Stable Carbon Isotope Ratio Measurements of the Carboxyl Carbons in Bone Collagen

by

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25/6/96

Abstract

The $^{13}C/^{12}C$ ratio of carbon in the biosphere varies slightly with source. The photosynthetic pathway of carbon fixation has the largest influence on this ratio but the environment (terrestrial or marine) and other factors also induce smaller variations. These shifts in the isotopic ratio can be traced through the food chain. Crucial dietary information can be obtained by measuring the $^{13}C/^{12}C$ ratio of animal tissues such as bone collagen from archaeological samples. In this thesis, a method was developed and tested to compare $^{13}C/^{12}C$ ratios between the total carbon in collagen and the carboxyl carbons in the amino acids of collagen hydrolysates. It was hoped that differences in these ratios might provide additional dietary information and thus be another tool for nutritional analysis of archaeological samples. The carboxyl carbons were selectively removed by decarboxylation with ninhydrin (2,2-dihydroxy-1,3-indanedione). A survey of the differences in $^{13}C/^{12}C$ ratios between carboxyl and total carbons in bone collagen from several modern and archaeological samples revealed that the carboxyl carbons were usually isotopically heavier than total carbon. This difference in $^{13}C/^{12}C$ ratios ranged from -0.9 to 4.0 parts per thousand. The average error (1 standard deviation) for replicate measurements was less than 0.5 parts per thousand. Animals at higher trophic levels usually had smaller differences than lower animals. Humans had the largest range of differences, possibly due to a larger variability in their diets. An earlier study found the $^{13}C/^{12}C$ ratio of collagen in the extinct European cave bear (*Ursus spelaeus*) increased with age. The present study found even larger increases with age for the carboxyl carbons. Possible sources of the differences in $^{13}C/^{12}C$ ratios between total and carboxyl carbons are discussed.

The shells upon the warm sands
have taken from their own lands
the echo of their story
but all I hear are low sounds . . .

Enya, from "Anywhere is"

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List of Abbreviations

ALA	Alanine
ASP	Aspartic acid
B.P.	Before Present
CAM	Crassulacean Acid Metabolism
GC	Gas Chromatograph
GLU	Glutamic acid
GLY	Glycine
HPLC	High Performance Liquid Chromatography
HYP	Hydroxyproline
IRMS	Isotope Ratio Mass Spectrometer
LEU	Leucine
PDB	Pee Dee Belemnite
PRO	Proline
PTFE	Polytetrafluoroethylene
SD	Standard Deviation
SER	Serine
THR	Threonine
VAL	Valine

1 INTRODUCTION

1.1 *Stable Carbon Isotopes*

Carbon has two stable isotopes, ^{12}C and ^{13}C, with relative natural abundances of approximately 98.9% and 1.1% respectively. Although the magnitude of the variation in these values is usually very small in nature, this variation can be used as a powerful indicator of carbon flow in the biosphere. A delta (δ) notation has been adopted to indicate the small differences in the stable isotope ratios between samples and a standard. The $\delta^{13}C$ value of a sample is defined by the stable carbon isotope ratios of sample and standard as follows:

$$\delta^{13}C = [\{(^{13}C/^{12}C)_{sample} / (^{13}C/^{12}C)_{standard}\} - 1] * 1000 \text{ (‰ PDB)}.$$

Thus, $\delta^{13}C$ is the difference in the $^{13}C/^{12}C$ ratio of the sample relative to the standard in parts per thousand (‰). By international convention, $\delta^{13}C$ values are calculated using one standard, originally based on a limestone fossil of *Belemnitella americana* from the Cretaceous Pee Dee formation in South Carolina (abbreviated PDB) which has a $^{13}C/^{12}C$ ratio of 0.0112372 (1). Because changes in the $^{13}C/^{12}C$ ratio are so small, absolute ratios such as this are rarely reported. The range of $\delta^{13}C$ values for biological samples is about 30‰.

1.2 *Isotopic Fractionation in the Biosphere*

Variations in the isotopic ratios of natural materials result from isotopic fractionation in physical, chemical and biological processes. These variations and their application to dietary reconstruction are very well described in the literature (2-5), and briefly summarised in Appendix A. In general, isotopically, you are what you eat. Thus, the $\delta^{13}C$ of an animal's tissue reflects the $\delta^{13}C$ of the primary producers in the food web to which the animal belongs. For example, a human eating salmon is isotopically distinguishable from one eating deer. However, there are some shifts in $\delta^{13}C$ between an animal and its diet. Although poorly understood, these shifts depend partially on what animal and what tissue in the animal are measured (6). Measuring $\delta^{13}C$ provides significant dietary and nutritional information about an organism.

1.3 *Carbon Isotopes and Archaeology*

Archaeologists attempting dietary reconstruction of ancient humans are faced with a few bones and artifacts from which to deduce a diet. Fortunately, bones are often relatively intact and are directly related to the individuals being studied. Approximately 25% of bone weight is organic matrix of which over 90% is the protein *collagen* (7). This is a type I collagen, which is also found in skin, tendon, and dentin (8). Collagen consists of a triple chain helix. Each chain contains about 1000 amino acids (95,000 Daltons) in a repeating glycine–X–Y triplet, where X and Y vary (9). Collagen is distinct from most other proteins due to its high glycine content (about one third) and the presence of large quantities of proline and hydroxyproline. Because collagen has a slow turnover in the living individual (10-30 years in large mammals) (2), it is almost

an ideal substance for looking at the long term dietary intakes of ancient organisms.

