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A REVIEW OF CURRENT APPROACHES IN THE PRETREATMENT OF BONE FOR RADIOCARBON DATING BY AMS

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ABSTRACT. Although the reliability of ^{14}C dates of bone has increased greatly since AMS methods permitted better pretreatment on smaller samples, most old, badly contaminated or severely weathered bone still give serious problems. Several groups have recently proposed improvements to sample purification methods, often supported by a number of ^{14}C measurements. We present here an overview of these improvements. The issue is complicated by the following:

1. Different problems are presented depending on age, preservation and degree of contamination of bone.
2. Methods may or may not be developed with routine application in mind.
3. Determining the conditions for which any method can be regarded as reliable is not at all straightforward.

INTRODUCTION

Several published papers, referred to below, describe problems and results in the pretreatment of bone for radiocarbon dating. Usually, they describe methods in one laboratory, and set the results in the context of the work of other laboratories. They are essential reading for knowledge of the subject, and contain important general and specific points for which there is not room here. In this review, we consider especially the development of recent methods, and hope to provide a simplified account for non-specialists, and an overview for those in the field.

The *attractions* for archaeology of the radiocarbon dating of bone are as follows:

1. Compared to most organic remains, bone intrinsically contains a high degree of additional archaeological (or environmental) information, *e.g.,* the species of animal, the bearing of cut-marks or decoration.
2. Bone may be fragmented, but generally occurs in pieces large enough to be far less mobile stratigraphically than, say, charcoal. Also bone is a common find on a large range of sites.
3. The main organic constituent of bone is protein, which has at least initially, a well-defined chemical structure. Further, the organics in bone are the subject of independent study, *e.g.*, for diet, disease and paleogenetics.

The *problems* in dating bone arise from the difficulty in extracting material containing carbon atoms that 1) are indigenous, 2) can be demonstrated not to include any exogenous carbon, and 3) are available in sufficient quantity. These problems were recognized in radiometric ^{14}C dating, but the requirements of gram-sized samples placed restrictions on the degree of chemical sophistication in pretreatment, and on the range of material available, making the reliability of dates on bone hard to assess and often suspect. AMS methods have enabled more elaborate chemistry to be used, and many bone dates to be critically evaluated. However, problems remain, particularly where diagenesis is severe.

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These problems take different forms, and require different kinds of solutions, depending upon the bone and the context of dating. Note that analogous problems, although often quantitatively different, also occur with stable isotope measurements on bone (Ambrose 1990; DeNiro 1985).

In this review, we first categorize and consider the various types of problem that attend the dating of bone. Next, we discuss ways of categorizing the bone samples themselves, with particular emphasis on analytical studies that reveal aspects of diagenesis. With these clarifications in mind, we discuss the various chemical approaches, and their implications, that are taken in pretreatment. We consider the general problem of validating these approaches, and we summarize the situation, and offer some recommendations.

Classification of Problems

Age

Figure 1 shows the extent to which contamination by modern carbon can reduce the age of the bone. A 1% level of modern contamination produces serious systematic errors (more than 2 standard deviations (σ)) for bones older than about 8 ka, with the bias being roughly constant (in terms of quoted error) from 15 ka to 30 ka. For such an error to be insignificant (*i.e.*, <0.5), contamination should be kept to less than 0.2–0.1% of equivalent modern carbon. The older the bone, the greater the probability that actual contamination will be appreciably younger than the age of the bone.

