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DNA damage and DNA sequence retrieval from ancient tissues

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ABSTRACT

Gas chromatography/mass spectrometry (GC/MS) was used to determine the amounts of eight oxidative base modifications in DNA extracted from 11 specimens of bones and soft tissues, ranging in age from 40 to >50 000 years. Among the compounds assayed hydantoin derivatives of pyrimidines were quantitatively dominant. From five of the specimens endogenous ancient DNA sequences could be amplified by PCR. The DNA from these specimens contained substantially lower amounts of hydantoins than the six specimens from which no DNA could be amplified. Other types of damage, e.g. oxidation products of purines, did not correlate with the inability to retrieve DNA sequences. Furthermore, all samples with low amounts of damage and from which DNA could be amplified stemmed from regions where low temperatures have prevailed throughout the burial period of the specimens.

INTRODUCTION

When DNA is extracted from archaeological remains it is generally found that only a small fraction of specimens contain endogenous, ancient DNA sequences that can be amplified by the polymerase chain reaction (PCR). For example, among 35 samples of late Pleistocene animal remains recently analysed only two yielded DNA sequences that were genuinely old (1). The likely reason for this is damage to the DNA that accrues over time and eventually renders the DNA unable to serve as a template for PCR.

Mainly two types of damage are likely to affect DNA in archaeological deposits. First, hydrolytic damage will result in deamination of bases and in depurination and depyrimidination (2). Second, oxidative damage, caused by the direct interaction of ionizing radiation with the DNA, as well as mediated by free radicals created from water molecules by ionizing radiation, will result in modified bases (2,3). Other mechanisms, for example alkylation or UV irradiation, are less likely to affect buried remains. However, the knowledge of damage actually present in ancient DNA is very limited. Several studies have shown that ancient DNA is degraded to a small average size (see for example 4) and in one study (4) enzymatic assays were used to show that abasic sites and oxidation products of pyrimidines were present in two ancient DNA samples.

Gas chromatography/mass spectrometry (GC/MS) is particularly suited to identify and quantify modifications in DNA (5). Here we describe the detection and approximate quantification of different types of oxidative base damage in samples of different age and the correlation of such damage with the inability to retrieve ancient DNA sequences by PCR.

MATERIALS AND METHODS

DNA extraction and amplification

DNA extraction and amplification by PCR were performed as described in Höss and Pääbo (6). To obtain the amounts of DNA needed for the GC/MS analysis (≥ 1 µg), 20 separate extractions, each from ~0.4 g bone tissue and 0.2 g soft tissue respectively, were performed from each sample. From five of the samples DNA sequences could be determined and were shown to be reproducible. Except for the ~40-year-old remains of a horse the investigations of these specimens have been published (1,6–8). For the remaining six specimens no endogenous DNA sequences could be amplified by PCR, despite repeated attempts where primers for short mitochondrial sequences that are conserved among vertebrates (16s6 and 16s7 in 1) were used under conditions that allow amplification from single template molecules (data not shown).

Gas chromatography/mass spectrometry (GC/MS)

Modified DNA bases, their stable isotope-labelled analogues and other materials for GC/MS were obtained as described (9). Aliquots of stable isotope-labelled analogues of modified DNA bases were added as internal standards to DNA samples. Samples were then dried under vacuum in a SpeedVac. Dried samples were hydrolyzed in 50 µl 60% formic acid in evacuated and sealed tubes at 140°C for 30 min. The hydrolysates were lyophilized and derivatized with 50 µl of a mixture of bis(trimethylsilyl)trifluoro-

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Table 1. List of archaeological samples used in this study

Nr.	Species	Age B.P.(years)	Tissue type	Location	Reference
1	Horse (Equus caballus)	40	bone	California, USA	–
2	Aurochs (Bos primigenius)	6,500	bone	Germany	–
3	Baboon (Papio cf. cynocephalus)	2,300	bone	Egypt	–
4	Vertebrate (–)	60,000	bone	Border Cave, South Africa	–
5	Horse (Equus ferus)	5,500	bone	Reusten, Germany	–
6	Onager (Equus cf. hemionus)	27,000	bone	Fairbanks, AK, USA	6
7	Ground sloth (Mylodon darwinii)	13,000	bone	Patagonia, Chile	1
8	Cave bear (Ursus spelaeus)	–	bone	France	–
9	Mammoth (Mammuthus primigenius)	≥ 50,000	soft tissue	Kathanga, Siberia	7
10	Ground sloth (Megalonyx sp.)	13,000	tooth	Florida, USA	1
11	Horse (Equus ferus)	35,000–40,000	soft tissue	Selerikan, Siberia	8

