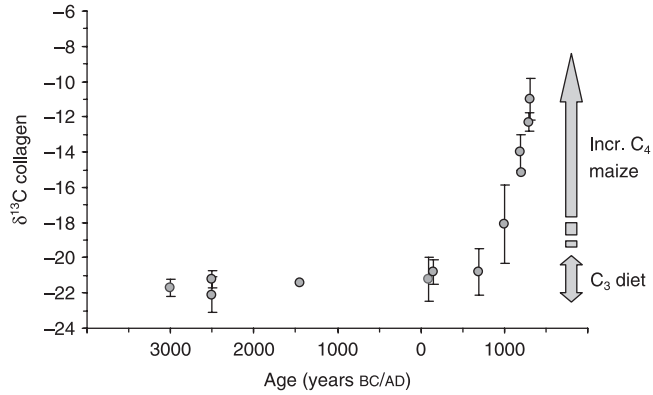

Figure 1 *Shifts in bone collagen $\delta^{13}C$ values of skeletons from Archaic and Early and Late Woodland sites in Northeastern America over* c. *5000 years. Age is given in calibrated years* BC/AD*, and the $\delta^{13}C$ data are shown as means and standard deviations. The data are from Vogel and van der Merwe (1977) and van der Merwe and Vogel (1978).*

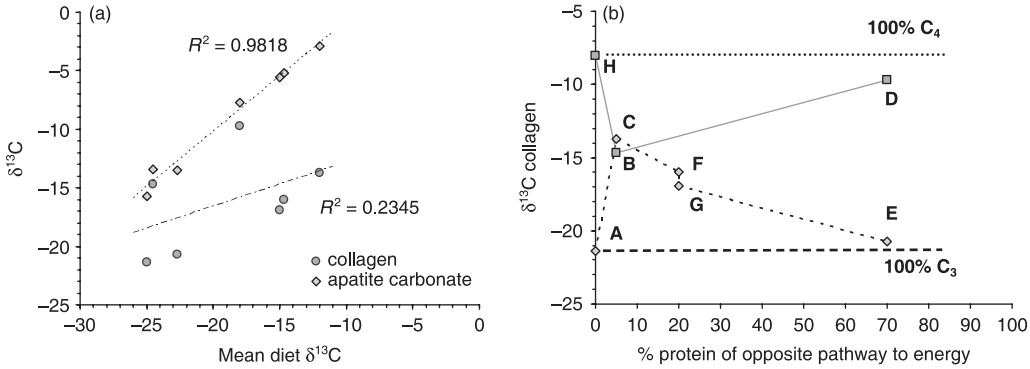

Figure 2 *The results of the Ambrose and Norr (1993) controlled feeding study. (a) A plot of $\delta^{13}C$ in bone collagen and apatite carbonate expressed against mean dietary $\delta^{13}C$ shows significant deviations for the collagen/diet expression. (b) Bone collagen $\delta^{13}C$ plotted against the percentage of protein of opposite pathway to energy components (lipids and carbohydrates). Each point represents a particular diet: A has 20% C_3 protein and C_3 energy, B 5% C_4 protein and C_4 energy, C 5% C_3 protein and C_4 energy, D 70% C_4 protein and C_3 energy, E 70% C_3 protein and C_4 energy, F and G both have 20% C_3 protein and C_4 energy, and H' is a hypothetical 100% C_4 diet. The results show that the protein pathway has a disproportionate effect on bone collagen $\delta^{13}C$.*

The results sparked a long-running debate about whether or not dietary proteins are preferentially routed to collagen, because if they are, foods high in starch and/or lipids (such as maize), could be greatly under-represented in collagen $\delta^{13}C$. Alternatively, all dietary macronutrients might contribute to construction of collagen, known informally as the 'scrambled egg' model (van der Merwe 1982). Since it is known that several essential amino acids present in collagen cannot be manufactured *in vivo* in mammals, the odds seemed weighted towards the former model. But hard evidence was lacking. Krueger and Sullivan (1984) put together a model based on first principles and their observations for differences between collagen and bone apatite carbonate $\delta^{13}C$ from animals at different trophic levels, and humans, which suggested that collagen $\delta^{13}C$ preferentially reflected the dietary protein, while bone carbonate $\delta^{13}C$ reflected rather the energy components. Data from a larger number of free-ranging herbivores, omnivores and carnivores were consistent with this idea (Lee-Thorp *et al.* 1989). Only after two carefully designed controlled feeding studies were carried out, however, did it become very clear that dietary protein was indeed preferentially routed to collagen, and also that bone apatite carbonate almost perfectly reflected the entire diet (Fig. 2 (a); see also Ambrose and Norr 1993; Tiezsen and Fagre 1993). The rat study used extreme differences in the isotope composition of proteins and energy (starch and lipid) sources to demonstrate that even small amounts of protein of opposite C_3 or C_4 pathway to the rest of the diet forced large shifts in collagen $\delta^{13}C$ (Fig. 2 (b); see also Ambrose and Norr 1993). The apatite results demonstrate that bone carbonate is strongly influenced by catabolism of *all* dietary macronutrients in both studies. Two later compound specific studies of the same material showed that (i) $\delta^{13}C$ of cholesterol, which is closely related to carbon oxidation pathways, is directly related to the bone carbonate $\delta^{13}C$ pattern (Jim *et al.* 2004) and (ii) modelling of the $\delta^{13}C$ results for individual amino acids suggests that, minimally, over 50% of dietary proteins are routed directly to collagen (Jim *et al.* 2006). In high-protein diets, more non-essential amino acids will be directly routed to collagen with no fractionation, while in low-protein diets, conditionally and non-essential amino acids would need to be synthesised *de novo* from non-protein sources and would thus be more dissimilar to dietary protein $\delta^{13}C$ composition (Jim *et al.* 2006).

