Preliminary Results for the Analysis of Lipids in Ancient Bone

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Lipid extracts were prepared for a number of samples of modern and ancient bones of humans and other animals (pig, cow, horse and whale). Aliquots of the total lipid extracts were trimethylsilylated and analysed directly by gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS) without prior fractionation or purification. The analysis of archaeological bones and samples of adhering soil showed that contamination of the bone by migration of lipids from the burial environment was not a serious problem. Analyses of modern reference materials showed the lipid extracts of bone possessing adhering marrow fat to be dominated by acyl lipids, e.g. triacylglycerols and free fatty acids. In contrast the lipid extract of a sample of the modern cow bone with the marrow fat removed was dominated by cholesterol; acyl lipids were present at low abundance. The GC and GC/MS analyses of the ancient bones of varying age and context of recovery also showed only a very low abundance of acyl lipids. Most significant was the finding of an appreciable concentration (1.5-46.5 μg g⁻¹) of cholesterol together with its diagenetic degradation products, in all the samples that were examined. The possibility of using the cholesterol (and its degradation products) as a palaeodietary indicator is discussed. Evidence is also presented for the occurrence of bacterial marker compounds in the bone total lipid extracts.

Keywords: BONE, HUMAN, ANIMAL, LIPIDS, GAS CHROMATOGRAPHY, GC, GAS CHROMATOGRAPHY/MASS SPECTROMETRY, GC/MS, CHOLESTEROL, DIAGENESIS, SOIL, PALAEODIETARY INDICATOR, BACTERIAL LIPIDS, HOPANOIDS.

Introduction

The bones recovered from archaeological sites have long been the focus of scientific investigations and have proved to be the source of much information of value to archaeological investigations. For example, amongst the organic components of archaeological bone, collagen is routinely recovered for the purpose of ¹⁴C dating (Bowman, 1990). In addition, after hydrolysis and separation, individual amino acids and small peptides can also be submitted to ¹⁴C dating (van Klinken & Hedges, 1992). The extent of racemization of individual amino acids has been proposed as a dating technique. Although problems have been encountered in applying this
technique to date collagen-derived amino acids (Bada, 1991) scope exists for the use of this technique in connection with the minor, more robust, proteins associated with the biomineralisation process (Ulrich et al., 1987). Estimations of the nitrogen ($\delta^{15}$N) and carbon ($\delta^{13}$C) isotope compositions of bone collagen have been conducted for the purposes of estimating palaeodiet (Vogel & van der Merwe, 1977; DeNiro, 1987; Schwarcz, 1991).

The number of non-collagenous proteins have also been detected in archaeological bones, most commonly using immunological techniques. Non-collagenous proteins, including osteocalcin (Ulrich et al., 1987), albumin (Tuross, 1989) and haemoglobins (Ascenzi et al., 1985; Smith & Wilson, 1990) have also been detected in archaeological and palaeontological bone by application of immunological techniques such as immunoblotting and the enzyme-linked inhibition assay (ELISA) (for a recent review, see Childs & Pollard, 1992). The recovery of DNA from bones by use of the polymerase chain reaction raises many exciting possibilities for anthropological and palaeontological investigation (Hagelberg et al., 1989; Hedges & Sykes, 1992; Brown & Brown, 1993).

Although lipids are known to survive in association with many archaeological materials (for a review, see Evershed, 1993), up until now detailed analyses of lipids from archaeological bones have not been performed. Evidence that lipids survive in human remains comes from the relatively few analyses that have been performed on mummies (Kuksis et al., 1978; Gülaçar et al., 1990) and bog bodies (Evershed, 1990, 1992; Evershed & Connolly, 1987, 1994). These studies have shown that lipids, particularly the steroidal compounds, i.e. cholesterol (see structure 1 of Figure 1), 5α- and 5β-cholestanols (structures 2 and 3), 5α- and 5β-cholestan-3-ones (structures 4 and 5) and bile acids, are well preserved at both waterlogged and arid sites. The preservation of this class of molecules stems from their inherent resistance to microbial and chemical degradation combined with their hydrophobicity which means they are not readily leached from tissues in waterlogged burials (Evershed, 1990; Evershed & Connolly, 1987, 1994). The finding in these latter investigations of cholesterol together with the 5α- and 5β-cholestanol and 5α- and 5β-cholestan-3-one degradation products is fully consistent with nature of decay that would be anticipated to arise from burial in an

![Figure 1. Steroid and hopanoid structures referred to in the text and other figures.](image-url)
anaerobic environment (Gaskell & Eglinton, 1976). The principal degradation products were the 5α- and 5β-cholestan-3β-ols. The 5α- and 5β-cholestan-3-ones that were detected are likely intermediates in the reduction of cholesterol to the 5α- and 5β-cholestan-3β-ols respectively.