Archaeologists use dietary information for more than just understanding diet. The diets of ancient animals can provide information about their migration, range, inter-animal and inter-group variation, and even the climate in which they lived. In addition, the diets of ancient humans might provide significant clues about technology, trade, and culture. In combination with other archaeological techniques, stable isotope analysis of ancient tissues is a very powerful tool for archaeologists. For example, researchers have used the $\delta^{13}C$ of bone collagen to investigate the introduction of agriculture into Eastern North America (10), weaning diets of prehistoric maize horticulturists (11), and the quantity of marine versus terrestrial protein ingested by ancient human populations in coastal British Columbia (12). Humans are not the only animals to be studied. For example, the diet and habitat use of African elephants were examined through the use of $\delta^{13}C$ (13).

An animal's tissues are built from the proteins, carbohydrates, and fats it eats. In addition, these tissues are constantly being turned-over and replaced by newly synthesised tissue incorporating carbon from the old tissues as well as recently consumed food. Bone collagen protein, therefore, is a complex mixture of amino acids obtained from many different carbon sources during a long period in the animal's life. It is apparent from the literature that much is still unknown about the routing of carbon that ultimately becomes bone collagen. The amino acids in bone collagen are thought to be derived directly from dietary protein unless protein intake is very limited. Recent research (14) has shown that 91% of the collagen carbon in laboratory mice originated from dietary

protein when 64% of dietary carbon was protein. Another study found that rats fed a very low protein diet probably incorporated dietary carbohydrate carbon into their collagen through amino acid biosynthesis (2). Until the process of carbon routing from diet to collagen is fully explained, even an empirical understanding of how an animal's bone collagen $\delta^{13}C$ changes with diet is useful to archaeologists for dietary reconstruction.

1.4 *Intramolecular Distribution of $\delta^{13}C$ in Collagen*

Normally, isotopic analysis for dietary reconstruction is conducted on collagen as a whole. The $\delta^{13}C$ is measured on CO_2 from the quantitative combustion of collagen (see Appendix B for an overview of the measurement of $\delta^{13}C$). With the hope of gleaning more dietary information from collagen, researchers have begun to take collagen apart and look at the intramolecular distribution of $\delta^{13}C$.

The most obvious way of taking collagen apart is into its component amino acids. Differences in $\delta^{13}C$ among amino acids might arise from the different pathways of amino acid biosynthesis, different dietary sources of particular amino acids, or amino acid routing and turnover in the organism itself. Since essential amino acids (accounting for about 17% of the total carbons and 11% of the carboxyl carbons in collagen) cannot be synthesised by the organism; they must be incorporated into collagen relatively unfractionated directly from dietary protein sources. Non-essential amino acids can be synthesised in the organism from other carbon sources if dietary intake of these amino acids are insufficient. Thus, the $\delta^{13}C$ values of essential amino acids

should more closely follow dietary protein, whereas non-essentials might provide additional information about the non-protein portion of the diet.

To date, limited data are available for the $\delta^{13}C$ of individual amino acids in collagen because of practical difficulties in separating the individual amino acids. Two general methods have been used: preparative separation of underivatized amino acids by HPLC using cation-exchange resins with inorganic solvents (15-18), and gas chromatography-combustion-isotope ratio mass spectrometry of derivatized amino acids (19-21). The first method is very tedious and is limited by column capacity and resolution (the collection of a complete, non-overlapping peak for each amino acid is necessary to prevent isotopic fractionation during chromatography). After separation, the individual amino acids are combusted to CO_2 and their $\delta^{13}C$ measured by an Isotope Ratio Mass Spectrometer (IRMS). In the second method, the derivatized amino acids (typically as N-trifluoroacetyl isopropyl esters) are separated by gas chromatography and the effluent is combusted on-line to CO_2 and introduced directly into the IRMS. This method is inherently more sensitive and practical but has the disadvantage of carbon addition (with different $\delta^{13}C$) and isotopic fractionation during derivatization; the measured amino acid derivatives are isotopically lighter than would be expected by mass balance. Although the derivatizing steps are quantitative for the amino acids, it appears that the derivatizing agents (in excess) are being fractionated. Thus, empirical corrections to $\delta^{13}C$ are required.

A few studies have shown that the $\delta^{13}C$ values of individual amino acids in collagen hydrolysates are markedly different from total collagen (17, 18). Using young and rapidly growing laboratory pigs fed controlled diets, Hare *et al.*

(17) found the $\delta^{13}C$ of each amino acid in pig collagen closely followed the $\delta^{13}C$ of the same amino acid in the diet. However, there were significant changes in the $\delta^{13}C$ of the pigs' aspartic and glutamic acids from these amino acids in the diet. This may suggest that these amino acids were fractionated differently in the growing pigs than the other amino acids. The $\delta^{13}C$ of the individual amino acids in collagen ranged over 17‰ about the value for collagen itself (17, 18). Much information would be anticipated if the separation and $\delta^{13}C$ measurement of individual amino acids was not so tedious.

