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Intra-bone Variability of Collagen and Apatite Isotopic Composition Used as Evidence of a Change of Diet

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A study of intra-individual variability of the isotopic composition of mineralized tissues was conducted on five modern steers (*Bos taurus*) whose diet shifted in the middle of their life time from a C_3 milk-based diet to a C_4/C_3-herbivorous diet. This dietary change was reflected in $\delta^{15}N$ and $\delta^{13}C$ values of collagen and carbonate hydroxylapatite from both tooth and bone. Material from the top and the bottom of the second lower molar gave isotopic signals of collagen and apatite synthesized exclusively before and after the change of diet. In the jawbone, the change of diet was reflected in a difference of isotopic composition of material sampled in the first molar socket zone (M1-bone) on the one hand, and in the dental bud socket zone (db-bone) on the other. M1-bone and db-bone were both found to be made of a mixture of ante- and post-dietary change material. However, the amount of newly synthesized material was clearly higher in db-bone, which was expected because of the remodelling of this part of the jawbone, to allow dental eruption.

Keywords: CARBON AND NITROGEN ISOTOPES, BONE, TOOTH, INTRA-INDIVIDUAL VARIABILITY, DIETARY CHANGE, WEANING, *BOS TAURUS*.

Introduction

The intra-individual variability of the isotopic composition of the mineralized tissues of vertebrates has been reported in many studies on palaeodiet and palaeoenvironment. A high variability of isotopic composition has been noticed at the individual level, between different kinds of mineralized tissues (dentine/bone: Bocherens, Fizet & Mariotti 1992, 1994; Bocherens *et al.*, 1995; Fizet *et al.*, 1995; Sealy, Armstrong & Schire, 1995; enamel/bone: Grupe *et al.*, 1997), between different anatomical elements made of the same tissue (enamel from different teeth: Bryant *et al.*, 1996*a*; Fricke & O'Neil, 1996; Lee-Thorp, Manning & Sponheimer, 1997; dentine from different teeth: Balasse, 1996; bone from different pieces of the skeleton: Rodière, 1995) and between different parts of an anatomical element (intra-tooth isotopic variations of the mineral fraction of dentine or enamel: Koch, Fischer & Dettman, 1989; Bryant *et al.*, 1996*b*; Fricke & O'Neil, 1996; Stuart-Williams & Schwarcz, 1997; Lee-Thorp, Manning & Sponheimer, 1997; intra-bone isotopic variations of collagen: Fizet, 1992).

This variability has been used for a few years as a valuable source of information. Intra-individual variations in $^{15}N/^{14}N$ ratio between dentine collagen from early-formed teeth and bone collagen may be the consequence of the change of diet occurring with weaning. The use of this difference has been suggested as a means of testing the preservation of the isotopic signal during diagenesis (Bocherens *et al.*, 1995). Intra-individual variation of the isotopic composition of dental tissues (dentine or enamel) on the one hand and bone on the other might reflect residential mobility (Sealy *et al.*, 1995; Grupe *et al.*, 1997). Differences in oxygen or carbon isotopic composition of enamel from different teeth of a single individual are linked to the timing of teeth growth. They can give information about seasonality of birth (Bryant *et al.*, 1996*a*, *b*) or show seasonal dietary shifts (Lee-Thorp *et al.*, 1997). Intra-tooth variations of the enamel or dentine oxygen isotopic composition result from the seasonal cycle and can be used as a measure of the season of death (Koch *et al.*, 1989); such variations have also been used to estimate the rate of enamel growth (Fricke & O'Neil, 1996).

However, each time intra-individual variability in the isotopic composition of bone collagen has been noticed, the need for an awareness of this has been emphasized when defining the sampling strategy, to avoid introducing any methodological bias in inter-individual comparisons (Fizet, 1992; Rodière, 1995). This potential bias could nevertheless be turned into an advantage with a better knowledge of the way a change of diet can find expression in intra-bone variability of nitrogen and carbon isotopic signatures of collagen and carbonate hydroxylapatite. Such is the aim of our study.