In contrast to the findings from bog bodies, an investigation of the lipids from a Nubian mummy recovered from an arid site showed a much greater abundance of oxidized lipids reflecting the contrasting burial environment (Gülaçar et al., 1990). The steroidal compounds that were recovered included: cholesterol from the skin and skull samples, with interthoracic compounds that were recovered included: cholesterol from the skin and skull samples, with interthoracic compounds that were recovered included: cholesterol from the skin and skull samples, with interthoracic compounds that were recovered included: cholesterol from the skin and skull samples, with interthoracic compounds that were recovered included: cholesterol from the skin and skull samples, with interthoracic compounds that were recovered included: cholesterol from the skin and skull samples, with interthoracic compounds that were recovered included: cholesterol from the skin and skull samples, with interthoracic compounds that were recovered included: cholesterol from the skin and skull samples, with interthoracic compounds that were recovered included: cholesterol from the skin and skull samples.

In one instance (9th–10th-century human mid-shaft femur, CHF 89 T–10) the whole bone (c. 2 g sample) was pre-washed with a mixture of chloroform and methanol (2:1 v/v; 10 ml) prior to crushing and extraction. The solvent washings were evaporated, trimethylsilylated as described below and analysed by GC and GC/MS. Two other bone specimens (4th–6th century AD tibia and 17–35,000 BP bone from cave site) were crushed and de-mineralized by treatment with aqueous 1 M HCl. After neutralization the aqueous solution was extracted with diethyl ether, evaporated, trimethylsilylated and analysed by GC.

The contemporary bovine bones were washed in distilled water and then oven dried at about 60°C for 1–2 h. The sample with the absorbed marrow fat was a section taken from a region of spongy bone near the jointed end where the marrow fat could be seen intimately buried in the tissue. The sample without the marrow fat corresponded to a section taken from the mid-shaft where the endosteal surface was smooth and readily mechanically cleaned from adhering marrow fat.

Two procedure blanks were prepared without bone tissue to check for the possibility of lipid contamination being introduced from apparatus or reagents during the sample work-up. The blanks used identical apparatus, internal standards, reagents and procedures as were used with the bone extractions described above.

Trimethylsilylation

Aliquots of the total lipid extracts obtained from above were derivatized in screw-capped vials by treatment (60°C for 10 min) with N,O-bis(trimethylsilyl)trifluoroaceticamide (BSTFA, 20 μl) containing 1% v/v trimethylsilylchloride (Pierce Chemical Co.). The resulting trimethylsilyl (TMS) derivatives were diluted with cyclohexane and analysed by GC and GC/MS.

Gas chromatography (GC)

High temperature GC and GC/MS (see below) were performed largely according to the procedures
Table 1. Descriptions of the archaeological bone specimens subject of this investigation

<table>
<thead>
<tr>
<th>Sample description</th>
<th>Context description</th>
</tr>
</thead>
<tbody>
<tr>
<td>U nidentified 1 (VP86A B 5099 2586) Small, unidentified shattered frass. Vale of Pickering, U.K.</td>
<td>M esolithic 7500 BC. Bones lay on the surface of sands/clay. Originally islands within wetlands later covered by 1-2 m of organic rich peat. Recent drainage/aquifer extraction has caused shrinkage and drying of peat.</td>
</tr>
<tr>
<td>W hale bone. Fragmentary pieces could not be identified to genus. Spitzburgen, Norway</td>
<td>Permafrost deposit.</td>
</tr>
<tr>
<td>Compact bone from mid-shaft of human femur (CHF 89 T-10)* Chimney farm, Bampton, Oxon, U.K.</td>
<td>Late Roman/early Saxon, dated by association to 300-500. This bone has not been studied morphologically, but closely associated bones show extensive micromorphological destruction, presumably due to microbiological attack. Relatively oxic, neutral well-drained soil subject to periodic flooding.</td>
</tr>
<tr>
<td>Compact bone from mid-shaft of human femur (DBP 81 (site 1,4))* Dorchester by-pass, Oxon, U.K.</td>
<td>Late Roman/early Saxon, dated by association to 300-500. This bone has not been studied morphologically, but closely associated bones show extensive micromorphological destruction, presumably due to microbiological attack. Relatively oxic, neutral well-drained soil subject to periodic flooding.</td>
</tr>
<tr>
<td>Compact bone from human tibia (DBP 81 (site 1,18))* Dorchester by-pass, Oxon, U.K.</td>
<td>M icromorphology is well-preserved; collagen content is also relatively high. Cool, oxic and dry conditions in presence of considerable concentration of other human bone. Dated to c. 1840 on historical records. The outer portions are poorly preserved micromorphology, indicating microbiological attack. Oxic, well-draining soil conditions.</td>
</tr>
<tr>
<td>Compact bone of unidentified species (L N(N P431))* Little Hoyle Cave, Gwent, U.K.</td>
<td>U pper Palaeolithic dated by association to 23-29 ka B.P. Not studied micromorphologically. Limestone sediments, oxic, neutral/alkaline.</td>
</tr>
<tr>
<td>Compact bone from mid-shaft of human femur (P3519) Rossberga passage grave, Sweden</td>
<td>N eolithic. Dated by radiocarbon to 4590 ± 120 B.P. M icromorphology is well-preserved; collagen content is also relatively high. Cool, oxic and dry conditions in presence of considerable concentration of other human bone. Dated to c. 1840 on historical records. The outer portions are poorly preserved micromorphology, indicating microbiological attack. Oxic, well-draining soil conditions.</td>
</tr>
<tr>
<td>Compact bone from human mid-shaft of femur (R 2)* Romsey Abbey, Hampsire, U.K.</td>
<td>Dated to c. 1840 on historical records. The outer portions are poorly preserved micromorphology, indicating microbiological attack. Oxic, well-draining soil conditions.</td>
</tr>
</tbody>
</table>

