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FUNDAMENTALS OF BONE DEGRADATION CHEMISTRY: COLLAGEN IS NOT "THE WAY"

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ABSTRACT. Collagen-based pretreatment methods for bone yield inconsistent results for those samples where protein preservation is low, as frequently found in bones from the semi-arid zones of Australia and North America. New methods for dealing with low collagen bones are needed, and this paper suggests that the non-collagenous proteins, particularly the blood proteins, may offer advantages for AMS dating because of their better preservation. Amino-acid profiles of collagen and non-collagenous proteins suggest that such differential preservation may be due to physico-chemical differences, and help to explain the poor results from dating low-collagen bones.

INTRODUCTION

Libby (1970) once remarked on the differences between the chemistries of life and death, with the radiocarbon pretreatment procedures acting as a kind of laundering process for separating the two. Bone has long been a problematic sample material for radiocarbon dating, and the best explanation of the variable results appears to be a failure in the laundry. The chemistry of life is biochemistry, and it is becoming necessary to apply more of the methodology developed in biochemistry to the pretreatment of dead bones we want to date – the palaeobiochemistry advocated by Jope (1980). This paper suggests that bone dating can benefit greatly from the characterization, isolation and purification of proteins by using the tools of biochemistry, suitably adapted for AMS radiocarbon dating and for the different properties of modern and fossil protein mixtures. It also implies that proteins other than collagen may have advantages, especially where bones are degraded, because of structural differences leading to preferential survival.

CURRENT METHODS

All current bone pretreatments utilizing the organic phase are based on the reasonable assumption that collagen is the target protein. Bone collagen pretreatments fall into three basic styles: 1) those leaving the protein largely intact in its three-dimensional architecture; 2) those seeking to reduce it to its smallest units, with of course, 3) some middle-ground styles based on solubilization.

Most common has been the *dilute acid insoluble residue,* which removes the inorganic bone matrix and some of the smaller organic molecules; sometimes this is followed by an alkaline treatment, which removes humic materials. These standard methods do not always work because of incomplete separation of sample and contaminant carbon, the most common error is to yield dates that are younger than expected. Likely contaminants include humic acids, amino acids or peptides foreign to the bone, micro-organisms

and their byproducts. An improvement on this approach is to solubilize the collagen by heating in very dilute acid, which, after filtering off the insoluble materials, produces a product usually called *gelatin*. It is probable that some modern contaminants such as bacterial or fungal cell remains, which may be resistant to hydrolysis, are effectively removed by this treatment. More complex pretreatments, such as complete hydrolysis of the acid insoluble residue or gelatin fraction, which can then be further purified by chromatographic means, yield the *total amino acid mixture* or a *single amino acid* from this mixture. These methods have all yielded useful data, particularly for the well-preserved bone from cave sites in northern Europe, as summarized by Gowlett and Hedges (1986).

But it is still not good enough in some circumstances, as shown by the extensive series of dated fractions reported by Stafford *et al* (1987) and Gowlett *et al* (1986) on low-collagen bones, and the comparative studies by Gurfinkel (1987) and Brown *et al* (1988) on gelatin and other fractions. Stafford (1988) reported that even single amino-acid dates are no guarantee of good results for degraded bones. Taylor (1987) has proposed a set of criteria and a protocol for the dating of bone, based on a sub-set of the current methods for collagen: a minimum of three separate organic fractions are to be dated for each 'critical' bone, and then only after stringent precautions have been taken to ensure clean apparatus and non-contaminating methodology is employed, and an amino-acid analysis is to be run on both total and dilute acid-insoluble fractions to determine if there is still collagen present. The details suggested in some cases are those employed routinely at some AMS laboratories, eg, Gillespie and Hedges (1984), Gillespie *et al* (1986), Vogel *et al* (1987) and Stafford *et al* (1987). Aside from the prohibitive cost, it is not clear that this approach will succeed generally, because in those bones where the simpler collagen-based methods fail, there is usually very little collagen left. There are also some misconceptions inherent in the methodology proposed by Taylor (1987) concerning amino-acid analysis (which will be discussed below). The time seems ripe for a closer look at the bone organic phase.