Ages, burial locations, tissue type, and, where applicable, references to publications of DNA sequences are given

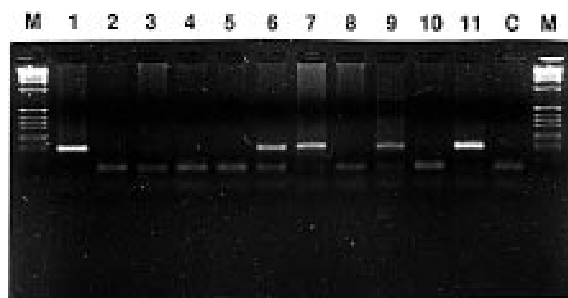

Figure 1. Agarose gel showing amplification products of an ~140 bp fragment of the mitochondrial 16S rDNA. M refers to size markers and C to a PCR control. Numbers 1–11 refer to the samples listed in Table 1.

acetamide (with 1% trimethylchlorosilane) and acetonitrile (80:20 v/v) at 120°C for 30 min in vials sealed under nitrogen with Teflon-coated septa. The derivatized samples were analysed by GC/MS with selected-ion monitoring (SIM) as described (10). The quantification of modified DNA bases was performed by isotope dilution mass spectrometry using stable isotope-labelled analogues as internal standards (9). The total amount of undamaged DNA was estimated from the amounts of unmodified thymine in each sample. Trace amounts of 8-hydroxyguanine (8-OH-Gua) may be generated during trimethylsilylation (11). To avoid this, derivatization reagents were stored under nitrogen and bubbled with nitrogen prior to use, and the derivatization was performed under nitrogen. In spite of this, a portion of the 8-OH-Gua detected may be due to the analytical procedure.

RESULTS AND DISCUSSION

Samples of 4–8 g tissue were removed from 11 tissue specimens that vary in age from 40 years to >50 000 years. These specimens represent different burial conditions and tissue types (Table 1). DNA was extracted from the samples and the total amount of nucleic acids extracted in each case was estimated by measurement of absorbance at 260/280 nm to be 2–6 µg. An aliquot of each extract was used to amplify an ~140 bp fragment of the mitochondrial 16S rRNA gene, using primers that are conserved among vertebrates and able to amplify single template molecules under the conditions used. As can be seen in Figure 1, five of the samples yielded amplification products. For four of these samples results demonstrating that the DNA sequences retrieved are reproducible and genuinely old according to a number of criteria have been published (1,6–8). In the case of the fifth sample, an ~40-year-old horse skull, DNA sequences were determined and found to be of horse origin (data not shown). For the six samples yielding no amplification products amplifications from multiple extracts, in addition to the ones shown (Fig. 1), were attempted. In no case were specific amplification products achieved.

The DNA extracts from all 11 specimens and from modern calf thymus DNA were analysed for base damage by GC/MS. Since the archaeological and palaeontological specimens used are valuable and available in limited quantity, the amounts of DNA that could be extracted allowed only two analyses by GC/MS per specimen. This obviously limits the possibility of estimating the variance of the quantification of the modified bases, as well as the number of compounds that can be analysed. However, from nine base modifications monitored, four modified pyrimidines [5-hydroxy-5-methylhydantoin (5-OH-5-MeHyd), 5-hydroxyhydantoin (5-OH-Hyd), 5-hydroxyuracil (5-OH-Ura) and 5,6-dihydroxyuracil (5,6-diOH-Ura)], as well as four modified purines [4,6-diamino- 5-formamidopyrimidine (FapyAde), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua), 8-hydroxyadenine (8-OH-Ade) and 8-OH-Gua; Fig. 2] occurred in amounts that allowed them to be reliably detected and quantified in at least one of the samples. Table 2 summarizes the results.