* Bones assumed to have been deposited with flesh adhering.

Described previously by Evershed et al. (1990). The GC analyses were performed on a Hewlett-Packard 5890A gas chromatograph, coupled to an Opus V PC using HP Chemstation software, which provided instrument control, data acquisition and post-run data-processing facilities. Samples were introduced by on-column injection into a 600 × 0.53 mm i.d. retention gap (deactivated polyimide clad fused silica capillary; Phase Separation, U.K.) connected to the analytical column via a light-weight, glass-lined, stainless-steel union of 0.8 mm i.d. (S.G.E.). The column used was a polyimide clad 12 m × 0.22 mm i.d. fused-silica capillary, coated with BP-1 stationary phase (immobilised dimethyl polysiloxane, OV-1 equivalent, 0.1 µm film thickness; S.G.E.). Helium was used as the carrier gas at a column head pressure of 20 psi, producing a linear flow velocity (v) of c. 100 cm s⁻¹. The temperature programme consisted of a 2 min isothermal hold at 50°C immediately after injection, followed by an increase in temperature from 50°C to 350°C at 10°C min⁻¹. The temperature was then held at 350°C for 10 min. Flame ionization detection was used to monitor the column effluent.

Gas chromatography/mass spectrometry (GC/MS) Two GC/MS instruments were used in this work: (i) a Carlo Erba 5160 Mega Series GC coupled to a Finnigan 4500 single stage quadrupole mass spectrometer; and (ii) a Pye Unicam 204 GC equipped with an S.G.E. OCI III on-column injector. The chromatograph was linked to a VG 7070H double-focusing,
magnetic-sector mass spectrometer. In both instances data acquisition and processing were on a Finnigan INCOS data system. An Opus V personal computer equipped with Finnigan DataMaster software was linked to the INCOS data system on instrument (ii) to provide an additional data-processing facility. Both mass spectrometers were operated in the electron-ionization mode (70 eV) while maintaining an ion source block temperature of c. 300°C. Full scan mass spectra were recorded over the m/z range 40–700. The same GC conditions were employed as described above.

Results and Discussion

The total lipid extracts of a range of bone samples were investigated by high-temperature GC and high-temperature GC/MS. Samples included modern reference materials, corresponding to sections of bovine femur (both with and without absorbed marrow fat; see Materials and Methods above) and a variety of ancient bone samples. The analyses focused on total lipid extracts of bone chips/powder obtained by crushing in a pestle and mortar. Demineralization was not performed in the majority of these initial investigations in order to preserve the integrity of the lipid extract, largely by avoiding chemical degradation (hydrolysis) of any intact acyl lipids that might be preserved in the ancient bone. The possibility of releasing additional lipid by demineralization of the bone matrix was tested in the case of two bone specimens (see below).

Figure 2 shows the high-temperature GC analysis of the trimethylsilylated total lipid extract of contemporary bovine bone both with (Figure 2(a)) and without (Figure 2(b)) marrow fat. Irving & Wuthier (1968) have reported that the total lipid extract of demineralized bovine bone contain similar proportions of non-polar and polar lipids. The methods employed in this preliminary investigation will only provide information on the composition of the non-polar lipid fraction. Information on the polar lipids, e.g. phospholipids, will require the use of an alternative approach, e.g. chemical or enzymatic hydrolysis, to facilitate the release of simpler lipid moieties, e.g. diacylglycerols, prior to GC and GC/MS analysis.