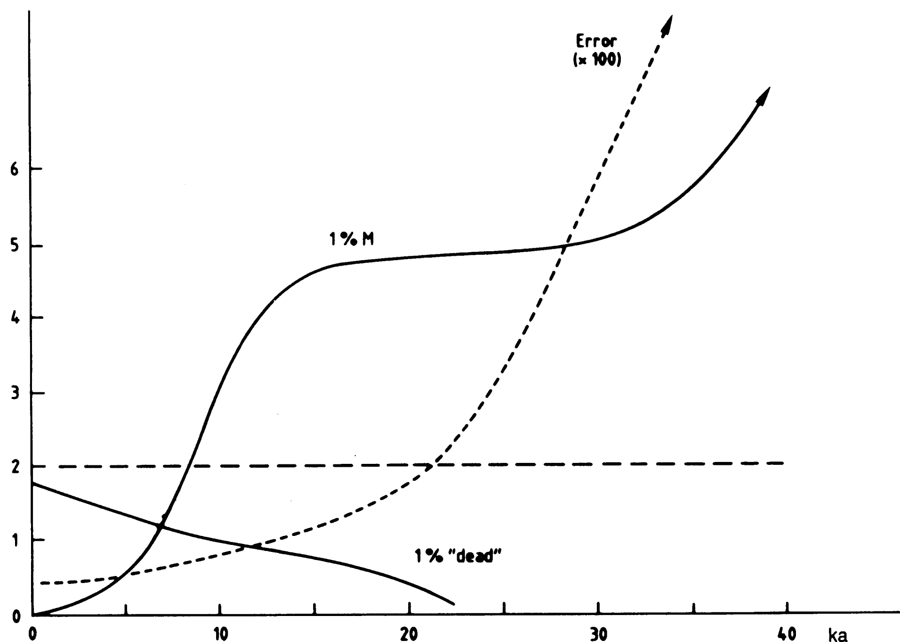

Fig. 1. A plot of the systematic error incurred on a date as a result of contamination. The horizontal axis is the true ^{14}C age of the sample in ka. The vertical axis is in multiples of the standard deviation of the "typical best" quoted laboratory random error made for samples corresponding to that particular age. The two continuous curves show how the systematic error compares with a 2 σ error (— — —) for contamination of the sample at the level of 1 pMC and 1% "dead" carbon. The systematic error can be regarded as highly significant when it is above the 2 σ line. The line labeled Error (× 100) (- - -) is the actual systematic error for 1 pMC contamination, for which the vertical axis is now in units of 100 yr.

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Preservation

This has two aspects: 1) as the original organic content diminishes, problems arise from the need to process larger amounts of sample, increasing the proportion of exogenous material in the sample; 2) as well as loss of protein, chemical degradation of the surviving protein will have occurred to a greater or lesser extent. This greatly increases the difficulty in purifying and characterizing the indigenous material.

Contamination

By this we mean the incorporation of exogenous material. Contamination only affects the radiocarbon date if the contaminant's ^{14}C content differs from that of the indigenous material. This is usually hard to predict; it is likely that most contamination is only a little younger than the sample, so that in practice high levels of contamination may not necessarily be detected through dating comparisons. Common sources of exogenous material are:

- applied consolidants and preservatives
- mobile organic molecules in the enclosing sediment
- metabolic products from microorganisms.

The difficulty of separating exogenous material becomes especially acute when the contamination bonds chemically to partially degraded bone material. Although difficult to identify, such a process occurs in tanning, where collagen becomes cross-linked to polyphenols. Similar reactions can take place during burial and laboratory pretreatment, *e.g.*, the Maillard reaction between amino acids and saccharides. In this sequence of reactions glycosyl amino compounds are formed from sugar-amino condensations. Further reaction, involving rearrangements, subsequent dehydration, fission, degradation and polymerization leads to relatively stable brown pigmented complexes (Berk 1976).

Economics

Most refinements in method bring additional costs, usually in manpower and expertise. Thus, any method selected must be matched to the importance of the potential date, as well as take into consideration the likelihood of error for a particular sample from a given context. The sample and its context should be characterized beforehand, so that the appropriate decision can be made.

Sample Size

If the standard sample for AMS dating is taken as 1 mg carbon (accepting that 100 μg is also possible, on occasion, 20–50 mg of well-preserved bone should be adequate (for dating collagen). If preservation is poor, however, 1 g of bone may not contain sufficient carbon. Further, in poorly preserved bone, it is desirable to extract highly specific fractions, increasing by a large factor the quantity of bone required. Available samples for bone dating vary from a few milligrams (of bone) to tens of grams. In practice, therefore, a balance must often be struck between the specificity of extract and the quantity of bone consumed.

Any approach to the pretreatment of bone must take account of all of these considerations. A specific sample or project will tend to have 1 or 2 factors dominant, which will then determine the particular choice of method. Thus, the sample itself needs to be considered.

Classification of the Bone Sample and Context

How the factors cited above influence a given date depends upon the assessment made for the particular bone sample. This assessment comes both from *initial* information and from *analytical* measurements.

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Initial Information

Geologic Age. Although in principle not known beforehand, a good estimate of age is usually possible from the context or reason for dating. We find it convenient to make a dichotomy defined by the Last Glacial Maximum (18 ka). Bones older than this are more difficult to date accurately, not only because of sensitivity to modern contamination, but because they are less common, often small, and usually contain less protein.

Value of Date. For example, is it worth measuring multiple dates on different fractions? Is it worth using much more elaborate pretreatment techniques, and thus, much more sample?