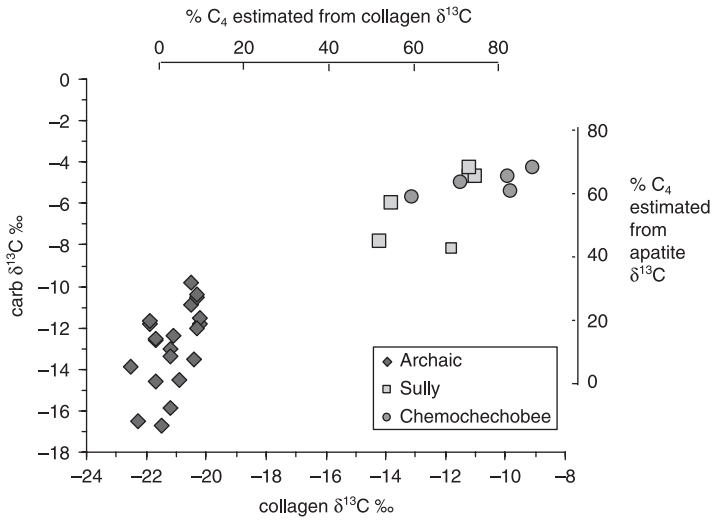

Figure 3 *A bivariate plot of $\delta^{13}C$ from bone carbonate and bone collagen for skeletons from Archaic (early, non-maize) sites, and from the sites of Sully and Chemochechebee (both later sites with maize). The bone carbonate $\delta^{13}C$ values used here are the results obtained using 0.1 M acetic acid pretreatment. A scale showing the percentage of C_4 carbon in the diet, as estimated from both fractions, is shown on the opposite axis. Data are from Koch* et al. *(1997).*

While rats are not humans and the experimental diets forced large differences, clearly these data have important implications for human dietary studies. In the maize case, the strong implication is that small amounts of maize in the diet will not be detectable by $\delta^{13}C$ analysis of collagen. In the course of a study on possible diagenesis and the effects of sample pretreatment on bone apatite, Koch *et al.* (1997) obtained a suite of $\delta^{13}C$ data on the collagen and bone carbonate from skeletons in Archaic and later sites. The results for the Archaic material suggest that up to 20% of C_4 carbon estimated from the apatite data remains 'invisible' in collagen (Fig. 3). Large inputs of C_4 maize carbon in the more recent sites were reflected in both collagen and apatite, but small differences nevertheless suggest that the two components are reflecting slightly different dietary macronutrient sources (Fig. 3).

Ambrose *et al.* (2003) were able to reveal status-related differences amongst individuals from Cahokia in access to dietary protein (Ambrose *et al.* 2003), using combined collagen and apatite isotope analysis. Status-related differences were apparent from grave goods, stature and pathologies amongst two groups, suggesting that the low-status group subsisted on very nutrient poor, possibly very high maize, diets. However, collagen $\delta^{13}C$ showed little difference between the two and the $\delta^{15}N$, while higher in the high-status individuals, was not very illuminating (Fig. 4). Bone carbonate analysis showed clearly a much larger proportion of C_4 in the low-status individuals' diets compared to that of the high-status individuals (Fig. 4).

Given the invisibility of low levels of maize in bone collagen, and in spite of the greater dangers of alteration of the bone carbonate, more recently several researchers have adopted the principle of analysing both tissue components to study dietary differences and address questions such as intensification (e.g., Harrison and Katzenberg 2003). Application of bone carbonate $\delta^{13}C$ data has revealed dietary components that have no protein whatsoever—in one case, cane sugar in the diets of Marianas Islanders (Ambrose *et al.* 1997). Clearly, this dual approach is

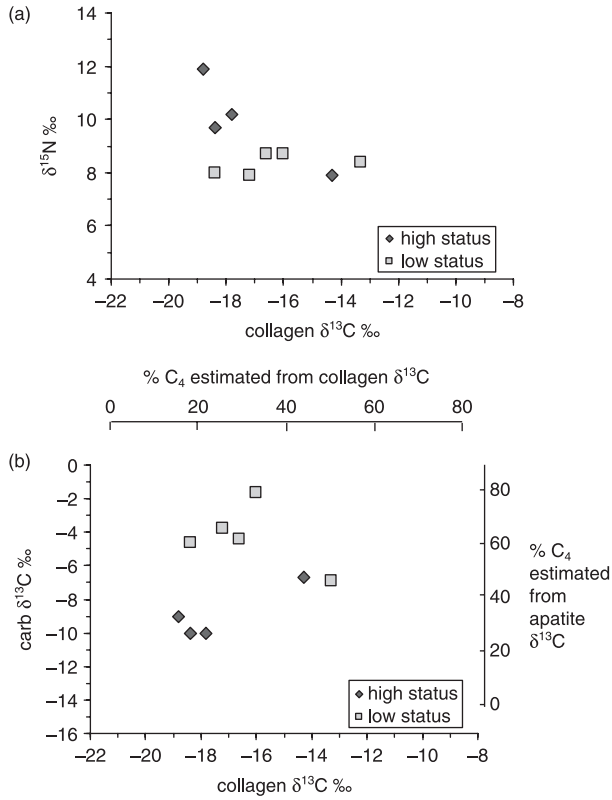

Figure 4 *Standard bivariate plots of (a) $\delta^{15}N$ and $\delta^{13}C$ from bone collagen for high- and low-status individuals from Cahokia Mound 72 and (b) $\delta^{13}C$ for bone carbonate plotted against collagen $\delta^{13}C$ for the same individuals. The data are from Ambrose* et al. *(2003).*

useful for scenarios where low-protein plants might form an important dietary component, because they would tend to be invisible in both the conventional archaeological record, and in the more standard collagen isotope approach.