Material

The study was conducted on five mandibles from modern steers (*Bos taurus*) which, once weaned, underwent a transition from a C_3 to a C_4/C_3 mixed diet. They

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Table 1. Carbon and nitrogen contents (%), $\delta^{13}C$ and $\delta^{15}N$ values of grass from meadows and of each component of the fattening diet of the steers

	Component	Weight (kg)	% C	% N	% C total diet	% N total diet	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)
Meadow	Grass	*ad lib.*					−26·5	+1·0
Stall 5	Maize silage (C_4)	7·0	44·6	1·1	65·6	36·6	−12·1	+4·4
	Maize grain (C_4)	2·5	44·6	1·4	23·4	16·6	−11·6	+5·0
	Soya bean (C_3)	1·2	43·5	8·2	11·0	46·8	−25·1	+0·7
	Total						**−13·4**	**+2·8**
Stall 6	Maize silage (C_4)	7·0	44·6	1·1	63·0	34·5	−12·1	+4·4
	Soya bean (C_3)	1·2	43·5	8·2	10·5	44·0	−25·1	+0·7
	Wheat (C_3)	3·0	43·8	1·6	26·5	21·5	−25·4	+4·2
	Total						**−17·0**	**+2·7**

came from an experimental farm (Vendée, France) where their diet had been very carefully controlled from birth to slaughtering. Until they were 7 months old, the calves were raised essentially on milk, while their mothers were fed with a C_3 fodder. Between 7 and 9–10 months, the calves followed their mothers on meadows and grazed C_3 grass: they were still suckling and were progressively weaned. Weaning was effective when the calves were separated from their mothers and brought to stall, where they were fattened with a C_4/C_3 mixed diet until they were slaughtered, 8 months later. The calves labelled V2, V4 and V6 were kept in stall 6, where they were raised on maize silage (a C_4 plant), soya bean and wheat (C_3 plants). In stall 5, where V3 and V5 were kept, wheat was replaced by maize grain. In each case, the C_4 proportion of the total diet was predominent, even if it was lower in stall 6 (Table 1).

Sampling Strategy

Weaning coupled to a transition from a C_3 to a C_4/C_3 mixed diet should be reflected in both nitrogen and carbon isotopic composition of organic (collagen) and inorganic (carbonate hydroxylapatite, called apatite in the following text) fractions of bone tissue. From the trophic step down occurring during weaning there is a change in the nitrogen isotopic composition of collagen (Schoeninger & DeNiro, 1984; Fogel, Tuross & Owsley, 1989; Katzenberg, Herring & Saunders, 1996). As C_3 and C_4 plants have different carbon isotopic compositions linked to their different photosynthetic modes (Deines, 1980; O'Leary, 1981), a change from a C_3 to a C_4 diet will also be reflected in the carbon isotopic signature of collagen and apatite.

The dietary change occurred in the middle of the steers' lifetime, at a period of fast growth. At death, the bone was expected to contain a mixture of material synthesized before and after the dietary change. However, different parts of a bone piece are not affected in the same way by growth, so that, from one zone to another, the proportion of newly synthesized material may not be the same. A double sampling was performed on each jawbone: a first chunk of bone was sampled at the first molar socket zone (M1-bone) and a second one in the third molar socket zone (db-bone). The third molar was present as a dental bud, around which bone was being remodelled to allow dental eruption. It was thus expected to contain a high proportion of newly synthesized material. As the first molar is already erupting at birth, the alveolar bone was expected to have a lower turnover rate, and to contain less newly synthesized material.

To assess the extent to which the isotopic composition of a given bone zone had been affected by the dietary change, we also analysed enamel and dentine from the second molar, to determine the isotopic compositions of collagen and apatite synthesized exclusively before and after the dietary change. The lower second molar starts growing during the second month after birth. Its crown is not complete before the end of the first year (Brown *et al.*, 1960), and its roots were still growing at the steers' death. Dental tissues are not renewed once formed and preserve an isotopic composition linked to the diet at their time of growth. Dentine samples from the top (M2-t) and from the bottom (M2-b) of the tooth, and enamel from the top (M2-te) and from the bottom (M2-be) of the crown, were thus expected to contain, respectively, collagen and apatite formed before and after the dietary change.