Quantity Available. Although defined by the submitted sample, whether there is sufficient quantity for a given method will not be known until the preservation of organic content is measured. This will depend on analytical information.

Analytical Chemistry of Bone

We consider here the analyses that help to define the state of preservation of the bone organic content. We treat later how measurements during the course of pretreatment can help in validating the chosen method.

Total Collagen Content. Since nearly all present bone dating is done on collagenous extracts, the preservation of collagen is of greatest interest. Collagen is the native, biochemically intact triple-helical macromolecule. Following DeNiro and Weiner (1988a), we will use "collagen" to refer to collagen that has undergone a degree of diagenesis. Alterations during diagenesis are believed to include random cross-linking, humification of parts of the molecule, attachment of exogenous humic materials, and hydrolysis with preferential loss of some amino acids.

In most laboratories, "collagen" is converted to gelatin, using slightly acidic hot water, in which the triple helical structure has become unfolded. There will be some differences between laboratory gelatins because of different "collagen" extraction and gelatinization procedures. In particular, since there is a compositional difference between proteins that are soluble under the conditions of bone decalcification (usually cooled dilute HCl), and insoluble protein, the composition of "collagen" (*i.e.*, the insoluble residue) will depend on the conditions used when decalcifying (*i.e.*, strength of acid, length of time, temperature), and also on the degree of diagenetic alterations of the collagen.

"Collagen" can be estimated by %N in the whole sample, or, much more relevantly, on estimating the decalcified extract (for total N, or better still, protein content or amino acid content) (Gillespie, Hedges & Humm 1986). Such numbers are used in classifications (Stafford, Brendel & Duhamel 1988; Hedges & Law 1989), but give only a rough idea of potential dating difficulties. They do not indicate whether the nitrogen is wholly present as collagen, nor do they indicate the extent of non-nitrogenous organic material. However, low apparent values of surviving "collagen" (*e.g.*, <10% of the "modern" value of 200 mg collagen g^{-1} of dry bone) certainly indicate poor preservation, and strongly suggest the need for further analytical information.

Further Analysis

C/N Ratios. These may be measured on whole bone, or some extract thereof. They have been shown to be useful in $\delta^{13}C$ studies (Ambrose 1990; DeNiro 1985). High values (*i.e.*, >>4) indicate either extensive diagenesis (*e.g.*, deamination), or a high proportion of exogenous carbon (*e.g.*, as humics), which may become cross-linked to the residual "protein." In practice, a very wide range of values is encountered, although this is liable to depend upon the stringency of sample preparation. (See, for a recent example, Ambrose (1990), where C/N values from gelatinized

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"collagen" tend to fall outside the range of 2.8–3.5 for bones whose extractable collagen is < *ca.* 10% of modern levels.)

Infrared Spectrum. The infrared spectrum of collagen or gelatinized collagen, even after substantial diagenesis, is usefully diagnostic (DeNiro & Weiner 1988a; Law *et al.* 1991), and should reveal and help to identify many classes of impurities at the >5–10% level. It has been used, for example, to estimate the degree of contamination of collagen with polyvinyl acetate preservatives as well as by humics. Infrared spectrometry has also been used to assess the degree of recrystallization of hydroxyapatite (Weiner & Bar-Yosef 1990), which is an additional measure of the diagenetic alteration of the sample.

Stable C and N Isotope Ratios. In most cases, for protein, $\delta^{13}C$ values are only likely to be shifted by 0.5–0.8 for a 10% contribution from soil humics since the $\delta^{13}C$ for soils rarely differs by more than 8% from that of protein. However, deviations from the C_3 range of –19 to –22‰ of >2‰ are not uncommon in gelatin extracts, and strongly signal the presence of exogenous carbon. Thus, anomalous $\delta^{13}C$ values are valuable in indicating gross contamination. The *change* of isotopic value during sample preparation (discussed below) can be a very sensitive test for the removal of contaminating material. Nitrogen isotopes can also be measured, but because values for uncontaminated bone collagen are rather difficult to predict, again only gross contamination is likely to be evident. However, our impression in practice is that there is often a strong correlation between seriously aberrant $\delta^{15}N$ values (*i.e.*, outside the range of +5 to +15 (w.r.t. atmospheric N)) and presumed contaminated preparations.