The chromatograms presented in Figure 2 show that the relative proportions of the various acyl lipids and cholesterol reported by Irving & Wuthier (1968) are comparable to the distribution observed in the case of the contemporary bovine bone containing absorbed marrow fat (Figure 2(a)). The cholesteryl fatty acyl esters (structure 6 of Figure 1) were present at lower abundance than reported in the earlier study and were only revealed in high-temperature GC/MS analyses by use of mass chromatography (m/z 368). The identities of the other components represented by the various peaks in the GC profile were also confirmed by high-temperature GC/MS from full scan spectra obtained using electron ionization (see caption to Figure 2).

Interestingly, the concentration of the cholesterol (Table 2) is similar in the bovine femur sample both with and without marrow fat present, which might suggest that this compound is more closely associated with the bone matrix itself rather than the marrow fat, a situation that contrasts directly with that seen for the acyl lipids. The free fatty acids palmitic (C16:0), stearic (C18:0) and oleic (C18:1) acids were evident at shorter retention time than cholesterol, while at longer retention times cholesteryl esters were detectable; their presence being confirmed by GC/MS. Although electron-ionization mass spectrometry was sufficient to confirm the presence of cholesteryl fatty acyl esters through detection of ions arising from the sterol nucleus, determination of the fatty acyl moiety associated with each of the ester peaks will require the use of chemical ionisation mass spectrometry (Evershed & Goad, 1987; Evershed, 1992). Further investigation of the cholesteryl fatty acyl ester fraction in this manner is currently in progress. The minor component eluting immediately before the internal standard in both Figures 2(a) & 2(b) corresponds to cholest-5-en-3β-ol-7-one (structure 7 of Figure 1) which is a well-established oxidation product of cholesterol (Smith, 1981).

GC and GC/MS analyses of archaeological bones

Subsequent analyses focused on ancient bones (see Table 1 for a description of finds) including those of humans, cow, pig, whale, horse and two samples that could not be readily assigned for the reasons discussed above, and soil samples taken from the same context of recovery as two of the bones samples. Significantly, the composition of the total lipid extracts of the archaeological bones most closely resembled that of the contemporary bone free from marrow fat. Although cholesterol was detected in all the archaeological bones studied notable variations were seen in the degradation (diagenetic) products which may relate to the burial environment and possibly the physical state of the bones at their time of deposition.

The high-temperature GC analysis of the Roman horse metapodial bone recovered from excavations at Richborough is shown in Figure 3. The profile obtained is dominated by a compound of comparable retention time to cholesterol. GC/MS confirmed this assignment.
(M+·458, [M-TMSOH]+ m/z 368) in addition to revealing the presence of several degradation products of cholesterol at slightly shorter and longer retention times. Eluting as a shoulder on the cholesterol at longer retention time is 5α-cholestanol (M+·460, [M-TMSOH]+ m/z 370) the expected anaerobic reduction product of cholesterol (Gaskell & Eglinton, 1976). At shorter retention time than cholesterol and in lower abundance is a peak corresponding to 5β-cholestanol (coprostanol; M+·460, [M-TMSOH]+ m/z...
Co-eluting with \(5\alpha\)-cholestanol as a shoulder at shorter retention time is the corresponding \(C_{27}\) steroidal ketone, \(5\alpha\)-cholestan-3-one (M\(^+\)·386). Eluting between this latter component and the cholesterol is a peak corresponding to \(5\beta\)-cholestan-3-one (M\(^+\)·386).

The biochemical (diagenetic) relationships between cholesterol, the two steroidal ketones and the \(5\alpha\)- and \(5\beta\)-cholestanols will be discussed further below.

