Degradation Patterns in Archaeological Collagen: New Evidence from Pyrolysis GC-MS and Solid State ^{13}C NMR

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ABSTRACT

Collagen is the most abundant protein found in calcified tissues and is used as a substrate in important applications such as radiocarbon dating and stable isotope reconstruction of diet and environment. In an archaeological context, collagen degrades, but the type and rate of degradation mechanisms on the molecular level are poorly understood. This study uses stable isotope mass spectrometry, amino acid analysis, protein sequencing, infrared spectroscopy, pyrolysis GC-MS, and solid state ^{13}C NMR to analyze the structure and alteration of the collagen molecule in different states of preservation. Major chemical transformations occur in samples with C/N_m greater than 3.1 (atomic 3.6). These data suggest that collagen degradation involves bacterially driven denaturation and deamination of R group nitrogen, followed by hydrolysis, deamination of peptide nitrogen, and formation of Maillard-type condensation products. These hydrolyzed peptide fragments, condensation products, and bacterial biomarkers are preserved in close association with each other by clay.

INTRODUCTION

Collagen extracted from bones collected at archaeological sites provides dietary information about ancient humans, because the carbon and nitrogen isotopic composition of bone collagen reflects an animal's trophic level, marine versus terrestrial diet, and relative contributions of C3 and C4 plants (Neuberger and Richards, 1964; DeNiro and Epstein, 1981; Schoeninger and DeNiro, 1984). However, performing isotopic analysis on ancient collagen is problematic because of degradation.

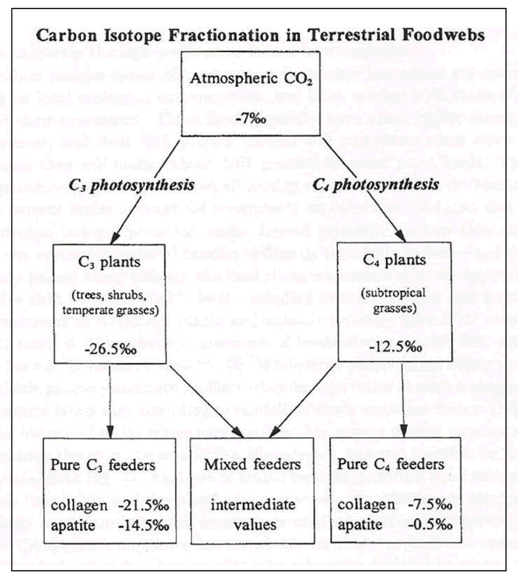


Fig. 1. Carbon Isotopic Fractionation in Plants and Animals

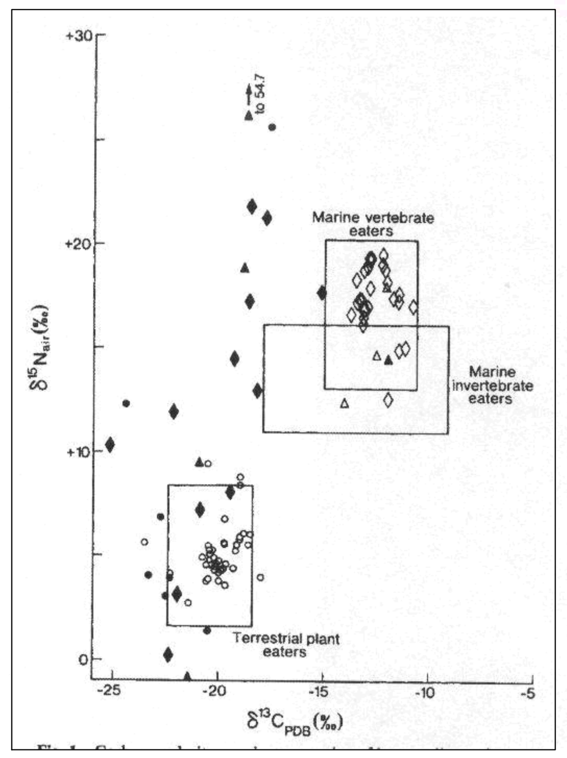
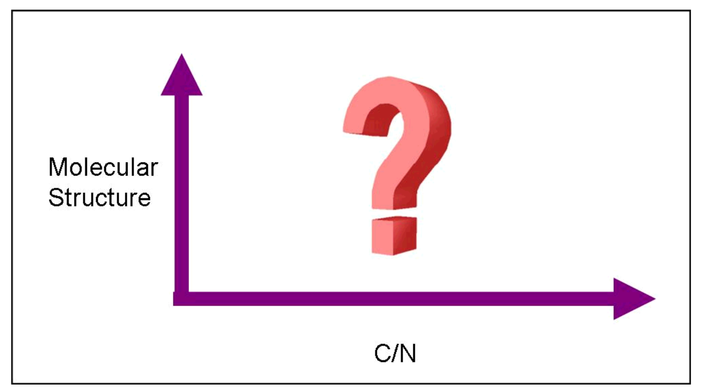