BONE STRUCTURE AND PROTEIN REACTIVITY

The organic portion of modern bone contains ca 90% collagen and it is no surprise that most effort has been directed toward isolating and purifying this dominant protein. Many other proteins, including osteocalcin, osteonectin and other phosphoproteins, proteoglycans and glycoproteins have been detected in modern bone. Also, blood proteins are present in bone, such as hemoglobin, serum albumin and the immunoglobins. These non-collagenous bone and blood proteins have not yet been utilized for dating bones (at least not intentionally), but may constitute a valuable source of AMS-datable sample materials for bones so severely degraded that the majority of the collagen has disappeared. To appreciate why non-collagenous proteins could survive better than collagen, we must consider their physico-chemical structures and the most likely conditions encountered in a fossil bone environment.

Different affinities for water or other solvents, conferred by the protein primary structure, lead to variations in solubility and reactivity, reflected in their different roles in the living body. Some of the non-collagenous proteins, particularly the phosphoproteins, are strongly associated with collagen and the hydroxylapatite mineral phase of bone, but all have primary structures very different to that of collagen. Only 1 or 2 of the phosphoproteins contain hydroxyproline, which constitutes >10% of the collagen monomer, and none contains as high a proportion of glycine as collagen (Termine, 1983; Price, 1983). Collagen is very easily hydrolyzed under acid, neutral or basic conditions, leading to many free polar end groups; the gelatinization procedure used by some laboratories is a partial acid hydrolysis. It is the polar residues that cross-link the collagen polymer, and they can also combine (when freed by hydrolysis) with other amino acids, sugars, etc from the environment, yielding a contaminated protein not amenable to purification by current methods. Although it was earlier believed that amino acids do not reversibly exchange their carbon, evidence now supports exchange by reversible decarboxylation (Barrett, 1985).

The globular proteins, eg, hemoglobin and serum albumin, tend to have more hydrophobic residues (which interact to stabilize the ovoid shape of the molecules) than collagen, and less of the more reactive polar amino acid residues. Although the blood proteins are soluble in aqueous solutions in their modern, native state, they are less easily degraded by the hydrolysis reactions that destroy collagen so efficiently under certain conditions because of partial denaturation and aggregation on dehydration (Lumry & Biltonen, 1969). Hemoglobin has been isolated from human bones up to 4500 yr old, in quantities of 1–4gm/100gm of bone, by Ascensi *et al* (1985). Loy (1987) has demonstrated that some blood proteins survive on stone tools for >75,000 yr, that the species of origin of the blood can be identified, and suggests that the clay/silt-sized fraction of the burial soil may be important to protein survival by providing a protective barrier. Blood residues from excavated human bone have been subjected to blood group typing (Brooks *et al,* 1977), and both collagen and blood proteins from bone have been used in immunoassay procedures for species identification and the tracing of genetic linkages between species (Lowenstein, 1985). The complex biochemical reactions involved in these techniques are highly specific at the molecular level; antibodies will usually only bind to a specific amino acid sequence of one particular protein. So long as the binding site is preserved, the protein can be detected in, and potentially isolated from, a fossil bone.

There is abundant evidence that low-collagen bones display amino-acid compositions quite different from those of modern collagen (see eg, Hare, 1980; Kessels & Dungworth, 1980; Armstrong *et al*, 1983) which may relate to different preservation characteristics of the noncollagenous proteins. Bada (1985) showed that some low-collagen bones have increased acidic amino acid and decreased hydroxyproline content, but enhanced aspartic acid racemization. This may be due to a greater stability toward hydrolysis of the non-collagenous proteins, some of which have increased rates of racemization (Masters, 1983). It is also possible that absorption of amino acids from the environment has radically changed the proportions of amino

acids, and that these foreign amino acids were already racemized to a significant extent, or that the amino acids freed by hydrolysis racemize faster than those still bound in the protein. Just what the racemization results are telling us is not at all clear, but may be related to the degree of degradation of the bone proteins, particularly collagen.