Another way of looking at the intramolecular distribution of $\delta^{13}C$ in collagen is to look at specific carbons in the molecule if these atoms could be selectively removed and measured. The intramolecular distribution of $\delta^{13}C$ in fatty acids has been investigated previously by Monson *et al.* (22) by converting the carboxyl carbon in a fatty acid to CO_2 via the Schmidt rearrangement. They found the carboxyl carbons to be up to 13‰ lighter than the total carbon and an alternating pattern of $\delta^{13}C$ values along the hydrocarbon chain. Analogously, ninhydrin (2,2-dihydroxy-1,3-indanedione) reacts quantitatively with an amino acid to produce CO_2, an aldehyde, and Ruhemann's purple (see Figure 1.1) (23-25). The carbon in the evolved CO_2 is derived specifically from the carboxyl carbon of the amino acid (both carboxyl carbons with aspartic acid). This reaction has long been used to measure (either manometrically or photometrically) the quantity of amino acids in a sample.

Because the release of CO_2 is specific to amino acids, ninhydrin has recently been used for radiocarbon dating by liberating carbon (as CO_2) derived from the amino acid carboxyl carbons in ancient bone collagen

hydrolysate (26). The use of ninhydrin has advantages for radiocarbon dating collagen samples contaminated with organic, non-proteinaceous material of different radiocarbon age. In other studies (27, 28), ninhydrin was used to liberate ^{13}C enriched carboxyl carbons from leucine for labelling studies.

Although very little work has been done using ninhydrin to measure the natural $\delta^{13}C$ of carboxyl carbons ($\delta^{13}C_{COOH}$), the results so far suggest there are significant differences between $\delta^{13}C_{COOH}$ and the $\delta^{13}C$ of total carbon ($\delta^{13}C_{TOTAL}$). This difference is defined here as follows:

$$\Delta\delta^{13}C = \delta^{13}C_{COOH} - \delta^{13}C_{TOTAL}.$$

Abelson and Hoering (15) found the carboxyl carbons of most amino acids in protein derived from photosynthetic organisms (algae) had much more positive

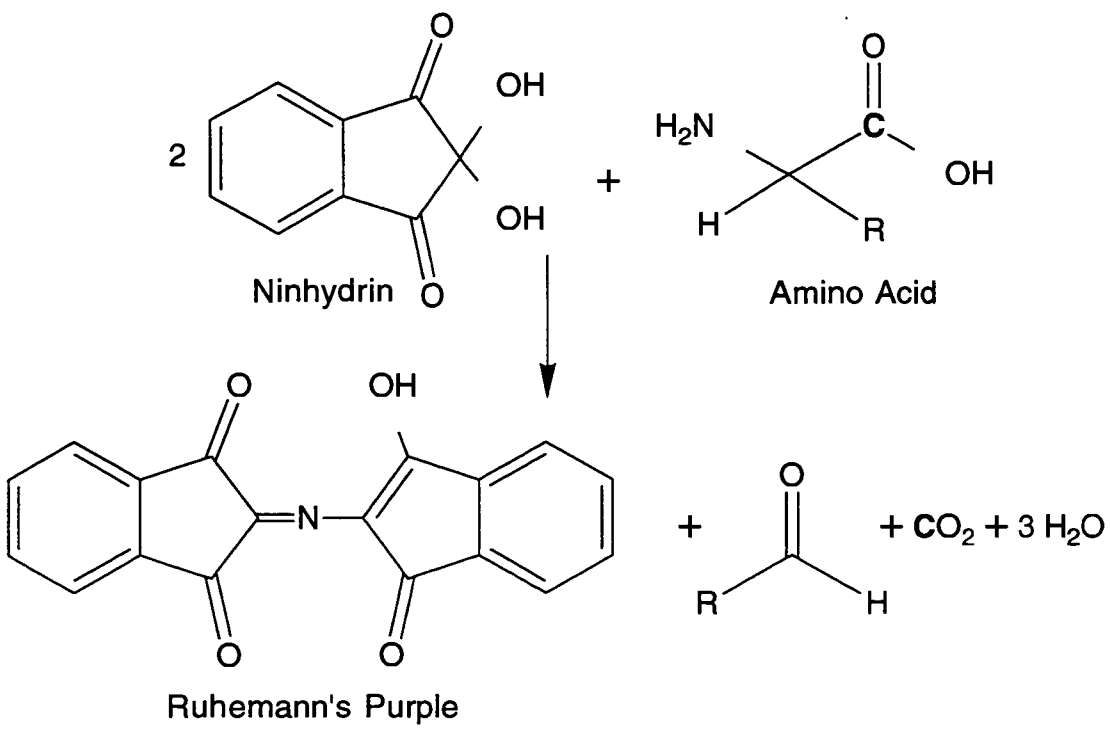


Figure 1.1:
Decarboxylation of an amino acid with ninhydrin.