Fig. 2. Distribution of $\delta^{13}C$ and $\delta^{15}N$ for archaeological collagen with C/N less than or equal 3.6 (open symbols) and C/N greater than 3.6 (solid symbols) in relation to modern collagen (boxes) (DeNiro, 1985)

One common indicator for the extent of collagen degradation is the atomic carbon-to-nitrogen ratio (C/N). Mass spectrometers measure a mass-based ratio, C/N_m, which is converted to an atomic ratio by multiplying by 1.1667 (White and Schwarz, 1989). Modern vertebrate collagen has C/N_m of 2.8. When C/N_m of archaeological collagen is greater than 3.1 (atomic 3.6), the isotopic values generally fall outside the observed ranges for modern animals, meaning that the collagen has degraded sufficiently that it is no longer an accurate reflection of diet (DeNiro, 1985). These changes in C/N_m must be associated with changes in molecular structure. However, the nature of the collagen degradation products and pathways is still largely unknown. Infrared spectroscopy and amino acid analysis have shown loss of amide bonds and of net amino acid content in degraded collagen, but they do not reveal new organic constituents generated by the degradation process (DeNiro and Weiner, 1988; Tuross, 2002).

This study uses high resolution chemical techniques to compare the molecular structure of modern and archaeological collagen samples. The goal is to understand the molecular structural changes that occur in Type I collagen during the degradation process and to relate the observed molecular changes to stable isotopic ratios.



METHODS

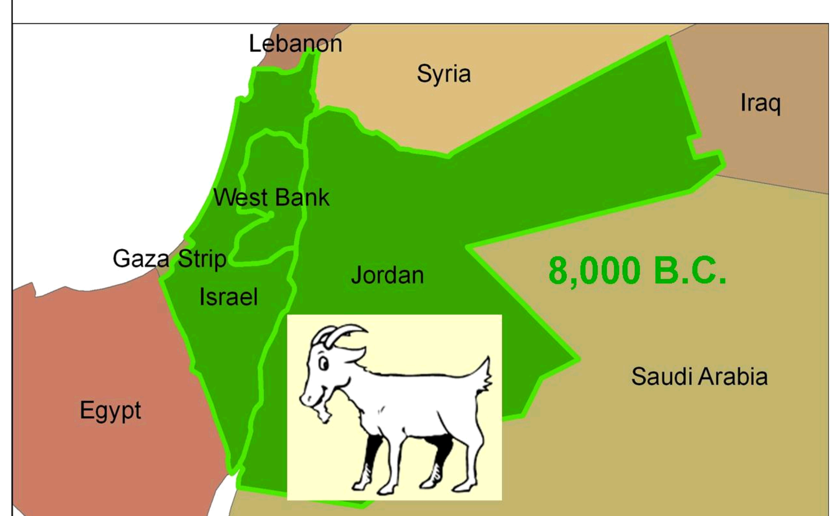


Fig. 4. Source of collagen samples

This study uses modern pig collagen and 378 archaeological collagen samples from goats excavated at Pre-Pottery Neolithic sites in Israel and Jordan. Collagen samples were prepared by demineralizing bones in EDTA (Tuross et al., 1988). We determined elemental and isotopic composition by mass spectrometry; protein content by amino acid analysis and protein sequencing; and molecular structure by infrared spectroscopy, pyrolysis gas chromatography/ mass spectrometry, and solid state ^{13}C NMR.

RESULTS

Theoretical Calculations

In order to assess how changing C/N_m might affect isotopic ratios, we determined what type and magnitude of molecular change could cause the observed changes in C/N_m. Deamination to remove all free amino group nitrogen results in C/N_m of 3.1, while deamination of all non-peptide bonding nitrogen results in C/N_m of 3.3. Therefore samples with C/N_m greater than or equal to 3.3 are no longer protein. Those samples are also darker, less dense, and less cohesive.

