Journal of Archaeological Science 1991, **18**, 277–292

The Isotopic Composition of Carbon and Nitrogen in Individual Amino Acids Isolated from Modern and Fossil Proteins

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(*Received 1 April 1990, revised manuscript accepted 1 September 1990*)

Pigs were reared in laboratory pens on controlled diets that consisted of either 100% C_3 plants or 100% C_4 plants. Carbon and nitrogen isotopic compositions of the diets, and the resulting pig products, purified collagen and muscle tissue, were measured to determine isotopic fractionation during growth and metabolism. Total collagen from pigs grown on C_3 diets was enriched in ^{13}C by 3·2‰ and in ^{15}N by 2·2‰, whereas that from pigs reared on C_4 diets was enriched in ^{13}C by 1·4‰ and in ^{15}N by 2·3‰. In addition, fractionation between pigs and their diets was determined at the molecular level on individual amino acids separated by ion exchange chromatography. The carbon isotopic compositions of separated amino acids from the C_3 and C_4 diets were transferred to amino acids in bone collagen. For nitrogen, the isotopic compositions of all non-essential amino acids were enriched in ^{15}N relative to those amino acids in the diet. Threonine, an essential amino acid, behaved oppositely, in that its isotope ratio ($\delta^{15}N$) was depleted by an average of 6‰ from the $\delta^{15}N$ of the whole collagen. Similar isotopic patterns were analysed in collagenous amino acids extracted from field specimens that included both herbivores and carnivores; marine animals and terrestrial animals; and C_3 and C_4 feeders. Amino acids from two fossil bones, a bison (4500 years old) and a whale (70,000 years old), recorded the same isotopic signals as modern collagen. The ubiquity of these isotopic patterns at the molecular level suggests that distinct biochemical mechanisms control the metabolism of amino acids in animals rather than random synthesis.

Keywords: CARBON ISOTOPES, NITROGEN ISOTOPES, BONE, COLLAGEN, AMINO ACIDS, DIET, DIAGENESIS, FOSSIL PROTEIN.

Introduction

Stable isotope ratios of both modern and fossil animal tissues have been used widely by ecologists, archaeologists, and geologists for assessing the diets of animals. In laboratory and field experiments, isotope ratios (δ) of animal tissues parallel closely the analogous signals in their diets to within a few per mil (‰)(DeNiro & Epstein, 1978; Fry & Parker,

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1978; Wada, 1980). This simple observation, however, may be complicated by several factors. Isotope changes or fractionations may occur when dietary protein is transformed into animal collagen and during diagenesis.

$$\delta \text{ dietary protein} \rightarrow \delta \text{ bone collagen} \rightarrow \delta \text{ fossil bone}$$

The exact magnitude of the small change in the isotopic ratio from diet to animal must be known in order to calculate percentages of any particular food source in an animal's diet. Fractionation will result as amino acids cycle through numerous biochemical pathways involved in metabolism. Furthermore, bone is a complex tissue of proteins, carbohydrates, and fats, all of which may have different isotopic compositions.

To interpret the isotopic ratios of fossil material one must recognize the effects of diagenesis on the structure and chemical composition of proteins, such as bone collagen. Collagen in bone experiences a range of post-mortem processes such as subaerial weathering, leaching by groundwater, and microbial attack. With time, bone may become contaminated by sedimentary organic matter. Thus, the isotopic and chemical composition of archaeologically-derived material may in some cases be compromised. Collagen itself undergoes partial hydrolysis that can affect the isotope ratio of the residual protein (Stafford *et al.*, 1988; Tuross *et al.*, 1988; Bada *et al.*, 1989). By analysing bone at the molecular level, biochemical pathways in the present and the past may be understood.

In order to assess the factors that influence the isotopic composition of bone, we examined collagen at the level of individual amino acids, thus reducing the chemical heterogeneity of samples. This molecular level approach to stable isotope analysis has been used with great success. The first experiments with nitrogen isotopes in separated amino acids demonstrated isotope fractionation during metabolism from diets to bones of laboratory-reared rats (Gaebler *et al.*, 1963, 1966). Subsequently, Macko *et al.* (1987) showed that amino acids from micro-organisms had distinct carbon and nitrogen isotope patterns that were associated with known biochemical pathways. In general, amino acids synthesised via transamination reactions had lower $\delta^{15}N$, relative to the whole protein. For carbon, amino acids with hydrocarbon R-groups had more negative carbon isotope ratios ($\delta^{13}C$) than total cells, which confirmed results by Abelson & Hoering (1961). Investigations have also been extended to the study of both carbon and nitrogen isotopes from amino acids separated from purified collagen (Stafford *et al.*, 1982, 1988; Hare & Estep, 1983; Tuross *et al.*, 1988).

Our approach was to raise domestic pigs on diets with known compositions, and then analyse $\delta^{13}C$ and $\delta^{15}N$ in individual amino acids from the food and in each pig's bone collagen. Pigs were chosen because of similarities in their digestive physiology and metabolism to those of humans (Kidder & Manners, 1978). To model the dietary causes of different carbon isotopic ratios in bone, we used diets composed of the endmembers of two photosynthetic plant systems: 100% C_3 or 100% C_4 plant diets. Most plants fix CO_2 during photosynthesis via a reaction that results in the formation of 3-carbon compounds and are termed C_3 plants. All woody plants, many shrubs, and some grasses are C_3 plants that are generally adapted to cooler, wetter environments. Other plants, notably many grasses and corn, initially fix CO_2 into 4-carbon compounds and are termed C_4 plants. C_4 plants are better adapted to drier and hotter environments. Carbon isotopes are discriminated uniquely by plants with these two photosynthetic modes (Smith & Epstein, 1971). The average carbon isotopic composition ($\delta^{13}C$) for C_3 plants is $-26{\cdot}5$‰ and ranges from -20 to 35‰, whereas C_4 plants average $\delta^{13}C$ is $-12{\cdot}5$ with a range of -9 to -16‰.

We also studied several herbivores and carnivores from modern and ancient environments to test the effects of diet and diagenesis on isotope signals. These field specimens are examples of materials that have been subjected to possible nutritional or water stresses or, in the case of the fossils, to diagenesis.