**Table 2. Total lipid yields and steroid concentrations for contemporary and archaeological bones**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total lipid yield ((\mu g\ g^{-1}))</th>
<th>Concentration of (C_{27}) steroids ((\mu g\ g^{-1}))</th>
<th>Concentration of cholesterol ((\mu g\ g^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contemporary</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cow femur</td>
<td>155.4</td>
<td>74.0</td>
<td>74.0</td>
</tr>
<tr>
<td>Cow femur with absorbed marrow fat</td>
<td>2500.9</td>
<td>78.6</td>
<td>78.6</td>
</tr>
<tr>
<td>Archaeological</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Horse</td>
<td>80.4</td>
<td>58.6*</td>
<td>46.5</td>
</tr>
<tr>
<td>Pig</td>
<td>61.3</td>
<td>41.4*</td>
<td>24.6</td>
</tr>
<tr>
<td>Cow</td>
<td>56.7</td>
<td>40.9*</td>
<td>19.8</td>
</tr>
<tr>
<td>U nidentified 1 (VP86A B 5099 2586)</td>
<td>139.3</td>
<td>32.7*</td>
<td>26.8</td>
</tr>
<tr>
<td>U nidentified 2 (VP86A B 2711 1016)</td>
<td>41.4</td>
<td>3.9*</td>
<td>1.8</td>
</tr>
<tr>
<td>Whale bone from permafrost</td>
<td>n.q.</td>
<td>n.q.</td>
<td>n.q.</td>
</tr>
<tr>
<td>H uman mid-shaft femur (CH F 89 T-10)</td>
<td>21.0</td>
<td>6.3†</td>
<td>6.3</td>
</tr>
<tr>
<td>H uman mid-shaft femur (DBP81)</td>
<td>172.0</td>
<td>16.9†</td>
<td>1.5</td>
</tr>
<tr>
<td>H uman tibia (DBP81)</td>
<td>118.2</td>
<td>12.7†</td>
<td>9.9</td>
</tr>
<tr>
<td>Bone from cave site (LH (N P431))</td>
<td>117.0</td>
<td>10.9†</td>
<td>9.6</td>
</tr>
<tr>
<td>H uman mid-shaft femur (P3519)</td>
<td>54.4</td>
<td>7.1†</td>
<td>5.8</td>
</tr>
<tr>
<td>H uman mid-shaft femur (R 2)</td>
<td>90.8</td>
<td>34.5†</td>
<td>28.7</td>
</tr>
</tbody>
</table>

n.q. = Not quantified.

*, Samples in which \(5\alpha\)- and \(5\beta\)-cholestanols and \(5\alpha\)- and \(5\beta\)-cholestan-3-ones are the major cholesterol degradation products.

†, Samples in which cholest-5-en-3\(\beta\)-ol-7-one is the major cholesterol degradation product.

**Figure 3. Partial gas chromatogram of the trimethylsilylated total lipid extract of the horse metapodial bone from the Roman excavation at Ribchester, U.K.**
The lipids of two substantially older (Mesolithic) samples of bone recovered from excavations at the Vale of Pickering were also studied. These samples fell into the category of "unidentified," largely due to the poor preservation they exhibited. The lipid profile (data not shown) for the Mesolithic "Unidentified 1" bone was somewhat analogous to that observed for the Roman horse metapodial. Most significant was the high abundance of cholesterol (26.8 μg g⁻¹) present. Although cholesterol degradation products, i.e. 5α-cholestan-3-one, 5β-cholestan-3-one, 5α-cholestanol and 5β-cholestanol, were present, they were in lower relative abundance than the cholesterol that was seen in the two lipid extracts of the Roman horse metapodial. At short retention time free fatty acids were again evident. Cholesteryl fatty acyl esters and a number of unidentified lipids were seen in low abundance at longer retention times. All these assignments were confirmed by GC/MS.

In contrast, the second Mesolithic bone ("Unidentified 2") showed a much lower abundance of cholesterol (1.8 μg g⁻¹). In this instance 5α-cholestanol was present in greater abundance than cholesterol. 5β-Cholestanol, 5β-cholestan-3-one and 5α-cholestan-3-one were detected at lower abundance than cholesterol. Free fatty acids were again present at short retention time. Although cholesterol fatty acyl esters were present no other acyl lipids were detected. The chromatograms (data not shown) obtained for the pig and cow bones were largely analogous with those obtained above in the analyses of the Roman and Mesolithic bones. Cholesterol was the major lipid detected together with smaller amounts of the 5α- and 5β-cholestanols and cholestan-3-ones. The concentration of the cholesterol and the other steroidal compounds in these two samples are given in Table 2.

The remaining six bone specimens that were examined ranged in age from 19th century AD to c. 70,000 BP. GC and GC/MS analysis of the total lipid extracts showed all the bones to contain an appreciable concentration of cholesterol. In contrast to the specimens examined above they lacked any of the 5α- and 5β-cholestanols and 5α- and 5β-cholestan-3-ones. For example, Figure 4 shows the GC chromatogram of the trimethylsilylated total lipid extract of a human tibia dated to 4th-6th century AD. Cholesterol was present at a concentration of c. 10 μg g⁻¹ dry wt of bone, while at longer retention time, eluting just before the internal standard peak, was a component that produced a mass spectrum congruent with that of cholest-5-en-3β-ol-7-one. Analysis of the lipid extract of another bone specimen (upper mid-shaft femur) from the same site also showed the presence of cholesterol and the 7-keto compound, albeit at lower concentration. Although neither of these latter two bones were examined histologically others found alongside showed poor preservation.