Elemental and Isotopic Analysis

We determined the mean carbon content (%C), nitrogen content (%N), carbon isotopic ratio ($\delta^{13}C$), and nitrogen isotopic ratio ($\delta^{15}N$) for collagen samples with C/N_m of 2.8 to 3.5. Percent C and percent N decrease as C/N_m increases; there is a significant difference in both measures between samples with C/N_m of 2.8 to 3.1 and samples with C/N_m of 3.2 to 3.9. Mean $\delta^{13}C$ values are also slightly depleted for collagens with higher C/N_m (Fig. 2).

Amino Acid Analysis

Archaeological collagen samples have a similar proportion of ASX, GLX, and GLY to modern collagen, slightly more PRO and ALA, and slightly less HPR and ARG. These differences in amino acid composition do not account for the differences in C/N_m.

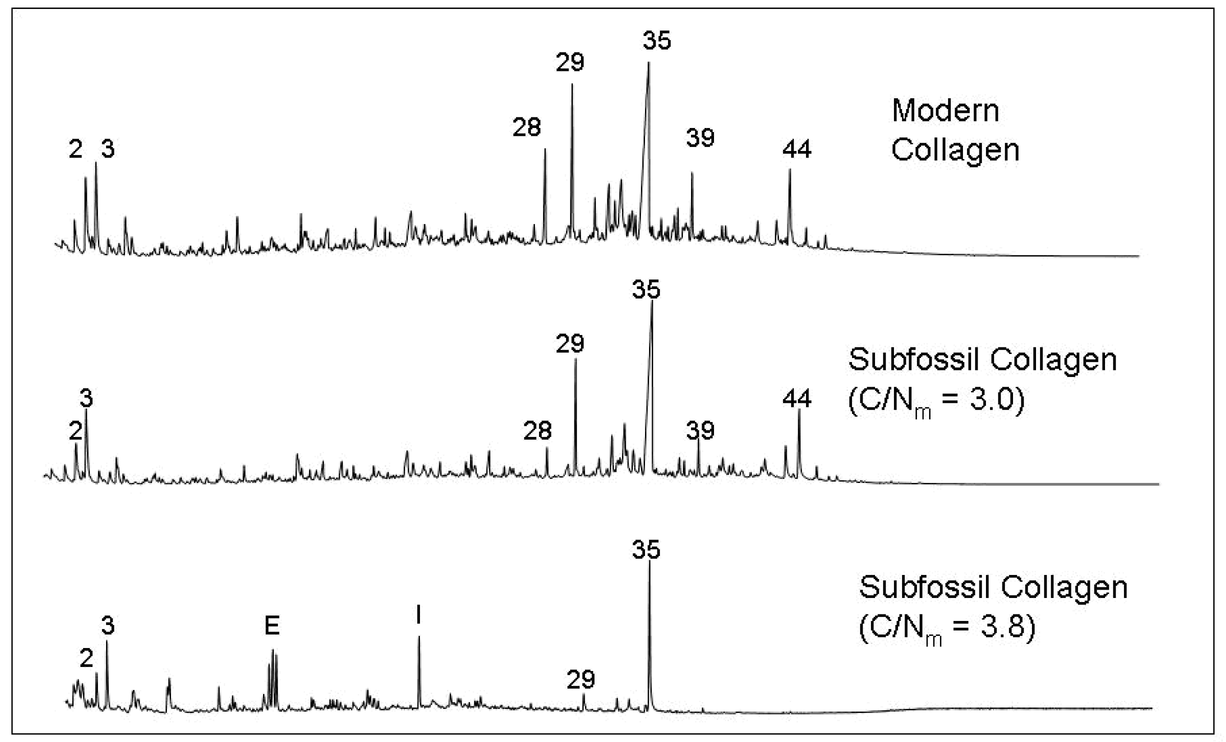


Fig. 6. Pyrolysis GC-MS elution profiles of modern, intact subfossil, and degraded subfossil collagen.