Many difficulties arise in comparing amino-acid analyses from different laboratories, which may use very different methods of analysis. Dedicated ion-exchange systems employing liquid chromatography have been the traditional mainstay, but there are many less expensive attempts to emulate this high performance, with varying degrees of success. Some systems, such as those employing OPA for derivatization and detection, do not measure the secondary amino-acids proline and hydroxyproline well or at all. Other derivatives such as the PITC, Dansyl, Ninhydrin and FMOC systems do measure all amino acids, but linearity of response for the wide range of amino-acid concentrations found in bones is often a problem and it is not straightforward to get results that are directly reproducible by other laboratories. Similar remarks apply to analyses using gas chromatographic techniques, which have their own idiosyncrasies.

Taylor (1987) gives examples of partial amino-acid profiles where proline and hydroxyproline are not measured (OPA method), and yet considers some of these to be collagen-like because of similar ratios of glycine to glutamic acid. Such ratios could well be fortuitous because of contamination and/or degradation, and without hydroxyproline measurement we cannot say whether the composition is collagen-like or not. It is essential to determine the full amino-acid profile for comparative studies (there are 20 amino acids commonly found in proteins), comparisons between incomplete results are not productive. It is not unusual to find peaks in a bone amino-acid profile that do not belong to collagen. Some possibilities for these extraneous peaks include tryptophane and cysteine (amino acids not found

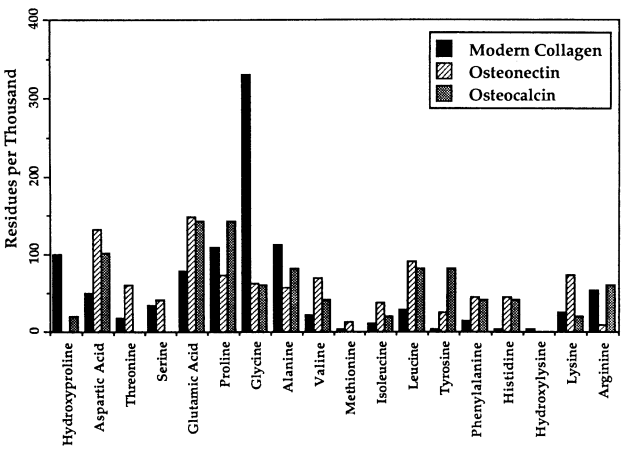

Fig 1. Modern collagen and bone phosphoproteins. Collagen data from Hare (1980) fetal phosphoprotein data from Termine (1983) and Price (1983).

in collagen but present in most other proteins), amino sugars, fulvic acids, small peptides, amino-acid degradation products and microbial metabolites. These are not normally reported because of the difficulty and expense of structural determinations. Such problems of comparability are not unique to amino-acid analysis; all analytical techniques (including radiocarbon dating) are subject to inter-laboratory bias and other variations, depending on the methodology used and the degree to which factors influencing the reproducibility of results are overcome.

Figures 1, 2, 3 and 4 show the approximate amino-acid composition of representative bones with both good and poor protein preservation, compared with some non-collagenous bone and blood proteins.

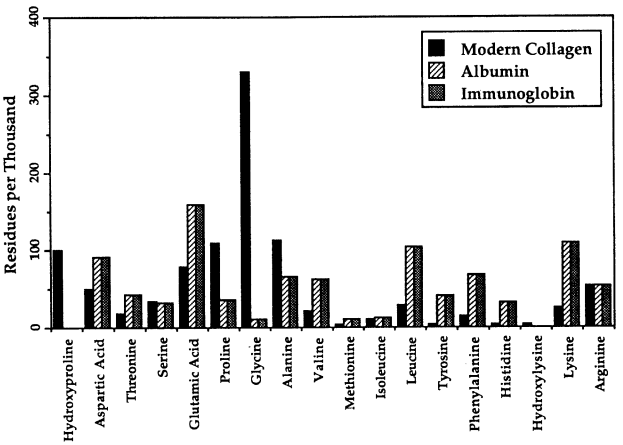

Fig 2. Modern collagen and blood proteins. Human serum albumin and immunoglobin G data from Turner and Hulme (1971).