The oldest specimen examined in this preliminary study of bone lipids was that of 70,000 BP whale found in a permafrost deposit which was also found to contain appreciable amounts of cholesterol with the 7-keto compound the most abundant component in this instance (see Figure 5). The total lipid yields and the concentrations the C27 steroidal compounds, and cholesterol in all the bones subject of this investigation are summarised in Table 2.
While the proteins and DNA of archaeological and palaeontological bones have been the focus of a number of chemical/biochemical investigations, the lipid components of ancient bones have up to now been largely neglected. It is clear from the data reported herein that lipids do indeed persist in ancient bones recovered from a range of contexts. The presence of significant amounts of cholesterol, together with its degradation products, \( \Delta^\alpha \) and \( \Delta^\beta \)-cholestanols, \( \Delta^\alpha \) and \( \Delta^\beta \)-cholestan-3-ones and cholest-5-en-3\( \Delta^\beta \)-ol-7-one is of particular interest. The presence or absence of these degradation products, or their relative abundances compared to cholesterol, or perhaps other bone lipids, may provide a measure of the diagenetic history of the bone. Substantial work will be required in order to determine this possibility. Brief descriptions of the contexts of recovery of the bones studied in this work are given in Table 1. It would seem inappropriate at this stage to attempt a detailed correlation of the diagenetic changes that have occurred in the bone lipids until more thorough assessments have been made of the physico-chemical conditions that prevailed in their respective burial environments. At this stage it is probably sufficient to note that the bones from the Ribchester and Vale of Pickering sites were recovered from waterlogged deposits, while the remaining specimens (with the exception of the whale bone) were recovered from relatively well-drained sites, including a dry limestone environment, a cave site and a 4th–6th century cemetery. On this basis the following general observations can be made.

The presence of \( \Delta^\alpha \) and \( \Delta^\beta \)-cholestanol and \( \Delta^\alpha \) and \( \Delta^\beta \)-cholestan-3-one, together with cholesterol, in the bones from the waterlogged deposits, is the result of two distinct diagenetic pathways of microbial reduction under anaerobic conditions having operated using cholesterol as the substrate. The \( \Delta^\alpha \) and \( \Delta^\beta \)-cholestan-3-one represent the intermediates in the pathways of conversion of cholesterol to \( \Delta^\alpha \) and \( \Delta^\beta \)-cholestanol respectively. In a recent report of the occurrence of these compounds in the skin of Lindow Man (Evershed & Connolly, 1994) it was reasoned that the gut flora migrating from the body, during the early phase of decay, were responsible for the somewhat unexpected occurrence of \( \Delta^\beta \)-cholestan-3-one and \( \Delta^\beta \)-cholestanol in high abundance. Although \( \Delta^\alpha \)-cholestanol was seen in five of the 12 ancient bones studied herein, its relatively low abundance (compared to that observed in the bog body skin) may be more consistent with the natural abundance that might be expected to arise through the reduction of cholesterol by soil microorganisms during burial (Gaskell & Eglinton, 1976). The finding of cholest-5-en-3\( \Delta^\beta \)-ol-7-one, together with cholesterol and none of the reduced compounds, in the bones from the well-drained or dry sites, is strongly indicative of oxidising conditions having prevailed.
either pre- or post-burial of these specimens. Oxidation of lipids, including cholesterol, occurs in two distinct ways: (i) autoxidation involving attack of singlet oxygen which gives rise to keto, e.g. cholest-5-en-3\beta-ol-7-one, and hydroxy derivatives via hydroperoxides; and (ii) lipoxygenase-catalysed oxidation. Enzymes with lipoxygenase or lipoxygenase-like activity are found in many micro-organisms, plant and animal tissues.

The decay processes operating on the organic components of bones include: (i) the action of cellular enzymes or micro-organisms active in the tissue at the time of death; (ii) the activities of micro-organisms present in the burial environment, and (iii) chemical processes, e.g. hydrolysis and oxidation. As alluded to above the relative contributions of these chemical and biochemical decay processes will be highly dependent on the conditions that prevailed in the burial environment. In addition the extent of preservation of biomolecules will be influenced by a range of pre-burial factors, such as the physical state of the bone at the time of burial. For instance, in the case of many animal bones recovered from archaeological sites considerable uncertainty will exist as to whether or not connective tissue or musculature were associated with the bone at the time of burial or discard; this will exert a strong influence on the potential for recovering biomolecules due to the differing extents of fungal or microbial growth.