Pyrolysis GC-MS

The pyrolysis GC-MS elution profiles demonstrate that intact archaeological collagen is similar to modern collagen and that the structure of archaeological collagen changes gradually with increasing C/N_m, although there are particularly significant changes for C/N_m greater than 3.1 (atomic 3.6). Intact archaeological collagen shares all of the major pyrolysis products of modern collagen, while degraded archaeological collagen shares some major products but also yields new products. The most likely identifications for major peaks are shown in Fig. 5. Evolution in collagen composition can be quantified by determining the quantity of major products relative to pyrrole. This shows, first, that there are significant differences between samples with C/N_m of 2.8 to 3.1 and those with C/N_m of 3.3 to 3.9. Second, pyrolysis of collagen samples with higher C/N_m produces fewer amino acid dimers compared to single amino acids. Third, branched alkanols appear in collagen samples with C/N_m greater than 3.1 (atomic 3.6).

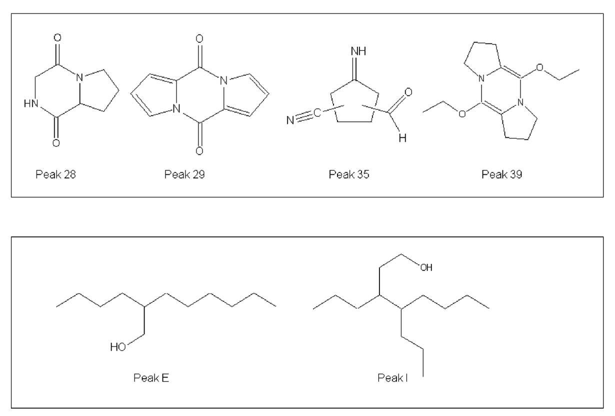


Fig. 7. Pyrolysis products: amino acid condensation dimers in intact and degraded collagen (top) and branched alkanols in degraded collagen (bottom).

C/N_m	Source	No. of Sequences Recovered	Percent Deamination of ASX and GLX
2.8	Modern	173	12
2.9	Subfossil	60	67
2.9	Subfossil	111	48
3.1	Subfossil	78	65
3.3	Subfossil	59	60
3.3	Subfossil	92	53
3.4	Subfossil	67	65
3.5	Subfossil	57	55
3.8	Subfossil	39	54

Table 1. Protein sequences and deamination in modern and subfossil collagen.

Protein Sequencing

The number of protein sequences recovered from archaeological samples was only 22%-64% of that recovered from modern collagen. In addition, whereas only 12% of ASX and GLX residues in modern collagen are deaminated, 48%-67% are deaminated in archaeological samples.

