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Carbon isotope analysis of bulk keratin and single amino acids from British and North American hair†

James S. O. McCullagh[1], Jennifer A. Tripp[2]* and Robert E. M. Hedges[1]

[1]University of Oxford, Research Laboratory for Archaeology, 6 Keble Road, Oxford, OX1 3QJ, UK
[2]Department of Chemistry, University of Scranton, 223 Monroe Avenue, Scranton, PA 18510, USA

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The reconstruction of ancient diets using isotopic measurements of bone collagen, and other tissues, which survive in archaeological contexts, relies on known isotopic relationships between diet and body tissues. Examination of these relationships often requires the study of modern human and animal subjects. While hair keratin can act as a useful proxy for bone collagen in isotopic studies on living humans, where it is inappropriate to sample tissues such as collagen, it can, in addition, act as a chronological indicator of dietary change. This study investigates hair keratin $\delta^{13}C$ values from current residents of the UK and the USA. Residents in the USA showed a clear bulk hair $\delta^{13}C$ enrichment of approximately 3‰ over UK individuals, attributed to an elevated C_4 dietary input from maize fed to livestock in North America. The keratin $\delta^{13}C$ of subjects who moved between the UK and USA showed a pronounced change after relocation, taking approximately 4 months to reach isotopic equilibrium. To investigate these differences further, we measured $\delta^{13}C$ values of dispensable and indispensable amino acids as a group, and selected individual amino acids. As a group, enrichment of dispensable amino acids compared with indispensable amino acids occurred in samples from both continents, averaging 7.2‰ in the UK and 7.9‰ in the USA. Dispensable and indispensable amino acids, as well as all individual amino acids measured, were enriched in samples from the USA compared with those from the UK. Copyright © 2005 John Wiley & Sons, Ltd.

The study of ancient diets through the isotopic analysis of archaeological biogenic remains is based on the principle, 'you are what you eat'. That is, the material from which your body is formed originates in the diet.[1–4] During life almost all human and animal body tissues are in a state of breakdown and re-synthesis, incorporating molecules from the diet on a continual basis.[5] Stable isotopic measurements of body tissues such as bone collagen, which often survives intact long after death, reflect dietary isotope values and can be used to estimate the types of foods from which they derive.[6]

Animal-feeding studies have provided evidence of diet-body tissue isotopic relationships and demonstrated that body tissue isotope values are inextricably linked to those of the diet.[6,7] The $\delta^{15}N$ of collagen, for example, shows enrichment from dietary protein indicating trophic level position in the food chain.[6,7] $\delta^{13}C$ values, on the other hand, correlate with dietary carbon from all macronutrients, reflecting types of photosynthetic mechanisms at the base of the food chain. However, feeding studies used to develop and understand these isotopic relationships with respect to collagen would be invasive and ethically problematic. In order to understand human-specific metabolic effects, proxies for collagen must be used.[8–13] Hair keratin is particularly suitable as it has similar molecular chemistry to collagen and can be easily sampled. Keratin is one of the few proteins in the body to not undergo further metabolic remodelling after biosynthesis, so the isotopic values along the length of the hair shaft reflect a linear record of diet. The growth rate of hair is approximately 1 cm per month,[14] so a chronological dietary record can be determined by measuring isotopes in hair from the proximal (most recent) to the distal end.

Like collagen, keratin largely reflects the protein component of the diet and exhibits 1–2‰ enrichment for carbon and a 2–3‰ enrichment for nitrogen.[15–18] Collagen shows a relatively larger enrichment for carbon, generally explained by its differing amino acid content. Glycine contributes approximately a third of the residues in collagen and tends to be enriched in ^{13}C compared with other amino acids,[19–22] leading to a relative enrichment for the bulk collagen in comparison with other proteins such as keratin. For palaeodietary studies, bone collagen is most often used because of

**Correspondence to*: J. A. Tripp, University of Scranton, Department of Chemistry, 223 Monroe Avenue, Scranton, PA 18510, USA.
E-mail: trippj2@scranton.edu
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its availability and preservation, but comparison data between collagen and hair have been published by O'Connell *et al.*, quantifying the isotopic spacing between bone and hair within the same individual both from modern and from archaeological populations.[10,11]

Previous work on the isotopic composition of human hair keratin addressed differences between individuals from different geographical locations,[23,24] and on different diets within the same population.[10,25,26] A number of animal-feeding studies have also been conducted.[15–17] O'Connell and Hedges found that the hair of vegans was significantly depleted in ^{15}N compared with that of omnivores and ovo-lacto vegetarians.[12] In addition, the $\delta^{13}C$ values of body proteins can be used to indicate the types of plants in an individual's diet.[2,25] The C_4 photosynthetic pathway produces enriched $\delta^{13}C$ values in subsequent metabolites compared with C_3 plants, and this is reflected in the isotopic composition of keratin and other body proteins of animals and humans that consume those plants. For instance, one would expect a difference in $\delta^{13}C$ of hair keratin from the USA and UK where livestock tend to be fed primarily maize (a C_4 plant) and C_3 grasses, respectively.