In relation to the above, examination of lipids extracts for molecular indicators of saprophytic organisms may be of value in assessing the extent and nature of decay, and maybe relevant to all classes of biomolecule that are currently being investigated in ancient bones. The normal practice in this investigation was to remove outer surface of the bone in order to eliminate soil contaminants prior to powdering and solvent extraction. In the case of one bone (9th–10th century human mid-shaft femur, CHF 89 T–10) the possibility was considered of detecting the lipid remains of saprophytic organisms by washing the intact bone with solvent prior to undertaking the normal cleaning procedure (although visible soil deposits were removed with a scalpel prior to washing). The resulting GC trace obtained (Figure 6) reveals a markedly different composition to that seen in the solvent extracts of cleaned bones. Cholesterol was present as one of the major components as indicated in Figure 6. Interestingly, cholesteryl fatty acyl esters were seen at long retention time in high abundance. Both the cholesterol and these latterly mentioned fatty acyl esters are presumed to have been endogenous to the bone at the time of death and subsequent burial. The presence of cholesteryl fatty acyl esters in association with the mineral phase of fresh bone has been reported (Shapiro, 1973), however, their relatively high abundance in the surface washings would seem to be consistent with them.
having precipitated from the blood that would have been associated with the bone at the time of death and burial. Of relevance in the search for lipid indicators of saprophytic organisms is the compound eluting at c. 23.5 min in similar abundance to cholesterol. The mass spectrum of this substance shown in Figure 7 is consistent with that of hopan-22-ene (Structure 8 of Figure 1; M⁺/410, major ions M/z 189 and 191; see inset structure for the origin of the fragment ions). Although hopanoids with 30 carbon atoms or less occur in higher and lower plants, there is compelling evidence that most hopanoids in sedimentary materials are of microbial origin (Ourisson et al., 1984). In view of this the hopan-22-ene is likely derived from saprophytic organisms originating from the soil and that have been involved in the decay of bone, and possibly any tissues and musculature that were associated with the bone at the time of burial. At least two other hopanoid components were present at longer retention time, however, further work is required to fully confirm their structures. Further work is also required to elucidate the structures of a range of homologous complex lipids that were seen eluting prior to the cholesteryl fatty acyl esters (see Figure 6). The identities of these components will be reported in a future publication.

The GC profile for the total lipid extract of CHF 89 T-10 obtained using our normal cleaning procedure is shown in Figure 8(a). In contrast to the situation observed for the solvent washings (see above) of the intact bone the lipid extract of the cleaned and powdered bone contained relatively little of the hopan-22-ene and cholesteryl fatty acyl esters compared to cholesterol. This observation would appear to lend further credence to the arguments that the cholesteryl fatty acyl esters and the hopan-22-ene are more closely associated with the surface of the bone rather than the mineral phase. Work is currently in progress in this laboratory to more fully elucidate the origins and distributions of lipids in the various parts of buried bones.

In view of the high abundance of lipid that was seen in the modern cow bone containing absorbed marrow fat (Figure 2(a)), the low abundance of acyl lipids, notably free fatty acids, mono-, di- and triacylglycerol, seen in ancient bones is worthy of some discussion. Two possibilities exist for the low recoveries of lipid: (i) the bone(s) contained low concentrations of lipid at the time of burial or discard, cf. to the low concentration of lipid seen in the section of contemporary cow femur lacking marrow fat (Figure 2(b) and Table 2); or (ii) the bone(s) contained high concentrations of lipid at the time of burial or discard but it was chemically and/or microbiologically degraded during burial. Drawing clear distinctions between these two possibilities on the basis of chemical analyses of ancient bone is unlikely to be successful. It may be possible to draw some conclusions by visual examination as to type of bone.
and its physical condition at the time of discard or burial giving due consideration to the nature of cutmarks or breaks. It can be envisaged that bones would have been broken or boiled to remove marrow fat for human consumption. This practice would have obvious implications for the reduction in the lipid content of some archaeological bones. Samples of two of the bones were demineralized by treatment with dilute acid prior to solvent extraction. Although an enhanced yield of cholesterol was achieved in both instances following demineralization (Table 3) no significant increase was seen in the recovery of acyl lipids.

The finding of cholesterol and its degradation products, in all the bone samples so far studied (11 in total) is especially significant, as it raises the possibility for its use as a new palaeodietary indicator in studies involving the measurement of $\delta^{13}C$ values. In view of the relatively high concentration of cholesterol detected ($1.5-46.5 \mu g \, g^{-1}$) and the availability of on-line GC/isotope ratio monitoring-MS instruments it will be possible to perform measurements of the $\delta^{13}C$ on the cholesterol preserved in archaeological bones and use
Measurements on the C27-5 measurements on collagen to draw conclusions concerning the various contributions to diet, e.g. C3 versus C4 plants. The possibility of using a molecule such as cholesterol is particularly attractive due to the fact that we can be completely assured of its integrity, i.e. the structure of the molecule can be confirmed unambiguously, such that we can be confident that its carbon skeleton has been completely unaffected by diagenesis. The possibility also exists for performing δ13C measurements on the C27, 5α- and 5β-cholestanols and cholestan-3-ones, or the 7-keto compound, where they are present in sufficient abundance. Even though these are degradation products of cholesterol the integrity of their carbon skeletons has been retained fully intact, the δ13C measurements can be recorded and compared directly with those of the endogenous cholesterol. Investigations aimed at developing the use of cholesterol is particularly attractive due to the fact that we can be completely assured of its integrity, i.e. the structure of the molecule can be confirmed unambiguously, such that we can be confident that its carbon skeleton has been completely unaffected by diagenesis.