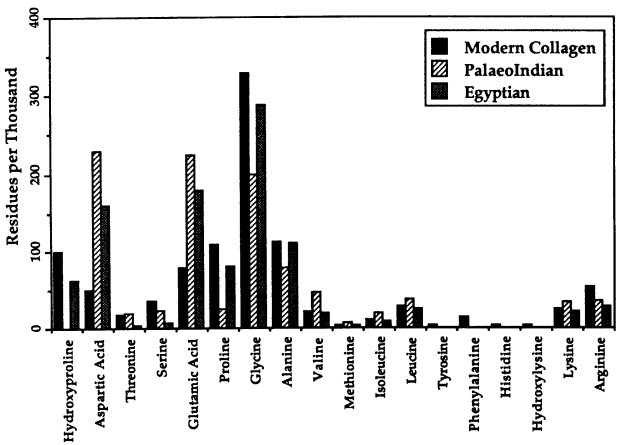

Fig 3. Modern collagen and fossil human bones. Fossil bone data from Hare (1980); not all Holocene age bones are as degraded as these specimens.

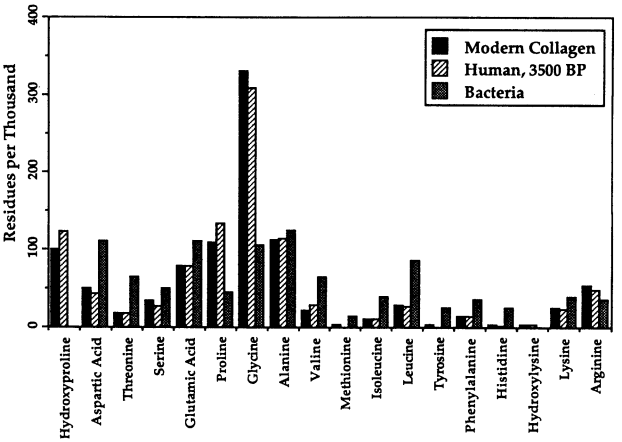

Fig 4. Modern collagen, fossil bone and bacteria. Bacteria and fossil human bone (ca 3500 BP) data from Armstrong *et al* (1983).

NEW ISOLATION TECHNIQUES

In the soluble middle ground, Nelson *et al* (1986) have shown the way using blood residues taken from a stone tool; they minimized modern contamination by dating only the >30,000 molecular weight portion of a soluble protein residue. The techniques used in this study are quite simple. Blood protein was extracted using water and a detergent, centrifuged and filtered to remove macroscopic insoluble particles, then separated by ultrafiltration into two fractions based on molecular weight. Another salutary feature of this work was the sample identification procedure – electrophoresis. In this example, the contamination was either in the fungal debris and/or in the low-molecular weight material; it will not necessarily always be this easy to separate the contaminants from the desired fossil protein. No such technique has yet been reported for bone, but work in progress at ANU has shown that blood cells and blood protein residues remain on fossil bones (ages estimated from similar material as >25,000 yr) from the Australian semi-arid zone, whilst collagen has all but vanished. This is precisely the kind of sample on which traditional methods fail – hydrolysis and other processes combine to degrade the collagen and effectively destroy the chances for collagen-based pretreatments to succeed. In these samples, human serum albumin was positively identified in an extract from a skull fragment using a monoclonal antibody technique (Benjamin *et al*, 1987), and hemoglobin was identified by the Ames Haemastix reaction. Hemoglobin was also crystallized from several bones, yielding characteristic crystal shapes for human and extinct megafauna in samples from appropriately identified bones (TH Loy, pers commun, 1988; Gillespie & Loy, 1988). In all the bones tested, the nitrogen content was <0.1%, indicating very low protein content. Gowlett and Hedges (1986) and Stafford (1988) consider that bones with <0.2% nitrogen are not suitable for collagen-based pretreatments.