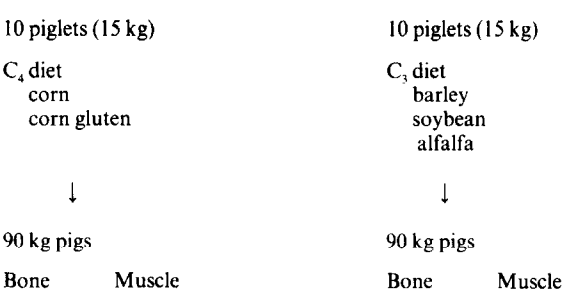

Figure 1. Experimental design for pig growth. Weanlings were born from several different mothers.

Table 1. Compositions of the C_3 and C_4 plant diet of pigs reared at Beltsville, MD. Compositions are based on dry weight and do not reflect % nitrogen or protein in the diet

Diet	Plant	% of diet	$\delta^{15}N$	$\delta^{13}C$
C_3	Soybean meal	10·0	−0·1	−24·0
	Barley	77·55	2·6	−25·3
	Selenium premix	0·05		
	Alfalfa	10·0	0·7	−26·0
	Calcium phosphate	1·7		
	Vitamins	0·1		
	Trace minerals	0·1		
	Iodized salt	0·5		
Total		100	1·8	−25·3
C_4	Ground yellow corn	67·35	6·3	−11·3
	Corn gluten meal	30	3·0	−13·2
	Lysine HCl	0·3	0·3	−12·4
	Selenium premix	0·05		
	Calcium carbonate	1		
	Calcium phosphate	0·6		
	Vitamins	0·1		
	Trace minerals	0·1		
	Iodized salt	0·5		
Total		100	3·2	−12·4

Methods and Materials

Growth of pigs

Pigs were reared in pens at the U.S. Department of Agriculture farm in Beltsville, MD, U.S.A. After weaning at 15 kg, 20 pigs were divided into two equal groups: one group was fed a diet consisting totally of C_3 plants, while the other was fed one of C_4 plants (Figure 1). Animals were raised until they reached 90 kg total weight. The muscle and whole bone were frozen immediately after the animals were killed. The composition and isotope ratios of the whole diet and dietary components are presented in Table 1. The dietary composition is reported strictly on a weight basis and does not reflect the relative amounts of dietary protein or calories. Lysine was added to the C_4 diet, because corn is notably deficient in this essential amino acid.

Bone samples

The modern whale bone was obtained from the Smithsonian Institution at autopsy and kept frozen until analysis. The fossil whale bone (70,000 years old) was obtained from Gifford Miller, University of Colorado. It was collected from Baffin Island, Canada. A North American lynx (*Lynx rufus*) was obtained from Margaret Schoeninger, Harvard University. Recent zebra and lion bones were collected at Lake Turkana, Africa, by A. K. Behrensmeyer, Smithsonian Institution. Fossil bison bone (4500 years old) was collected by T. W. Stafford from the Lubbock Lake site in the Texas Panhandle (Stafford, 1981).

Extraction of proteins from bones

Adhering muscle and fat were physically scraped from pig bones. Small chunks (3 g total weight) were then extracted with 250 ml of 4 M guanidine and 0·5 M EDTA at 4°C for 1 week. The solution, which contained non-collagenous proteins (Termine *et al.*, 1981), was decanted. A second solution of 0·5 M EDTA (tetrasodium salt at pH 7·2) was added, and the bones were further demineralized for an additional 7 days. Following non-collagenous-protein extraction and demineralization, the insoluble collagen was washed 14 times with distilled water over the next 7 days. Amino acid composition confirmed that the material was purified collagen.

Samples of other modern and fossil bones were demineralized in 1 M HCl at 4°C for 1–3 days. The samples were washed with distilled water until the wash reached neutrality. Material extracted with HCl conceivably contained some non-collagenous proteins, however, Tuross *et al.* (1988) have shown that collagen comprised the majority of residual protein.

Amino acid separation and analysis

Collagen and dietary proteins (approximately 100 mg dry weight) were hydrolysed in 6 M HCl at 105°C for 24 h. The acid was evaporated under vacuum, and an aliquot of the extract was analysed by HPLC (Hare, 1980) to confirm complete hydrolysis. Amino acids were redissolved in 0·05 M HCl (pH 2·0) and loaded onto a 0·9 × 50 cm glass column packed with cation-exchange resin that consisted of closely-sized, spherical resin beads, 10–15 μm diameter (St John's and Associates, Adelphi, MD). The column was packed in sections by a slurry technique with an inert solvent reservoir containing 2·5 M HCl under 400 pounds per square inch (psi) helium. The packed column was then equilibrated with 0·6 M HCl before applying the amino acid mixture. A sample loop of teflon tubing with a 1 ml volume was filled with the sample, which was then injected onto the top of the column. A series of stepwise-gradient changes in HCl concentration up to 2·5 M HCl were used to elute the entire range of amino acids from the column. Separation was monitored with a UV detector set at 214 nm (Figure 2). Pooled fractions (4–20 ml) of each amino acid were subsequently evaporated to dryness. Each fraction was analysed by HPLC analysis to prove its purity.