This study investigated carbon isotopic differences in hair keratin from individuals in the USA and the UK at both bulk and single amino acid levels. The first part of the project was to measure bulk keratin $\delta^{13}C$ from individuals residing permanently in the USA and UK and three individuals who relocated, one from the USA to the UK and the other two from the UK to the USA, each for extended stays in their new locations. This part of the project had two aims: first, to measure $\delta^{13}C$ values from representatives of both countries to ascertain systematic isotopic differences, and, second, to record the change and isotopic equilibrium time for individuals moving between locations. Lengths of hair corresponding to growth covering the period of change were obtained and multiple isotopic measurements made to estimate the length of time taken to reach isotopic equilibrium for the new diets in both cases. The second part of the project was aimed at understanding how these isotopic differences were manifested at the bulk dispensable and indispensable, as well as individual, amino acid level. We measured single amino acid values from the hair keratin of residents from both continents and from a single individual before and after emigrating to the USA from the UK.

We hypothesised that a distinction in $\delta^{13}C$ of human hair would reflect the consumption of foods with different $\delta^{13}C$, in particular the difference in meat isotope values given that livestock in the USA are predominantly fed on maize (incorporating a C_4 signal) and in the UK on C_3 wild grasses. Corn syrup, used predominantly in the USA as a sweetener, might also contribute a C_4 component to the diet and, thus, also to body tissues. We suspected the latter to be of little importance, however, as its incorporation into body proteins, such as keratin, would be contingent upon the biosynthesis of dispensable amino acids utilising significant carbohydrate as a source of carbon. As most people have many dietary sources of carbohydrates, and biosynthesis of dispensable amino acids is not always nutritionally required, we thought that the impact of C_4 corn syrup in the diet was likely to be minimal.

EXPERIMENTAL

Sample information

All 14 individuals providing hair samples were non-vegetarian omnivores and had not travelled outside of the continents where they resided within the previous year. This was with the exception of the three individuals who relocated between continents. Five individuals with long hair provided samples of several strands of hair for bulk isotopic measurements. These were cut as close to the scalp as possible and sliced into 1 cm sections, each representing approximately 1 month of growth.[14] Two of these five individuals lived in the USA and one in the UK. The two remaining long-haired individuals had moved within the year prior to sample collection: one from the USA to the UK and the other from the UK to the USA.

Eight individuals with short hair, three from the USA and five from the UK, were asked to save removed hair from their most recent haircut for analysis. Another individual who moved from the UK to the USA had his hair cut and collected for analysis three times throughout his stay in the UK and USA: approximately 1 year and 1 month prior to the move, and 6 months after the move.

Bulk isotopic analysis

All samples were sonicated for 30 min in, and rinsed twice with, 50:50 methanol/chloroform. Each hair portion was wrapped in aluminium foil and submitted for $\delta^{13}C$ analysis. Stable isotope mass spectrometric analyses were undertaken using a Europa (now SerCon Ltd, Crewe, UK) ANCA Roboprep CHN analyzer interfaced to a Europa 20/20 mass spectrometer operating in continuous-flow mode. $\delta^{13}C$ values in this paper are reported with reference to VPDB.[27] Typical errors for replicate measurements of $\delta^{13}C$ are $\pm 0.3‰$.

Isotope analysis of single amino acids

To hydrolyse the keratin, approximately 1 g of washed hair was placed in a flask under a stream of nitrogen, and 50 mL of 6 M HCl were added. The mixture was stirred for 24 h at 110°C. The resulting hydrolysate was cooled, washed, and the acid evaporated on a rotary evaporator almost to dryness before being lyophilised. Deionised water was added and the solution was then frozen until use.

The high-performance liquid chromatography (HPLC) method used for amino acid separation has been reported in detail elsewhere.[22] A two-step separation method involving reversed-phase and ion-pair chromatography allows for the separation and collection of amino acids for isotopic analysis. After the amino acids had been collected, the solvent was removed by lyophilisation, and the dry samples weighed into tin capsules for continuous flow isotope ratio mass spectrometry (CF-IRMS) analysis, as described above.