The three base modifications 5-OH-5-MeHyd, 5-OH-Hyd and 8-OH-Gua were detected in all samples analysed. This is consistent with the observation that 5-OH-5-MeHyd is one of the major degradation products of thymine in γ-irradiated DNA (12), whereas 8-OH-Gua is one of the major oxidation product of guanine upon hydroxyl attack (3). Of these three lesions the amounts of the hydantoins 5-OH-5-MeHyd and 5-OH-Hyd were 2.7–20.2 and 5.1–29.2 times lower, respectively, in the samples that yielded DNA sequences than in those that did not (Fig. 3). The amounts of 8-OH-Gua, in contrast, showed no such correlation. Thus an inverse correlation exists between the amount of oxidized pyrimidine bases in these samples and the ability to amplify ancient DNA sequences. Since the hydantoins are likely to block strand elongation by DNA polymerases, it is reasonable that they would make enzymatic amplification of DNA sequences impossible. In contrast, miscoding lesions, such as 8-OH-Gua, are not expected to impede PCR.

Among the samples that produced DNA sequences two were from soft tissues and three from bones. For those that did not work

Table 2. Amounts of damaged nucleotide bases (nmol/mg) in the samples analyzed. Sample numbers refer to Table 1

Sample	5-OH-5-MeHyd	5-OH-Hyd	5-OH-Ura	5,6-diOH-Ura	FapyAde	8-OH-Ade	FapyGua	8-OH-Gua
C	0.42 (0.06)	0.16 (<0.01)	0.05 (0.01)	N/A	0.07 (0.01)	0.15 (<0.01)	0.11 (0.01)	0.75 (0.01)
1	1.82 (0.65)	1.20 (0.49)	0.58 (0.30)	N/A	0.33 (0.05)	0.22 (0.11)	0.42 (0.06)	2.63 (1.59)
2	9.11 (1.46)	8.85 (1.20)	<DL	<DL	<DL	<DL	<DL	0.98 (0.01)
3	8.22 (1.09)	9.07 (2.10)	<DL	<DL	<DL	0.58 (0.22)	<DL	1.46 (0.40)
4	11.17 (0.81)	10.53 (2.92)	<DL	<DL	<DL	<DL	<DL	0.65 (0.03)
5	15.81 (5.21)	17.09 (1.18)	<DL	<DL	<DL	<DL	<DL	1.53 (0.28)
6	2.08 (0.43)	1.19 (0.16)	0.86 (0.18)	N/A	0.21 (0.10)	0.13 (0.05)	0.29 (0.11)	0.44 (0.12)
7	3.09 (0.27)	1.72 (0.43)	0.67 (0.22)	N/A	0.23 (0.01)	0.27 (0.02)	0.44 (0.08)	0.94 (0.18)
8	14.69 (2.59)	19.10 (6.38)	<DL	<DL	<DL	<DL	<DL	1.31 (0.10)
9	1.25 (<0.01)	0.70 (<0.01)	0.20 (<0.01)	N/A	0.23 (<0.01)	0.27 (0.12)	<DL	1.67 (0.51)
10	12.73 (1.88)	9.86 (1.71)	<DL	<DL	<DL	<DL	<DL	0.76 (0.23)
11	0.78 (0.35)	0.66 (0.27)	N/A	1.10 (0.20)	0.51 (0.13)	0.67 (0.23)	<DL	0.93 (0.32)

Standard deviations are given within parentheses. N/A designates compounds that were not analyzed in the samples indicated and <DL indicates that a compound was below the detection limit (0.005 nmol/mg). In addition to the compounds shown, 5-hydroxycytosine was analyzed and was found to be below the detection level.