MARINE-RICH DIETS

Radiocarbon chemists first observed that bone collagen $\delta^{13}C$ values from coastal people were high. The first significant publications to exploit these observations documented diachronic shifts from high-$\delta^{13}C$, marine-rich diets in the Danish Mesolithic to low-$\delta^{13}C$, terrestrial diets in the Neolithic (Tauber 1981; see also Fig. 5), and the exploitation of salmon and other marine resources in the American Northwest Pacific (Chisholm *et al*. 1982). These developments were followed shortly afterwards by the demonstration of $\delta^{15}N$ distinctions between marine and terrestrial foods (Schoeninger and DeNiro 1984), including a survey of historic and archaeological human groups following different subsistence patterns, which incorporated Tauber's Mesolithic samples (Schoeninger *et al*. 1983). The isotope distinctions between marine and terrestrial C_3 diets have since been applied around the world: just two study areas are discussed here.

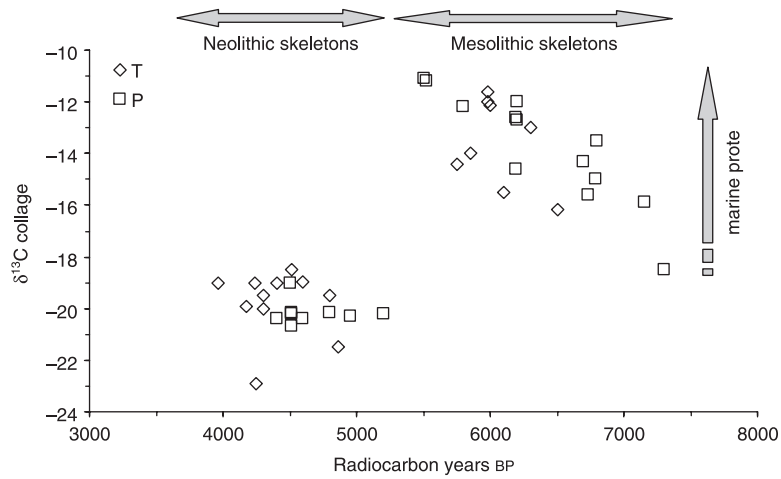

Figure 5 *A plot of radiocarbon ages (in radiocarbon years BP) and associated human collagen $\delta^{13}C$ values from Mesolithic and Neolithic contexts in Denmark, using data combined from Figures 1 and 2 in Richards* et al. *(2003a), using data from work by Persson (marked P, as squares) and Tauber (marked T, as diamonds) in the diagram. In the case of the Tauber data, the radiocarbon ages were converted from calibrated ages BC to uncalibrated radiocarbon years BP for comparability (Richards* et al. *2003a).*

Sealy applied these stable isotope differences to identify group distinctions and to test models of hunter–gatherer seasonal mobility during the Holocene in the southwestern Cape, South Africa (Sealy and van der Merwe 1985, 1986, 1988). One of the distinctive features of this study was that the interpretations of human diets were based on an extremely thorough isotopic survey of the regional terrestrial and marine foodwebs, rather than on global averages. Carbon isotope analysis of collagen showed clearly that skeletons buried at the coast were uniformly higher in ^{13}C than those buried inland of the coast (Fig. 6). Many collagen $\delta^{13}C$ values were so high (−11 to −12‰) that they resembled values of marine mammals, suggesting that human diet was often completely dominated by marine foods. The exact interpretation of these data has been disputed, as well as a question about how significant were the isotopic and dietary differences between the coastal and inland skeletons (Parkington 1991).

When nitrogen isotopes were included further important points emerged. First, many $\delta^{15}N$ values for small local game animals were anomalously high, probably due to the effects of low mean annual rainfall (<400 mm a^{-1}; see Sealy *et al.* 1987), and, second, components of the marine foodweb of known importance as food items, such as shellfish, were quite low in $\delta^{15}N$. These results showed that the terrestrial/marine cutoff point of +10‰ proposed by Schoeninger and DeNiro (1984) does not always hold in all environments. In an expansion of the study to the southern Cape coast, where there are modest components of C_4 in the ecosystem and moister conditions, the combination of $\delta^{13}C$ and $\delta^{15}N$ proved more useful, showing again that inter-group dietary distinctions were maintained (Sealy 1997).