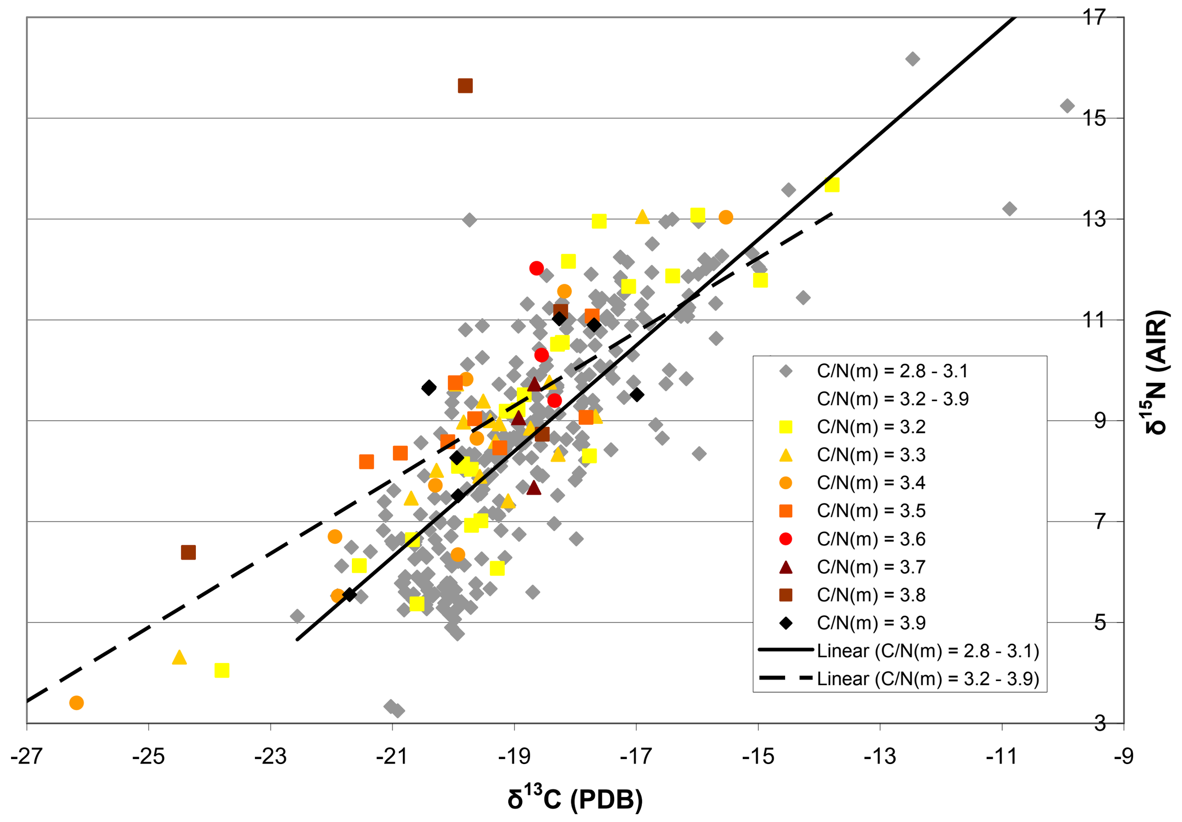


Fig. 5. $\delta^{13}C$ and $\delta^{15}N$ values of subfossil collagen samples as a function of C/N_m

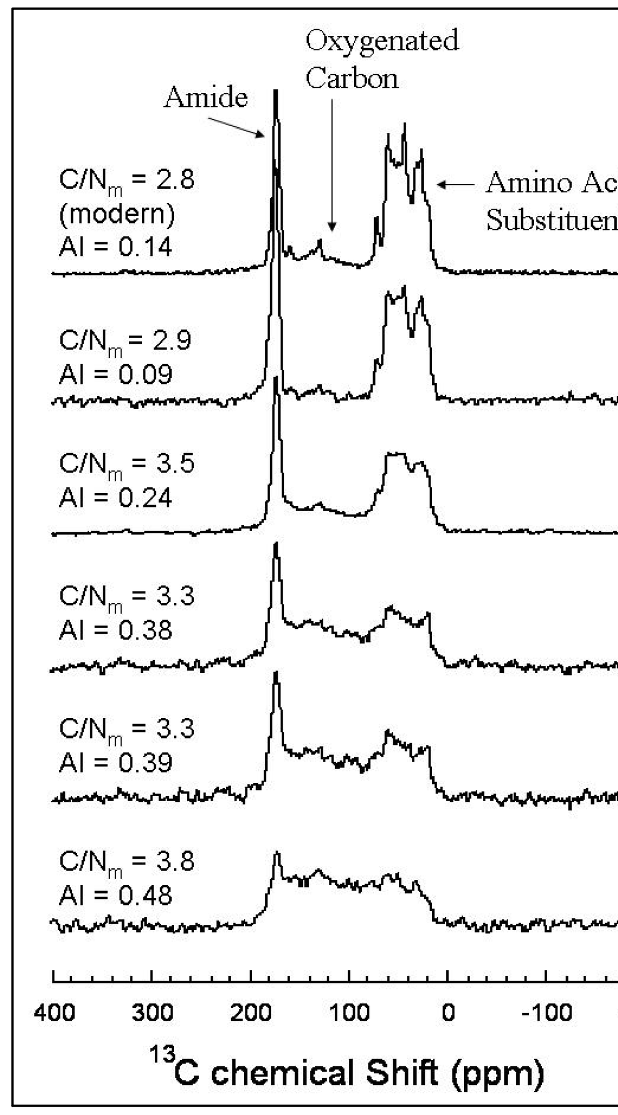


Fig. 8. Solid state ^{13}C NMR spectra of modern and subfossil collagen.

Solid State ^{13}C NMR

Solid state NMR reveals that degraded collagen maintains some characteristics of intact collagen but also contains additional functional groups (Fig. 7). The NMR spectrum of modern collagen has a strong amide peak at 161.7 to 208.6 ppm and peaks characteristic of amino acid substituents at ~10 to 80 ppm. NMR spectra of archaeological collagen samples with high C/N_m still reveal a prominent amide/carboxyl peak and amino acid subsituent peaks, but there is also signal in the alcohol and olefinic/aromatic region from 80 to 161.7 ppm. The extent of alteration from intact collagen can be quantified based on the NMR spectra by determining the ratio of signal area in the oxygenated carbon region (80 to 161.7 ppm) to the total signal area (-5.7 to 208.6 ppm). This NMR-derived alteration index ranges from 0.09 to 0.48 as C/N_m increases from 2.9 to 3.8. Thus NMR reveals the appearance of oxygenated carbon compounds in collagen samples with C/N_m greater than 3.1 (atomic 3.6) and the increase in quantity of such compounds compared to protein as C/N_m increases.

