

## AMS RADIOCARBON DATING OF BONES AT ARIZONA

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**ABSTRACT.** Modern bone contains ca 25% protein material, most of which is collagen. Amino acids separated from collagen isolated from bone are suitable for  $^{14}\text{C}$  dating of fossil bone, but attempts to carry out this procedure on bones seriously depleted in protein can yield erroneous  $^{14}\text{C}$  dates. Amino-acid analysis of fossil bone gives quantitative information on the degree of preservation of its organic component. Also, the relative abundance of the amino-acid components reveal the degree to which the collagen-like pattern has been altered. Alteration may be caused by addition of extraneous material. A 1mg sample of bone material is sufficient for this preliminary analysis. We have developed a series of acceptance criteria for whether a particular specimen is likely to yield the correct  $^{14}\text{C}$  age.  $^{14}\text{C}$  dating of fossil bones not seriously depleted in protein is a straightforward procedure and yields reliable dates.

### INTRODUCTION

Radiocarbon dates are often the most direct chronological information obtainable on geological and anthropological events. The availability of reliable dates on bones can bypass questions of stratigraphic correlation and coevality of a cultural or paleontological event and an associated sample of wood or charcoal, which would be easier to date. Thus, it is important that reliable  $^{14}\text{C}$  dates be obtainable on bone. Unfortunately, the history of  $^{14}\text{C}$  dates on bones has been less than satisfactory. Although many measurements have yielded dates concordant with stratigraphic order and cultural expectation, others do not. Bone dates often elicit undisguised skepticism and rebuke. And until recently, a sizable portion of a valuable bone specimen was often sacrificed for a questionable  $^{14}\text{C}$  date. A generalization has developed on the quality of bone dates. Well-preserved bone specimens, eg, those from dry caves, produce acceptable dates. Specimens having resided in moist and warm soil for thousands of years usually yield poor dating results. The hypothesis has emerged that if original carbon in the bone could be isolated, purified and analyzed for  $^{14}\text{C}$ , the correct age of the bone could be calculated. The development of accelerator mass spectrometry (AMS) presents the opportunity to analyze small chemical fractions isolated and purified by modern microchemical processes. Recent work on  $^{14}\text{C}$  dating of bone has focused on proteinaceous components of bones, as previous studies have demonstrated that the inorganic fractions yield less reliable dates. We have thus attempted to develop protocol for proper dating of original bone organic matter. Here we present the results of efforts of the Arizona TAMS research group to realize the goal of reliable bone dates.

## THE PROBLEM

Simply stated, the unreliability of bone dates results from entirely expected chemical and physical processes in soil. Fresh bone containing protein (mostly collagen) and inorganic minerals (primarily apatite), when exposed to moist, oxidizing conditions, will begin to change in response to its environment (Tuross, Fogel & Hare, 1988). The protein will, with the help of water and micro-organisms, oxidize and degrade to smaller, more soluble molecules. Water flowing through the system will help remove both organic and inorganic components of the bone, leaving a porous, high-surface area structure. This inorganic structure can adsorb organic matter produced in the soil, which would most likely be younger than the bone and have a different  $^{14}\text{C}$  content. Soil chemical processes may cause precipitation of new minerals, eg, calcite, in the bone, thus sealing the soil-derived organic carbon in the bone. If this extraneous organic matter is still in the bone when processed for  $^{14}\text{C}$  dating, and if it contains dilute acid-insoluble polypeptides, it would affect the apparent age of the amino-acid fraction. Thus, the reality emerges that bone specimens for  $^{14}\text{C}$  dating exist in a continuum of quality, ranging from well-preserved, uncontaminated with more recent carbon, to completely devoid of original amino acids, and consequently undatable. The challenge to the radiocarbon community is to develop criteria for recognition of properly datable bones and extract and date the original organic fraction.

## THE SOLUTION

Reliable  $^{14}\text{C}$  dates on soil-process-degraded bones can become a reality if original carbon from the bone can be isolated, purified and analyzed for  $^{14}\text{C}$ . The solution has four parts: 1) determining where on the datable/undatable continuum each specimen lies, 2) identifying original organic components in those deemed datable, 3) isolating and purifying the original components, and 4)  $^{14}\text{C}$  analysis of these components.