In Europe, the original Tauber data have been augmented, and the geographical area expanded to other parts of Scandinavia, to Britain, and southwards to Brittany and Portugal. The pattern of a sharp shift in human bone collagen $\delta^{13}C$ from the Mesolithic to the Neolithic has remained intact, and is also reflected in a shift to lower $\delta^{15}N$ values (Richards and Hedges 1999a,b; Schulting and Richards 2001; Richards *et al.* 2003a). While some doubts were raised about the spread of radiocarbon dates (Milner *et al.* 2004), almost all newer data with calibrated

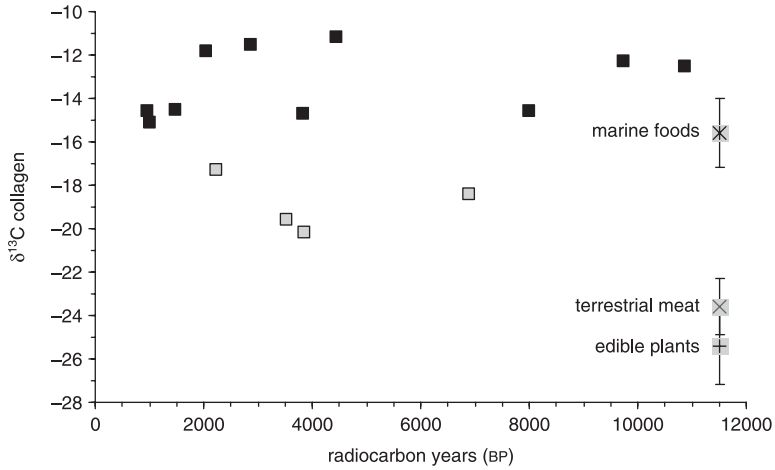

Figure 6 *Bone collagen $\delta^{13}C$ values obtained from skeletons buried at the coast (solid squares) and in inland locations (grey squares) of the southwestern Cape, South Africa, plotted against age in radiocarbon years. Means and standard deviations are shown on the right-hand edge of the diagram for the following classes of foods: marine foods, terrestrial meat and terrestrial plants, all from the same region. The data are from Sealy and van der Merwe (1985).*

dates fits the same pattern. Redating of three burials from Dragsholm, which were in close proximity to each other and had originally given close ages although assigned as Mesolithic (two females) and Neolithic (one male), effectively provided greater separation in time (Price *et al*. 2007). It is clear that a sharp, culturally related economic shift occurs, from a hunter–gatherer subsistence mode that included a good deal of marine fish and shellfish in the Mesolithic, to a terrestrial diet, focused rather on cereals and domestic animals, as Richards and co-workers have argued (Richards *et al*. 2003a; Richards and Schulting 2006). The observation about the high marine content of coastal Mesolithic diets is not in dispute; rather, it is the magnitude and apparent completeness of the shift that occurs with the Neolithic. Archaeologists have argued that modest amounts of fish bone and shell at Neolithic sites show a pattern in which fish continue to be exploited, and have argued that alternative explanations should be sought (Milner *et al*. 2004; Fischer *et al*. 2008). So the argument in this case is not so much about whether there was over-representation of marine foods in the Mesolithic (although this should be subject to scrutiny) but, rather, whether there is some way in which marine fish is under-represented in Neolithic bone collagen.

Both the European Mesolithic/Neolithic and the South African coastal hunter–gatherer studies have raised important questions and debates that partly concern the intrinsic meaning of the isotope data, and partly concern the 'fit' with other contextual archaeological evidence (and its interpretations). Are the very large amounts of marine foods represented in the southwestern Cape coast bone collagen $\delta^{13}C$ values reasonable, or is marine food greatly over-represented? It is now understood that collagen $\delta^{13}C$ preferentially reflects the protein component of the diet, and that the relationship shifts with the amount of protein (Fig. 2; see also Ambrose and Norr 1993; Tieszen and Fagre 1993), strongly suggesting that the latter is the case, especially if the terrestrial component of the diet was low in protein. In the southwestern Cape, the Fynbos biome is poor in large game, and stable terrestrial sources available to foragers were seasonally abundant starchy corms and other plant foods (Sealy and van der Merwe 1986). A limited study on the bone apatite carbonate of some of the human skeletons suggested that some ^{13}C-depleted

components of the diet were not well-represented in collagen $\delta^{13}C$. The effect of this difference (high collagen $\delta^{13}C$/low apatite $\delta^{13}C$) is a small difference between the tissues, or small $\Delta_{collagen\text{-}apatite}$ (Lee-Thorp *et al.* 1989). These insights, however, do not change the finding that coastal and inland foragers differ in bone collagen isotope composition.

Is there some way in which the European Neolithic bone collagen $\delta^{13}C$ can be reconciled with inferences from the contextual evidence? The issue of shellfish consumption can be relatively easily explained; shellfish residues are over-represented in the archaeological record because they generate a very large amount of debris for caloric return, whereas, because of their low trophic position, they have relatively low $\delta^{13}C$ and $\delta^{15}N$ flesh values compared to fish and marine mammals. Another issue is that the fishbone residues found in modest amounts at Neolithic sites are apparently not reflected in human bone collagen isotope values. However, if one considers that some of these residues are from freshwater or anadromous species such as eel, they may represent foods low in ^{13}C (Dufour *et al.* 1999), rather than high as is the case for marine fish. It has been suggested that a combination of modest inputs of marine and freshwater fish, in combination, would effectively cancel each other (Fischer *et al.* 2008). One problem with this neat solution is that freshwater fish are also high in $\delta^{15}N$, which is not consistent with the Neolithic human bone collagen values. Given the consistency of the Mesolithic to Neolithic pattern, and the tight clustering of Neolithic human bone collagen $\delta^{13}C$ and $\delta^{15}N$, it seems clear that even if modest amounts of shellfish and fish, marine or freshwater, were consumed, the dietary shift observed in the skeletal collagen isotope values marks a sharp and distinct economic and cultural change. Interestingly, at least in the British Isles, marine foods only re-appear as regular items in human diets during medieval times (Müldner and Richards 2005).