Amino-Acid Composition. Collagen has a characteristic amino-acid composition, containing unusually high abundances of glycine (Gly), proline (Pro) and hydroxyproline (Hyp). This facilitates the determination of the proportion of surviving "collagen" in a proteinaceous mixture by amino-acid analysis. However, a number of factors make interpretation problematic; these are:

1. The process of extraction will alter the total amino-acid composition of the bone. (*e.g.*, the composition of the acid-insoluble component will, in general, be different from the acid-soluble component.)
2. Even if all the amino acids analyzed are derived from bone collagen, the resulting composition is not necessarily accurately collagenous because differential loss of amino acids and peptides can take place during collagen diagenesis.
3. Some amino acids may be intrusive, and their proportion increases as the "collagen" content of the bone decreases.
4. Some amino acids may derive from non-collagenous protein within the bone, which have been better preserved.

Our own practice is to perform amino-acid analyses on material that has been extracted as the insoluble component on decalcifying bone with dilute acid, carefully washed in alkali and subsequently gelatinized and filtered. We find that, provided the total extractable "collagen" is at least 10 mg g^{-1} of bone (*i.e.*, 10% of the level found in modern bone), the amino-acid composition of such material is usually within 10–20% of that expected for collagen. Thus, whereas a collagenous composition can be expected from most bones in which at least 5% of the original protein appears to have survived, the situation is more confused for bones with worse organic preservation (Weiner & Bar-Yosef 1990; Stafford *et al.* 1991). In such cases, non-collagenous amino-acid compositions may indicate indigenous non-collagenous protein surviving better than collagen, or exogenous proteinaceous material, or both. A useful index for a non-collagenous composition is the Gly/Asp ratio, because glycine is unusually abundant in collagen, whereas

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aspartate is abundant both in bone non-collagenous proteins and in most (including bacterial) protein (Taylor 1980; Long *et al.* 1989; Weiner & Bar-Yosef 1990; DeNiro & Weiner 1988a; Law & Hedges 1989). Most bones, for which analysis indicates non-collagenous amino-acids, have total insoluble protein contents of less than 1 mg g^{-1}. Relatively few analyses exist for the range 1 mg g^{-1} to 10 mg g^{-1}, yet it is this range where some "collagen" or collagen-derived amino-acids is probably available for radiocarbon dating (provided it can be purified), but where the composition is far from collagenous. This area could benefit from further work; for example, few analyses of Hyp have been made for such bones.

Summary

Despite differences in analytical method and in agreement on how degraded "collagen" is characterized, laboratories generally agree in recognizing:

- "good" preservation (>20% original collagen remaining)
- "poor" preservation (<5% original collagen remaining)
- "non-collagenous" preservation (<0.5% collagenous composition remaining).

There is a close, but not well-understood or quantitatively definable, relation between collagen survival and the efforts that are required to remove contaminants.

If the degree of actual contamination is substantial (say >5–10%), it can often be recognized analytically on bones with good or intermediate preservation. Contamination must always be suspected for poor and non-collagenous preservation, and it is probably not possible to use chemical analysis to show that significant contamination is not present. In any case, if no recognizable "collagen" signature remains, there are no longer good grounds for believing that extracted organic material is mainly indigenous.

Nature of Contamination

The detailed chemical nature of contamination depends upon the particular environment. Any bone is liable to contain exogenous soluble and insoluble organic materials – ranging from rootlets and soil particles to humics and other molecules mobilized in groundwater. Bone has an enormous surface area (10 m^2 g^{-1}), and a correspondingly high potential for adsorption of molecular species onto hydroxyapatite, and of reaction with collagen fibrils. Relatively little work has been done beyond the obvious remarks above to identify possible contaminating species to be found in the burial environment. A "humic" fraction is nearly always extractable from bone with alkali, and generally gives a similar or younger date. Note that humic acids contain a few per cent of amino acids, as well as other carboxylic groups. Humic complexes with clays are to be expected, and these are likely to release soluble components slowly during pretreatment. Many of the contaminants can be regarded as chemical species (polyphenols, polysaccharides, lignins) in addition to the degraded protein and other bone organic component mixture, which should be removable by physico-chemical separation techniques. However, at least two kinds of contamination are particularly difficult to combat. These are the occurrence in bone "collagen" extracts of exogenous amino acids of a different age (Stafford *et al.* 1991), and the cross-linkage, presumably of humics, to extracted albumin (revealed by gel electrophoresis) (Tuross 1989). Whereas these were detected in admittedly "difficult" contexts (very low collagen survival), such contexts are quite common, and similar contamination is potentially possible in any environment. Chemical pretreatment methods must take such contamination into account.

