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BONE PRESERVATION AND DNA AMPLIFICATION

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The use of ancient DNA has increased during the past two decades in several scientific disciplines. However, the underlying mechanisms of DNA degradation in bone tissue are poorly understood. Here we address the importance of hydroxyapatite and collagen for DNA preservation in bone. We used two series of bones and teeth, one set of modern experimentally degraded bovid bones and one set of ancient horse bones/teeth. From these samples, we measured crystallinity, DNA presence and extracted collagen. The mtDNA fragments, parts of cytochrome b and the D-loop were amplified and sequenced. Our results show that presence of DNA was strongly related to the crystallinity in the hydroxyapatite and to the amount of collagen. This suggests that the hypothesis that hydroxyapatite has a crucial role in DNA preservation in calcified tissue is valid; and hydroxyapatite and collagen can be used to indicate whether DNA is present in the material. This is what would be expected if DNA is adsorbed to and stabilized by hydroxyapatite in calcified tissue, and collagen is part of the complex system that preserves DNA in bone tissue. Further, since collagen is the preferred material for radiocarbon dating, such bones may be a starting-point for a DNA analysis.

KEYWORDS: COLLAGEN, HYDROXYAPATITE, aDNA, XRD, DEGRADATION

INTRODUCTION

The use of ancient DNA has increased enormously during the past two decades in a multitude of scientific disciplines. Increasing claims for antideluvian DNA (Golenberg *et al*. 1990; Cano *et al*. 1993; Poinar *et al*. 1993; Zou *et al*. 1995) ceased in the mid-1990s with the introduction of greater controls (Lindahl 1993a,b). However, surprisingly few studies on DNA degradation in bone tissue have been published and the knowledge is limited. Even though the pattern of DNA degradation in bone tissue is not understood, the method is used on some of the most precious and valuable fossils (Krings *et al*. 1997; Ovchinnikov *et al*. 2000; Scholz *et al*. 2000). Rates of depurination and deamination in the DNA molecule that causes base substitution and other artefacts in amplifications and sequencing reactions are well known. The knowledge of DNA degradation is, however, currently restricted to physiological conditions applied during the tests and cannot be applied directly to DNA degradation in bones (Lindahl 1993a). Furthermore, factors specific for the bone matrix, such as the adsorption of DNA to hydroxyapatite or the three-dimensional packaging of the macromolecule within the bone, may influence the degradation of DNA (Collins *et al*. 2000), something that has yet to be studied. Here we focus on finding a reliable, fast and fairly non-expensive way of predicting the preservation of DNA in ancient bones and teeth. We will further discuss the role of hydroxyapatite and collagen during degradation of ancient bone tissue.

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MATERIAL AND METHODS

We used two series of bones and teeth, one modern experimentally degraded set and one set of ancient bones (Table 1). The ancient samples consisted of horse bones or teeth from different geographical locations and time periods, where the main part is from Early Iron Age Scandinavia. The modern samples consisted of bovid bones that had been homogenized prior to analysis. They were also incubated under different humidity conditions (the presence or absence of water) in order to disturb the three-dimensional packing (Waite *et al*. 1997; Collins *et al*. 1998, 2000).

The bones and teeth were powdered in a mortar prior to X-ray diffraction analysis (XRD), on a Siemens Diffraktometer D5000 Kristalloflex, and the crystallinity index for hydroxyapatite was calculated according to Bartsiokas and Middleton (1992). Collagen from the archaeological samples was extracted according to Brown *et al*. (1988) where high molecular weight (>30 kD) remnants of bone collagen are selected for ultrafiltration. Collagen yield was calculated as the percentage of whole bone (the obtained amount of collagen divided by powdered bone). The collagen content in the modern samples was determined as described in Collins and Galley (1998). Briefly, bones were pulverized in a freezer mill and defatted with acetone, and thereafter they were demineralized overnight in 0.6 M HCl with agitation at 4°C. The collagen present in the insoluble fraction was estimated from the residual nitrogen content, measured using a CarloErba Carbon Hydrogen Nitrogen Sulphur analyser.