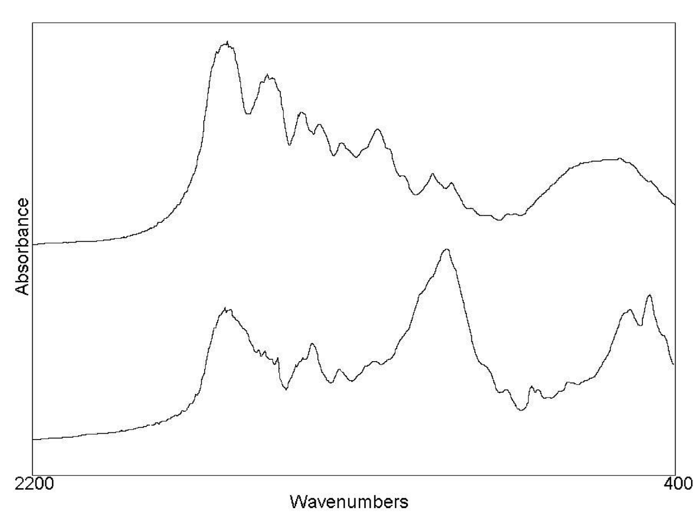


Fig. 9. Infrared spectra of intact collagen (top) and degraded collagen (bottom).

Infrared Spectroscopy

IR spectra for intact and degraded collagen show that degraded collagen samples have lost much of their amide bond character and also contain clay.

DISCUSSION

These analyses provide evidence that major structural changes have taken place in collagen with C/N_m greater than 3.1 (atomic 3.6) and suggest a potential pathway for the degradation of collagen on archaeological sites.

Extent of Degradation

Theoretical calculations show that collagen with C/N_m greater than or equal to 3.3 has degraded such that it is no longer protein, decreased percent C and percent N suggests that such samples contain inorganic material like clay, and isotopic analysis shows depletion in ^{13}C. Since differences in amino acid composition do not account for changing C/N_m, degradation must be spatially heterogeneous.

Deamination and Hydrolysis

Protein sequencing suggests that deamination of free amino groups may account for C/N_m increases up to 3.1 and that much of the protein in degraded collagen is so altered that it is no longer recognizable as protein. Based on pyrolysis GC-MS data, degradation also involves denaturation, hydrolysis of amide bonds, and deamination of formerly peptide-bonding nitrogen

Branched Alkanols and Complex Oxygenated Compounds in Degraded Collagen

The NMR alteration index indicates the increasing proportion of oxygenated carbon compared to protein present in collagen samples with higher C/N_m. The suite of complex oxygenated carbon compounds present in the NMR spectra is consistent with Maillard-type condensation reactions between amino acids and sugars or, more likely, between amino acids and keto acids formed by oxidative deamination of amino acids (Ikan et al., 1996). The two specific branched alkanols that are pyrolysis products of degraded collagen samples are likely bacterial biomarkers of sediment bacteria driving collagen degradation in order to scavenge nitrogen. The alkanols may be fatty alcohol side products of bacterial fatty acid synthesis, possibly even using amino acids from degraded collagen as primers (Kolattukudy, 1971; Rock, 1978; Kaneda, 1991).

A Degradation Pathway

The molecular data suggest a possible process by which collagen degrades. Between C/N_m of 2.8 and 3.1 (atomic 3.2 to 3.6), collagen undergoes denaturation, deamination of R group nitrogen, and the beginning of peptide bond hydrolysis. Starting at C/N_m of 3.2 (atomic 3.6), collagen undergoes more complete hydrolysis, deamination of formerly peptide bonded nitrogen, and Maillard-type condensation reactions of hydrolyzed amino acids and keto acids formed by oxidative deamination of amino acids. The hydrolysis, deamination, and condensation reactions appear to be components of a concerted process driven by sediment bacteria which leave biomarkers in degraded collagen. Clay may contribute to the preservation of degraded collagen by holding together hydrolyzed fragments and condensation products. Note that since several different reactions take place simultaneously during collagen degradation, samples with identical C/N_m may not have undergone exactly the same degradation process.

