^{14}C DATING OF BONE USING γ-CARBOXYGLUTAMIC ACID AND α-CARBOXYGLYCINE (AMINOMALONATE)

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ABSTRACT. Radiocarbon determinations have been obtained on γ-carboxyglutamic acid [Gla] and α-carboxyglycine (aminomalonate) [Am] as well as acid- and base-hydrolyzed total amino acids isolated from a series of fossil bones. As far as we are aware, Am has not been reported previously in fossil bone and neither Gla nor Am ^{14}C values have been measured previously. Interest in Gla, an amino acid found in the non-collagen proteins osteocalcin and matrix Gla-protein (MGP), proceeds from the suggestion that it may be preferentially retained and more resistant to diagenetic contamination affecting ^{14}C values in bones exhibiting low and trace amounts of collagen. Our data do not support these suggestions. The suite of bones examined showed a general tendency for total amino acid and Gla concentrations to decrease in concert. Even for bones retaining significant amounts of collagen, Gla (and Am extracts) can yield ^{14}C values discordant with their expected age and with ^{14}C values obtained on total amino-acid fractions isolated from the same bone sample.

INTRODUCTION

Discussions concerning the reliability of radiocarbon-based age determinations on bone have occurred essentially from the beginning of ^{14}C research. Libby (1952) commented on the problematical nature of this sample type for ^{14}C analysis even before the first measurements on bone were undertaken. The long-term difficulties with bone in ^{14}C studies have borne out his concerns and reservations. Despite the great amount of attention given to the exclusion of contamination by isolation and purification of specific chemical and molecular fractions of bone, there continues to be a tradition of skepticism concerning the general reliability of ^{14}C values on bone (*e.g.*, Brown 1988; see Taylor (1987, 1992) for literature).

Despite this tradition, *for samples containing significant quantities of well-preserved collagen*, it is now generally agreed by ^{14}C laboratories working with bone that appropriate physical and chemical pretreatment can, in most cases, effectively isolate and purify the *in-situ* residual collagen, and accurate ^{14}C age estimates can be obtained. Many laboratories now have a series of explicit acceptance/rejection criteria to determine which bones can and cannot be expected to yield accurate ^{14}C age specimens. These criteria currently include the proportion of the original collagen remaining (Hedges and Law 1989), the degree to which a bone exhibits a collagen-like amino-acid pattern (Taylor 1992) and the presence of anomalous concentrations of certain amino acids (Long *et al.* 1989).

For well-preserved bone samples, the isolation of a total amino-acid fraction has become widely employed along with the use of chromatographic methods to remove humate compounds (Stafford *et al.* 1987, 1988; Gillespie 1989; Long *et al.* 1989; van Klinken and Mook 1990). Another approach adds an ultrafiltration step designed to exclude low molecular weight components under the assumption that most of the exogenous contamination will be contained in this fraction (Brown *et al.* 1988). Still another strategy, originally developed to purify collagen for stable isotope analyses, involves the use of collagenase, which preferentially isolates peptides of known length from the surviving collagen fragments (DiNiro and Weiner 1988).

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In contrast to the various techniques generally employed successfully to purify collagen where it is retained in appropriate quantities, a continuing problem is the challenge of obtaining reliable ^{14}C-based age estimates on collagen-degraded bone. For many regions, including many temperate and most tropical areas, some of the most interesting archaeologically related bone exhibits low (<5%) or trace (<1%) amounts of collagen. Currently, no consensus exists on which biogeochemical methods can be used on these types of bones to distinguish indigenous amino acids, peptides and other products of collagen diagenesis from external contamination from various sources.

Approaches to ^{14}C Dating of Collagen-Degraded Bone

Because of the difficulty of identifying and isolating non-diagenetically affected or autochthonous organics in collagen-deficient bone, several researchers have suggested targeting one or more major (>1%) non-collagen components of bone. Sometimes characterized as "matrix proteins," they include osteocalcin, osteonectin and other phosphoproteins, proteoglycans, and sialo- and glycoproteins (Gundberg *et al.* 1984; Hauschka and Wiams 1989). Other non-collagen components include blood proteins such as hemoglobin, serum albumin and the immunoglobins (Long *et al.* 1989; Gillespie 1989; Nelson *et al.* 1986; Masters 1987). The UCR Radiocarbon Laboratory has previously been involved in examining the biochemical and isotopic integrity of osteocalcin ^{14}C values in fossil bone.