Isotope analysis

Amino acid hydrochlorides were redissolved in distilled water, and between 1–3 mg of each was loaded into a quartz tube (6 mm O.D. × 4 mm I.D.) in a volume of 0·5 ml. The samples were freeze-dried; CuO and metallic copper were added as described previously (Macko *et al.*, 1987). Evacuated tubes were combusted at 900°C for 1 h. Gases resulting from combustion were purified by cryogenic distillation on a vacuum line. The isotopic composition of carbon dioxide was analysed on a Nuclide 6-60-RMS, whereas that of nitrogen was analysed on a 6 in-60° sector field mass spectrometer constructed at the Geophysical Laboratory. Isotope ratios are defined as follows:

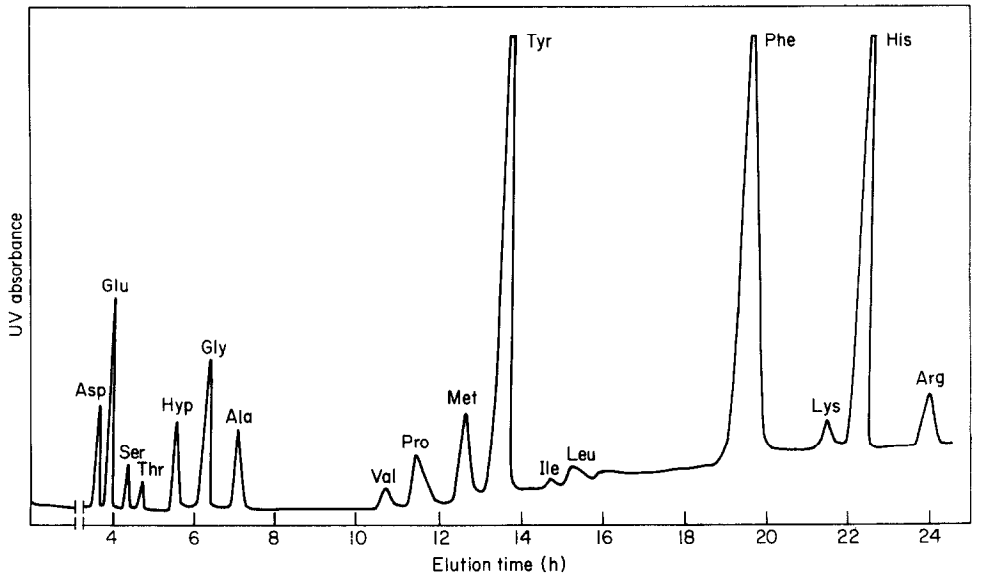

Figure 2. Separation of individual amino acids from collagen on cation exchange resin column with helium pressure elution with a gradient of 0·6 M–2·5 M HCl. Detection is at 214 nm wavelength. Absorbance at 214 nm is not indicative of absolute concentration, because each amino acid has different molar absorption.

$$\delta X = (R_{sample}/R_{standard} - 1) \times 10^3,$$

where $X = {}^{13}C$ or ${}^{15}N$ and $R = {}^{13}C/{}^{12}C$ or ${}^{15}N/{}^{14}N$.

To check for possible isotope fractionation during the separation of amino acids by column chromatography, a suite of controls were analysed before and after chromatography. Isotope ratios post-separation for both carbon and nitrogen were within ±0·4‰ of the initial values. In addition, to demonstrate the possible isotope fractionations that can occur during chromatography, fractions of a large amount of glycine were collected and analysed as above (Figure 3). Carbon isotope fractionation was minimal, but nitrogen isotope fractionation ranged over 30‰. The fractionations during chromatography demonstrated clearly that baseline separation and complete collection of individual amino acids must be accomplished for accurate and precise measurements of their isotopic compositions.

Results

Isotope composition of pigs and their diets

Whole tissues: Pigs reared on either C_3 or C_4 diets had $\delta^{13}C$ values of muscle and bone collagen that were similar to their diets (Tables 1 and 2). Nitrogen isotope ratios of bone collagen were about 2‰ more positive than that of the diet, which is within the range reported in other studies (e.g. Schoeninger & De Niro, 1984). The difference or isotope fractionation between the $\delta^{13}C$ of collagen and the $\delta^{13}C$ of the diet is + 1·4‰ in C_3 pigs and + 3‰ in C_4 pigs. These fractionations are several parts per mil less than the + 5‰ reported in the literature from field studies (e.g. van der Merwe & Vogel, 1978). This isotopic fractionation is commonly used to assess the nature of animal and human diets. Isotopic fractionations in laboratory experiments with rodents, either mice or gerbils whose

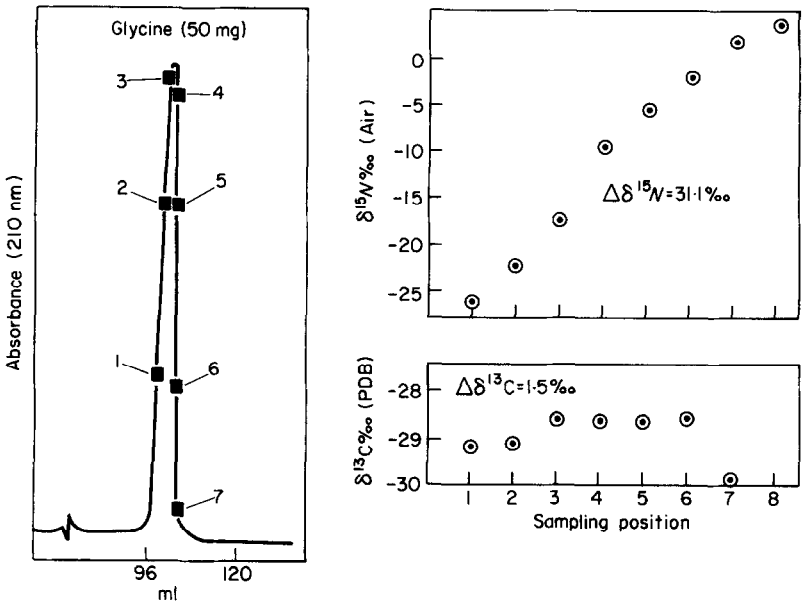

Figure 3. Isotope fractionation during the separation of glycine from an ion exchange column. A 50 mg quantity of glycine was loaded onto the ion exchange column. Fractions were collected across the peak. Each individual fraction was dried, combusted, and analysed as in Materials and Methods.