Diagenetic Changes

The study of contamination would be greatly simplified if the diagenetic changes to bone collagen,

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and indeed other bone proteins, *e.g.*, glycoproteins, mucopolysaccharides, were understood. Although this is a most important subject, clear evidence is insufficient for more than an outline of the principal areas of ignorance.

For all but the most poorly preserved bone, the acid-insoluble extract remains largely collagen-like in amino-acid composition, but spans a wide molecular weight range. Cleavage at specific amino-acid positions within the peptide chain (*e.g.*, by CNBr, trypsin or collagenase) is possible with a reduced yield. Amino acids susceptible to, *e.g.*, oxidation or deamination, are generally reduced in abundance. It is not known to what extent "collagen" consists simply of a range of shorter lengths of collagen, retaining the overall tertiary structure, and to what extent novel cross-linkage between collagen peptide residues and with other molecular species (both indigenous and exogenous) takes place. C/N ratios from diagenetically altered bone indicate that, frequently, the extracted protein is accompanied by material with much lower N content; and also that nitrogen can be present in bones in forms other than amino acids. This material has not been identified, even to the extent of whether it is indigenous (the result of deamination, *etc.*) or exogenous (humic acids, *etc.*). As a general rule, measures indicating the extent of degradation of "collagen" (yields of specific reactions, infrared spectra, composition, ease of purification) show it to correlate much more closely with the fraction of surviving "collagen" in a bone than with the duration of burial.

The acid or EDTA-soluble fraction contains material that is adsorbed or stabilized by the inorganic matrix as well as "collagen" in the process of being leached away. There is increasing evidence that non-collagenous proteins are preferentially preserved (Masters 1987), and that these dominate when very little collagen survives. Aspartate racemization is faster in the soluble fraction than in the acid-insoluble fraction, and particularly if the soluble composition is markedly non-collagenous. Cross-linking of surviving serum protein mentioned above may well occur during the diagenesis of other NCPs. Such cross-linking is likely to help retain in the bone normally soluble proteins that would otherwise be leached away. The study of NCPs tends to be concerned with a higher grade of diagenesis than for collagen studies, and chemical changes to the protein (deamination, oxidation, hydrolysis) will be more substantial; but very little is in fact known of their extent. A degree of antigenicity is retained for many proteins, but again, nothing is known of the extent of epitope alteration through diagenetic changes in secondary or tertiary structure.

THE APPROACHES TAKEN IN PRETREATMENT

Carbonate

No one so far has demonstrated that the indigenous carbonate in the bone inorganic matrix can be extracted reliably and separated from diagenetic carbonate (see Stafford *et al.* 1991, for greater detail). The demonstration that careful etching with acetic acid can enable the residual carbonate to retain (albeit with some modification) a biogenic $\delta^{13}C$ signal (Lee-Thorp, Sealy & van der Merwe 1989) gives some hope, however, that such a component might still be useful in ^{14}C dating.

Collagen

Nearly all bone-dating procedures have concentrated on isolating collagen or its components, because collagen is the dominant protein, at least until more than 90% has been lost.

Initial mechanical separation of the bone from rootlets and from superficial infiltration of silt, is, of course, important and useful. A refinement of this is chemical fractionation to isolate the "aggregates" (DeNiro & Weiner 1988b) identified as having potentially a better protected environment for collagen survival, with advantages for stable isotope measurement. (Aggregates are a fraction of the bone, *ca.* 10%, relatively resistant to dissolution by the action of sodium

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hypochlorite.) Our own work (ms.) in collaboration with Weiner strongly indicates that the dating of aggregates from low collagen bone does not give reliable results.