DNA was extracted as described by Lidén *et al*. (1997), where detergents and caothrops were used to removed proteins and lipids. DNA was extracted with a phosphate buffer and purified by silica binding. The DNA was amplified with amplitaq gold™ (Perkin Elmer). The 25 µl reactions contained 50 mM KCl, 10 mM Tris-HCl pH 8.3, 2.25 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 µM of each primer, 0.75 U Taq-polymerase and 5 µl template DNA (Bovine Serum Albumine was excluded to avoid contamination in the bovid samples). Hot start was performed automatically due to the enzyme used. The amplification cycles were

Table 1 *Data table. Collagen values for the ancient bones were measured as obtained collagen by weight divided by the weight of whole bone, whereas for modern bones the collagen index was measured according to Collins and Galley (1998), and the crystallinity index was measured according to Bartsiokas and Middleton (1992). The bone types were ancient horse (A) and modern cow (M)*

Sample	*Collagen (%)*	*Collagen index*	*XRD 0-X*	*DNA presence*	*Bone type*	*Estimated age*	*Site*
1	3.45		1.5	+	A	Viking age	Alsike
2	3.56		1.56	+	A	Viking age	Alsike
3	7.1		1.48	+	A	Viking age	Alsike
4	4.59		1.48	+	A	Viking age	Alsike
5	4.07		1.46	+	A	Viking age	Alsike
6	3.83		0.91	+	A	Viking age	Birka
7	4.3		1.7	+	A	Viking age	Birka
8	2.12		2.35	+	A	Iron Age	Pada
9	0.59		1.05	+	A	Iron Age	Asula
10	6.63		1.22	+	A	Iron Age	Asula
11	2.1		1.67	+	A	Palaeolithic	Siberia
12	1.9		1.82	+	A	Palaeolithic	Siberia
13	2.1		2.4	–	A	Viking age	Alsike
14	4.79		1.56	–	A	Viking age	Alsike
15	2.31		2.1	–	A	Viking age	Alsike
16	2		3.79	–	A	Viking age	Birka

Table 1 *(continued)*

Sample	*Collagen (%)*	*Collagen index*	*XRD 0-X*	*DNA presence*	*Bone type*	*Estimated age*	*Site*
17	7.07		2.4	–	A	Viking age	Birka
18	0.41		2.92	–	A	Viking age	Birka
19	7.45		2.11	–	A	Viking age	Birka
20	1.53		1.82	–	A	Viking age	Birka
21	1.63		3.21	–	A	Viking age	Birka
22	2.48		2.18	–	A	Viking age	Birka
23	1.47		3.22	–	A	Viking age	Birka
24	0.2		2.38	–	A	Viking age	Birka
25	0.44		2.96	–	A	Viking age	Birka
26	0.29		2.98	–	A	Viking age	Birka
27	0.69		3.62	–	A	Viking age	Birka
28	2.75		2	–	A	Viking age	Birka
29	0.24		3.61	–	A	Viking age	Birka
30	0.73		3.02	–	A	Viking age	Birka
31	0.7		2.97	–	A	Viking age	Birka
32	0.34		4.12	–	A	Viking age	Birka
33	1.92		3.11	–	A	AD 500–750	Valsgärde
34	2.28		2.38	–	A	Viking age	Old Uppsala
35	1.41		2.48	–	A	Iron Age	Pöide
36	1.67		2.63	–	A	Iron Age	Asva
37	1.7		2.56	–	A	Iron Age	Asula
38	1.12		2.98	–	A	AD 500–750	Valsgärde
39	4.4		2.61	–	A	Iron Age	Asula
40	2.73		3.32	–	A	Iron Age	Asula
41	2.01		3.79	–	A	Iron Age	Asula
42	2.4		2	–	A	Iron Age	Pöide
							Dry/wet
43		0.89	1.37	+	M		Dry
44		ND	1.07	+	M		Dry
45		0.79	ND	+	M		Dry
46		0.95	1.08	+	M		Dry
47		ND	1.44	+	M		Dry
48		0.97	1.49	+	M		Dry
49		ND	1.3	+	M		Dry
50		0.19	1.03	+	M		Dry
51		0.19	1.17	+	M		Dry
52		0.03	1.35	+	M		Wet
53		0.00	1.43	+	M		Wet
54		0.00	2.27	+	M		Wet
55		0.19	0.67	+	M		Wet
56		ND	1.54	+	M		Wet
57		0.63	0.9	–	M		Dry
58		0.36	ND	–	M		Dry
59		ND	3.79	–	M		Wet
60		0.00	2.38	–	M		Wet
61		ND	ND	–	M		Wet
62		0.00	ND	–	M		Wet
63		0.00	4.8	–	M		Wet
64		0.00	4.23	–	M		Wet