DIETS IN DEEP TIME

A good deal of effort has gone into extending isotope analyses to more remote time periods in order to address dietary questions during earlier periods of human evolutionary history. As can be seen in the foregoing sections, most of the existing isotope research has concentrated on bone collagen $\delta^{13}C$ and $\delta^{15}N$. In order to push further back in time, researchers have had to either extend collagen-based methods or develop methods based on the mineral phase. They have done both. Recent progress in extracting good-quality collagen from older material has shown that it can survive under the right conditions for up to 200 000 years (Ambrose 1998; Richards and Hedges 1999a; Jones *et al.* 2001). This has made it possible to analyse the bone collagen of Late Pleistocene hominins in Europe, where temperatures have been low for most of this time. Applications deeper in time have required the development and testing of apatite-based methods, which quickly settled on tooth enamel as a far more reliable material that retained biogenic isotope compositions (Lee-Thorp and van der Merwe 1987; Ayliffe *et al.* 1994; Wang and Cerling 1994; Lee-Thorp 2002). These developments, coupled with improvements in mass spectrometry that greatly reduced sample size requirements and increased throughput, opened the way to apply isotope methods to very old fossil teeth of australopiths and early *Homo*. One advantage is that enamel apatite $\delta^{13}C$ reflects the composition of the entire diet.

Late Pleistocene Neanderthal diets

Stable isotope studies of Neanderthal diets began with analysis of a single 40 000-year-old Neanderthal individual and associated fauna from Marillac, France (Bocherens *et al.* 1991).

Although the authors in this first study relied for quality control on amino acid profiles that might be considered inadequate today, and not much can be deduced from one individual, later analyses at this site (Fizet *et al*. 1995) showed that the original data were robust. The first Marillac study, and analyses of older faunal material from Vindija, Croatia and other sites (Ambrose 1998) paved the way for subsequent analyses of Neanderthal specimens at Marillac (Fizet *et al*. 1995), Scladina, Awirs and Betche-al-Roche Caves in Belgium (Bocherens *et al*. 1997, 2001) and Vindija (Richards *et al*. 2000).

In the mono-isotopic C_3 European environment, bone collagen $\delta^{13}C$ reveals little about Neanderthal diet, except that there is no evidence of a preference for dense, forested environments (Bocherens *et al*. 1997; Richards *et al*. 2000). The focus has been entirely on $\delta^{15}N$ composition, which has been used to address the question of trophic level and meat consumption. Given the frequency of injuries, evidence for close contact hunting, and the frequency of stress episodes (in the form of enamel hypoplasias) amongst Neanderthals (Trinkhaus 1995), their hunting (or scavenging) success has been the subject of a great deal of debate. One hypothesis was that Neanderthals had lower hunting success and trophic levels compared to Upper Palaeolithic modern humans (Ambrose 1998).

All isotopic data in the literature show that Neanderthals have high $\delta^{15}N$ compared to contemporaneous (or near-contemporary) herbivores such as horse (*Equus caballus*), reindeer (*Rangifer tarandus*) and bison (*Bison priscus*), and similar to carnivorous wolves (*Canis lupus*), hyenas (*Crocuta spelaea*) and lions (*Panthera spelaea*) (Bocherens *et al*. 1991, 2001, 2005; Fizet *et al*. 1995; Richards *et al*. 2000). When the data from all western and central European sites are combined, Neanderthal $\delta^{15}N$ is significantly higher than that of herbivores and also slightly higher than that of carnivores (Fig. 7). The mean difference between average herbivore and Neanderthal $\delta^{15}N$ is about +5‰ and sometimes higher. Richards *et al*. (2000, 2001) and

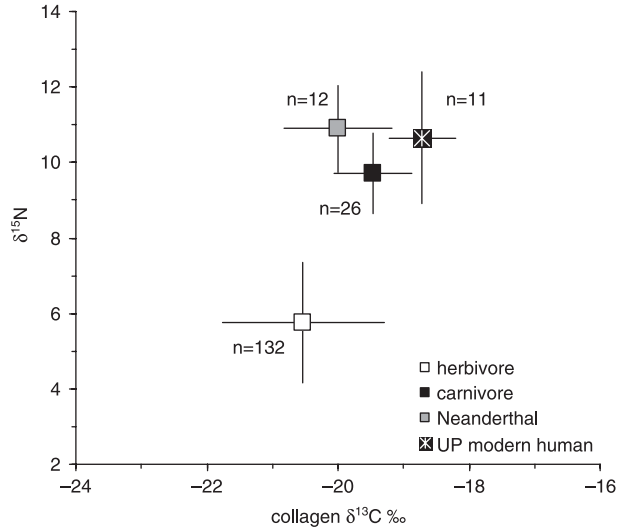

Figure 7 *A bivariate plot of the means and standard deviations for $\delta^{15}N$ and $\delta^{13}C$ for herbivores, carnivores, Neanderthals and Upper Palaeolithic modern humans from Glacial-period European sites. The data for the sites of Marillac, La Berbie, Scladina, Vindija and Carniac have been combined from Bocherens* et al. *(1991, 1997, 2001), Fizet* et al. *(1995) and Richards* et al. *(2000, 2001), while the UP modern human data are drawn from several sites, using data from Richards* et al. *(2001), Pettit* et al. *(2003) and Schulting* et al. *(2005).*

Bocherens *et al.* (2005) have argued that Neanderthals were high-level carnivores, with little of their dietary protein coming from plant foods, and, further, that they relied on herbivores with relatively high $\delta^{15}N$, such as mammoths (*Mammuthus primigenius*), or even the consumption of omnivorous bears (*Ursus* spp.) (Richards *et al.* 2000; Bocherens *et al.* 2001). Bocherens *et al.* (2005) applied a mixing and resource partitioning model developed in modern ecosystem studies to calculate a statistical probability that a major component of Neanderthal diet was mammoth. There are significant constraints, however, to the application of such a statistical model in ancient ecosystems where there are large numbers of unknowns, which were not met in this case.