"Collagen" is normally extracted as the insoluble residue upon decalcifying bone. (But see Stafford *et al.* (1991) for the "non-destructive" extraction of solubilized "collagen" from solid bone by hot water.) Decalcification of ground bone by 1 M HCl is quite fast, but arguably, the degraded "collagen" and non-collagenous proteins in very low collagen bone can be extracted in higher yield using EDTA (Masters 1987). (EDTA is usually "dead", so that its removal beyond 99% is not critical.) The "collagen" residue may then be purified from other insoluble materials by solubilizing to form gelatin (Longin 1971). Many laboratories treat the gelatin with alkali at this stage, with the aim to remove humic components. The purity of the gelatin produced at this stage depends on the techniques used, *e.g.*, the degree of filtration, or the extent to which material is trapped within collagen fibrils. Thus, it is rather difficult to compare the performance among different laboratories. For example, at Oxford, we noticed a significant improvement in the separation from many contaminants, on changing to our automated continuous flow system (Law & Hedges 1989), even without any significant change to the chemistry of the process. The purity of the gelatin can be assessed by amino-acid analysis on the hydrolyzate, and by infrared spectrometry. In many laboratories, the pretreatment outlined above is taken as the routine method for bones in "good" chemical condition (Longin 1971; Olsson *et al.* 1974; Taylor 1980; Stafford, Brendel & Duhamel 1988; Stafford *et al.* 1991). Further purification of gelatin is possible, and some studies have demonstrated its advantages. Ultrafiltration (Brown *et al.* 1988) to retain MW >30,000 has been shown to increase the ^{14}C age of some gelatin extracts, presumably because lower MW fractions present are substantially too young. This approach will not remove high molecular weight humic acids, or complexes between humics and (partially degraded) gelatin, but the method has the advantage of being fairly easy. Ion exchange of the gelatin (Law & Hedges 1989) using AGMP-50 also "improved" the ^{14}C date on occasion, and yielded a product with a purer infrared spectrum (Law *et al.*, 1991). Probably the combination of both techniques would be more powerful still. Whereas the *validation* of such methods is discussed below, we should point out that the methods outlined have probably been taken as far as they can. At best (and with considerable effort), an analysis of amino-acid composition, C/N ratio and infrared spectrum could give reassurance that the preparation is very probably at least 90% "collagen"; beyond that, one has to rely on the methods themselves to deal with unknown forms of contamination. A higher level of reliability can only come from selecting material better defined at a molecular level. Nevertheless, we recommend this type of approach for bones with collagen preservation (defined in terms of the extracted gelatin) at levels of >5–10 mg g^{-1} (*i.e.*, >2–5% remaining).

Peptides of Collagen

The main choice for specific cleavage of collagen is to employ collagenases. DeNiro & Weiner (1988c) explored the potential for determining stable isotope ratios from degraded bone. We have extended this work, using HPLC to separate the peptides produced, and in particular, to isolate and purify the principal tri-peptides (Gly-Pro-Hyp,.....) (van Klinken & Hedges 1992a, b). Undoubtedly, the specificity of [Gly-Pro-Hyp?] gives a much needed confidence in the material being analyzed. The disadvantage is that the theoretical maximum yield of Gly-Pro-Hyp is only about 10% of the total collagen. Also, results so far indicate that yields strongly depend on the state of collagen preservation, presumably because enzymatic cleavage demands a rather specific set of steric conditions. Thus, the technique may well not be suitable for "low collagen" bones unless a very large amount of material (at least 100 g) is available. The possibilities of this method are currently being investigated.

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Mixed Amino Acids from Collagen

Several researchers have chosen to date purified total amino acids, after hydrolyzing extracted gelatin (Gillespie, Hedges & Humm 1986; Gurfinkle 1987; Long *et al.* 1989). The amino acids may be separated from contaminating humics by the use of XAD resin (Stafford, Brendel & Duhamel 1988; Gillespie, Hedges & Humm 1986) from general contamination by using charcoal (Gillespie & Hedges 1983); and by adsorption on and subsequent elution from ion-exchange columns (Gillespie, Hedges & Humm 1986). Although the method is attractive, it is not clear that it offers advantage over well-purified gelatin, and we have now abandoned it. One reason is that it is difficult to avoid forming condensation products with impurities such as carbohydrates during hydrolysis (*via* the Maillard reaction). These are not easily separated by ion exchange. This was demonstrated in a model experiment carried out in Oxford, in which an equal quantity of modern sucrose was added to "collagen" (dated at >45 ka) extracted from a well-preserved bone. The mixture was subjected to the standard procedures of gelatinization, hydrolysis and ion exchange. The final product was measured for ^{14}C content, and proved to contain about 5 pMC contamination, presumed to be in the form of amino-sugars that were not separated from amino-acids by the ion exchange column.

A recent refinement of this approach is to extract chemically the carboxylic carbon by reaction with ninhydrin (Nelson 1991). This effectively deals with most condensation products, but might still include carbon from exogenous carboxylic groups. An important attraction of this refinement is its simplicity (with some cost in yield).