$\delta^{13}C$ values than both the total protein ($\Delta\delta^{13}C$ = 6 to 14‰) and the individual amino acids ($\Delta\delta^{13}C$ = -3 to 24‰). The only published $\Delta\delta^{13}C$ values of fossil bone collagen appear incidentally in a ^{14}C dating study by Nelson (26). Values of approximately 7‰ were measured on only three samples. Bada *et al.* (29) have specifically used ninhydrin to measure the $\Delta\delta^{13}C$ of modern moose and wolf dentin (tooth) collagen. The average for the five samples measured was 3.1‰. The existence of $\Delta\delta^{13}C$ in bone collagen is not well understood. Bada *et al.* suggest $\Delta\delta^{13}C$ reflects the trophic level of the animal due to the mixing of plant, animal, and internally biosynthesized amino acids.

What then, is $\Delta\delta^{13}C$ measuring? Carboxyl carbons account for approximately 27% of the carbon in collagen. $\delta^{13}C_{TOTAL}$ is the weighted average of the $\delta^{13}C$ values of all constituent amino acids in collagen whereas $\delta^{13}C_{COOH}$ is the weighted average of the $\delta^{13}C$ values of all amino acids' carboxyl carbons. Because each amino acid has a different number of carbon atoms, these averages are not weighted equally. Amino acids with few carbon atoms are over-represented in $\delta^{13}C_{COOH}$ and amino acids with many carbon atoms are under-represented. For example, glycine accounts for 17% of the total carbon in collagen but 32% of the carboxyl carbons. This suggests $\Delta\delta^{13}C$ of collagen might be non-zero even if the $\Delta\delta^{13}C$ of every individual amino acid is zero because of the differences in $\delta^{13}C$ among amino acids in collagen. Of course, as found previously by Abelson and Hoering (15), individual amino acids do have non-zero $\Delta\delta^{13}C$ values and thus the $\Delta\delta^{13}C$ of collagen is most

likely not zero. $\Delta\delta^{13}C$ of collagen, therefore, reflects differences in both $\delta^{13}C_{COOH}$ and $\delta^{13}C_{TOTAL}$ of the amino acids in collagen. This composite measurement might provide information that otherwise would require tedious, preparative amino acid separations.

The limited studies so far suggest large variations in $\Delta\delta^{13}C$ among protein samples (15, 26, 29). If these variations are due solely to the samples themselves, they might provide insight into the diet or nutritional status of the organism. Unfortunately, all of the previous studies have not described either the method or the testing of the method (for isotopic fractionation) in sufficient detail to follow or assess the reproducibility of their methods.

1.5 *Scope of this Study*

The aim of this investigation was to measure the $\delta^{13}C_{TOTAL}$ and $\delta^{13}C_{COOH}$ of many modern and archaeological bone collagen samples from several species to provide a preliminary survey of the range of $\Delta\delta^{13}C$ in natural samples. Such an empirical study is necessary to see if significant differences between individuals can be observed, whether this measurement can be correlated with the diet or nutrition of the animal, and whether further study is warranted.

This required a method for measuring $\delta^{13}C_{COOH}$ that was adequately tested to have confidence in the results. Although the method developed for labelling studies (27, 28) has the advantages of smaller sample size and inherent convenience, it is troubled by residual CO_2 in the apparatus and requires immediate access to a Gas Chromatograph-Isotope Ratio Mass

Spectrometer (a recent and not readily available combination of instruments). Instead, Nelson's method (26) was developed further and tested for yield, contamination, and isotopic fractionation.

This thesis is therefore divided into two sections. The first describes the method for measuring $\Delta\delta^{13}C$, and the testing of the method to minimise isotopic fractionation during preparation of CO_2. The second describes the application of this method to several samples of modern and fossil bone collagen from a wide variety of animal species.

2 METHOD DEVELOPMENT

2.1 *Overview*

A few bone samples were chosen to develop and test the method. Collagen was extracted and purified from each. The collagen was hydrolyzed and the resulting amino acids subsequently reacted with ninhydrin to release the carboxyl carbons as CO_2. After purification, the $\delta^{13}C$ was measured on the CO_2 and compared with the $\delta^{13}C$ measured for the collagen as a whole. Although most of the above steps were relatively straightforward, stable isotope analysis requires a method free from isotopic fractionation or contamination. Measuring differences of less than 1‰ in $\delta^{13}C$ is not trivial. Isotopic fractionation can occur when a process occurs faster with one isotope than the other. Since isotopic fractionation is not likely if a process is 100% complete, the yield at each step in the method was monitored.