RESULTS AND DISCUSSION

Bulk isotope results

Using isotope measurements of bulk hair keratin, the aim was to measure geographical differences in $\delta^{13}C$ values of hair keratin between individuals living in the UK and the USA. Table 1 lists the $\delta^{13}C$ measurements on bulk keratin from all individuals in the study according to their place of residence

Table 1. Carbon isotope measurements of bulk hair keratin from subjects residing in the USA or the UK in January 2005

$\delta^{13}C_{PDB}$, ‰	USA residents	UK residents
	−18.1	−20.7
	−18.1	−20.8
	−17.2	−20.3
	−17.8	−20.0
	−16.5	−20.5
	−17.7	−20.4
	−17.7	−20.8
Mean	**−17.6**	**−20.5**
Std. Dev.	**0.6**	**0.3**

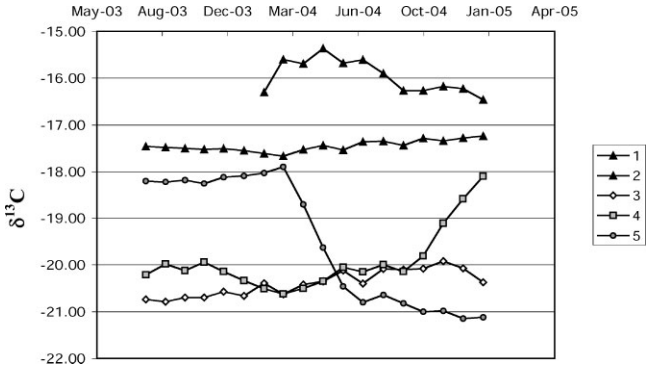

Figure 1. $\delta^{13}C$ of subjects with long hair. Samples were taken down the length of the shaft with 1 cm representing 1 month of hair growth. Individuals 1 and 2 reside in the USA. Individual 3 resides in the UK. Individual 4 moved from the UK to the USA in July 2004. Individual 5 moved from the USA to the UK in February 2004.

in January 2005. Our results clearly show a 3‰ difference between the mean values of residents of each continent. The USA values are enriched, as would be expected from the increased C_4 input to the diet. The USA values do not, however, reflect a complete dependence on C_4 plants but instead demonstrate that the livestock are fed a mixture of C_3 and C_4 plant products, and that the individuals themselves have varied diets incorporating food resulting from plants with both photosynthetic mechanisms.

Figure 1 shows the carbon isotope results from contiguous 1-cm sections from subjects with long hair. Each measurement thus reflects the dietary input over approximately 1 month.[14] Subjects were asked about their diets and stated that they did not change their diet significantly over the period of hair growth (approximately 18 months); thus any isotopic variation is likely to be the result of geographic differences. The hair from subjects who moved between the UK and the USA shows a clear adjustment in $\delta^{13}C$ during the time after relocation; commensurate with that between indigenous individuals. The time of isotopic adjustment to the new diet was about 4 months (assuming a growth rate of 1 cm/month) in both cases.

Relatively large variations were observed in $\delta^{13}C$ between the USA and UK values but also within the USA samples. Only general information was obtained about the diet and geographical movement within the home continent of the individuals studied, which leaves a potential unidentified contribution to isotopic variation from these inputs. Some of this can be hypothesised to be the result of unknown travel within the respective continents and concomitant variation in diet.

The $\delta^{13}C$ equilibrium time measured in this study is believed to be the first reported for human subjects moving between Europe and North America. A study by Jones *et al*. that examined isotopic equilibrium time after a change in the diet of cattle (C_3 to C_4) reported an equilibrium time of 74 days.[28] This is about half the time demonstrated by our findings, and the difference may largely be explained by the fact that the 1981 study was measuring a total dietary change from C_3 to C_4, whereas this present study was only of a partial change in the protein portion of the diet. Differences in metabolism and hair growth between cattle and humans may also contribute to the observed difference in hair isotope equilibration time but further comparative studies are needed to determine the significance of these factors. A study by O'Connell and Hedges examined equilibration of $\delta^{15}N$ in the hair keratin of an individual who changed from an omnivorous to a vegan diet.[12] They observed a rapid equilibration over the first 5 months after the dietary change, followed by a slower but still evident isotopic equilibration taking place up to 1 year after the change in diet. In our subject who moved to the UK, the effect is similar. For the subject who relocated to the USA, isotopic measurements were not taken for long enough to determine if a similar effect took place.