Specific Individual Amino Acids

Hyp accounts for about 10% of the amino-acid content of collagen, and is rarely found in abundance elsewhere. It can be isolated chemically (Stafford *et al.* 1982; Gillespie, Hedges & Wand 1984), but the effort involved has prevented any frequent practice of this method. Examples are known where the dates have been "improved" (Hedges *et al.* 1988; Stafford *et al.* 1987). However, Hyp *may* be exogenous (*e.g.*, it is found in animal urine, fungal cell walls, some plant structural proteins and in some microorganisms). In "low collagen" bone, Hyp tends to be less than 10% of the total collagen, so that 20–50 g may be required of a bone in which 0.5% of collagen survives to produce 1 mg carbon from extracted Hyp. But as mentioned, data on Hyp from such bones are scarce. To our knowledge, measurement on Hyp have been confined to bones with much higher levels of surviving collagen than this.

Recently, it has become possible to separate and purify amino acids by using preparative HPLC (Stafford *et al.* 1991; van Klinken & Mook 1990). Such work requires an impressive level of funding and expertise, although once developed, is reasonably straightforward. At this stage, the main use has been to investigate the concordance of dates between different amino acids in selected specimens. Where collagenous compositions are found, concordance is probable, and becomes very probable when more than 10% of collagen survives. Stafford *et al.*(1991) give a detailed account of the major work so far in this field, including 8 Hyp measurements. In only one case (bone from Pyramid Lake) is there a discrepancy between Hyp and other amino acids (especially glycine). In such a case, a question must be placed against any date. The power of dating individual amino acids is to indicate the validity of the pretreated sample, as well as to ensure that a well-defined molecule is dated. Compared to other methods so far developed, it enables lower collagen bones to be tackled with greater confidence, provided adequate sample is available. The most abundant amino acids in collagen are Gly, Pro, Hyp, alanine and glutamic acid (33%, 12%, 10%, 10% and 7%, respectively, although low collagen bones may well show different patterns in their extracts). Note, however, that in a bone (Escapule mammoth) with a non-collagenous composition, no sur-

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viving Hyp, and total N content equivalent to 1.3 mg g^{-1}, the five amino acids that could be measured (Hyp of course was not) gave concordant values, but dates that were far too young.

Non-Collagenous Proteins

Bone containing very low levels of collagen often contain amino acids with a non-collagenous composition. Some of this is evidently due to the better survival of plasma proteins, such as albumin and of bone-specific proteins, such as osteocalcin (Masters 1987; Tuross 1989; Cattaneo *et al.* 1990; Ulrich *et al.* 1987; Ajie *et al.* 1992). If such proteins can be extracted and purified, they may be suitable candidates for dating (Hauschka 1980; Gillespie 1989).

It is difficult to conceive circumstances in which such proteins are *preferable* to collagen, which, after all, has a very recognizable structure and composition, and is insoluble during decalcification. Proteins might survive better to the extent that ultimately more material for dating might be obtained from them, and they may have undergone less diagenesis, making them more amenable to purification and characterization.

Some work has been done with osteocalcin, which, being of low MW, should be separable from other proteins by ultrafiltration. In modern bone, osteocalcin is present at about 1% of collagen. Ajie *et al.* (1990) claim that the same (modern) level is extractable from archaeological bone, so that where the collagen level is reduced to below 1% of its original abundance, osteocalcin is more abundant. However, the extracted osteocalcin has only about 5–10% of the immunological activity of modern osteocalcin. Less than 10 g of bone should yield sufficient osteocalcin for dating. Measurements reported so far (Ajie *et al.* 1990) on protein (extracted and purified using EDTA, dialysis, gel filtration and ion exchange, but not preparative HPLC) have confirmed that, if "collagen" dates are possible, "osteocalcin" dates are in agreement. However, so far, no osteocalcin dates have been produced from bone of known age but with insufficient collagen for a collagen date. Nevertheless, this is a most important and hopeful development, provided the extracted protein can be characterized well enough to be compromised by exogenous material through admixture of peptides or cross-linkage. Similar possibilities may well apply to albumin.

THE ASSESSMENT OF PRETREATMENT METHODS

How reliable is any method in a particular circumstance? Where the pretreated sample is most characteristic of bone, *e.g.*, as protein, adequate purity cannot be guaranteed; where purity can be guaranteed, *e.g.*, as in HPLC separation of single amino acids, the sample is not uniquely characteristic of bone. Thus, any method so far developed cannot guarantee the complete removal of contamination, and those that come closest are liable to require unrealistic quantities of material.