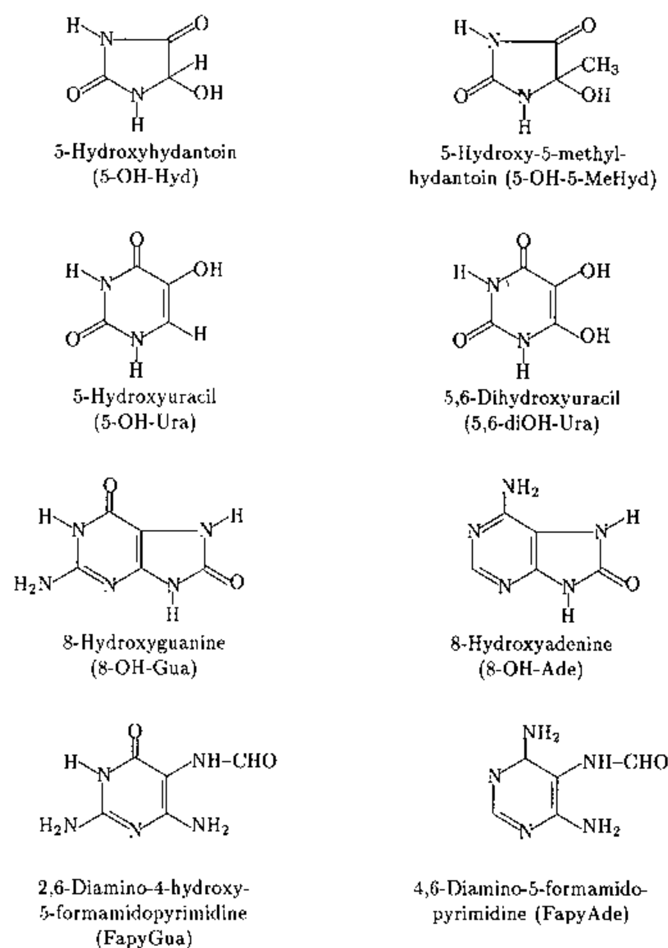

Figure 2. Structures of eight oxidative base modifications detected in the ancient DNA. For 8-OH-Gua and 8-OH-Ade the keto forms, which predominate in aqueous solution, are shown.

all five stemmed from bones. Thus no correlation could be seen between DNA preservation and tissue type. However, it is noteworthy that all samples that have a low amount of base damage and allow amplification by PCR stem from arctic and subantarctic regions. The mammoth sample and the Selerikan horse are from permafrost deposits in Siberia, the onager comes from a frozen deposit in Alaska and the giant ground sloth (*Mylodon darwinii*) is from a cold cave deposit in Southern Chile. In contrast, the samples from Egypt, Europe, South Africa and warm regions of the Americas had large amounts of DNA damage and yielded no DNA sequences. It therefore seems that for the long-term preservation of DNA a cold environment is of critical importance. This is consistent with the fact that a decrease in temperature of 20°C is expected to result in a 10- to 25-fold reduction in the rate of chemical reactions such as the decay of nucleotide bases. Freezing, as is the case for the permafrost remains, is expected to further reduce the decay rate. Thus over the time period considered here (40–50 000 years) low temperature seems to be of great importance for slowing down the processes responsible for post-mortem degradation of nucleic acids in archaeological remains.

It has been shown for two of the samples studied here, a mylodont ground sloth (1) and a mammoth (13), that in the order of only 1/1000 of the extracted DNA hybridizes to total DNA of related extant species. Furthermore, a fraction of the DNA is of microbial origin, as judged by PCR assays. It may therefore seem surprising that a negative correlation between the amount of base damage in the total DNA preparation and amplifiability of the endogeneous mammalian DNA exists. However, much of the ancient mammalian DNA may be too damaged to hybridize to modern DNA under standard conditions. This is likely in view of the fact that the amounts of DNA damage measured are low estimates of the total extent of damage, since not all types of chemical modifications are monitored with the technique used. Also, the microbial DNA sequences amplified from these extracts may stem either from recent microorganisms or from microorganisms whose DNAs are partially protected against damage due to spore formation (2,14,15). A second, not mutually exclusive, possibility is that much of the DNA in the specimens may stem from microorganisms that colonized the carcasses shortly after death. Such microbial DNA would be of ancient origin and could be expected to be damaged to a similar extent to the endgenous DNA. Further work is needed to clarify this.