Although direct stable isotope data comparisons between Neanderthals and Upper Palaeolithic *Homo sapiens* (UP humans) from similar periods and places are not possible, one can compare average values. Richards *et al.* (2001) argued that since $\delta^{15}N$ for a suite of near-contemporary ~30 000-year-old Upper Palaeolithic modern humans was even higher than that for the Neanderthal data existing at the time, in addition to a dependence on animal foods they might also be incorporating freshwater fish and fowl resources in their diets. If that was the case, it would indicate an early broad-spectrum foraging base. However, the addition of new Neanderthal and Upper Palaeolithic human data shows that any $\delta^{15}N$ differences are not statistically significant (Sponheimer and Lee-Thorp 2007). Little attention has been paid to the small difference in $\delta^{13}C$ between Neanderthals and UP humans (Fig. 7); it may reflect differences in preferred environments or prey, or simply that climate conditions differed.

The main rationale behind the application of stable isotope analyses to Neanderthal and UP human diets is related to the question of trophic level, but this is also where we have the greatest interpretive problems. Interpretations offered so far for the large enrichment in ^{15}N between the mean for hominins and for associated herbivores are that they preferred to exploit the game that happened to be relatively high in ^{15}N. This interpretation assumes a 'standard' trophic enrichment of +3‰, yet, as pointed out by Hedges and Reynard (2007), we do not know that this is the correct offset for humans. It is also not at all clear that the relationship between dietary and collagen $\delta^{15}N$ is linear (Hedges and Reynard 2007), meaning that one cannot readily determine 'shades' or degrees of carnivory, or more properly, high-protein diets. Controlled feeding studies have suggested that the amount and quality of protein in the diet may affect the diet-tissue $\delta^{15}N$ spacing (Δ) and that Δ is higher in herbivores fed protein in excess of their requirements (Sponheimer *et al.* 2003). Therefore, thresholds may operate. The isotope data for Glacial-age Neanderthals and UP humans in Europe illustrate the problems for interpreting $\delta^{15}N$ data in a palaeo-ecosystem for which we have no modern analogues. In spite of these caveats, however, it would appear that both Neanderthals and UP humans almost certainly consumed large quantities of protein-rich animal foods.

Early hominin diets

Isotopic studies of early hominins are grounded primarily upon the $\delta^{13}C$ distinctions between C_3 and C_4 plants, since, in the African savannah environments that they occupied, all carbon dietary sources from trees, bushes, shrubs and herbs are distinct in ^{13}C compared to those from tropical grasses and some sedges. $\delta^{13}C$ analysis provides opportunities to test hypotheses about their primary dietary habits. Amongst the South African hominins, where a good deal of the dietary research has taken place, it was widely believed that *Australopithecus africanus* consumed primarily fruits and leaves, and some animal foods, while *Paranthropus robustus* concentrated more on plant foods that tended to be small, hard items and that caused greater occlusal enamel pitting (summarized in Lee-Thorp and Sponheimer 2006).

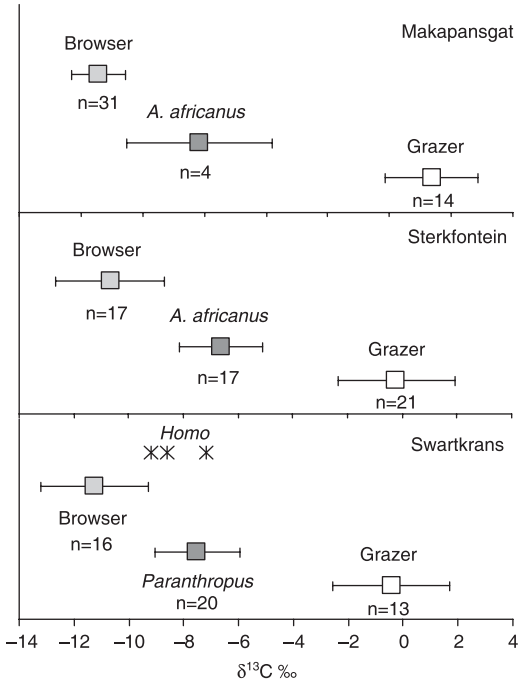

Figure 8 *The distributions of $\delta^{13}C$, shown as means and standard deviations, in the enamel of C_3-feeders (typical browsers), C_4-feeders (typical grazers) and hominins from the sites of Makapansgat (≥ 3 Ma), Sterkfontein Member 4 (*c. *2.4–2.6 Ma) and Swartkrans (*c. *1.7 Ma). The figure is redrawn from Lee-Thorp* et al. *(2003).*