Table 2. Isotope ratios of total protein in diet, bone and muscle of C_3 and C_4 pigs

	Isotopic compositions of controlled-diet pigs			
	$\delta^{13}C$	$\Delta^{13}C$	$\delta^{15}N$	$\Delta^{15}N$
C_4 pigs				
Diet	−12·4		+3·2	
Muscle	−11·4	1·0	+5·0	1·8
Collagen	−9·2	3·2	+5·5	2·3
Faeces	−12·8	−0·4	+2·3	−0·9
C_3 pigs				
Diet	−25·3		+1·8	
Muscle	−23·8	1·5	+2·7	0·9
Collagen	−23·9	1·4	+4·0	2·2
Faeces	−25·7	−0·4	+5·1	3·3

$\Delta = \delta$ pig or faeces sample $- \delta$ diet.

digestive systems are not similar to humans, were in the order of 2–4·5‰ (DeNiro & Epstein, 1978; Tieszen *et al.*, 1983).

Separated amino acids, carbon isotopes: A wide range in isotopic compositions in amino acids from both the diet and the resulting pig bone collagen was measured (Tables 3 and 4; Figure 4). For carbon, a comparison of the $\delta^{13}C$ values for amino acids of the diet and in

Table 3. Isotopic ratios of amino acids in pig diets

Amino acid	C_3 diet		C_4 diet	
	$\delta^{13}C$	$\delta^{15}N$	$\delta^{13}C$	$\delta^{15}N$
Asp	−24·1	4·0	−11·6	3·4
Glu	−25·2	2·5	−12·9	1·1
Ser	−13·8	−1·6	1·3	2·1
Thr	−14·8	−1·3	−1·9	−0·1
Gly	−17·3	0·4	−5·7	3·6
Ala	−23·4	2·8	−11·9	2·5
Val			−16·7	5·6
Pro	−27·4	3·7	−13·2	4·6
Total diet	−25·3	1·8	−12·4	3·2

Table 4. Isotope ratios of amino acids separated from the collagen of laboratory-reared pigs grown on controlled diets. Comparisons of isotope ratios of animal amino acids to those in the diet

Amino acid	Pigs grown on C_3 diet				Pigs grown on C_4 diet			
	$\delta^{13}C$	$\Delta^{13}C^*$	$\delta^{15}N$	$\Delta^{15}N^*$	$\delta^{13}C$	$\Delta^{13}C$	$\delta^{15}N$	$\Delta^{15}N$
Asp	−21·5	2·6	5·7	1·7	−8·1	3·5	7·0	3·6
Glu	−19·2	6·0	7·4	4·9	−5·5	7·4	7·8	6·7
Ser	−12·1	1·7	1·6	3·2	2·5	1·2	4·7	2·6
Thr	−14·1	0·7	−5·7	−4·4	−1·9	0·0	−6·2	−6·1
Gly	−16·9	0·4	1·2	0·8	−4·8	0·9	5·8	2·2
Ala	−22·0	1·4	4·7	1·9	−8·5	3·4	6·1	3·6
Val	N.D.	N.D.	N.D.	N.D.	−15·4	1·3	9·7	4·1
Pro	N.D.	N.D.	N.D.	N.D.	−11·2	2·0	8·2	3·6
Hyp	−22·9	N.D.	7·2	N.D.	−10·6	N.D.	8·1	N.D.

$\Delta^{13}C^* = \delta^{13}C$ collagen amino acid $- \delta^{13}C$ diet amino acid.
$\Delta^{15}N^* = \delta^{15}N$ collagen amino acid $- \delta^{15}N$ diet amino acid.
N.D. = not determined.

the collagen shows the effects of both incorporation of dietary isotope signals and fractionations during biosynthesis in the pig. For example, dietary glycine had a carbon isotope ratio 8‰ more positive than the total C_3 diet (Table 3). This isotopically-enriched glycine was incorporated almost directly into the pig's bone collagen. Glycine is the most abundant amino acid in collagen (33%), which causes the bulk isotopic composition to be several ‰ more positive than the diet.

Two other major constituents of collagen are glutamate (7%) and aspartate (5%). The carbon isotope compositions of these amino acids in collagen from both C_3 and C_4 pigs were enriched in ^{13}C relative to glutamate and aspartate in the diet by 6 and 3‰, respectively (Table 4). Thus, fractionation during metabolism is an additional reason why the $\delta^{13}C$ of total collagen is enriched relative to the bulk diet.

Proline, a secondary amino acid (imino acid), is synthesized by ring closure of glutamate. Hydroxyproline is derived directly from proline by enzymatic hydroxylation.

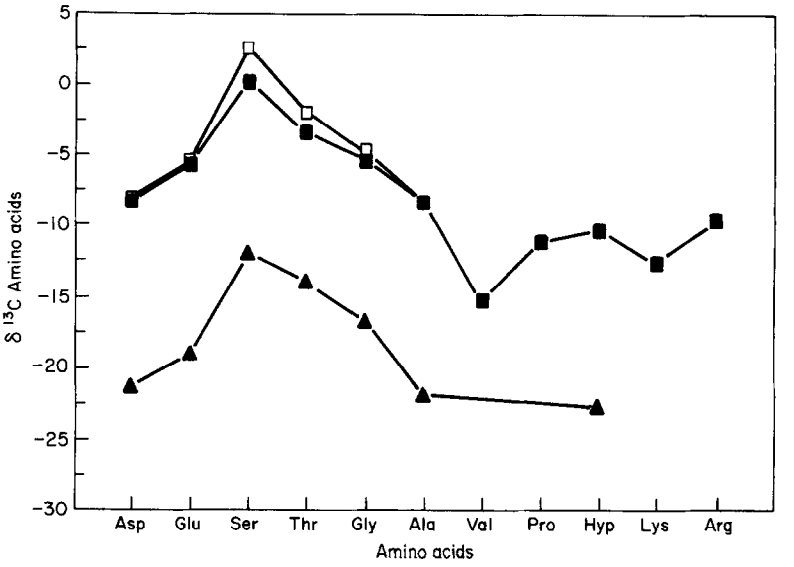

Figure 4. Carbon isotope ratios of individual amino acids separated from bone collagen of C_3 and C_4 pigs. (—□—), C_4 pig no. 1; (—▲—), C_3 pig; (—■—), C_4 pig no. 2.