2.2 *Materials/Equipment*

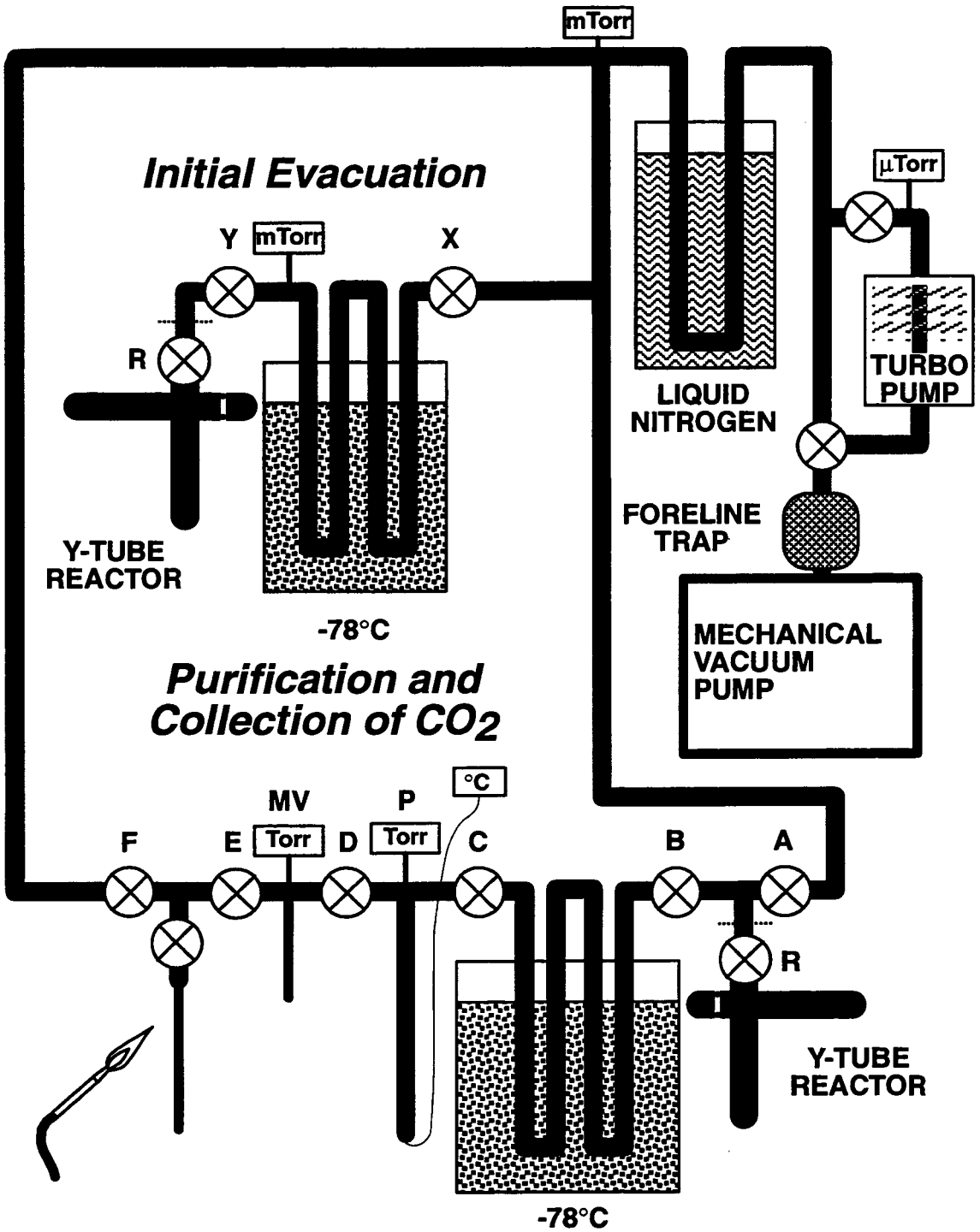
Tap water was purified by passing it through two ion-exchange filter cartridges (Cole-Palmer Ion-X-Changer, Universal) followed by a water purification system (Millipore Alpha-Q Reagent Grade Water System). Ninhydrin (analytical reagent), trisodium citrate (analytical reagent) and citric acid (anhydrous analytical reagent) were obtained from BDH. Phosphoric acid (85%, analytical reagent) was obtained from Mallinckrodt. The calcium carbonate used was a laboratory test material (99.9%, supplier unknown). Hydrochloric acid (37%, reagent grade, from Fisher) was diluted with purified water to 5.5 M. This solution was then glass distilled to collect the constant

boiling fraction for use in collagen hydrolysis. This acid was also used to prepare the more dilute solutions used in collagen extraction. The individual (L)-amino acids were obtained from Sigma (Sigma Grade). Several bone samples were used for method development and testing: CBB-1, T-53, and T-636 were modern samples; PRB was about 10,000 years old. These samples are described further in chapter 3 and Appendix C.

The vacuum line used to purify and collect the CO_2 evolved from the ninhydrin reaction was constructed of Pyrex glass and stainless steel tubing, Cajon Ultra-Torr connectors and Nupro valves (Figure 2.1). All o-rings were Viton. Apiezon N high vacuum grease was used where necessary. Pressures down to 2.0×10^{-6} Torr (at pump inlet) were achieved using a turbomolecular pump (Varian Turbo-V60) backed by a mechanical pump (Varian SD 40). Pressures were measured at various points along the vacuum line: a cold cathode gauge (MKS Instruments SensaVac Series 421) at the turbomolecular pump's inlet, thermal conductivity gauges (Varian type 0531) along the main vacuum manifold and in the trap used for initial evacuation of the reactors, and pressure sensors (Micro Switch (Honeywell) 140 PC pressure sensors) at traps P and MV. A thermocouple (Omega, Type T (copper-constantan)) was attached to the very bottom tip of trap P to monitor its temperature.