Analytical Procedure

Defleshed mandibles were boiled and the remaining muscle removed by an enzymatic treatment (papaine). Each bone sample is a 2-cm^2 plate, taken off with a circular saw from the vestibular side of the jawbone. After the external surface (2 mm) and the spongiosa had been removed, the bone was powdered to less than 0·7 mm. Collagen was extracted from about 100 mg of this powder as previously described by Bocherens *et al.* (1991). One lower second molar from each steer was demineralized (EDTA solution, 0·5 M, pH = 7·4). Dentine was sampled from the 4 uppermost and lowermost

Table 2. $\delta^{15}N$ and $\delta^{13}C$ values of collagen from jawbone sampled in the first molar socket zone (M1-bone) and in the dental bud socket zone (db-bone), and from dentine from the top (M2-t) and the bottom (M2-b) of the second lower molars of the steers; $\delta^{13}C$ values of apatite from the jawbone and enamel from the top (M2-te) and the bottom (M2-be) of the second molar crown

		$\delta^{15}N$ collagen (‰)				$\delta^{13}C$ collagen (‰)				$\delta^{13}C$ apatite (‰)			
Steer	Stall	M1-bone	db-bone	M2-t	M2-b	M1-bone	db-bone	M2-t	M2-b	M1-bone	db-bone	M2-te	M2-be
V2	6	+5·7	+4·9	+6·9	+5·6	−16·9	−13·5	−19·7	−14·2	−8·4	−5·1	−9·7	−3·7
V4	6	+6·0	+5·6	+7·8	+5·4	−17·9	−15·8	−21·1	−13·6	−9·7	−6·7	−13·3	−2·5
V6	6	+6·8	+6·0	+8·0	+5·7	−19·2	−14·7	−20·6	−14·1	−10·9	−6·1	−13·1	−3·5
V3	5	+6·8	+6·3	+8·4	+5·2	−17·3	−14·0	−21·1	−12·3	−9·3	−4·8	−12·2	−0·6
V5	5	+6·9	+6·6	+8·1	+5·7	−18·1	−14·7	−21·2	−10·5	−9·5	−4·5	−11·8	0·0

millimetres of the organic frame of the tooth. Dentine samples were homogenized by solubilization (HCl 10^{-2} M, 100°C, 17 h), filtered and freeze-dried. Nitrogen and carbon isotopic compositions of collagen from bone and dentine were measured by isotopic ratio mass spectrometry (Rodière *et al.*, 1996). The analytical precision is 0·2‰ for $\delta^{15}N$ and 0·1‰ for $\delta^{13}C$ analyses.

An aliquot of 100 mg of bone powder was used for apatite extraction. The powder was soaked in a 2–3% NaOCl solution for 3 days at room temperature, in order to remove organic matter. It was then rinsed with distilled water, treated with a 1 M acetic acid–Ca acetate buffer (pH=4·75) for half a day, then thoroughly rinsed with distilled water. Finally, the powder was dried overnight at 60°C. Enamel was collected from the teeth by drilling about 40–50 mg of powder. Enamel samples underwent the same treatment as bone, but spent only 2 days in the NaOCl solution. Apatite was isotopically analysed by mass spectrometry as previously described (Iacumin *et al.*, 1996) with an analytical precision of 0·1‰.

Each component of the fattening diet was sampled, as well as a representative sample of grass from the meadows. These samples spent 24 h at 60°C. A handful was then ground to less than 0·3 mm and homogenized. A few milligrams (4–6 mg) were analysed for carbon and nitrogen content and isotopic composition. The weighing and analysis were repeated five times, allowing the determination of mean values of the isotopic composition for each component.

Results

Carbon content, nitrogen content, and carbon and nitrogen isotopic compositions of each component of the calves' diet are presented in Table 1. The isotopic composition of the whole diet was calculated for each stall according to the proportional contribution of each component (maize silage, maize grain, wheat and soya bean) to the total C and N contents of the diet. The nitrogen isotopic composition of the whole diet is similar in both stalls ($\delta^{15}N=+2·8$‰ and $\delta^{15}N=+2·7$‰ for stalls 5 and 6, respectively). The contribution of the C_4 component (maize) is higher in stall 5, so that the whole diet is more ^{13}C-enriched in stall 5 ($\delta^{13}C=-13·4$‰) than in stall 6 ($\delta^{13}C=-17$‰). These global values for the diet are presented as estimations since we are aware of the real carbon and nitrogen assimilation processes that can modify this isotopic signal.