In addition, the hydroxylation of proline occurs after its translation into the collagen molecule (Udenfriend, 1966). Therefore, hydroxyproline in bone is synthesized solely in the animal. Both these imino acids had different isotope ratios from glutamate in both C_3 and C_4 grown pigs (Table 4 and Figure 4). An animal obtains some proline from its diet. However, because the concentrations of imino acids in collagen are greater than the diet, a portion of proline in addition to all of the hydroxyproline must be synthesized by the animal. Accordingly, in the pig the isotope ratios of imino acids have isotopic compositions based on composite isotope values of both proline and glutamate.

Nitrogen isotopes: The positive isotopic fractionation between bone and diet is displayed on the molecular level for all amino acids except threonine (Figures 5 and 6). Isotope fractionation between amino acids in bone relative to the amino acids in the diet was greatest for glutamate, a key amino acid in numerous transamination reactions. Its amino group is donated to keto acids to form other amino acids, i.e. aspartate, alanine, and valine (Cammarata & Cohen, 1950). In addition, the first step in the formation of urea occurs during the deamination of glutamate by glutamate dehydrogenase. Although the isotope fractionation by this enzyme has not been determined directly, excreted nitrogen products from a diverse group of animals are generally isotopically depleted in ^{15}N relative to the whole organism (Kreitler, 1975; Checkley & Miller, 1990).

Threonine was consistently depleted in ^{15}N relative to both the diet and all other amino acids in collagen. Threonine is an essential amino acid in all mammals. Therefore threonine in the pig's proteins must originate from the diet. Obviously, threonine in animal collagen has undergone some catabolism, otherwise the isotope ratios in diet and bone should be identical within experimental error ($\pm 0{\cdot}5‰$). Threonine has been shown to donate some of its nitrogen into the common nitrogen pool of an animal (Elliot & Neuberger, 1950; Meltzer & Sprinson, 1952) but the enzymes necessary to reaminate the resulting keto acid are not present. Most enzymatic reactions are associated with normal

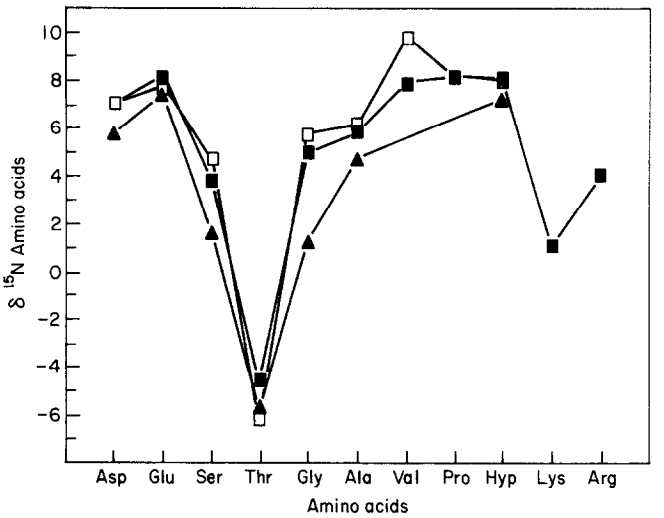

Figure 5. Nitrogen isotope ratios of individual amino acids separated from bone collagen of C_3 and C_4 pigs. Key as for Figure 4.

isotope effects in which the product is enriched in the lighter isotope, while the residual reactant becomes enriched in the heavier isotope (e.g. Macko *et al.*, 1986). Threonine deaminase may be an unusual enzyme having a reaction mechanism with an inverse isotope effect.

Muscle tissue: Two samples of muscle tissue were analysed for four individual amino acids: glutamate, aspartate, threonine, and serine. For these particular amino acids, the isotopic compositions in muscle were similar to those in bone. From this limited sampling, synthesis of amino acids in muscle and bone tissues appeared to be catalysed by similar enzymatic reactions.

To conclude, whatever the processes, similar metabolism of a C_3 and a C_4 diet occurred in the gut and tissues of the pigs. Accordingly, the pattern of isotopic distributions in both pigs is almost identical with only a shift in the absolute ratios (Figures 4 and 5).

Isotope ratios of amino acids separated from field specimens

Nitrogen and carbon isotope distributions in herbivores and terrestrial and marine carnivores are consistent across trophic levels, environments, and diets (Figures 7, 8 and 9). For example, both the lion (African) and the lynx (North American) had identical patterns of carbon isotopic fractionation in their separated amino acids even though the absolute values differed. Lions feed on animals subsisting on C_4 plant species, while the lynx relies on a diet of small mammals eating primarily C_3 plants.

Similar patterns for isotopic fractionation among amino acids were measured within both modern and fossil bone collagens and can be directly compared with the pig data (Figures 4 and 5). Threonine gave the most unusual isotopic ratio for both carbon and nitrogen. Threonine and lysine are essential amino acids for most animals, and participate in the internal metabolic nitrogen cycle by only donating nitrogen into a common pool (Foster *et al.*, 1939; Schoenheimer *et al.*, 1939; Cammarata & Cohen, 1950). Most other amino acids are able to accept and to donate nitrogen from a common pool.

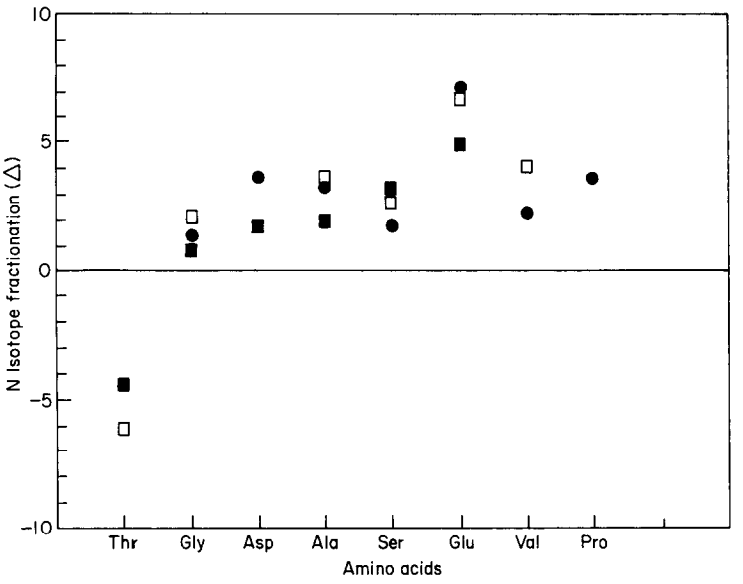

Figure 6. Nitrogen isotope fractionation between amino acids from bone and those in the diet. Fractionation is defined as $\delta^{15}N$ bone $-\delta^{15}N$ diet. (□), C_4 pig no. 1; (●), C_4 pig no. 2; (■), C_3 pig.