Other pretreatment approaches will depend on the biological properties exhibited by all proteins, such as their ability to selectively bind other

molecules. These specific binding reactions could also be used for the separation and isolation of pure proteins suitable for dating, with affinity chromatography or bead-linked immunoassay techniques. Electrophoresis and gel filtration, which have been carried out on fossil bone at analytical scale, can also be adapted to isolating proteins for AMS samples, allowing both positive identification of target molecules and their separation from other molecules which may have a quite different origin and radiocarbon concentration. These biochemical techniques have well-developed methodologies, though modifications will be necessary to minimize contamination from the chemicals and biological reagents used to create and maintain the right conditions for protein separations. It is also clear from published work that fossil collagen does not behave like modern collagen, probably due to degradation and diagenetic changes. The partial success mentioned above in using antibody and other reactions on blood proteins lends support to this line of research.

CONCLUSION

We need to know precisely what we are dating at the macromolecular level. This has not yet been possible for the collagen-based pretreatments because of the lack of applicable positive identification procedures for collagen or its derived fragments. Perhaps the days of collagen dating are numbered, at least for badly degraded bones where the newer biochemical techniques can be applied to the non-collagenous bone and blood proteins. The physicists have given us a wonderful tool in AMS, which allows, even encourages, the dating of 50–500μg of carbon. The radiocarbon chemists must respond in kind by putting the developed tools of biochemistry to work on the elusive residues in, or on, fossil bone – so that the integrity of the molecules used to prepare the AMS dating target is unquestioned. In this way, it may yet be possible to provide solutions to the problems posed by the dating of, for instance, the Neanderthals or the early colonization of, and the extinct megafauna in, Australia or the Americas.

REFERENCES

Ascenzi, A, Brunori, M, Citro, G, and Zito, R, 1985, Immunological detection of hemoglobin in bones of ancient Roman times and of Iron and Eneolithic Ages: Natl Acad Sci, Proc, v 82, p 7170–7172.

Armstrong, WG, Halstead, LB, Reed, FB and Wood, L, 1983, Fossil proteins in vertebrate calcified tissues: Royal Soc [London] Philos Trans, v B301, p 301–343.

Bada, JL, 1985, Amino acid racemization dating of fossil bones: Ann Rev Earth Planetary Sci, v 13, p 241–268.

Barrett, GC, ed, 1985, The chemistry and biochemistry of the amino acids: London, Chapman and Hall, 684 p.

Benjamin, DC, Herr, JC, Sutherland, WM, Woodward, MP, DeCourcy, K and Condon, TP, 1987, A unique epitope on human serum albumin recognized by monoclonal antibody HSA-1: a probe for identification of the human origin of blood or tissue: Hybridoma, v 6, no. 2, p 183–190.

Brooks, ST, Heglar, R and Brooks, RH, 1977, Radiocarbon dating and palaeoserology of a selected burial series from the Great Cave of Niah, Sarawak, Malaysia: Asian Perspectives, v XX, no. 1, p 21–31.

Brown, TA, Nelson, DE, Vogel, JS and Southon, JR, 1988, Improved collagen extraction by modified Longin method: Radiocarbon, v 30, no. 1, p 171–177.

Gillespie, R and Hedges, REM, 1984, Laboratory contamination in radiocarbon accelerator mass spectrometry: Nuclear Instruments & Methods, v B5, p 294–296.

Gillespie, R, Hedges, REM and Humm, MJ, 1986, Routine AMS dating of bone and shell proteins, *in* Stuiver, M and Kra, R S, eds, Internatl ^{14}C conf, 12th, Proc: Radiocarbon, v 28, no. 2, p 451–456

Gillespie, R and Loy, TH, (ms) 1988, The preservation of soluble proteins in ancient bones: implications for radiocarbon dating and species identification of prehistoric blood residues: Paper presented at Joint Australia, New Zealand, USA Workshop on Pleistocene extinctions, Univ Sydney.

Gowlett, JAJ and Hedges, REM, 1986, Lessons of context and contamination in dating the Upper Palaeolithic, *in* Gowlett, JAJ and Hedges, REM, eds, Archaeological results from accelerator dating: Oxford Comm Archaeol, Mono no. 11, p 63–72.