The biochemical quality of the extracted organic matter from bone and dental tissue identify it as collagen: carbon contents range from 39·8 to 43·6% (mean=41·7 ± 1·2%), nitrogen contents from 14·4 to 16% (mean=15·4 ± 0·5%), and C:N ratio ranges from 3·1 to 3·3 (data not shown). Results from carbon and nitrogen isotopic analysis of collagen and apatite are shown in Table 2 & Figure 1.

Intra-tooth variation of collagen and apatite isotopic compositions

Dentine collagen from the bottom of the tooth is systematically ^{15}N-depleted and ^{13}C-enriched compared with collagen from the top of the tooth (Figure 1(a), (b)). This reflects the change from the C_3-milk-based diet to the C_4/C_3-herbivorous diet. The difference in $\delta^{15}N$ values between M2-t and M2-b is around 2·3‰ (from 1·3 to 3·2‰), which corresponds to the ~3‰ expected difference for the step between two trophic levels before and after weaning (DeNiro & Epstein, 1981; Minagawa & Wada, 1984; Fogel *et al.*, 1989).

The $\delta^{13}C$ values of collagen from M2-t are around −21‰, whereas $\delta^{13}C$ values of collagen from M2-b are around −14‰ in stall 6, and around −11·4‰ in stall 5. The first values are linked to the C_3 diet, the second ones to the C_4/C_3 mixed fattening diet. Amongst these last values, the difference of isotopic signal between individuals from stall 6 and stall 5 reflects the different C_4/C_3 proportion in the diet, which was higher in stall 5. In both stalls, the difference ($\delta^{13}C$ diet − $\delta^{13}C$ collagen) does not reach the expected value of ~ 5‰ observed in numerous studies (e.g., Vogel, 1978; Sullivan & Krueger, 1981; Lee-Thorp, Sealy & van der Merwe, 1989), but is around 2·5‰. This observation is consistent with the conclusions drawn from experiments on the relationship between

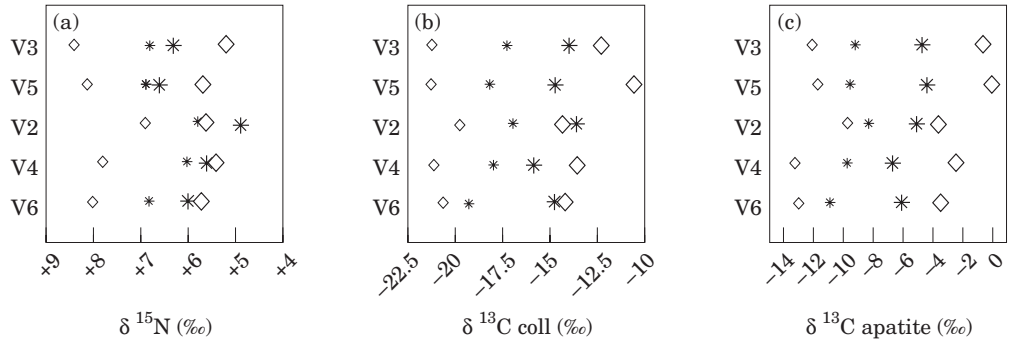

Figure 1. Intra-individual variability of nitrogen and carbon isotopic composition of bone and tooth collagen (a, b), and apatite (c) from the steers. ◇, M2-t; ✳, M1-bone; ✳, db-bone; ◇, M2-b.

diet and collagen carbon isotopic composition performed on rats and mice (Ambrose & Norr, 1993; Tieszen & Fagre, 1993). These experiments demonstrate that the collagen carbon isotopic composition reflects mainly that of the dietary proteins. Consequently, the $\delta^{13}C_{diet} - \delta^{13}C_{collagen}$ relationship depends on the difference between the carbon isotopic values of the protein and non-protein components of the diet (Ambrose *et al.*, 1997). It seems that such conclusions can also be applied to these steers. In stall 6 as well as in stall 5 (Table 1), the $\delta^{13}C$ value of the whole diet reflects the mixture of C_4 and C_3 plants from which it derives, but a large part of dietary protein comes from soya bean, which is a C_3 plant with a $\delta^{13}C$ value lower than that of the whole diet. This explains why the $\delta^{13}C$ value of the steers' collagen is lowered in a C_3 signal direction, thus decreasing the $\delta^{13}C_{whole\ diet}$–$\delta^{13}C_{collagen}$ difference.