Isotope measurements on selected single amino acids

The $\delta^{13}C$ values of individual amino acids and groups of amino acids were measured from individuals in the USA and UK to determine the distribution of the C_3–C_4 isotopic differences. Amino acids that can be synthesised in the body, via various biochemical pathways, are called dispensable (non-essential) amino acids. Those that must originate from protein in the diet and have no biochemical synthetic pathways in the body are called indispensable (essential) amino acids; these must be supplied from the diet. Table 2 lists the amino acids present in hair keratin along with their concentration. Comparing the dispensable and indispensable amino acids isolated from British and North American keratin offers a useful way to separate the signal that necessarily comes from just the protein portion of the diet; in the case of indispensable amino acids, from that of potentially the whole diet, where carbon utilised in biosynthesis of dispensable amino acids can originate from different sources of macronutrient. The HPLC method used

Table 2. Weight percents of amino acids in hair keratin

Indispensable amino acids	(%)	Dispensable amino acids	(%)
Leucine	10.1	Glutamate	14.2
Threonine	5.7	Cysteine	10.7
Valine	4.1	Arginine	9.3
Phenylalanine	3.2	Serine	8.9
Lysine	2.0	Proline	8.5
Tryptophan	1.6	Aspartate	6.4
Histidine	0.7	Glycine	5.9
Methionine	0.6	Tyrosine	4.1
		Alanine	3.8

Table 3. $\delta^{13}C$ of dispensable and indispensable amino acids in hair keratin from two individuals residing in the UK and two in the USA

	Dispensable[a] (‰)	Indispensable[a] (‰)
UK	−26.9	−19.7
UK[b]	−26.5	−19.4
USA[b]	−24.7	−16.5
USA	−23.6	−15.8

[a] The fraction of dispensable amino acids includes threonine, and the indispensable amino acids include tyrosine.
[b] These two measurements were from the same individual who moved from the UK to the USA. The measurements labelled 'UK' were from samples obtained 1 month before the move, and those labelled 'USA' were from samples obtained 6 months after the move.

Table 4. Average $\delta^{13}C$ of single amino acids from British and American residents

Amino acid	British residents (‰)	American residents (‰)
Tyrosine[b]	−26.5	−24.7
Phenylalanine[a]	−27.9	−25.8
Leucine[a]	−26.8	−25.2
Methionine[a]	−27.2	−23.1
Valine[a]	−27.8	−22.5
Tryptophan[a]	−32.1	−19.9
Serine[b]	−17.7	−16.7
Cysteine[b]	−21.4	−18.0
Glutamate[b] + threonine[a]	−17.8	−14.1

[a]Indispensable amino acids.
[b]Dispensable amino acids.

allows for easy separation of these two classes of amino acids, although threonine, an indispensable amino acid, is grouped with the dispensable amino acids in this analysis. Tyrosine, a dispensable amino acid, is grouped with the indispensable amino acids, an appropriate designation since its sole biosynthetic pathway is from phenylalanine, an indispensable amino acid.[29]

Our results show that indispensable amino acids are more depleted than dispensable amino acids in samples from both continents, but to a slightly greater degree for the American samples (Table 3). Enrichment of dispensable over indispensable amino acids averages 7.2‰ in the UK and 7.9‰ in the USA. This shows that enrichment in indispensable and dispensable amino acids is prevalent in USA samples compared with UK samples but that dispensable amino acids may have a proportionally stronger C_4 influence in the USA. This is somewhat surprising if the C_4 input is largely via dietary protein (consumption of corn-fed livestock) as presumed. Interpretations from isotopic feeding studies so far confirm that indispensable amino acids are directly routed from diet to body tissues; hence we would expect the indispensable amino acids to reflect the $\delta^{13}C$ values of dietary protein.[20,30,31] Dispensable amino acids, on the other hand, are potentially directly routed *or* biosynthesised using carbon from the whole diet (or carbohydrates, in particular cases) to a variable degree for different amino acids. Since the majority of C_4 input to North American body tissues is presumably from the animal products consumed, we would expect that the amino acids most likely originating from the protein portion of the diet (the indispensable amino acids) would be more influenced by C_4 values and thus relatively enriched. However, because we see this effect in the British values as well, where the diet should be almost entirely C_3, a metabolic explanation is likely.