initiated by a 2 min denaturation step at 93°C, followed by 15 s at 94°C, 30 s at 50°C and 15 s at 72°C. This cycle was repeated 50 times. Finally, an extension step at 72°C for 7 min followed. The modern bovid samples were amplified with primers based on cytochrome b, where KO1F (sequence TGACTAACATTCGAAAGTCC) was used as the forward primer and KOR2 (sequence AAATGTTCGATGGGGCT) was used as the reverse primer. These primers amplify an 81 BP long fragment, including the primer sites. The archaeological remains were amplified with HFORW1 (sequence GCCATCAACTCCCAAAGCT) and HREW1 (sequence CATAGGCCATTCATAAGATAT), a primer set that amplifies a fragment of HVR1 in *Equus*. This primer pair, which does not amplify DNA from any other taxon except for *Equus* as far as we know, amplifies a 165 BP long fragment, including the primer sites. The result was detected on a 2% ethidium bromide stained agarose gel and sequenced to confirm the result (sequencing was performed on all ancient results and on some of the modern ones) (BIG Dye terminator kit™, ABI). Several of the ancient horse sequences were used in a horse domestication study (Vilá *et al.* 2001) We avoided contamination by separating pre- and post-PCR areas. We also UV-irradiated surfaces and tools, washed tools in HCl, applied uracil-*N*-glycosylase methods and reproduced every extraction and amplification in the same laboratory (Götherström and Lidén 1998).

When we tested the experimental treatment of the modern bones, we used a two-way analysis of variance for crystallinity and the amount of collagen against treatment humidity conditions. We used a logistic regression to analyse the function of DNA presence on crystallinity and collagen in ancient and modern bones. That analysis generates a function that switches from 0 to 1 and is tested by a χ^2 test. Only significant results are considered ($p < 0.05$), and when $0.10 < p < 0.05$ we have discussed results as a trend or tendency. All statistical analyses were performed on the Statistica software (Statsoft Inc., 1999 version).

RESULTS

Two sets of bones were used for DNA extraction and amplification, a set of ancient horse bones and teeth, and a set of modern, synthetically degraded, bovine bones. Twelve out of the 42 ancient samples were successfully extracted, amplified and sequenced. Fourteen out of the 22 modern samples were successfully extracted, amplified and sequenced. These results were compared for crystallinity, the amount of collagen and the presence or absence of water in the samples.

The artificial diagenesis of the modern bones was influenced by the presence or absence of water. As expected, crystallinity and collagen were influenced by the presence of water (Table 2). However, there was only a tendency for the presence of water to affect the DNA extraction (Table 2). This can be summarized by saying that dry conditions were more

Table 2 *Results from analyses of the treatment of the modern bones with group averages and statistics. Bones were placed in wet or dry conditions. The effects on collagen index and crystallinity index were tested with a two-way analysis of variance (F-values). The effects on the presence of DNA were tested with logistic regression (χ^2-values). Bold averages were significant, $p < 0.05$*

		Dry/wet			
	N	*Dry*	*Wet*	F/χ^2	*P*
DNA (% with)	22	82%	45%	$\chi^2 = 3.25$	0.07
Collagen index	16	0.77	0.003	$F = 169.7$	<0.0000
Crystallinity (XRD)	18	1.10	3.02	$F = 7.13$	0.02

favourable to preservation in terms of improved yields of collagen, greater success with DNA amplification and reduced mineralogical changes (Table 2).

The preservation of collagen in the ancient bones was negatively correlated with the crystallinity index in the hydroxyapatite (Fig. 1; $r = -0.54$, $p = 0.0002$). This supports the view that the preservation of a macromolecule such as collagen is related to the preservation of hydroxyapatite. Although the trend was similar, we found no significant effect in the modern bones (Fig. 1; $r = -0.44$, $p = 0.13$). This may be due to sample size or stochastic mechanisms or, less likely, that it is not there.

The presence of DNA in the ancient bones was strongly related to the crystallinity in the hydroxyapatite, and a very similar pattern appeared for the modern bones (Fig. 2). The probability of extracting a sufficient amount of DNA for a successful amplification decreased