The vacuum line was protected from mechanical pump oil back-streaming with a foreline trap (MDC, stainless steel gauze) between pumps and a liquid nitrogen trap at the inlet of the turbomolecular pump. The mechanical pump was used for the initial pump-down. As the pressure dropped below 0.2 Torr, the turbomolecular pump was brought on-line to complete pump-down. All subsequent roughing of sample tubes and reactors were done slowly via the turbomolecular pump. These measures greatly reduced the risk of mechanical

Figure 2.1:
Schematic of the vacuum line. This system was used for initial evacuation of the reaction tube prior to ninhydrin reaction, and purification and collection of the CO_2 evolved after the ninhydrin reaction. Circles with X's represent valves. Small rectangles represent pressure gauges or thermocouple gauges. A dotted line indicates the point of attachment of reactor to vacuum line. A slush of 2-propanol and solid CO_2 was used in the -78°C traps.



pump oil back-streaming into the vacuum line and contaminating the sample gas.

The ninhydrin reaction was completed under vacuum in Y-shaped reactors constructed of Pyrex glass with a Viton o-ring sealed access port and a high vacuum valve (Kontes, PTFE with Viton seals). All glassware was baked for 4 hours at 550°C prior to use.

To monitor the purity of the evolved CO_2, a Hewlett Packard 5985 Gas Chromatograph/Mass Spectrometer (HP 5985 GC/MS) was used (electron impact, 70 eV) with a direct insertion probe for gas samples (samples emitted directly into mass spectrometer without passing through the gas chromatograph).

The $\delta^{13}C$ values were measured on a VG Isotech Prism IRMS at the Department of Oceanography, University of British Columbia. Solid collagen samples were combusted to CO_2 and purified in a Fisons NA 1500 C/N Analyser before entering the mass spectrometer. Gaseous samples were collected in sealed Pyrex sample tubes and introduced directly into the mass spectrometer by cracking the tube in a vacuum manifold attached to the mass spectrometer.

2.3 *General Method*

Collagen was extracted from both modern and ancient bones as follows using the method of Brown *et al.* (30). Each bone's surface was scraped with a scalpel to remove surface contaminants, and then drilled with a low speed hand drill. The resulting bone turnings (100-300 mg) were collected, weighed and then soaked in about 10 ml of 0.25 M HCl at room temperature for 2 days to demineralise the bone and remove acid soluble contaminants. After filtration

(Whatman, GF/A), the remaining residue was soaked overnight at 58°C in about 10 ml of 0.01 M HCl. This denatured the triple-helical collagen into single polypeptide chains, making them soluble. Unfortunately, these chains also begin to degrade during this soak. The solution was allowed to cool and then filtered (Whatman GF/A). The filtrate was then ultrafiltered (Amicon, Centriprep–30) to keep soluble fragments larger than 30,000 Daltons, and lyophilized (Savant Instruments Inc., Speedvac SS1). Depending on the condition of the original bone sample, the weight of lyophilized collagen was 1–15% of the weight of the bone drillings.

A portion of the lyophilized collagen (1-3 mg) was weighed and then hydrolyzed with 4 ml of constant boiling HCl (5.5 M) in a Pyrex hydrolysis tube purged with argon and evacuated following the standard method (31). Hydrolysis tubes were placed in a heating block at 110°C for approximately 24 hours.

The collagen hydrolysate was then transferred from the hydrolysis tube into one side of a Y-shaped glass reactor, frozen, and dried under vacuum over potassium hydroxide (Savant Instruments Inc., Speedvac SS1). When dry, 50 mg of citrate buffer powder (17.66 g tri-sodium citrate and 8.40 g citric acid ground with mortar and pestle to a fine powder) and 2.0 ml purified water were added to the side of the reactor not containing the hydrolysate. (When individual amino acids were reacted with ninhydrin, they were dissolved in purified water and pipetted into the reactor in place of the 2.0 ml water added above.) A glass boat was used to place 50 mg of ninhydrin in the dry side of the reactor. The access port and high vacuum valve (valve R) were closed and the reactor sonicated for 10 to 20 seconds to facilitate the dissolution of the citrate buffer and degas the solution.

The reactor was then attached to the vacuum line and slowly evacuated with valves R, X, and Y open for about 30 seconds (see top of Figure 2.1). To check for completion of evacuation, valves X and Y were closed, and the pressure in the trap was measured after gases condensable at –78°C (mostly water vapour) had frozen out. These two steps were repeated until the pressure in the trap, with valves X and Y closed, approached baseline. This procedure ensured air was thoroughly removed from the reactor without losing much water from the reactor. When evacuation was complete, valves R and Y were closed and the reactor removed from the vacuum line. The reactor was then tipped and shaken to mix the citrate buffered solution with the ninhydrin and hydrolysate or individual amino acid before being placed on a 105°C heating block. The reactor was shaken intermittently (approximately every 5 minutes) until removed from the heating block after 30 minutes.

The evolved CO_2 was then purified from water vapour and aldehyde byproducts as follows. Once the reactor was removed from the heating block after the ninhydrin reaction and allowed to cool, valves A and B were closed and the reactor attached to the vacuum line as shown on the bottom of Figure 2.1. Valves A through F were opened and the vacuum line was evacuated for 10 minutes. This removed air introduced when the reactor was attached to the vacuum line and removed volatile impurities remaining in the cold trap and trap P from previous samples.