## QUANTITATIVE CRITERIA OF DATE ACCEPTABILITY

To be declared datable, a bone specimen must pass a sequence of tests. We are still adjusting the acceptability parameters by comparing results we obtain with the presumed true age of the bone. In some cases, the true age is known quite well (eg, mammoth bones from Clovis sites), but most cases involve less secure arguments. The acceptability tests are: 1) visual appearance under optical microscope, 2) proportion of original protein remaining, 3) collagen-like appearance of the amino-acid chromatogram, 4) level of exotic amino acids appearing in the chromatogram. Visually, the specimen considered for dating must be free of obvious extraneous material, such as micro-rootlets and remains of micro-organisms. Its texture must be "tight", *ie*, have low porosity to solutions carrying organic matter from the soil. We maintain records of visual examination of all bones processed.

The three remaining criteria derive from the amino-acid chromatogram obtained in the following manner. A small sample of cleaned bone is heated

overnight with 6M HCl to hydrolyze the protein. The solution is evaporated to dryness, then redissolved with a solution containing an internal standard amino acid (norleucine). This solution is run through the analytical amino-acid analyzer, which produces peaks, the areas of which correspond to the concentrations of each amino acid. Thus, the ratio of the area of the norleucine (not a natural amino acid) peak to any other peak area (glycine, eg) in a fossil bone compared with the same ratio in modern bone, gives a quantitative appraisal of the degree of preservation of that particular amino acid in the specimen. Note that glycine is highly enriched in collagen; thus, glycine abundance is an index of collagen abundance. We are building a library of amino-acid depletion factors for all bones processed.

The amino-acid chromatogram of each processed bone not only allows quantitative appraisal of the degree of preservation, but also yields valuable information on the integrity of the specimen. Severe degradation of bone protein and addition of exogenous protein or amino acids can alter the relative proportions of amino acids in the chromatogram. A distorted pattern of amino acids is a signal that a date on the total amino acids may not be trustworthy. This is especially the case if the chromatogram shows the sample to contain an anomalously high amount of amino acid. Serine is such an amino acid. Its presence would suggest contamination from human sweat or from groundwater. High glutamic acid would suggest contamination with non-bone protein. We are maintaining records of amino-acid chromatograms of all bone specimens dated and developing quantitative criteria for acceptance or rejection of future samples.

#### IDENTIFICATION OF ORIGINAL ORGANIC COMPONENTS

Collagen is the major organic constituent of bones. Demineralization of well-preserved bones reveals a soft, gelatinous pseudomorph of the original bone. This bone gelatin may be dated with confidence. Collagen from less well-preserved bones will be more degraded, have more broken bonds and will more likely be contaminated with extraneous organic material. The more degraded the collagen, the more fragmented the collagen macromolecule will become and the more difficult it will be to separate collagen fragments from contaminant molecules. In these cases, it is appropriate to isolate identifiable organic molecules and address the question of their cogenesis with the bone.

The procedure we have used for only moderately degraded bones involves isolation of the total amino acids from bone. This process removes external substances (clays and authigenic carbonates) and humic material, and yields only amino acids and amino sugars. Total amino acids are assumed to derive from laboratory hydrolysis of bone protein if they exhibit a "collagen-like" chromatogram. In cases involving specimens with chromatograms not very collagen-like, it may be fruitful to isolate individual amino acids for separate  $^{14}\text{C}$  analysis.

#### THE MYTH OF THE HYDROXYPROLINE PANACEA

Proteins consist of sequences of amino acids. Collagen is unusual in that it contains a significant proportion of an otherwise relatively rare amino acid,

hydroxyproline. If hydroxyproline is an unlikely younger contaminant in bones, isolation and dating of this amino acid should yield the correct age of the bone. Recent work of Nagy *et al* (1988) has demonstrated that hydroxyproline is a major component in some humic material dissolved in natural waters. If humic acid remains on the bone specimen during the protein hydrolysis stage in the laboratory process, it, as well as other amino acids from the humic acid, could become part of the dated material, and affect the apparent age of the bone. It is risky to select a single amino acid for dating a bone when its chromatogram is suspect and degree of preservation poor. Further, bone samples seriously depleted in amino acids rarely contain significant quantities of hydroxyproline.

#### ISOLATION AND PURIFICATION OF THE DATABLE COMPONENT

In cases of reasonably good preservation, and a collagen-like chromatogram on whole bone, we employ the following procedure (modified from Gillespie *et al*, 1984): 1) Scrape or grind off outer layers of bone sample, or collect only the inner portion; 2) Break up the sample to mm size and place in ultrasonic bath with distilled water; 3) Decant and discard liquid with its suspended solids. Dry sample; 4) Pulverize sample to  $< 250\mu$ ; 5) Add 0.6M HCl carefully until no further bubbling, then demineralize with 0.6M HCl until pH is constant. Decant and discard dilute-acid-soluble components. Dry; 6) Hydrolyze with 6M HCl overnight at 105°C; 7) Evaporate HCl to dryness. Take up in distilled water; 8) Collect cations on ion exchange column; 9) Elute amino acids with 1.5M  $\text{NH}_4\text{OH}$ ; 10) Freeze-dry; 11) Run amino-acid chromatogram; 12) If second chromatogram passes acceptability test, measure  $^{14}\text{C}$  on this total amino-acid fraction.