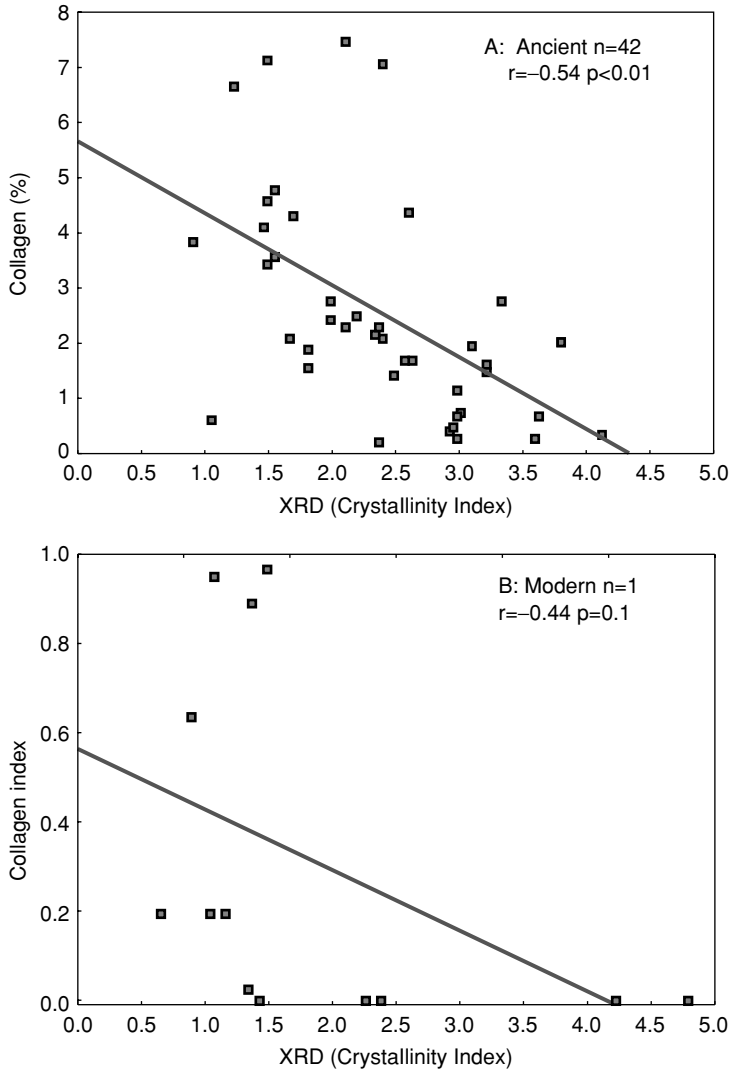

Figure 1 *The relationship between crystallinity in the hydroxyapatite (XRD) and the amount of collagen for ancient (A) and modern (B) bones.*