### Possible sources of contamination

In any chemical analysis of archaeological artefacts or palaeontological finds the possibilities for alteration ("contamination") of the original chemical composition through the introduction of substances of exogenous origin must be carefully considered. Four principal sources of contamination are recognized: (i) substances migrating or diffusing into the specimen during the period of burial; (ii) substances arising through the growth of fungi or bacteria on the specimen during the period of burial; (iii) inadvertent post-extraction contamination due to the handling of specimens and artefacts; and (iv) post-extraction contamination arising through the growth of bacteria and fungi on specimens due to storage under inappropriate conditions.

In spite of the rigor employed in the storage, handling and sample preparation of the specimens analysed during the course of this investigation, the possibility has been considered that the cholesterol observed in the samples may represent contamination. The possibility of inadvertently introducing contamination from finger skin lipids, can be dismissed on three accounts: (i) the two blank extractions that were performed yielded no significant cholesterol or other lipids of the type found in the ancient bones; (ii) squalene which is present at higher concentration in skin lipids than cholesterol was undetectable in all samples; and (iii) the presence of the cholesterol degradation products, i.e. C27 steroidal ketones and the 5α- and 5β-cholestanols, do not occur naturally in skin lipids but arise diagenetically from cholesterol only under anaerobic conditions (see above, and Evershed & Connolly, 1994).

The burial matrix, namely the soil, is another source of chemical contamination that must be carefully considered. The detection of the presence of lipids derived from the remains of saprophytic organisms originating from the burial environment and that have been active on the bone, has already been discussed in detail above. A related, although distinctly different mode of contamination, derives from the possibility for the migration (diffusion) of lipids from the soil into the bone during burial. It has been reasoned previously, in relation to the study of bog bodies (Evershed & Connolly, 1987, 1994; Evershed, 1990, 1991) and pottery (Heron et al., 1991), that if the migration of lipids into archaeological finds occurs to any appreciable extent, then a chemical signature representing the chemical components of the burial environment should be superimposed on the chemical signature for the endogenous lipid components of the find. This possibility was investigated here by an analogous procedure to that employed previously (e.g. Evershed & Connolly, 1987, 1994; Heron et al., 1991), namely by comparing the composition of total lipid extract of two bones with that of the immediately surrounding burial soil. Analogous results were obtained in both instances. Figure 8 shows the GC "fingerprints" obtained from an analysis of the total lipid extract of a human mid-shaft femur (CHF 89 T-10) together with the corresponding GC "fingerprint" from the lipids of the surrounding burial soil. The composition of the soil lipids is very typical of that we have obtained from a range of different agricultural and archaeological soils from Northern Europe (Heron et al., 1991; Evershed & Bethell, in press). The major components of the soil are long-chain alcohols, alkanes, fatty acids and wax esters. In contrast the major components of the bone correspond to the C27 steroidal compounds, comprising cholesterol and smaller amounts of its diagenetic products as indicated in the figure caption.

### Conclusions

Although the early work of Das et al. (1967) and Everts et al. (1968) on fossil bones showed the possible presence of substances of comparable chromatographic (TLC and GC) mobility to various acyl lipids and cholesterol, the results presented herein provide the first unambiguous chemical evidence, based on the
use of GC/MS, for the survival of lipids in archaeologi-
cal bones (and one palaeontological specimen). The
presence of lipids in ancient bones with compositions
and structures that are markedly different to those that
are seen in soil extracts indicates that the lipids in bone
are not derived from the soil by migration of diffusion.
In one bone specimen hophanoids components, com-
prising largely hopan-22-ene (diploptente), were
detected that represent the remnants of saprophytic
bacteria that originated in the soil and appear to have
been involved in the decay of the bone. Cholesterol was
seen in the lipid extracts of all the bones that were
examined. The presence of different diagenetic prod-
ucts of cholesterol in the various bone specimens raises
the possibility of drawing conclusions relating to the
conditions that prevailed in the burial environment.
Additionally, the survival of cholesterol, and its dia-
genetic products, provides a potential new source of
$\delta^{13}C$ information, accessible by means of GC/isotope-
ratio-M S, for use in palaeodiетary investigations.

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