Also known in the biomedical literature as "Gla-containing protein" and "bone Gla protein" (Termine 1988), osteocalcin was isolated as part of the search for the source of γ-carboxyglutamic acid (Gla), a calcium-binding amino acid (Hauschka, Lian and Gallop 1975; Hauschka 1977; Hauschka and Gallop 1977). Hauschka (1980) was the first to suggest the potential usefulness of osteocalcin in the dating of fossil bone. Osteocalcin is a low molecular weight protein (5200–5900 daltons) with 46–50 amino-acid residues per molecule, which contains 2 or 3 (depending on species) residues of Gla per molecule. Human osteocalcin has 2 Gla residues of Gla per molecule (Poser *et al.* 1980). In structure, Gla is similar to glutamic acid (Glu) except that it has an additional carboxyl group ($-COO^-$) attached to the gamma carbon atom (Fig. 1). Osteocalcin is formed with Gla and the carboxyl groups are added later enzymatically under the influence of vitamin K (Hauschka 1977). Another protein containing Gla—the Matrix Gla Protein (MGP)—with a molecular weight of 9000–11,000 daltons has also been identified in bone (Hauschka *et al.* 1989).

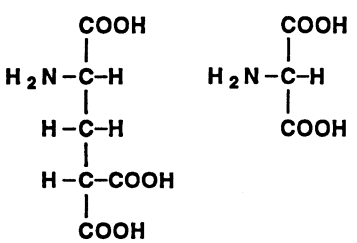

Fig. 1. Structure of gamma-carboxyglutamic acid (Gla) and alpha-carboxyglycine (aminomalonate) (AM)

Several properties of osteocalcin offered the possibility that it would be a potentially useful protein for ^{14}C dating of collagen-degraded fossil bones (Hauschka 1980). First, osteocalcin appeared to bind tightly to the hydroxyapatite crystals, the major mineral component of bone. In this bound form, it was argued that the protein should be well protected from biochemical degradation due to the buffering action of hydroxyapatite [$Ca_5(PO_4)_3OH$] and the decreased accessibility to exogenous

proteinases. Poser and Price (1979) reported that they could not decarboxylate Gla in its protein while it was attached to a hydroxyapatite crystal. Second, Gla has not been detected above instrument baseline levels (>0.2 residues of Gla 1000^{-1} residues of Glu) in any potential contaminants including other vertebrate and plant proteins or bacterial cell cultures (Hauschka 1977). Finally, it has been retained in bone of significant geological age. Ulrich *et al.* (1987) examined the osteocalcin content of six bovid bones recovered from geological contexts ranging in age from the Miocene (*ca.* 13 Myr) to the late Pleistocene (*ca.* 12 ka), and found that the osteocalcin antigen as well as its Gla residue remained detectable in all of the samples.

The studies previously reported in Ajie *et al.* (1990, 1991, 1992) undertook osteocalcin ^{14}C measurements, and some aspects of the results were interpreted to suggest that osteocalcin was a promising means of dating collagen-degraded bone. However, our current understanding of the earlier data plus the data reported here indicate that the use of osteocalcin for the dating of fossil bone poses many more problems than heretofore recognized. This should not have been unexpected since hydroxyapatite crystals have been noted for their ability to attract and hold anionic proteins. The surface area of such crystals in bone is immense—estimated to be in the range of 100 to 200 $m^2\ g^{-1}$ of apatite (Newesley 1989; Weiner and Price 1986). Bada (1985) suggested that this affinity for acidic amino acids and proteins may cause bone to attract such compounds from the environment and be a partial cause of degraded bone giving a non-collagen amino-acid profile. Weiner and Price (1986) even suggested using bone apatite crystals as the stationary phase in chromatographic columns because of their propensity to attract anionic compounds.