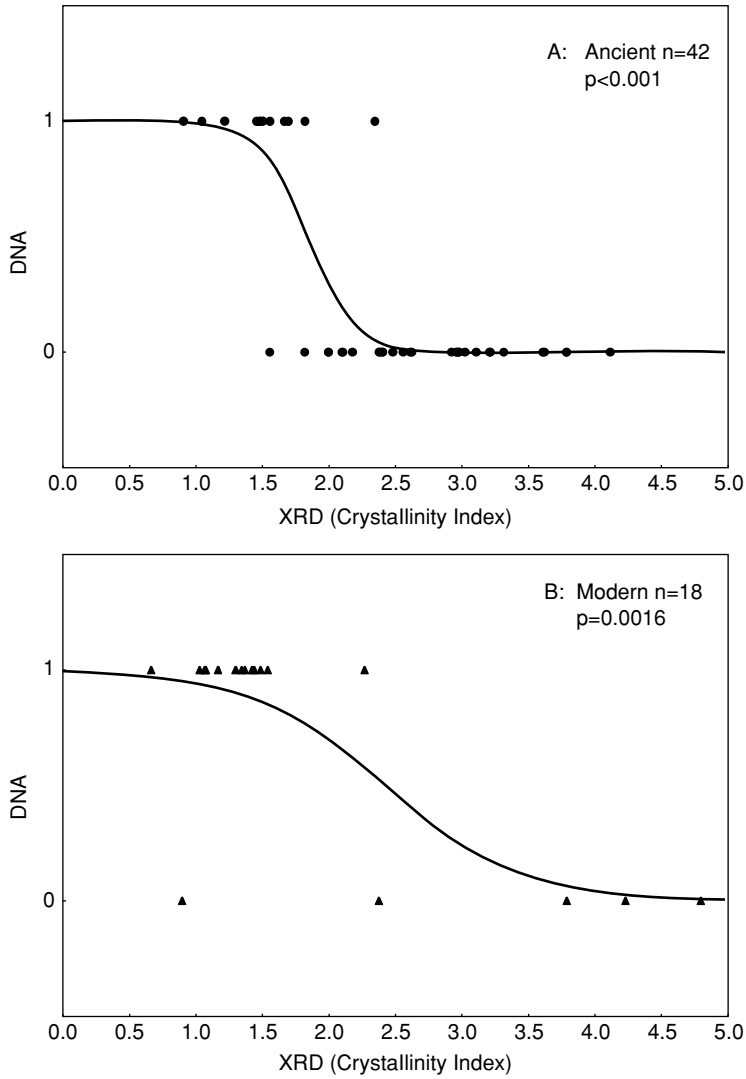

Figure 2 *The relationship between presence of DNA and crystallinity in the hydroxyapatite (XRD). The curves show the logistic regression curves: (A) for ancient bones,* $\chi^2 = 32.3$, $p < 0.00001$; *(B) for modern bones,* $\chi^2 = 9.94$, $p = 0.002$.

considerably at crystallinity values higher than 2.5. We found a similar relationship between the presence of DNA and the amount of collagen in the ancient bones. Although the trend was similar for the modern bones, there was no equally significant relationship (Fig. 3).

DISCUSSION

Bone is a composite of hydroxyapatite and collagen. The main question is how DNA degradation is related to these two molecules. The strong affinity of DNA for hydroxyapatite has led to the suggestion that aDNA degradation is related to strong mineral sorption (Hagelberg *et al*. 1989; Romanowski *et al*. 1991; Paget *et al*. 1992; Person 1992; Tuross 1993; Ogram *et al*.

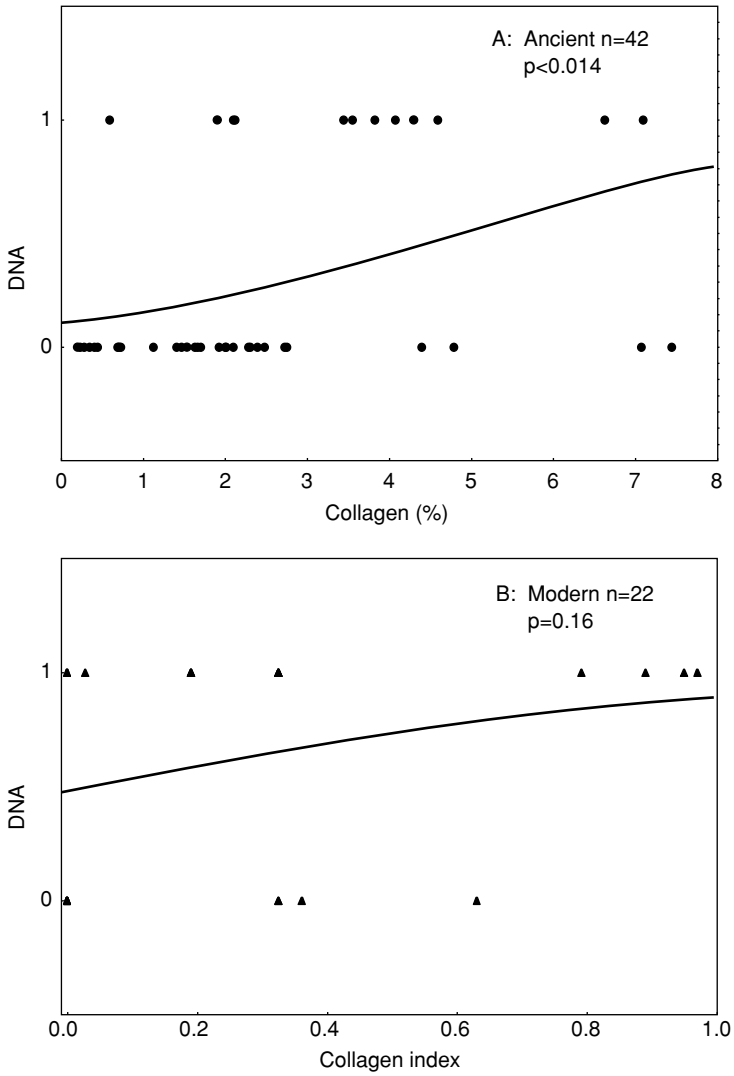

Figure 3 *The relationship between presence of DNA and the amount of collagen. The curves show the logistic regression curves: (A) for ancient bones,* $\chi^2 = 5.98$, $p = 0.01$; *(B) for modern bones,* $\chi^2 = 1.95$, $p = 0.16$.