The difference between dispensable and indispensable amino acids can be further studied by measuring $\delta^{13}C$ values for individual amino acids. Although not all keratin amino acids were measured, due to limitations of the analytical method, we found that every amino acid isolated and measured from the North American samples was more enriched in ^{13}C than the British samples (Table 4). Similarly, amino acids measured at different times in an individual who moved from the UK to the USA show a systematic enrichment in single amino acids, due to increased C_4 influence (Table 5). This implies that the C_4 influence in American diets is ubiquitous, and affects dispensable and indispensable amino acids. Again, we see that the values for the six indispensable amino acids measured are, on average, more depleted in ^{13}C than the two dispensable amino acids, reinforcing the results presented in Table 3.

Single amino acid analyses of bone collagen from animal-feeding experiments and archaeological human remains demonstrate the same occurrence (enrichment of dispensable amino acids) in both human and animal collagen.[19,20,31] Fogel and Tuross report a $\Delta^{13}C_{\text{dispensable-indispensable}}$ of between 3.6‰ and 12.8‰ in humans, and a similar enrichment in herbivorous animals.[19] They attribute the difference to isotope fractionation in the biosynthesis of the amino acids. For instance, the indispensable amino acids synthesised in the citric acid cycle in plants (such as phenylalanine, leucine, and valine) are depleted by enzymatic selection of light isotopes in plant biosynthesis. Dispensable amino acids tend to reflect the whole diet; thus any C_4 input from protein, lipids or carbohydrates (such as corn syrup or sugar cane, both of which are C_4 plants) will be reflected in those amino acids.

Several suggestions, outlined below, are put forward to help explain these results at the molecular level. One explanation may be that only dietary protein contributes to the dispensable and indispensable amino acids found in hair keratin. They would therefore reflect a roughly equal proportion of the C_4 input. This seems unlikely, however, as there is evidence that a number of dispensable amino acids found in relatively large quantities in keratin, such as glycine, alanine, and glutamate, are preferentially biosynthesised in general from intermediates of glycolysis or the citric acid cycle before being incorporated into body proteins.[5,29,32]

Table 5. $\delta^{13}C$ of bulk hair keratin and single amino acids from one individual who moved from the UK to the USA in July 2004

	December 2003 (UK resident) (‰)	January 2005 (USA resident) (‰)
Tyrosine	−26.1	−24.6
Phenylalanine	−27.0	−25.8
Valine	−24.5	−22.5
Methionine	−24.4	−23.1
Bulk collagen	−20.1	−18.1

An alternative explanation for enrichment of dispensable amino acids in keratin from both the USA and the UK is that C_4 carbon is common in other non-protein foods in the diet. This is possible as added sugar from sugar cane is present in foods on both continents, and corn syrup, already mentioned, is particularly prevalent in processed foods in the USA. A C_4 influence on dispensable amino acids from biosynthesis of these compounds could explain the enrichment in ^{13}C of these amino acids compared with the indispensable amino acids depending on their prevalence in the diet.

Additionally, the various pathways used to biosynthesise the dispensable amino acids are likely to lead to some degree of isotope fractionation. Kinetic isotope fractionation occurs in any reaction that does not reach completion, and in the various routes used to synthesise amino acids, only fractions of the starting materials (various intermediates in glycolysis or the citric acid cycle) are used to make amino acids; the remainder of these intermediates go on to form other compounds.[29] Therefore, it is also possible that the isotopic fractionation of these complex series of reactions leads to the 7‰ enrichment of dispensable amino acids observed.

CONCLUSIONS

We have demonstrated a significant enrichment in the carbon isotope values of bulk hair keratin from subjects in the USA compared with the UK. The isotopic difference can be at least partially attributed to the different diets of livestock on each continent: C_4 maize in the USA and C_3 grasses in the UK. Changes in hair isotope values of three individuals who moved between the continents showed an adjustment in the $\delta^{13}C$ with an equilibration time of approximately 4 months.

Isotopic measurement of all single amino acids measured showed a consistent enrichment of $\delta^{13}C$ in all USA samples compared with UK samples. Interestingly, dispensable amino acids were found to be depleted compared to indispensable amino acids in individuals from both countries. This contradicted our expectations that the indispensable amino acids, being directly routed from the protein part of the diet, would most reflect the differences in C_3 and C_4 content of livestock on each continent. A possible explanation is that a significant C_4 input via carbohydrate in the diet, from added sugar or corn syrup, could be enriching the dispensable amino acids through biosynthesis. In addition, the various biosynthetic pathways for dispensable amino acids may exhibit inherent kinetic isotope fractionation resulting in the observed differences.

Additional work is ongoing, utilising single amino acid $\delta^{13}C$ analysis, to investigate the causes of these isotope differences.

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