Tuross *et al.* (1989) studied bones collected over a 10-yr period, and reported that matrix proteins and collagen both decreased at roughly the same rate in test bones. However, the authors suggested that the loss of matrix proteins may have been more of a function of the extraction techniques employed—antibody recognition and electrophoeresis—rather than the intrinsic matrix proteins levels. In another study involving leaching experiments in water, King (1978) found that Gla, like collagen, went through an initial rapid decrease and then continued decreasing at an essentially constant rate. King suggested using the loss of Gla as a measure of the leaching history of a bone. In a later study of 12 well-dated bones, King (1980) clearly showed a relationship between collagen and retained Gla amounts. Ten of the 12 bones showed either a collagen profile with some Gla or a non-collagen profile and no Gla. One non-collagen bone contained a slight amount of Gla, while another with a collagen profile contained no Gla.

The earlier studies determined that in many fossil bones, osteocalcin typically exists as, or can only be extracted primarily as, polypeptide fragments rather than as an intact protein. In one case, concordant ^{14}C determinations were obtained on osteocalcin and collagen (extracted as gelatin) fractions from the same bone where the collagen was relatively well preserved. However, in other bones, there were significant discordances in the osteocalcin/collagen ^{14}C values with age offsets tending to increase as a function of the decreasing amounts of extractable collagen occurring in the bone. For all but one of the bones examined, the extractable collagen contents were depressed but amino-acid composition and C/N data indicated that the collagen was still largely intact (Ajie *et al.* 1992).

Interestingly, in several cases, the discordances involved osteocalcin ^{14}C values that were significantly *older* than expected based on both the collagen ^{14}C data and previously obtained ^{14}C evidence of age for the skeletal series. The typical expectation is that environmental or diagenetic contamination would cause the apparent osteocalcin age to be *younger* than expected. That the osteocalcin ^{14}C values tended to be older led to the suggestion that the extensive extraction procedures required for the isolation of the osteocalcin, including the use of several types of reagents, may perhaps have

been, in some manner, responsible for the anomalous ^{14}C results. This was the stimulus and context for the work reported here to isolate and obtain ^{14}C determinations on Gla as the characteristic amino acid of osteocalcin, by a process which should be less susceptible to contamination introduced during the processing of samples.

Extraction of Gla and Aminomalonate for ^{14}C Dating

The extraction of Gla was carried out by base hydrolysis followed by separation on a cation exchange column using Dowex-50W resin. Base hydrolysis is required since acid hydrolysis converts Gla to Glu. The Gla was identified by comparing the position and timing of its elution peak with that of a commercial Gla standard (Sigma No. C3767) on an HPLC chromatogram. Additional verification of the identity of Gla was attempted by applying a thermal decarboxylation test (Hauschka *et al.* 1980). Unexpectedly, both glutamic acid and glycine resulted from the decarboxylation of the eluted product. If only Gla were present in the elutant, there was the expectation that decarboxylation would have yielded only glutamic acid. The first buffer of the HPLC system was adjusted to a lower pH to lengthen the elution time. When the elutant was rerun, two separate peaks were resolved, indicating the presence of two separate amino acids.

By adjusting the cation exchange column elution procedures, it was possible to separate the elutant into two amino-acid solutions. Decarboxylation of one yielded only glutamic acid, indicating it to be Gla. The decarboxylation of the second elutant yielded glycine. Based on the previous work of Hauschka *et al.* (1980), it was suggested that the second peak contained Am. To test this possibility, Am was produced by base hydrolysis of both diethyl aminonomalonate hydrochloride (Sigma No. D7144) and acetamidomalonic acid diethyl ester (Sigma No. A6384). The product was applied to our ion-exchange resin and eluted in a single peak that had a reproducible characteristic retention time. No other peaks were observed. The second elutant exhibited the same characteristic retention time. The presence of Am in base hydrolysates of bone was not expected since Hauschka *et al.* (1980) had previously reported that an examination of various proteins and tissues had yielded no evidence of this amino acid. Details of our extraction procedure for all fractions and process of characterization of Gla and Am will be presented elsewhere (Burky *et al.*, in preparation).