The zebra from Lake Turkana is an extreme example and had the most positive $\delta^{15}N$ value for total collagen. However, the pattern for individual amino acids followed those of the other bones. The principal difference in the zebra amino acid $\delta^{15}N$ values was the greater enrichment ^{15}N in glutamate and aspartate. Interestingly, the carbon isotopic compositions of these amino acids [Figure 6(b)] were similar to those in pigs and carnivores. Because the patterns for carbon and nitrogen isotopes in eight amino acids were not random, we conclude that the enrichment in ^{15}N did not result from diagenesis.

The isotopic composition of proline and hydroxyproline was in most cases similar to that of glutamate, which is expected as proline is synthesized directly from glutamate. Accordingly, hydroxyproline is synthesized after translation in the collagen molecule. In certain instances, the isotopic composition of proline was not equal to that of glutamate. The differences may occur because of a direct input of proline into the diet of the animal.

The isotope compositions of amino acids from fossil material were comparable to those of modern collagen. In these bones, intramolecular isotope fractionations were preserved over time. As long as the collagen itself remains intact, we expect that the universal pattern measured in modern herbivores and carnivores from a wide variety of environmental settings will be found in fossil bone.

Discussion

The use of individual amino acids for stable isotope analyses has considerable advantages over analysis of bulk animal or plant tissue. Although separation of amino acids for isotopic analysis is a lengthy and tedious procedure, one amino acid in the diet can theoretically be traced throughout the food chain. Isotopic tracers are integrative, in that the isotopic composition of a molecule records its biochemical history in the food chain. A second benefit would result from an understanding of the isotopic fractionation between collagen and an animal's diet. Chromatographic isolation of individual amino acids is not

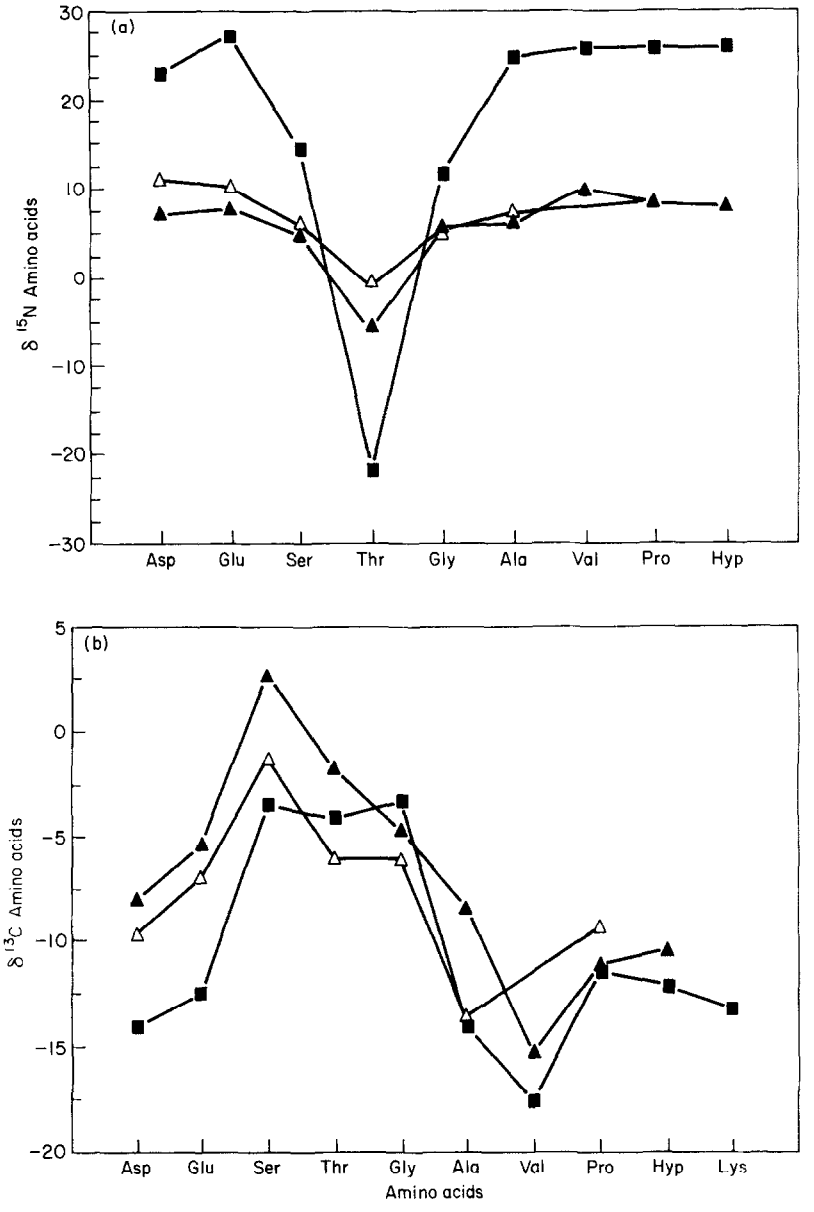

Figure 7. Carbon and nitrogen isotope ratios of terrestrial herbivores. (a) Nitrogen. (b) Carbon. (—▲—), C_4 pig; (—■—), zebra; (—△—), fossil bison.

routine, but this degree of purification eliminates lipids, carbohydrates, and other amino acids that are present in varying amounts in bone. As a result, processes that contribute to the isotopic fractionation between an animal and its diet can be evaluated. Factors that govern the isotopic composition of bone will be invaluable in the final interpretation of modern and fossil food webs.