The change from the pure C_3 diet to the mixed C_4/C_3 diet is also reflected in the $\delta^{13}C$ values of enamel apatite (Figure 1(c)). Considering the 11–13‰ difference observed between diet $\delta^{13}C$ value and apatite $\delta^{13}C$ value in herbivores (Krueger & Sullivan, 1984; Lee-Thorp *et al.*, 1989), the carbon isotopic composition of M2-te ($\delta^{13}C = -12 \pm 1{\cdot}3$‰) reflects a pure C_3 diet, whereas the carbon isotopic composition of M2-te ($\delta^{13}C$ mean $= -3{\cdot}2$‰ in stall 6; $-0{\cdot}3$‰ in stall 5) shows the expected values as regard the estimated $\delta^{13}C$ values of the whole diet. The range of intra-tooth variation of the isotopic signal is higher in enamel apatite (from 6 to 10·8‰ in stall 6 and around 11·7‰ in stall 5) than in dentine collagen (from 5·5 to 7·5‰ in stall 6 and from 8·8 to 10·7‰ in stall 5). This is due to the fact that, as previously mentioned, the collagen $\delta^{13}C$ value is linked to that of dietary proteins, which even after the dietary change come for a large part from C_3 plants, whereas the apatite $\delta^{13}C$ value reflects the carbon isotopic composition of the whole diet (Krueger & Sullivan, 1984; Ambrose & Norr, 1993; Tieszen & Fagre, 1993), which was exclusively composed of C_3 plants before the dietary change and is mainly composed of C_4 plants after the change (89% of the total amount of C comes from maize in stall 5, and 63% in stall 6).

Intra-bone variation of collagen and apatite isotopic compositions

Bone collagen and apatite from the dental bud zone are systematically ^{15}N-depleted and ^{13}C-enriched compared with collagen and apatite from the first molar zone (Figure 1(a), (b), (c)). The range of intra-individual variability of bone collagen and apatite isotopic composition is lower than that observed in the second molar dentine and enamel. No clear dichotomy appears between stall 5 and stall 6 nor between collagen and apatite $\delta^{13}C$ range of intra-individual variation, contrary to what is observed for intra-tooth variability.

Discussion

The top and the bottom of the second molar can be considered as reflecting the nitrogen and carbon isotopic composition of collagen and apatite exclusively synthesized, respectively, before and after the dietary change. Bone collagen and apatite $\delta^{15}N$ and $\delta^{13}C$ values are always included between these extreme isotopic values (Figure 1(a), (b), (c))—except for individual V2, for which it is not true concerning collagen carbon and nitrogen isotopic composition. This means that:

(1) M1-bone and db-bone are both made of a mixture of organic and inorganic material synthesized before and after the change of diet;
(2) the proportion of material synthesized after the change of diet is higher in db-bone.

This mixture of new and older material explains why the intra-individual and inter-stall isotopic variability observed in bone collagen and apatite is lower than that observed in dental tissues: isotopic signals are weakened.

The extreme isotopic values obtained from dentine and enamel analysis allow the estimation of the proportion of new material in bone—that is material synthesized after the dietary change. The results of these calculations are presented in Table 3. New collagen content can be estimated from both $\delta^{15}N$ and $\delta^{13}C$

Table 3. Amounts of collagen and apatite synthesized since the change of diet in both parts of the jawbone