We note that the measured concentration of Gla in our modern bone standard was 545 nmol g^{-1} while the concentration of Am was measured at 438 nmol g^{-1}. King (1980) had previously reported a Gla concentration of 1100 nmol g^{-1} in modern bone. This is about a factor of 2 larger than what we measured. One possible explanation of this discrepancy is that King used a HPLC system and procedures in which Gla and Am co-eluted in the same peak. Other factors, including incomplete hydrolysis, might also be partially responsible for the discrepancy.

Gla and Aminomalonate ^{14}C and Composition Values

We have obtained a suite of ^{14}C values from Gla and Am fractions isolated from a suite of bone samples from a series of localities ranging in age, previously estimated or directly determined by various means, from *ca.* 2.5 ka to >50 ka. In most cases, from the same sample, total amino-acid fractions obtained by both acid and base hydrolysis were also isolated and ^{14}C analysis undertaken (Burky 1996). Four samples of human bone, all of Holocene age, were included in the series. The expected age for each sample was estimated by various criteria including geological context, previously obtained ^{14}C data, and, in one case (UCR-3353), by a suggested historic association with a destruction layer dated to 612 BC at the late Assyrian site of Nineveh in Mesopotamia. Table 1 summarizes the expected and measured ^{14}C ages of these samples listed in order of decreasing expected age.

TABLE 1. Expected Ages (ka) and Measured Ages (^{14}C yr BP) of Different Fractions Isolated from Bone Samples from Archaeological and Paleontological Contexts

Location	Expected age (ka)	Measured ^{14}C age (ka BP) Total AA/acid	Total AA/base	Gla	Am
Abri Pataud, Les Eyzies, Dordogne, France	31.8*	31.1	31.1	15.4	14.4
Abri Pataud, Les Eyzies, Dordogne, France	20.8*	18.8	19.3	5.8	--
Titaluk River, North Slope, Alaska	20†	21.0	21.1	19.5	--
La Brea Tar Pits, Los Angeles County, California	15.5*	15.6	15.7	15.7	17.4
Colorado Creek Mammoth, Alaska	15.1*	16.4	16.3	15.6	16.3
Burning Tree Mastodon, Allen County, Indiana	11†	11.0	10.9	10.1	10.5
Dent Mammoth, Colorado	10.8*	10.8	11.0	8.8	7.6
Spirit Cave, Fallon County, Nevada‡	9.4*	9.5	9.3	--	9.9
Wizard Beach, Pyramid Lake, Nevada‡	9.2*	9.3	9.2	9.5	9.2
Haverty (Angeles Mesa), No. 4, Los Angeles County, California‡	4.0–5.2*	2.5	5.0	--	--
Mosul (Nineveh), Iraq‡	2.56§	2.6	2.5–2.8#	3.7	--

*Based on previous ^{14}C values
†Based on estimated age from geological association
‡Human skeletal sample
§Based on historical association (destruction layer at Nineveh dated to 612 BC)
#Range in values for two duplicate analysis

The original strategy of this study was to examine Gla ^{14}C values in bone samples containing significant amounts of collagen from a variety of environments and then analyze an equal number of Gla values in bones containing low or trace amounts of collagen. For reasons explained below, only the first segment was completed. Of the 15 bone samples selected for this study, 3, all bone from Clovis age sites in the U.S. Southwest (BLM, Blackwater Draw and Naco mammoths), contained <1% modern total amino-acid concentrations and no detectable amounts of Gla or Am. Of the remaining 12 bone samples (Tables 1 and 2), all but 2 of these bones exhibited Gly/Glu ratios >4 and all but 2 exhibited total amino acid content >25%. Previous studies have indicated that Gly/Glu ratios can be used as one means to characterize the degree to which bone samples have retained a collagen-like amino-acid pattern (Hare 1980). With the determination of the existence of Am in most of the bones examined, ^{14}C and compositional data were determined for this amino acid as well. Table 2 summarizes the total amino acid, Gla and Am content obtained on the bones used in this study normalized to the amount found in our modern bone standard.