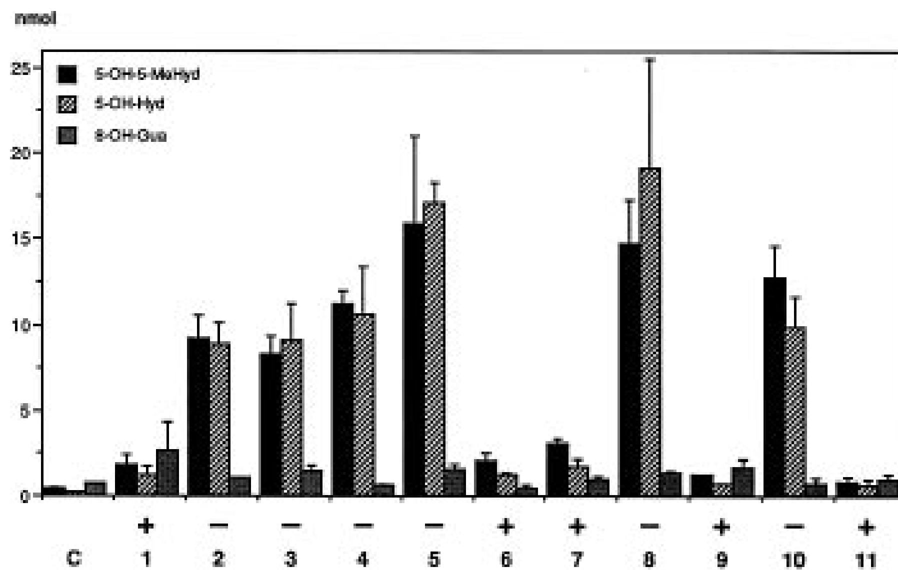

Figure 3. Bar chart of the results of the GC/MS analyses. The amounts of the three modified bases that could be detected in all samples are given in nmol/mg undamaged DNA, as estimated from the amount of unmodified thymine. The values for these, as well as the other five base modifications, are given in Table 2. Sample numbers refer to Table 1. As a control (C) modern calf thymus DNA was used. Standard deviations calculated from two runs of each sample are depicted as bars. + and – below the histograms refer to successful and unsuccessful amplifications respectively of ancient DNA.

In order to establish the generality of the negative correlation between amounts of hydantoins and DNA amplifiability in ancient remains larger numbers of samples will need to be studied. Unfortunately, such work is likely to proceed slowly, since the authenticity of the ancient DNA sequences retrieved needs to be rigorously established. This remains the major problem in the study of ancient DNA (16). However, if this correlation can be confirmed, hydantoins could serve as a useful indicator of DNA decay in archaeological remains from which large enough amounts can be extracted. Furthermore, a thorough understanding of the chemical lesions present in ancient DNA may allow repair strategies to be designed that would make a larger fraction of the DNA amenable to enzymatic amplification.

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REFERENCES

1 Höss,M., Dilling,A., Currant,A. and Pääbo,S. (1996) *Proc. Natl. Acad. Sci. USA*, **93**, 181–185.
2 Lindahl,T. (1993) *Nature*, **362**, 709–715.
3 Dizdaroglu,M. (1992) *Mutat. Res.*, **275**, 331–342.
4 Pääbo,S. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 1939–1943.
5 Dizdaroglu,M. (1991) *Free Radical Biol. Med.*, **10**, 225–242.
6 Höss,M. and Pääbo,S. (1993) *Nucleic Acids Res.*, **21**, 3913–3914.
7 Höss,M., Vereshchagin,M.K. and Pääbo,S. (1994) *Nature*, **370**, 333.
8 Taylor,P.G. (1996) *Mol. Biol. Evol.*, **13**, 283–285.
9 Dizdaroglu,M. (1994) *Methods Enzymol.*, **234**, 3–16.
10 Doetsch,P.W., Zastawny,T.H., Martin,A.M. and Dizdaroglu,M. (1995) *Biochemistry*, **34**, 737–742.
11 Wagner,J.R., Hu,C. and Ames,B.N. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 3380–3384.
12 Breimer,L.H. and Lindahl,T. (1985) *Biochemistry*, **24**, 4018–4022.
13 Höss,M. (1995) PhD Thesis, Ludwig-Maximilians University, Munich, Germany.
14 Setlow,P. (1992) *J. Bacteriol.*, **174**, 2737–2741.
15 Potts,M. (1994) *Microbiol. Rev.*, **58**, 755–805.
16 Handt,O., Höss,M., Krings,M. and Pääbo,S. (1994) *Experientia*, **50**, 524–529.