The experiments on laboratory-reared pigs provide a first step in tracing individual amino acids into an animal's diet. Because the C/N in amino acids of collagen is typically

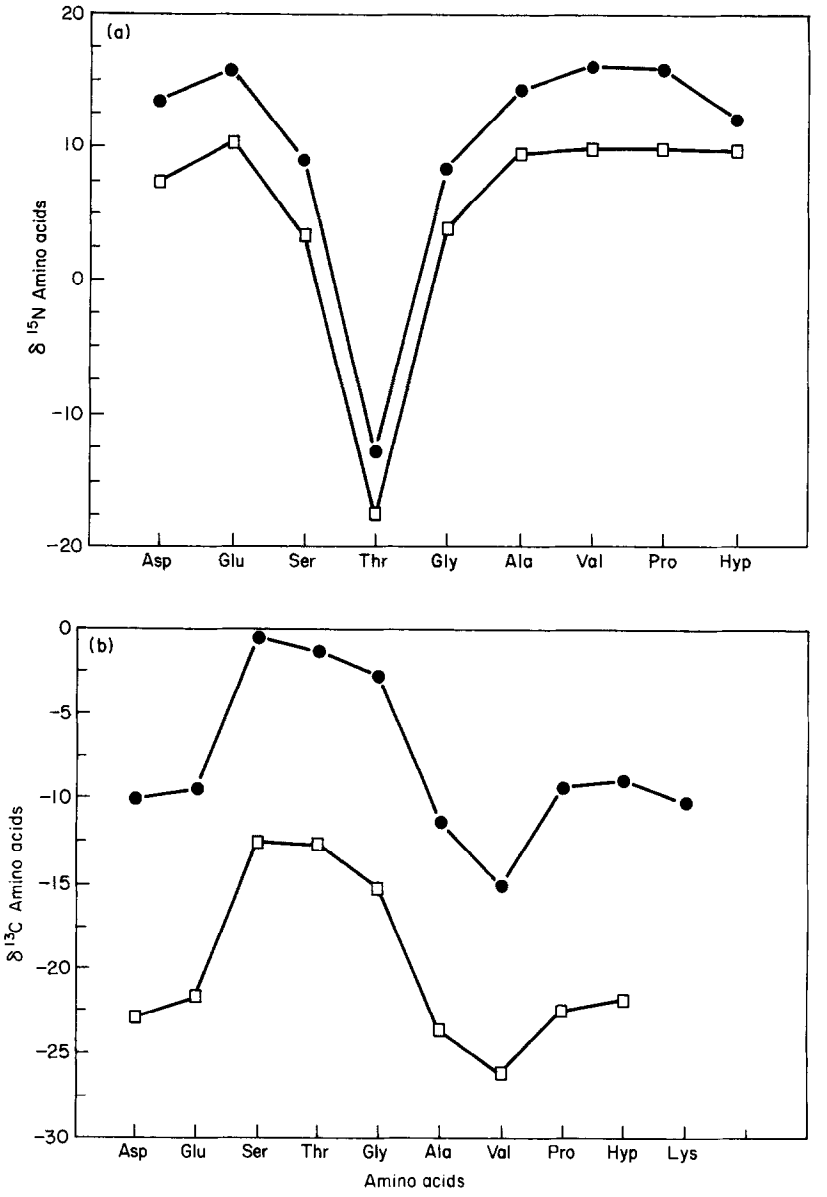

Figure 8. Carbon and nitrogen isotope ratios of terrestrial carnivores. (a) Nitrogen. (b) Carbon. (—●—), Lion; (—□—), lynx.

3/1, a single manipulation with nitrogen could cause an obvious isotope fractionation, whereas multiple enzymatic changes in amino acid structures must occur before changes will be measured in carbon isotopes. Therefore, carbon isotope ratios of amino acids were more consistent than nitrogen isotope ratios and have values almost identical to diet. The source of lysine in the diet of corn-dependent populations of humans would be a particularly interesting amino acid to study. Corn proteins contain inadequate amounts of lysine for human nutrition. Thus, lysine in collagen from human populations, that were dependent on corn, must have originated from the remaining constituents in the diet.