In many cases, ^{14}C determinations on the Gla and Am extracts required the use of a series of mass-balanced standards and backgrounds for the AMS-based measurements since, in several instances, the sample sizes were <0.3 mg of carbon. The requirement for mass-balanced samples results from observations, first examined in detail by Vogel, Nelson and Southon (1987), that for catalytically reduced graphitic carbon, there are significant increases in background blank ^{14}C levels for submilligram samples weights for samples <0.5 mg of carbon as well as decreasing ^{14}C activity in contemporary standards. As a result of previous UCR/LLNL CAMS collaboration, significant reductions in ^{14}C activity in our sample processing blanks have been achieved. These currently range from *ca.* 0.15 pMC for >0.1 mg (>100 μg) of graphitic carbon to 0.5 pMC for 0.02 mg (20 μg) samples obtained on wood from Pliocene sediments (Kirner, Taylor and Southon 1995; Kirner *et al.* 1996; Kirner *et al.* 1997).

TABLE 2. Total amino acids (total AA/acid hydrolysis), Gla and Am content and compositional data for bones used in study listed in order of decreasing expected age (Table 1). Total AA/acid, Gla and AM content expressed as percentage contained in our modern bone standard.*

Location	AA/acid (% mod)†	Gla (% mod)†	Am (× mod)†	Gly/Glu
Abri Pataud, Les Eyzies, Dordogne, France	30	38	4 ×	4.4
Abri Pataud, Les Eyzies, Dordogne, France	63	<1	4 ×	4.4
Titaluk River, North Slope, Alaska	104	65	4 ×	4.1
La Brea Tar Pits, Los Angeles, California	79	38	2 ×	4.5
Colorado Creek Mammoth, Alaska	109	42	14 ×	4.2
Burning Tree Mastodon, Allen County, Indiana	101	<1	5 ×	5.4
Dent Mammoth, Colorado	28	34	2 ×	4.5
Spirit Cave, Fallon County, Nevada	119	80	3 ×	4.2
Wizard Beach, Pyramid Lake, Nevada	93	52	6 ×	4.3
Haverty (Angeles Mesa) No. 4, Los Angeles County, California	2	<1	Trace	3.5
Mosul (Nineveh), Iraq	9	<1	0.7 ×	4.4

*Three samples—BLM, Blackwater Draw and Naco Mammoth bone—contained <1% modern total amino acids and no detectable amounts of Gla or Am.

†Modern bone used as standard for 100% total amino acids, Gla and Am with Gly/Glu = 4.1. Our modern bone standard exhibits same Gly/Glu value as that used by Hare (1980).

We note that standard screening tests of the resin used in our ion-exchange procedures identified a potential source of ^{14}C contamination by resin bleeding if rigorous cleaning protocols were not followed. While the uncleaned resin itself exhibited a ^{14}C activity indistinguishable from our Pliocene wood blank, elution tests on the resin revealed the presence of significant levels of contamination. A 50-ml elution was made from a 12-cm resin column using only ultra-pure water. The 50-ml elution was evaporated to dryness and combusted according to our standard procedures. We obtained the equivalent of 70 μg of carbon that exhibited a ^{14}C activity of *ca.* 10.5 pMC [apparent age: 18,110 ± 110 BP] (CAMS-25401). A larger elution (150 ml) yielded the equivalent of 120 μg of carbon with a ^{14}C activity of *ca.* 9.7 pMC [apparent age: 18,770 ± 110] (CAMS-25402).

Before the nature of the resin contamination problem was identified and more stringent resin cleaning and storage procedures employed, three ^{14}C determinations had been completed. Current protocol for the cleaning of the resin involves heating in 3M HCl at 80°C for several hours, discarding the acid and adding clean 3M HCl, agitating, and then repeating this process until the acid solution is clear. Resin is stored in 3M HCl. When needed, it is placed in a column and washed with 4–5 bed columns of ultra-pure water brought to pH 1.5.