Gowlett, JAJ, Hedges, REM, Law, IA and Perry, C, 1986, Radiocarbon dates form the Oxford AMS system: Archaeometry datelist 4: Archaeometry, v 28, p 206–221.

Gurfinkel, D, 1987, Comparative study of the radiocarbon dating of different bone collagen preparations: Radiocarbon, v 29, no. 1, p 45–52.

Hare, PE, 1980, Organic geochemistry of bone and its relation to the survival of bone in the natural environment, *in* Behrensmeyer, AK and Hill, AP, eds, Fossils in the making – vertebrate taphonomy and paleoecology: Chicago, Univ Chicago Press, p 208–219.

Jope, EM, 1980, Ancient bone and plant proteins: the molecular state of preservation, *in* Hare, PE, Hoering, TC and King, Jr, K, eds, Biogeochemistry of amino acids: New York, John Wiley & Sons, Inc, p 23–33.

Kessels, HJ and Dungworth, G, 1980, Necessity of reporting amino acid compositions of fossil bones where racemization analyses are used for geochronological applications: Inhomogeneities of D/L amino acids in fossil bones, *in* Hare, PE, Hoering, TC and King, Jr, K, eds, Biogeochemistry of amino acids: New York, John Wiley & Sons, Inc, p 527–541.

Libby, WF, 1970, Ruminations on radiocarbon dating, *in* Olsson, IU, ed, Radiocarbon variations and absolute chronology, Nobel symposium, 12th, Proc: New York, John Wiley & Sons, Inc, p 629–640.

Loy, TH, 1987, Recent advances in blood residue analysis, *in* Ambrose, WR and Mummery, JMJ, Archaeometry: Further Australasian studies, Canberra, ANU Printing Service, p 57–65.

Lowenstein, JM, 1985, Molecular approaches to the identification of species: Am Scientist, v 73, p 541–547.

Lumry, R and Biltonen, R, 1969, Thermodynamic and kinetic aspects of protein conformations in relation to physiological function, *in* Timashef, SN and Fasman, GD, eds, Structure and stability of biological macromolecules: New York, Marcel Dekker Inc, p 65–212.

Masters, PM, 1983, Stereochemically altered non-collagenous protein from human dentin: Calcified Tissue Internatl, v 35, p 43–47.

Nelson, DE, Loy, TH, Vogel, JS and Southon, JR, 1986, Radiocarbon dating blood residues on prehistoric stone tools: Radiocarbon, v 28, no. 1, p 170–174.

Price, P, 1983, Osteocalcin: Bone Min Research Ann, v 1, p 157–190.

Stafford, TW, Jr, 1988, Accelerator dating at the molecular level: Jour Archaeol Sci, in press.

Stafford, TW Jr, Jull, AJT, Brendel, K, Duhamel, RC and Donahue, D, 1987, Study of bone radiocarbon dating accuracy at the University of Arizona NSF Accelerator Facility for Radioisotope Analysis: Radiocarbon, v 29, no. 1, p 24–44.

Taylor, RE, 1987, AMS dating of critical bone samples: proposed protocol and criteria for evaluation: Nuclear Instruments & Methods, v B29, p 159–163.

Termine, JD, 1983, Osteonectin and other newly described protein of developing bone: Bone Min Research Ann, v 1, p 144–156.

Turner, MW and Hulme, B, 1971, The plasma proteins: an introduction: London, Pitman Med Sci Pub Co Ltd, p 31.

Tuross, N, Eyre, DE, Holtrop, ME, Glimcher, MJ and Hare, PE, 1980, Collagen in fossil bones, *in* Hare, PE, Hoering, TC and King, K, Jr, eds, Biogeochemistry of amino acids: New York, John Wiley & Sons, Inc, p 53–64.

Vogel, JS, Southon, JR and Nelson, DE, 1987, Catalyst and binder effects in the use of filamentous graphite for AMS: Nuclear Instruments & Methods, v B29, p 50–56.