Valves A and D were then closed and trap P was cooled in liquid nitrogen. Valve R was opened to allow the gases in the reactor to expand into the static vacuum between valves A and D. Water vapour and most of the aldehyde by-products were trapped in the –78°C cold trap. Those gases with appreciable vapour pressure at –78°C passed through the cold trap and

condensed in trap P. After 2 minutes, valves B, C, and R were closed.

While liquid nitrogen still cooled trap P, valve D was opened to allow gases not condensed at -196°C (i.e. N_2, O_2 etc.) to be pumped away. Very little of these non-condensable gases were present (<< 1 Torr in trap P). The pressure at the turbomolecular pump inlet returned to baseline within a few seconds of opening valve D. Valve D was closed after 5 minutes. Thus, trap P only contained gases that could be trapped at –196°C but not at –78°C.

Trap P was removed from the liquid nitrogen and warmed to room temperature after which the pressure of the gases in the trap was recorded. This pressure gave an indication of the quantity of gases present before the "warming trap transfer" which follows next. The very bottom tip of trap P was then cooled with liquid nitrogen in a half-filled Dewar (the bottom of trap P was just touching the surface of the liquid nitrogen) until the gases were frozen at the tip, and then a further 1.5 cm portion above the tip was cooled to –196°C. The CO_2 was thus frozen in a small area at the tip of trap P adjacent to the thermocouple. Valve E was then closed and trap MV cooled to –196°C.

Valve D was then opened and the half-filled Dewar around trap P lowered so that the surface of the liquid nitrogen was about 1 cm below the tip of the trap. Thus, the CO_2 distilled and was frozen in trap MV as the tip of trap P, surrounded by a cold Dewar, slowly warmed. CO_2 distilled before the impurities because of its much higher vapour pressure at such low temperatures (see section 2.6 for information about the impurities).

The temperature of the tip of trap P was monitored with a thermocouple gauge and the height of the Dewar adjusted if the temperature was rising too quickly or slowly. A typical warming curve is shown in Figure 2.2. Once transfer of CO_2 was complete (10 minutes), valve D was closed and both Dewars

(around traps P and MV) were lowered to allow both traps to reach room temperature.

The pressures in both traps were then recorded. (The pressure in trap MV indicated the quantity of clean CO_2, the pressure in trap P indicated impurities removed.) The actual yield of CO_2 was calculated from the pressure in MV (typically 20 to 150 Torr) by comparison to a calibration curve established by calibrating traps P and MV with CO_2 produced by reacting known quantities of $CaCO_3$ with 85% H_3PO_4 under vacuum. Quantities of material are often specified by the mass of the element of interest in isotope studies; this convention will be used here to refer to yields of CO_2. Sample size ranged from 50 to 450 μg C. Procedural blanks for the calibration and the ninhydrin reaction and subsequent purification and collection did not produce detectable levels of

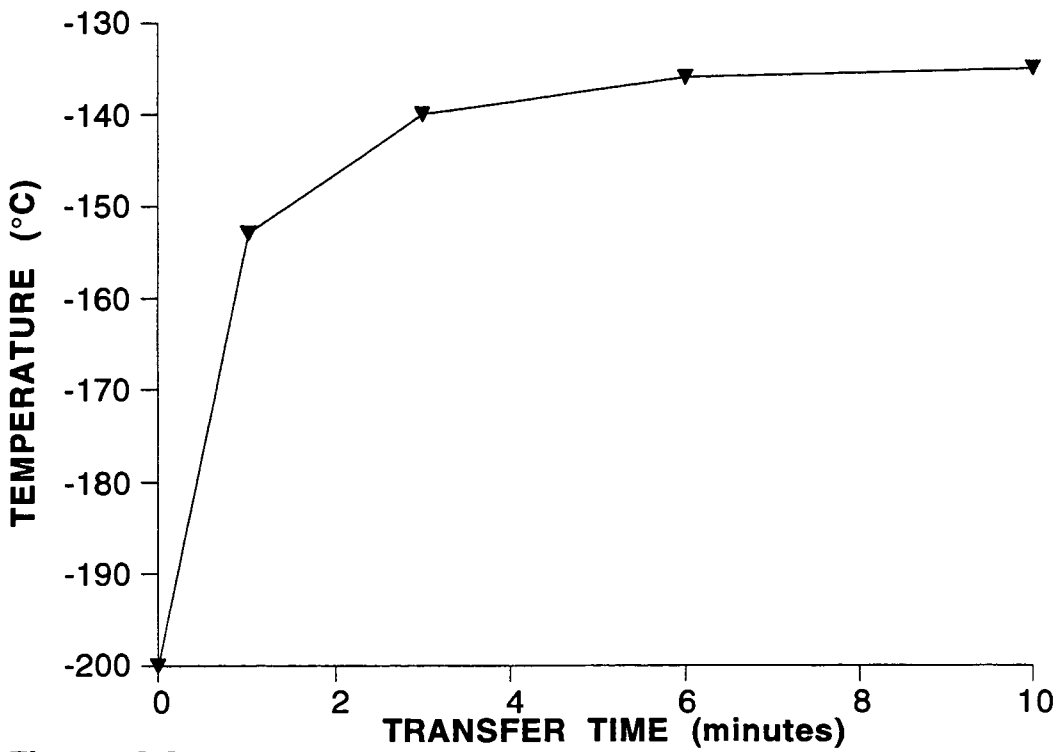


Figure 2.2:
Typical warming curve of trap P during the purification of CO_2. A thermocouple monitored the temperature at the very bottom of trap P.