The prediction, then, would be that *A. africanus* and *P. robustus* should have $\delta^{13}C$ values indistinguishable from those of C_3 browsers and frugivores. Analysis of more than 40 hominin specimens from the sites Makapansgat, Sterkfontein, Kromdraai and Swartkrans, spanning a period of about 1.5–3.0 million years, however, demonstrates that the $\delta^{13}C$ of both australopiths and the few early *Homo* individuals is very distinct from that of coexisting C_3-consumers such as browsers (Fig. 8). Furthermore, *A. africanus* and *P. robustus* mean values are indistinguishable in spite of the passage of time and shifts in environmental conditions (Lee-Thorp *et al*. 2003). If we take the mean $\delta^{13}C$ of C_4- and C_3-consuming herbivores as indicating the C_4 and C_3 'endpoints', we can estimate that, on average, both *Australopithecus* and *Paranthropus* obtained over 30% of their carbon from C_4 sources, while the estimate for *Homo* is possibly a little lower (although uncertain, given that $n = 3$). All taxa were eating considerable quantities of C_4 resources, which must have consisted of grasses, sedges, or animals that ate these plants.

This result was unexpected, since extant apes consume minimal or no C_4 resources even when they live in relatively open habitats, and suggests a fundamental niche difference between the australopiths and extant apes. The distinction between the hominins and other fauna cannot be ascribed to diagenesis, as there is no evidence that browser or grazer $\delta^{13}C$ is altered, and diagenesis should affect all fauna. The association with C_4 resources persists throughout environmental trends that sees shifts from relatively closed Pliocene habitats at the earlier sites (~2.4–3.0 Ma) through to more open environments after *c*. 1.7 Ma (Lee-Thorp *et al*. 2003; see also Fig. 8). Within each site, and where there are sufficient samples, australopith $\delta^{13}C$ data are

more variable than most modern and fossil taxa analysed in southern Africa to date, suggesting that they were opportunistic primates with wide habitat tolerances.

Alone, the $\delta^{13}C$ data allow firm conclusions to be drawn only about the proportions of carbon from C_3 and C_4 sources, but not about what the actual resources were. Lee-Thorp *et al.* (2000) argued that savannah grasses are unlikely staple foods for hominins, since they are relatively nutrient poor, small packages, and that consumption of C_4-consuming insects and vertebrates is a more plausible explanation. Closer examination of various possibilities, such as edible, starchy sedges and termites, suggests that none of them, on their own, offers a satisfactory explanation for the significant C_4 contribution. One other possible source of information may be found in enamel $\delta^{18}O$. As discussed above, $\delta^{18}O$ from apatite carbonate or phosphate is influenced by dietary ecology, including trophic behaviour. In two southern African modern ecosystems examined, suids (warthog), some primates and in particular all faunivores (i.e., carnivores and insectivores) have relatively low $\delta^{18}O$ compared to the herbivores (Lee-Thorp and Sponheimer 2005). The reasons are not entirely clear; in the case of suids, it may reflect reliance on underground storage organs, and for faunivores, a high proportion of dietary lipids and proteins or, equally, a heavy reliance on drinking water. Australopith $\delta^{18}O$ data from Makapansgat and Swartkrans overlap with those of carnivores in the same strata (Lee-Thorp *et al.* 2003). Although tantalizing, the interpretation of low $\delta^{18}O$ values for hominins is still obscure, and the topic requires further study.

Despite these uncertainties, the isotope data have shown that australopiths increased their dietary breadth by consuming C_4 resources, whatever those resources were. A fundamental difference between hominins and extant apes, therefore, might be that when confronted with increasingly open areas, apes continued to use the foods that are most abundant in forest environments, whereas early hominins began to exploit the new C_4 resources.

WHERE DO WE GO FROM HERE?

Clearly, the field of isotopic dietary reconstruction has taken enormous strides since the first applications in the mid-1970s. Many promising new developments, such as high-resolution intra-individual sampling and life history applications, were not included in the discussions above, because they are still under development. They are included in my admittedly subjective list of where I believe we should be heading in the future.

Isotope distributions in modern and palaeo-ecosystems

One area that requires more intensive and broader effort is to improve our understanding of the natural distributions of stable isotopes in different kinds of ecosystems, and under various conditions. In trying to understand the past, we apply, sensibly, principles of uniformitarianism, but our information is frequently garnered either at site-based or at global scales, and little at the regional scale. One of the distinctive features of the Sealy and van der Merwe study of Holocene coastal hunter–gatherers was the thorough coverage of the environmental and isotopic context across an entire region, for both the past and the present (Sealy *et al.* 1987; Sealy and van der Merwe 1988). We need more such regional contextual studies. Admittedly, accomplishing such a goal is frequently very difficult, because many regions have been completely altered by millennia of human agricultural activity. Furthermore, at many agricultural-era archaeological sites, the faunal material is often limited to a few domesticated animals, thus reducing our ability to capture elements of the broader ecosystem. However, refugia do remain even in heavily altered regions of the world.

In studying palaeo-ecosystems, we frequently encounter conditions very different to those of today, and we may need to be creative in locating modern ecosystems that are at least roughly comparable. A good example is the last Glacial period in Eurasia. Patterning of $\delta^{13}C$ and $\delta^{15}N$ in many of the fauna analysed in Neanderthal and palaeontological sites in south-western Europe suggests that the carbon and nitrogen cycles were (variably) significantly different under glacial conditions (e.g., Bocherens *et al.* 2005; Stevens *et al.* 2008), but in order to understand these patterns we may need to look elsewhere—for instance, towards the fringes of the tundras of Siberia.