In cases where a non-collagen-like chromatogram is obtained in step 12, but the degree of preservation of bone protein suggests that original organic carbon is still moderately abundant,  $^{14}\text{C}$  analysis of two or more individual amino acids may yield the correct age. This method of dating is still experimental, but our limited experience with it convinces us that this approach will not yield the correct ages in all cases, and it, too, is subject to strict acceptability criteria to be developed in future studies.

#### GLYCINE DEPLETION RATIOS

In order to determine where on the datable/undatable continuum a given fossil bone lies, we have developed the concept of a glycine depletion ratio (GDR). We define a GDR of 1.0 as having as much glycine as a sample of fresh cow bone we have prepared and use as a reference standard. A bone sample with a GDR of 2.0 would have half as much glycine per unit weight as our "cow standard." A specimen with a GDR of 1000, and we are sent samples even lower than this, would have only 1/1000 the glycine content of our standard. We use the GDR as an index of collagen degradation, and hence of our ability to obtain a reliable  $^{14}\text{C}$  date. We routinely determine GDR values on all bones submitted for dating and are gaining experience in assigning GDR ranges to bones which are likely to yield reliable dates, and the GDR value beyond which a bone should be considered undatable by our current technique. In practice, the GDR values have ranged from slightly

>1 to >3000. The relatively small effort required to obtain a GDR value saves wasted effort on undatable samples.

We also use a similar parameter, norleucine (NOR), as our internal standard, and the NOR equivalent is the area on our chromatograms of ASP + SER + GLU + GLY + ALA + ISOLEU + LEU + PHE expressed in terms of the area produced by a known quantity of NOR. This term is a function of the preservation of ASP + SER + GLU + GLY + ALA + ISOLEU + LEU + PHE in the bone or collagen samples. The higher the NOR equivalent per gram, the better preserved the fossil bone.

#### EXAMPLES OF REAL BONES

Figure 1 shows an amino-acid chromatogram for modern, non-degraded bone collagen. This is our reference to which we compare all bones consid-