DISCUSSION

In the bones we have examined, Gla tends to decrease in fossil bones in concert with the total amino-acid concentration, which, in general, reflects reductions in collagen content (Fig. 2). The suggestion that there would be a preferential retention of Gla in fossil bone is not supported by this data. There is no correlation with age, rather the Gla content and total amino acids as a percentage in our modern bone standard were, in general, highest in bones from environments characterized by constant cold and dry conditions regardless of age. We lack data on effective soil pH, but this also could be an important factor. In our suite of samples, the best preservation of Gla and collagen occurred in bone from a naturally desiccated early Holocene human "mummy" recovered from Spirit Cave, Nevada. The lowest concentrations were in human skeletons [Haverty No. 4 (California) and Mosul

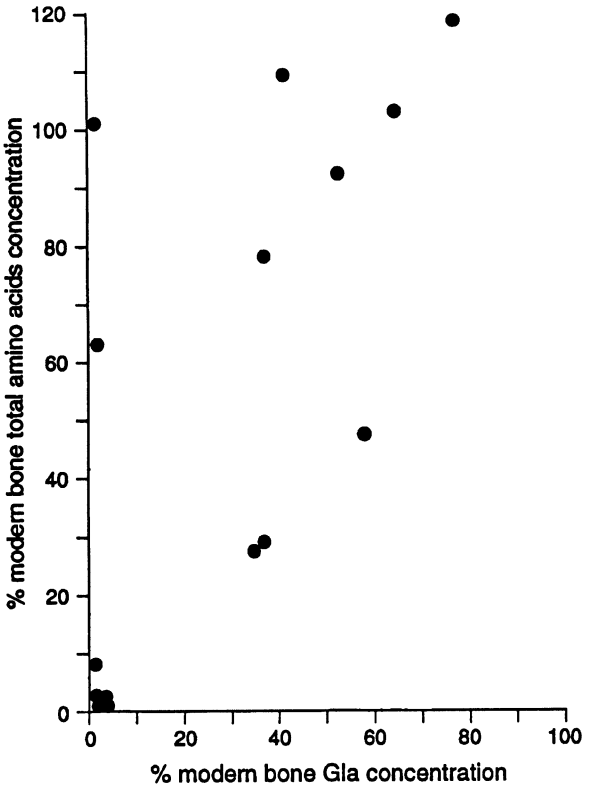

Fig. 2. Relationship of total amino acids and Gla concentrations in a series of fossil bones expressed as a percentage of total amino acids and Gla in a modern bone standard.

(Iraq)], where the burial environments were probably characterized by alternating wet and dry conditions. Interestingly, Am concentration typically exceeds that which is characteristic of modern bone, sometimes, as in the case in a bone of Middle Pleistocene age from Medicine Hat, Alberta, Canada, by a factor of >20. This pattern suggests that Am is produced as a breakdown product, perhaps through several diagenetic mechanisms.

To examine the effectiveness of our extraction procedures, a series of duplicate extractions were carried out on a sample of bone collected from sediments associated with the Yarmouth interglacial stage at Medicine Hat, Alberta, Canada. Since the assigned age of this deposit is Middle Pleistocene, bone from these sediments should be ^{14}C "dead," *i.e.*, exhibit ^{14}C activity indistinguishable from our background blanks. (As previously noted, these currently are at the level of the equivalent of *ca.* 52,000 BP (0.15 pMC) for >100 μg to *ca.* 42,000 (0.5 pMC) for 20 μg samples.) Although the Gly/Glu ratio is depressed (2.8), the Medicine Hat bone contains *ca.* 50% of the total amino-acid concentration of our modern bone standard and thus appreciable amounts of collagen have been retained. Although we were able to achieve >50,000 BP on four duplicate preparations in total AA/base extractions and achieved close to 50,000 BP on a total AA/acid extraction, other preparations, particularly of the total AA/base fractions, exhibited apparent ages ranging down *ca.* 40,000 BP. Factors contributing to this variability perhaps included continuing problems with small but still detectable resin contamination not entirely removed by the extended resin cleaning and storage procedures described above.

The age offsets reflected in the Gla and Am ^{14}C values indicate significant age anomalies reflected in these fractions (Table 1 and Fig. 3). Assuming that contamination is modern, Gla extracts experienced a greater degree of contamination than was reflected in the Am ^{14}C values. The same patterns in ^{14}C age offsets among total AA, Gla and Am extracts continue in all of the fossil bones examined where all three values were obtained.