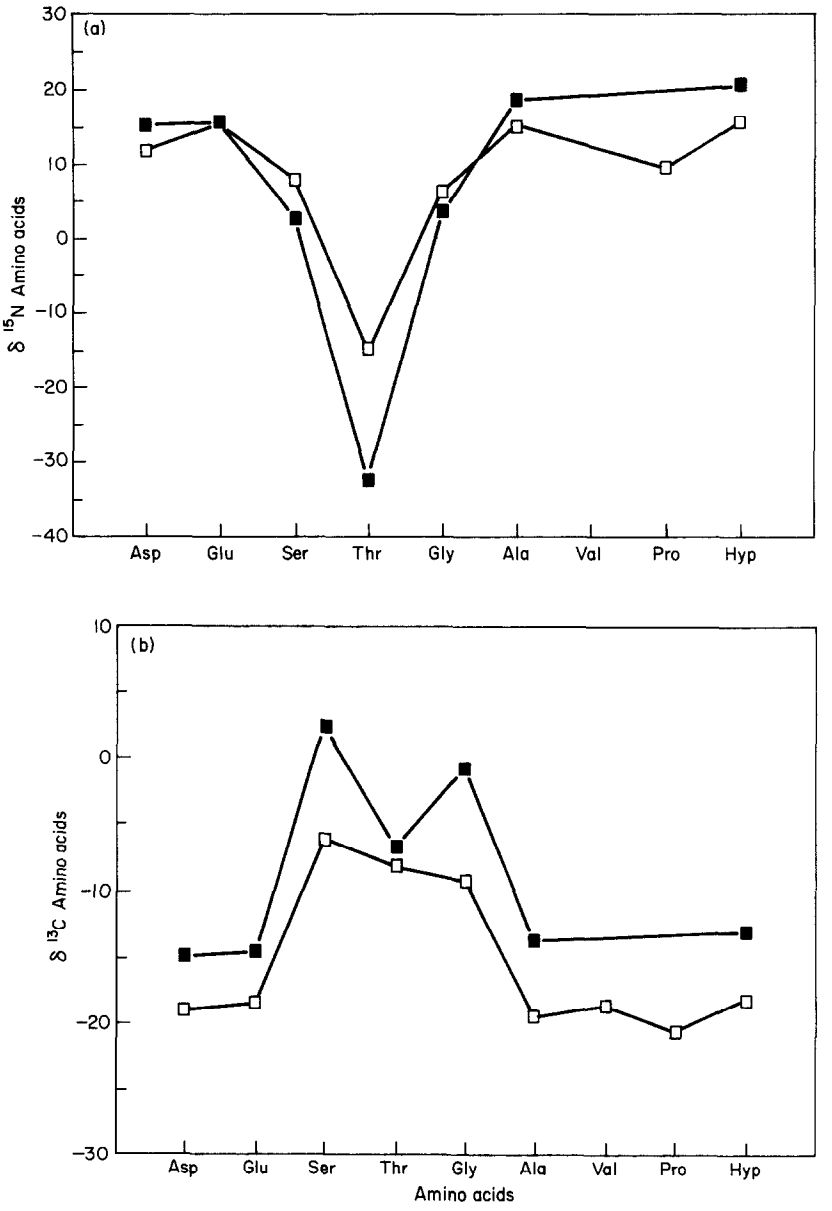

Figure 9. Carbon and nitrogen isotope ratios of marine carnivores. (a) Nitrogen. (b) Carbon. (—□—), Fossil whale; (—■—), modern whale.

A basic understanding of the isotopic fractionation from diet to bone has also resulted from the analysis of individual amino acid components. Our data show that the unusual amino acid composition of collagen affects its isotopic composition, because collagen contains more isotopically-distinct glycine than all other animal proteins. In pig bones, the $\delta^{13}C$ of collagen was enriched by 2–3‰, a fractionation similar to those measured in previous laboratory experiments. In field or in archaeological studies when the isotopic

composition of diet needs to be inferred from that in bone, this smaller, experimentally-determined isotopic fractionation has been ignored. It appears, however, that the fractionation determined from the field of 5‰ best describes the isotopic relationship of carbon from diet to bone.

Pigs in our experiments were young, rapidly-growing animals. Thus, gross synthesis exceeded net resorption, while rates of both synthesis and resorption are high in juvenile bone. Accordingly, incorporation of dietary amino acids was significantly more important than reincorporation of amino acids resulting from a lifetime of slow but significant collagen turnover. In contrast, collagen from archaeologically-derived humans or field specimens of animals, e.g. the African mammals (Heaton *et al.*, 1986; Sealy *et al.*, 1987), was sampled from organisms in which resorption and reincorporation of amino acids were dominant processes. Reworking of collagen in adult bone must therefore, result in additional isotopic fractionation of both carbon and nitrogen. The increased isotope fractionation can be seen on the molecular level in individual amino acids. In general, differences in isotope ratios of separated amino acids are consistent from one animal to the next (Figures 4–9). Glutamate, for example, which donates an amino group in the urea cycle, is always enriched in ^{15}N relative to the whole protein. In the zebra that suffered some dietary stress, the enrichment in ^{15}N of glutamate is twice that in pigs.

Threonine is another amino acid with an isotopic composition especially diagnostic of resorption or reincorporation processes. In either terrestrial herbivores or marine carnivores the isotopic ratio of threonine is severely fractionated relative to the whole protein. In fact, nitrogen isotope ratios of $-30‰$ have never been recorded in the biosphere (Owens, 1987; Fogel & Cifuentes, 1991). Because threonine transaminase does not occur in mammals (Elliot & Neuberger, 1950; Meltzer & Sprinson, 1952), the unusual isotopic ratio of this molecule must occur as a result of a degradative metabolic process rather than a synthetic one.

Diet determination with fossil material is even more difficult because of the following. Firstly, exogenous compounds, i.e. humic and fulvic acids and adsorbed proteins and peptides, can contaminate bone and skew isotopic ratios. Secondly, diagenetic alteration of the collagen molecule will shift the relative proportion of amino acids, which results in a different mass balance, and consequently a different total-protein isotopic composition. The technique of separating amino acids for isotopic analysis is one way of providing some insulation from diagenesis. If intact collagen exists, isotope ratios of the total molecule give reasonable and interpretable dietary signals. When the collagen molecule is partially hydrolysed, a residual protein with high concentrations of glutamate and aspartate and very little hydroxyproline remains (Masters, 1987; Stafford *et al.*, 1988; Tuross *et al.*, 1988). The effect from selective preservation of aspartate and glutamate, in greater proportions in non-collagenous proteins, would cause a total bone preparation to have more negative $\delta^{13}C$ and more positive $\delta^{15}N$. Isotope ratios of bone collagen with $\delta^{15}N$ values over 15‰ and $\delta^{13}C$ values less than $-24‰$ should be scrutinized carefully.

If intact collagen is not preserved, individual amino acid isotopic compositions may still be useful. The $\delta^{13}C$ of glutamate, proline and especially hydroxyproline should be compared, as they should all be similar. If they differ, the presence of exogenous material is indicated. Serine, a particularly labile amino acid, is frequently in much higher concentrations in contaminating proteins. Its isotope ratio could be a good indicator of either intact protein or contamination during diagenesis.

In conclusion, the isotopic composition of individual molecules can be followed from the diet into the bone, especially for carbon. Many animals have similar patterns in the composite isotopic labelling even though they varied in feeding habits. Exceptions include the essential amino acids that appear to be controlled by an individual animal's metabolism. From the data presented in this paper, separation and isotopic analysis of

the first seven amino acids in Figure 2 would provide enough information to study metabolism and diagenesis in any sample. The distinctive patterns measured in modern bones are also retained in well-preserved fossil bones. Thus there is an additional tool for detecting the diets of organisms that lived in the past.

Acknowledgements

This study was funded by a grant from the National Science Foundation to M. L. Fogel (Estep) and P. E. Hare (Grant No. EAR 83-13164). The authors thank David J. Velinsky, Paul L. Koch, and Noreen Tuross for critical reviews of the manuscript.

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