	$\delta^{15}N$ collagen		$\delta^{13}C$ collagen		$\delta^{13}C$ apatite	
Steer	% new material (M1-bone)	% new material (db-bone)	% new material (M1-bone)	% new material (db-bone)	% new material (M1-bone)	% new material (db-bone)
V2	92 ± 31	*154 ± 47*	51 ± 4	*113 ± 4*	22 ± 3	77 ± 3
V3	75 ± 17	92 ± 17	43 ± 3	71 ± 3	33 ± 2	61 ± 2
V4	52 ± 17	87 ± 17	22 ± 3	91 ± 3	23 ± 2	73 ± 2
V5	50 ± 13	66 ± 13	43 ± 2	81 ± 2	25 ± 2	64 ± 2
V6	50 ± 17	63 ± 17	29 ± 2	61 ± 2	20 ± 2	62 ± 2

For collagen, estimates were made according to both $\delta^{15}N$ and $\delta^{13}C$ values. Error (Δ) on calculations was estimated according to the following equation: $\Delta=\delta\ (|A-B|+|C-A|+|B-C|)/(A-B)^2$ where δ is the analytical precision; $A=\delta_{M2b}$; $B=\delta_{M2t}$ and $C=\delta_{M1\ bone}$ or $\delta_{db\ bone}$. Aberrant values for individual V2 appear in italics.

values. The estimates obtained from these two parameters are not always the same, and when they significantly differ, the estimates made from $\delta^{15}N$ are higher than those from $\delta^{13}C$. Several explanations are possible. First, the weaning process began several months before the change from the pure C_3 to the C_3/C_4 diet, so that ^{15}N-depleted collagen synthesis could have begun earlier than the ^{13}C-enriched collagen synthesis. Second, the presence of antenatal collagen in bone might disturb $\delta^{15}N$ values. Antenatal collagen might have the same isotopic composition as the mother's collagen, that is a C_3-herbivorous signature comparable to that of the newly synthesized collagen. No fractionation was observed in humans between the $\delta^{15}N$ values of mother and newborn (Fogel *et al.*, 1989). Some results obtained on archaeological bovine bones seem to agree with this scheme (Balasse *et al.*, 1997) but so far no measurements have been made on modern cows. Because of this uncertainty we chose to take into account only the results obtained from the $\delta^{13}C$ values.

The mean percentage of collagen synthesized since the change of diet is 34% (from 22 to 43%) in M1-bone, and 76% (from 61 to 91%) in db-bone (results from V2 are not taken into account, because of aberration): about twice as much new collagen has been accumulated in db-bone than in M1-bone. In the same way, the mean percentage of new apatite is 67% (from 61 to 77%) in db-bone, which is more than twice that estimated for M1-bone (mean 25%, from 20 to 33%). These figures are certainly not to be taken into account other than as indicating a trend. Eight months after the dietary change, the steers, despite the fact that they were still growing fast, retained in their bone tissues a substantial amount of material synthesized before this dietary change. However, this amount of old material is variable, and it is much less important in the dental bud zone.

Conclusion

A dietary change such as weaning or a change from a pure C_3 to a C_4/C_3 mixed diet is recorded in enamel apatite and/or dentine collagen from a tooth that was growing during this change. In bone, such a change of diet is expressed in the variability of the isotopic composition of apatite and/or collagen from zones that are not renewed at the same rate. We chose to work on the jawbone, where a comparison was made between the dental-bud socket zone and the first molar socket zone. It was demonstrated that during growth, the collagen and apatite turnover is faster in the dental bud zone than in the first molar zone. Such a study could be conducted on other bone pieces, for example on long bones, where a comparison of isotopic signal could be made between collagen and/or apatite from epiphysis and shaft.

This kind of double sampling procedure can be applied easily to archaeological bones in order to detect a dietary change. A decision has to be made about the choice of the zones to be sampled, which for instance will depend on the timing of tooth growth in the particular species, if sampling is done in the jawbone. As this kind of sampling strategy is based on the allometric nature of bone growth it will probably give poor results in adults, but it is more particularly adapted to the detection of a dietary shift in growing individuals. One of the dietary changes undergone by all mammals during growth is weaning. Our methodology allows the estimation, at a population scale, of the age of weaning; at the individual scale it allows a determination of whether an individual was weaned or not at the time of death. Applied to domestic dairy livestock, such a methodology can give valuable information on pastoral economies from the past (Balasse *et al.*, 1997).

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