Figure 3 plots the differences in ^{14}C ages between Gla and Am fractions compared with the total AA/acid fraction. The most serious discrepancy was found in the oldest bone sample from Abri Pataud where Gla and Am values were >15,000 yr younger than the total AA/acid and base fractions. The most concordant total AA and Gla ^{14}C values were obtained on bone from the La Brea Tar Pits. Presumably, this is due in part to the atypical protected biochemical environment of this site.

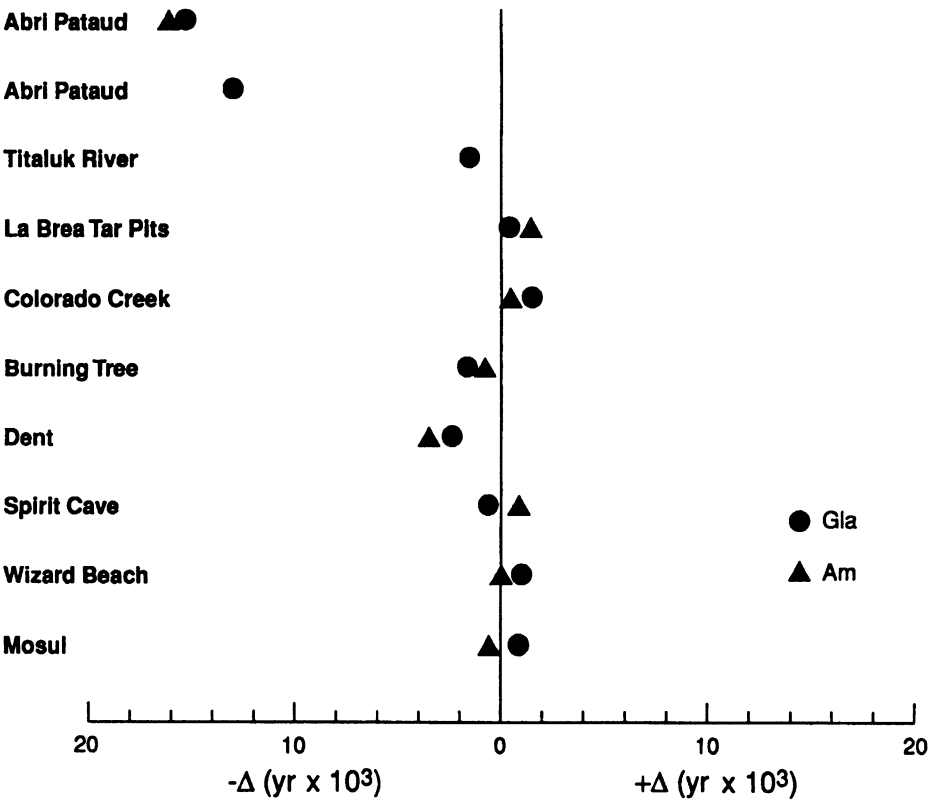

Fig. 3. Age offset (in yr × 10^3) between Gla and Am and total amino acid fractions

Conclusion

There now appears to be a general consensus among investigators concerning the reliability of bone ^{14}C values: first, where appropriate biochemical purification procedures are employed, generally accurate ^{14}C estimates can be obtained on bones retaining significant amounts of intact collagen; second, that bones seriously depleted in their original protein (mostly collagen) content can yield seriously anomalous ^{14}C values. We have examined the suggestion that a non-collagen amino acid, γ-carboxyglutamic acid (Gla), might be protected from diagenetic contamination in fossil bones characterized by low and trace amount of collagen. ^{14}C data on Gla obtained from a series of bones of varying age and degree of collagen retention does not support this suggestion. Discordant ^{14}C val-

ues on Gla (and on Am, newly identified as occurring in fossil bone) were obtained from bones retaining significant amounts of collagen. Also, in the bones we studied, Gla concentrations decreased in concert, or even more rapidly, with collagen depletion. Am concentrations increased, indicating that it is a breakdown product. Thus, we conclude that the proposal that stable Gla content and protection of isotopic integrity of Gla might be exhibited in fossil bone, regardless of collagen content, has not been confirmed.

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