1994; Collins *et al*. 1995; Götherström and Lidén 1996; but see Lindahl 1993a). This sorption is probably based on negatively charged phosphate groups in the DNA molecule and adsorption hydroxyl sites on the hydroxyapatite (Kawasaki *et al*. 1985). Crystal maturation in bone is assumed to proceed by a process of Ostwald ripening, with the concomitant loss of carbonate, which in hydroxyapatite occupies hydroxyl sites in the lattice. Biomolecules with high affinity for the hydroxyapatite surface will inhibit dissolution and reprecipitation reactions (Long *et al*. 1998). Correspondingly, under conditions in which dissolution and reprecipitation occurs, this is either driven by deterioration of the biomolecules or will result from desorption. DNA will no longer be protected by the hydroxyapatite and degradation can continue. The conceptual model described above is supported by our results; that is, a dependence between hydroxyapatite crystallinity and DNA preservation.

In addition to changes in crystallinity, DNA degradation may also be linked to losses of collagen. A number of studies have shown at least weak correlation between DNA amplification and protein content (Poinar *et al*. 1996; Colson *et al*. 1997; Cooper *et al*. 1997; Poinar and Stankiewicz 1999). Collagen is a triple helical molecule and the degradation processes may be similar to those of DNA. Collins *et al*. (2000) reported that survival of the bone-protein osteocalcin in animal bones, from a Neolithic site in France, declined with increasing crystallinity. The presence of organic molecules will slow the rate of mineral solution reaction (Kleter *et al*. 1994; Putnis and Putnis 1995). Conversely, dissolution of the mineral phase will expose collagen to degradation (Krane 1975), thereby increasing the rate of decomposition (Klont *et al*. 1991).

Our data set is suggestive in several ways. When the hydroxyapatite undergoes diagenesis, so does the DNA, supporting the view that DNA is stabilized and preserved by adsorption to bone apatite. If this is so, where is the DNA in the bone? If it is in the osteocyte lacunae, and vulnerable to autolysis and microbiological activity (Collins *et al*. 2002), it is difficult to understand the high correlation between hydroxyapatite preservation and DNA content in ancient samples. The correlation with collagen degradation is intriguing even though there are anomalies, which may in part be related to alternative mechanisms of collagen loss, such as microbial mineralization and differences in the temperature dependence of depurination and gelatinization. This is probably due to the fact that the presence of organic molecules will passivate the mineral surface (Putnis and Putnis 1995), slowing down the rate of mineral solution reaction (Kleter *et al*. 1994). Although the association between collagen and the mineral phase of the bone seems clear, the precise relationship is still debated (Traub *et al*. 1989; Glimcher 1990; Lee and Glimcher 1991; Fratzl *et al*. 1996; Landis 1996; Prostak and Lees 1996; Plate *et al*. 1998; Weiner and Wagner 1998; Weiner *et al*. 1999).

A number of other markers have been suggested for DNA presence in ancient bones, such as thermal age (Smith *et al*. 2001) and the racemization of aspartic acid (Krings *et al*. 1999; Kumar *et al*. 2000; Scholz *et al*. 2000). The use of thermal age is based on two assumptions: that depurination of DNA is the key pathway for DNA degradation; and that the activation energy of depurination is temperature related (Smith *et al*. 2001). However, the method is of limited use, since it only permits relative comparison between sites and the limit of DNA survival: furthermore, it requires an estimate of the thermal history of each site. The application of racemization as a screening tool in bone implicitly assumes a relationship between DNA degradation and collagen (Poinar *et al*. 1996). This assumes similar activation energy of DNA depurination and racemization of bound aspartyl and asparingyl residues (Geiger and Clarke 1987). The rate of formation of succinimide, and hence the rate of racemization, is potentially a consequence of the rate of collagen denaturation (van Duin and Collins 1998; Collins *et al*. 1999). Low racemization values may therefore indicate the presence of an intact collagen triple helix.

In conclusion, our results do not only suggest that hydroxyapatite plays a crucial role in DNA preservation in bone tissue, but also that hydroxyapatite and collagen can be used to indicate whether DNA is present in the material. There was a striking similarity between crystallinity indexes of the degraded modern and ancient samples. With the right equipment, crystallinity measurements are cheap, fast and easy. Furthermore, since collagen is the preferred material for radiocarbon dating, and there is a correlation between collagen and the presence of DNA, radiocarbon-dated bones may be a starting-point for selecting bones for DNA analysis. However, we want to emphasize that the only reliable source for DNA screening is the molecule itself. Thus, we suggest that DNA is adsorbed to and stabilized by apatite in bone tissue, and that collagen is part of the complex system that preserves DNA in bone tissue.

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