Assessment "In Theory"

Essentially, this is given in the chemical description of what each method is trying to achieve. For a single fraction, the isolation of Hyp, where possible, has been the theoretically preferred choice. The peptide, Gly-Pro-Hyp, should be even better, and work underway will show if it can be extracted with a comparable efficiency to that for Hyp alone. Such an approach will be confined to bone that is reasonably well preserved or available in large quantity.

In addition, model experiments can be carried out in which various potential contaminants are added to bones of a different age, and the success of the method in removing these is measured. Very little such work has been done, (see section above under mixed amino acids for one example) partly because the relevance of any particular artificial contaminant can be questioned. Possibly in the future, it will be an aspect of interlaboratory quality control to test methods against interlaboratory standards of massively contaminated bone.

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Assessment From Analysis of the Sample

Analytical methods described above indicate the degree of contamination and diagenesis of the sample itself. Unfortunately, the criteria are neither sufficient (too insensitive), nor necessary (contamination may not differ in ^{14}C). Nevertheless, such measurements are decisive for the choice of method, and also are inevitably used to indicate possible caveats concerning the reliability of the date.

Assessment From Analysis During Pretreatment and Dating

Analytical methods can be used more sensitively to monitor the effectiveness of the "clean-up" of samples during pretreatment. This is particularly the case for $\delta^{13}C$ measurements. For example, a sample with an initial value of −22.4‰ for extracted "collagen" (a marginally acceptable value) gave −20.5‰ for gelatin and −20.1‰ for ion-exchanged gelatin. Of course, it is not clear that *all* contamination has been removed. Similarly, Law *et al.* (1991) have used infrared spectra, and C/N ratios for successively purified fractions give valuable, independent information. This would be the ideal situation, which has not yet become part of standard practice.

Perhaps of greater effectiveness is the possibility of dating different fractions. These could be from different stages of pretreatment or different chemical species, such as individual amino acids. If all fractions agree in ^{14}C, much greater confidence can be placed upon them; where they differ, confidence in a particular result will depend on understanding the reasons. This again represents an ideal situation.

Assessment From Agreement with Known-Age Material

As already stated, the ability of a method to produce the correct date is not sufficient for its validation. The use of a more elaborate method needs justification by showing that the error in dating is actually reduced. Unfortunately, bone of known age, for which simple methods are inadequate, is not very common, except where very little collagen is preserved. Failing known-age material, recourse must be to old material (>30 ka), and to showing that better methods produce older dates, since such material is only sensitive to young contamination, or less satisfactorily, to showing that the bone dates achieved conform more closely with archaeological expectation. On the whole, this last approach is probably used most often to validate pretreatment methods. Although it provides a weak and uncertain criterion for a given sample, its general applicability allows such evidence to be usefully accumulated.

These methods have all been used in helping to develop the pretreatment approaches outlined above, and the references cited below give further details of the extent of validation on the basis of the results obtained. However, even where improvements are clear, it is difficult to generalize from one particular context, because the chemical nature of both contaminant and degree of diagenesis is seldom known. This means that the assessment of different methods remains rather anecdotal.

SUMMARY AND CONCLUSIONS

A variety of approaches to the pretreatment for AMS dating of bone is now available. We make no simple recommendation because:

1. No absolutely "sure-fire" method exists.
2. The best choice depends on the preservation, age and environment of the bone.

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3. Routine methods can be greatly improved through intelligent use of other analytical data from the sample before and during pretreatment.
4. Elaborate methods, which can give a greater measure of reliability, continue to be developed. They will usually exact the price of much greater sample quantity and/or cost of analysis.
5. There is still no reliable method for dating bones with insufficient extractable collagen. We see some reasons to hope that this situation is changing, but validation is a long way off.

In our view, the methods that date carefully extracted and purified gelatin, and can demonstrate analytically that the material dated corresponds to the composition expected for gelatin, are adequate for the great majority of bones that have lost up to 95% of indigenous protein. The approach taken at Oxford (*i.e.*, of ion-exchanged gelatin) is probably adequate for the majority of bones with a protein loss of up to 99%. However, at best, these statements have only a statistical truth, and even with support from subsidiary analyses, the accuracy of a given date may be difficult to assess.

For other than well-preserved bones, we strongly recommend that more attention than has been the case in the past is paid to the results of subsidiary analyses, which should come to be regarded as an essential part of the evidence leading to the results of a bone date. Multiple fraction dating, including, where possible, the isolation of purified amino acids (principally glycine and hydroxyproline), can provide very valuable internal control.

Bones containing less than about 1% of original protein, or, more specifically, in which no hydroxyproline can be detected, should not be attempted until better methods are developed.

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