Isotopic Trends

The molecular changes associated with collagen degradation suggest possible isotopic trends. Significant molecular changes are expected to affect the stable isotopic signatures of collagen samples with C/N_m greater than 3.1 (atomic 3.6). Predicting isotopic shifts may not be straightforward. In this study, formation of Maillard-type degradation products and incorporation of bacterial biomarkers in samples with high C/N_m appear to be associated with a slight reduction in $\delta^{13}C$. Further analyses of the precise molecular changes during degradation and the processes by which they might occur are necessary to predict more specific isotopic shifts.

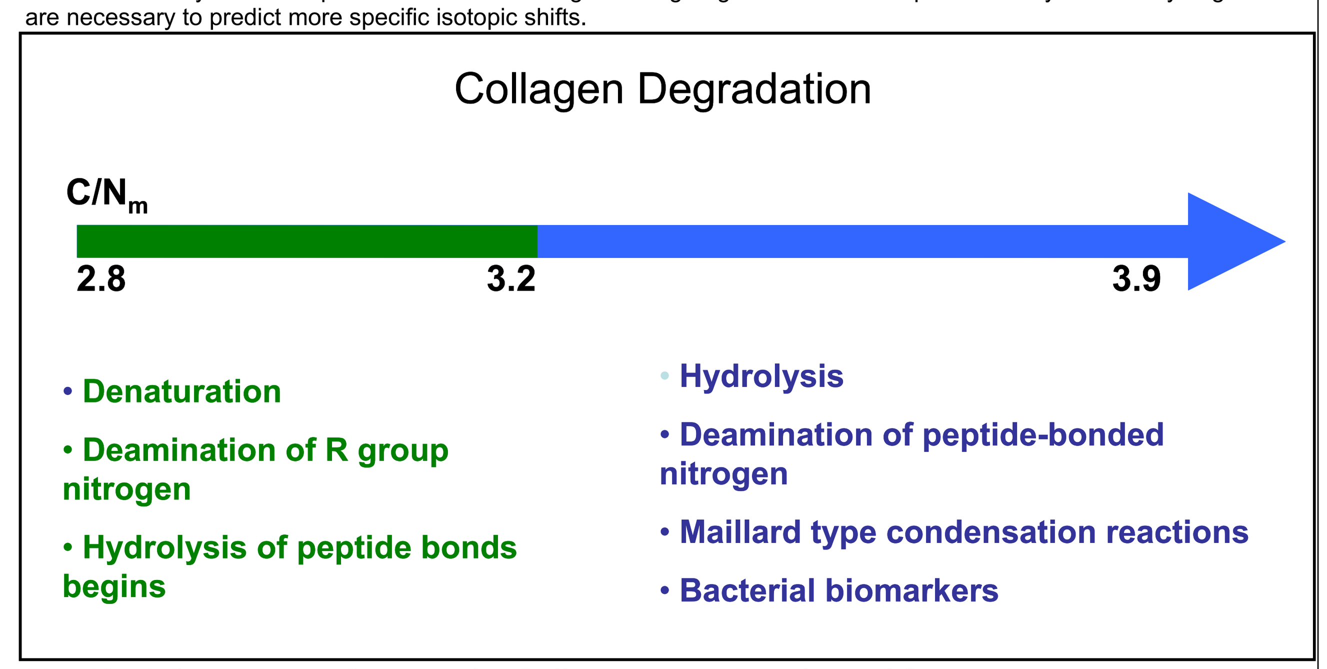


Fig. 10. A Degradation Pathway for Collagen on Archaeological Sites

CONCLUSION

This study provides a molecular perspective on the degradation pathway for bone collagen recovered from archaeological sites (see "A Degradation Pathway"). The characterization of degradation processes has practical implications for future diet studies. Because of molecular changes in collagen, samples with C/N_m greater than 3.1 (atomic 3.6) cannot be assumed to have carbon and nitrogen isotopic ratios that accurately reflect diet. However, studies such as this one that pinpoint the molecular changes taking place during degradation may allow prediction of isotopic shifts. At this point, quantitative prediction of shifts is not possible, and therefore degraded collagen samples with C/N_m greater than 3.1 (atomic 3.6) should not be used for isotopic analysis.

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