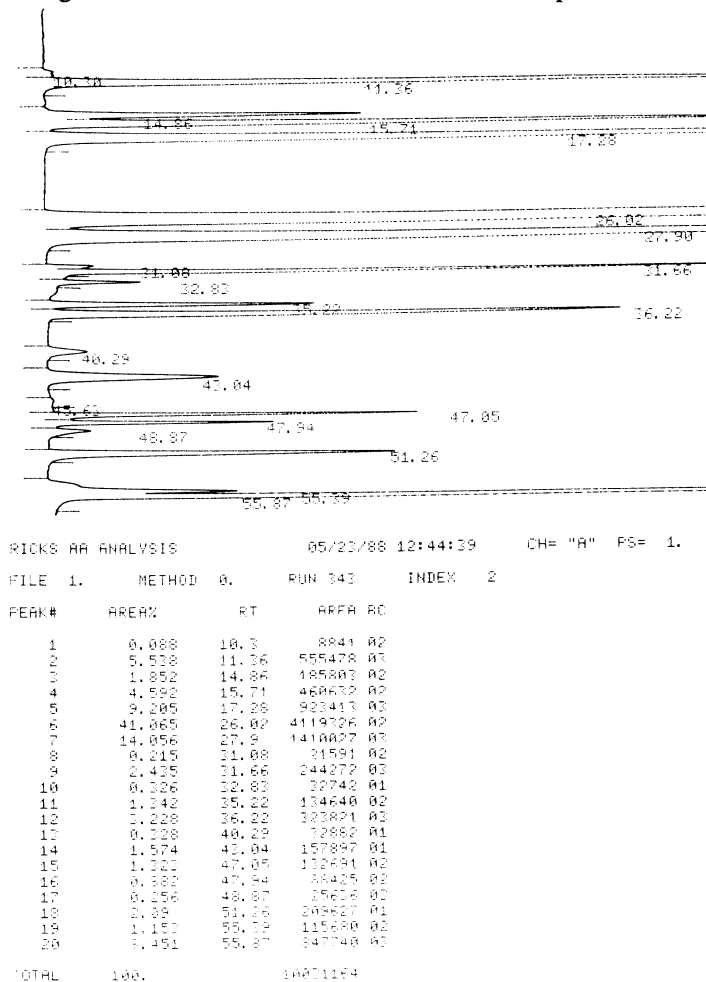


Fig 1. Amino-acid chromatogram of modern, non-degraded bone

## SIGNATURE CHANGE

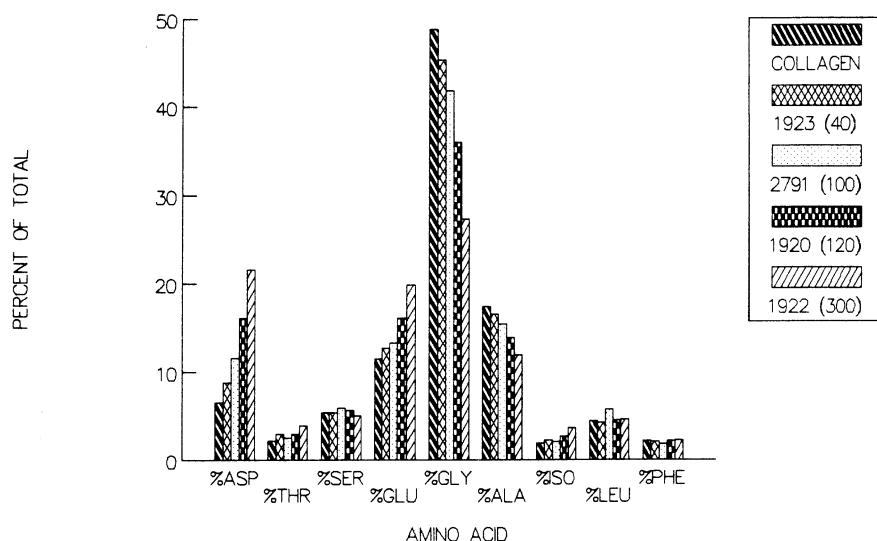


Fig 2. Comparison of selection amino acids in bone specimens of various degrees of degradation. Numbers in parentheses are the GDRs.

ered for dating by AMS. Chromatograms of modern bones of different species may show some variation, but the general patterns are similar. The data-output device on the amino-acid analyzer also measures the areas under each curve, which are the numbers shown below and indexed on the chromatogram. These areas are transferred to a spreadsheet program (LOTUS 1-2-3) for ease of comparison with other samples. Figure 2 shows a comparison of five bone specimens processed in our laboratory. Within each sample, the sum of the amino acids listed is 100%. Numbers preceded by AA – are the TAMS log number; those in parentheses are GDRs. Using as an index of collagen degradation, the relative proportion of glycine in these samples increases with degradation.

Figure 3 illustrates how the ratio of glycine to aspartic acid decreases with progressive degeneration of bone protein. Shown are all bones analyzed in this laboratory from October 1987 through June 1988. The plotted specimens are split between Figures 3A and 3B based somewhat arbitrarily on the visual appearance of their chromatograms. Those in Figure 3A are visually similar to our standard collagen; those in 3B differ visually from the standard. The horizontal coordinate indicates the degree of preservation represented by the NOR equivalent number; the better preserved the bone, the higher the NOR equivalent. Figure 3 illustrates that 1) the degree of preservation of bones submitted for dating range over 4 orders of magnitude; 2) a