Expanding the isotope toolkit

Most of this review has concentrated on developments related to $\delta^{13}C$ and $\delta^{15}N$ from collagen, and $\delta^{13}C$ from enamel apatite, but brief sections on hydrogen, oxygen and sulphur have shown that there is some rationale for expanding investigations of those isotope systems for palaeodietary purposes. From first principles and the existing studies, it could be argued that $\delta^{34}S$ is less promising as a palaeodietary indicator, because the main delineator is the singular value for marine organisms, while everything else is highly variable.

The pointers to trophic patterning in δD and $\delta^{18}O$, obtained independently in different contexts, offer promising new avenues for investigation. The inter-species variability in $\delta^{18}O$ in phosphate or enamel carbonate emerged unexpectedly in the pursuit of other goals. A handful of opportunistic comparative studies of suites of African fauna suggest that faunivory may be detectable based on distinct low $\delta^{18}O$ values for animals such as hyena and bat-eared foxes (Lee-Thorp and Sponheimer 2005), but there is clearly a good deal of overlap and variability. Furthermore, in order to determine whether the pattern holds more widely, the distribution of $\delta^{18}O$ amongst suites of fauna from cool, temperate environments should be tested. That δD in collagen holds potential for trophic-level information has only recently been reported, and the information obtained so far is based largely on a modest suite of mostly domestic animals (Reynard and Hedges 2008). The demonstration that δD holds trophic-level information, *and* that the patterns survive in archaeological biomolecules, shows great promise. Since the behaviour of hydrogen and oxygen isotopes is almost always linked, further steps could be directed at bringing the two together, based on biomolecules. So far the research has been completely decoupled, because $\delta^{18}O$ was studied in the mineral and δD in the organic phases.

More information from smaller biomolecular units

The discussion of routing of dietary components concluded with the outcome of compound-specific isotope analyses of amino acids and cholesterol from a controlled feeding study (Jim *et al.* 2004, 2006). Those two examples demonstrate that a good deal more detailed information about biochemical pathways can be extracted from compound-specific approaches, rather than (or in addition to) the bulk sampling approaches in broad use so far. Another promising example is the development and application of a $\delta^{13}C_{\text{glycine–phenylalanine}}$ index to detect the presence of marine foods in the diet (Corr *et al.* 2005). The advantage of this approach is that it is independent of the presence of C_4 plants in the environment, a major complicating factor in distinguishing marine from terrestrial diets using bulk collagen methods in certain regions. More broadly, compound-specific approaches, carried out in the context of carefully directed controlled feeding studies, remain the most promising avenues for providing the kind of detailed

coupled biochemical and isotopic information required to address those tricky, previously intractable problems of quantification of dietary elements (i.e., how much marine food, how much maize). Further promising avenues would include the means to detect previously undetectable dietary items, such as freshwater fish and even plant foods. That would constitute a very significant step.

Developments in sampling and analysis

Recent developments in mass spectrometry, and particularly in the automated delivery of sample to the mass spectrometer for isotopic analysis, mean that multiple, very small samples can be rapidly analysed. These developments have opened up many opportunities. High-resolution, serial analyses of vertical transects down tooth crowns are not new; Balasse pioneered manual high-resolution serial sampling of domestic and wild fauna in order to determine seasonal patterns and examine domestic animal management (Balasse 2003).

Laser-ablation sampling systems coupled to stable light isotope mass spectrometers, while not yet widely available, hold a good deal of promise for further reducing sample size requirements while still obtaining maximum intra-tooth information (Sharp and Cerling 1996). Laser ablation damage is minimal, a factor that makes it a much more attractive proposition for museum curators and could thus facilitate access to larger numbers of fossil specimens. There are, of course, many limitations. For instance, the gas released by laser ablation of tooth enamel contains oxygen from several sources, but it is mainly a mixture from the phosphate (~90%) and carbonate (~10%) compartments. Since $\delta^{18}O$ from phosphate and carbonate in the same tooth or bone differs by ~9‰, the gaseous mixture reflects both and is not directly comparable to existing data. An application to hominin teeth demonstrated, for the first time, the extent of intra-annual variability in contributions of C_4 resources to the diets of South African *Paranthropus* specimens (Sponheimer *et al*. 2006). High-resolution transects of tooth crowns, or of the dentine roots for isotope analysis, hold enormous potential for addressing questions about the life histories of individuals in the past. Several studies have investigated the age at which important culturally influenced biological events occur, particularly the duration of breastfeeding and the age of weaning, using mostly manual sampling methods (e.g., Wright and Schwarcz 1998; Fuller *et al*. 2003). Smaller sample size requirements and rapid, automated analysis now provide the means for greater resolution in such approaches, while minimizing damage. These are rosy possibilities, but in enamel there are inherent constraints in the amount of information that may be extracted, due to the intrinsic patterns of tooth crown formation. Maturation occurs for many months after primary enamel is laid down, thus dampening the isotopic 'input' signal (Balasse 2003; Passey *et al*. 2005a). Thus higher sampling and analytical resolution may not necessarily translate into a similar resolution of information.

Stable light isotope analysis is one of the very few methods capable of identifying events within the lives of individuals, and also for identifying dietary and life history differences between individuals, in addition to the opportunities for broader scale inter-group and even inter-species comparisons. Thus the approach can operate at many scales. So far, the broader scale has been favoured, partly because it allows statistical confirmation of trends and comparisons. With new developments in mass spectrometry enabling finer-scaled sample analysis, minimal damage and high sample throughput, not to mention the developments in analysis of compound specific amino acids and lipids, the forthcoming decade should see the realization of the full potential of stable light isotope approaches.

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