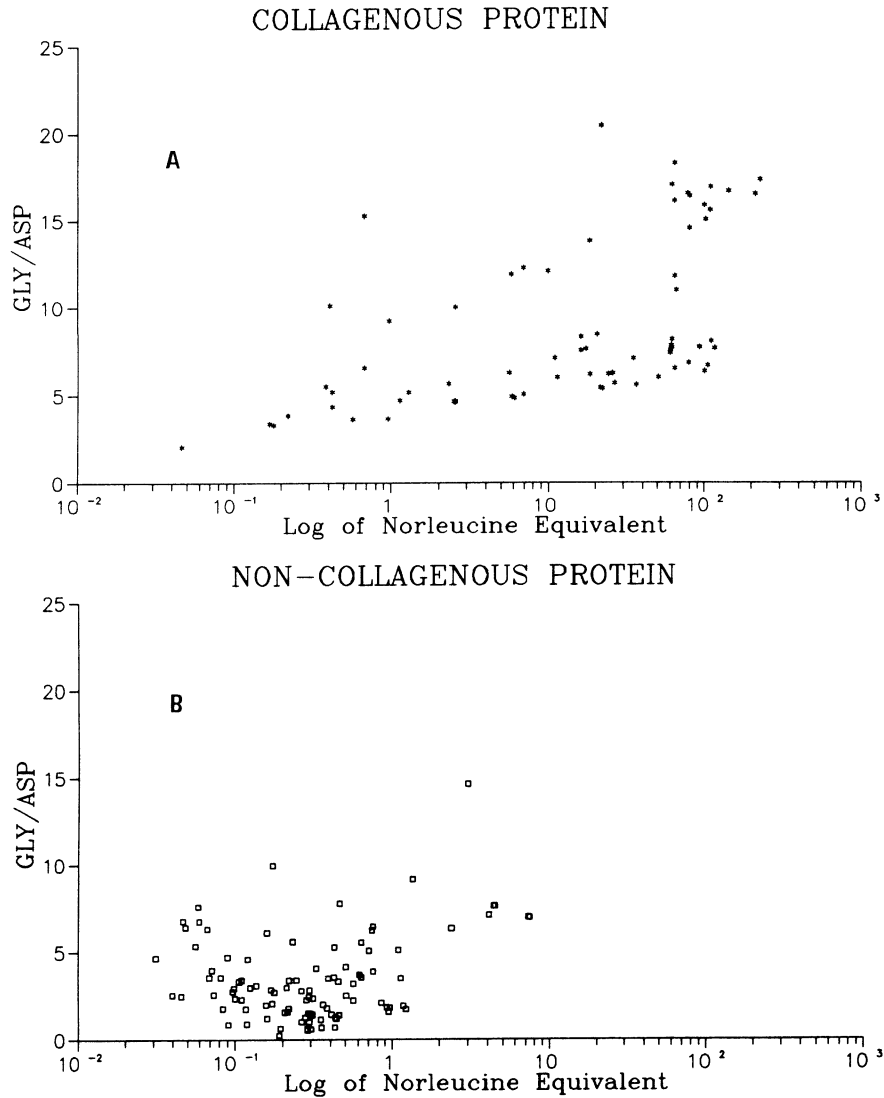


Fig 3. Glycine/aspartic acid ratio trend with degree of degradation on all samples analyzed. Higher norleucine equivalents correspond to better preservation.

- A. Specimens with amino – acid chromatograms visually similar to standard collagen  
 B. Specimens with amino – acid chromatograms visually unlike standard collagen

quantitative parameter (NOR equivalent) and a subjective parameter are in essential agreement; 3) the glycine/aspartic acid ratio decreases with greater degradation; 4) because of the overlap of visual and NOR equivalent criteria, a conservative approach to prejudge whether the bone specimen will

yield the correct  $^{14}\text{C}$  date would include a series of acceptability criteria, with both subjective and quantitative evaluations.

Thus, it is expedient, saving many unnecessary full-procedure preparations, to be able to develop multi-factor acceptability criteria based on a relatively simple test. We are still adjusting the levels of acceptability in each, and will soon add the second amino-acid chromatogram to the decision process. The second amino-acid analysis (see Isolation and Purification...) will help us decide if our separation procedure has made the amino-acid analysis more collagen-like.

#### EVIDENT SOURCES OF CONTAMINATION, AND BONE DATING CAVEATS

Bones have long been known to present special problems in  $^{14}\text{C}$  dating, so we should not have been surprised to learn that solutions will not come easily, even with AMS and microbiological technologies. Fossil bone organic matter is particularly chemically unstable when wet; it is also an excellent food source for microbiota. Percolating water in soils can dissolve and remove organic molecules produced during bone degradation. Particulate and dissolved organic matter can migrate through soils and adsorb onto bone surfaces which have previously lost their collagen. We now have evidence that these and other processes occur and affect the apparent ages of bone. Only with a better understanding of more details of the processes outlined above will we be able to determine whether a particular bone specimen is datable, and to carry out the dating with confidence.

#### FUTURE WORK

It has been fruitful to combine bone dating research with routine bone dating; both benefit from the relationship. On the one hand, the routine work keeps the research aimed at solving the practical problems such as reliability. Routine dating also identifies those samples that are valuable to understanding problems. On the other hand, the research undoubtedly contributes to the quality of the routine dating, especially if unusual samples are received.

We will continue our routine dating of clearly acceptable bone specimens received while further developing criteria for the identification of which non-routine specimens will yield reliable dates. Also, some work on apparently uncontaminated bones with high GDRs suggests that proteins other than collagen may yield reliable dates, eg, phosphoproteins may be protected from degradation because of their bonding to apatite structures in bone. We will pursue this possibility of dating poorly-preserved bone.

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