CO_2 (less than 2 μg of C).

For collagen hydrolysates, the pressures of the retained impurities were undetectable in trap P (<1 Torr). For alanine, the pressure of the retained impurities was significant. To quantify, in comparison with the purified CO_2, collagen hydrolysate impurities would be equivalent to less than 1% of the pressure of the purified CO_2, whereas alanine-derived impurities would be equivalent to about 12% of the pressure of the purified CO_2 (at room temperature in the same volume).

After this purification procedure, valve F was closed and valve E opened to allow the CO_2 to enter the sample storage tube (6 mm Pyrex). This tube was then cooled with liquid nitrogen to freeze the CO_2. Valve F was then opened and the tube flame sealed under vacuum. The $\delta^{13}C$ of this gas sample ($\delta^{13}C_{COOH}$) and the related unhydrolyzed collagen ($\delta^{13}C_{TOTAL}$) were measured on an IRMS and compared.

2.4 *Method Testing*

The above method involved many steps in which isotopic fractionation could occur. However, isotopic fractionation cannot occur if a step is 100% complete. To establish the extent of completion and isotopic fractionation at each major step, overall CO_2 yield and $\delta^{13}C_{COOH}$ were monitored as parameters were varied.

Isotopic fractionation might occur if hydrolysis does not go to completion. This would be particularly apparent with the carboxyl carbons as they are directly involved in the hydrolysis of collagen. A kinetic isotope effect during

acid hydrolysis might be detectable in $\delta^{13}C_{COOH}$. Also, as reviewed by Hill (32), the rate of peptide bond cleavage depends on the amino acids involved in cleavage. Those amino acids released easily during hydrolysis might have different $\Delta\delta^{13}C$ values than those more resistant to hydrolysis. To examine these possibilities, collagen was hydrolyzed for different times and changes in both yield and $\delta^{13}C_{COOH}$ were monitored.

A small portion of some of the collagen hydrolysates used in chapter 3, as well hydrolysates of collagen extracted in previous studies were derivatized with ethyl chloroformate for gas chromatographic amino acid analysis (33). These samples ranged in age from modern to 48,000 years old and included many different species. Analysing a range of samples checked whether the process of collagen extraction and hydrolysis yielded samples with consistent amino acid compositions irrespective of diagenesis (the gradual destruction of ancient material). In this application, comparing ratios among amino acids was sufficient and therefore no quantitization was attempted. Analysis was completed on a Varian 3400 gas chromatograph with a DB-23 column (30 m x 0.32 mm, 0.25 μm film) as follows: Injector (split) temperature 250°C; detector (flame ionisation) temperature 300°C; temperature program: 100°C to 200°C at 20°C/minute, then held at 200°C for 20 minutes.

Isotopic fractionation could occur during decarboxylation in the ninhydrin reaction since bonds to the carboxyl carbons are being broken. Overall yield and $\delta^{13}C_{COOH}$ of the evolved CO_2 were measured at several time intervals to establish how long the ninhydrin reaction took to reach completion, and how much isotopic fractionation occurred if the reaction was not complete. Ninhydrin was reacted with both proline and PRB collagen hydrolysate for 5, 10, 20, 30

and 60 minutes at 105°C in separate reactors. The first series determined when the reaction was complete for an individual amino acid. Isotopic fractionation prior to completion with proline would be due solely to the kinetic isotope effect in the ninhydrin reaction. The second series additionally measured fractionation of the resulting CO_2 due to the different amino acids in collagen hydrolysate (with different $\delta^{13}C_{COOH}$ values) reacting with ninhydrin at different rates.

The purification of evolved CO_2 involved two major transfer steps: the transfer through the -78°C cold trap from the reactor to trap P, and the "warming trap transfer". Overall yield and $\delta^{13}C_{COOH}$ were measured at intervals during both transfers.

The whole procedure from hydrolyzing the collagen to collecting the CO_2 involved many steps. Although most of the steps were studied to determine their individual influences on $\delta^{13}C_{COOH}$, establishing the overall reproducibility of the method was also desired. The reproducibility of yield and $\delta^{13}C_{COOH}$ using this method were tested several ways. The influence of sample size on overall yield and $\delta^{13}C_{COOH}$ was established for a collagen hydrolysate: samples of T-53 collagen between 0.5 and 3.2 mg were hydrolyzed individually and reacted. Replicate samples of proline and alanine as well as several collagen samples were measured for $\delta^{13}C_{COOH}$. The reproducibility of the method including and excluding hydrolysis could thus be established.

2.5 *Results of Method Testing*

Of all the steps tested, isotopic fractionation was largest during the hydrolysis of collagen. The amino acids released early in hydrolysis were much lighter in their carboxyl carbons than the rest. As can be seen in Figures 2.3 and 2.4, there were differences among different collagen samples. Experiments with PRB collagen (data not shown) indicated that yields after 73 hours of hydrolysis were no better than after 25 hours. If hydrolysis is continued too long, some amino acids start to degrade before others have completely

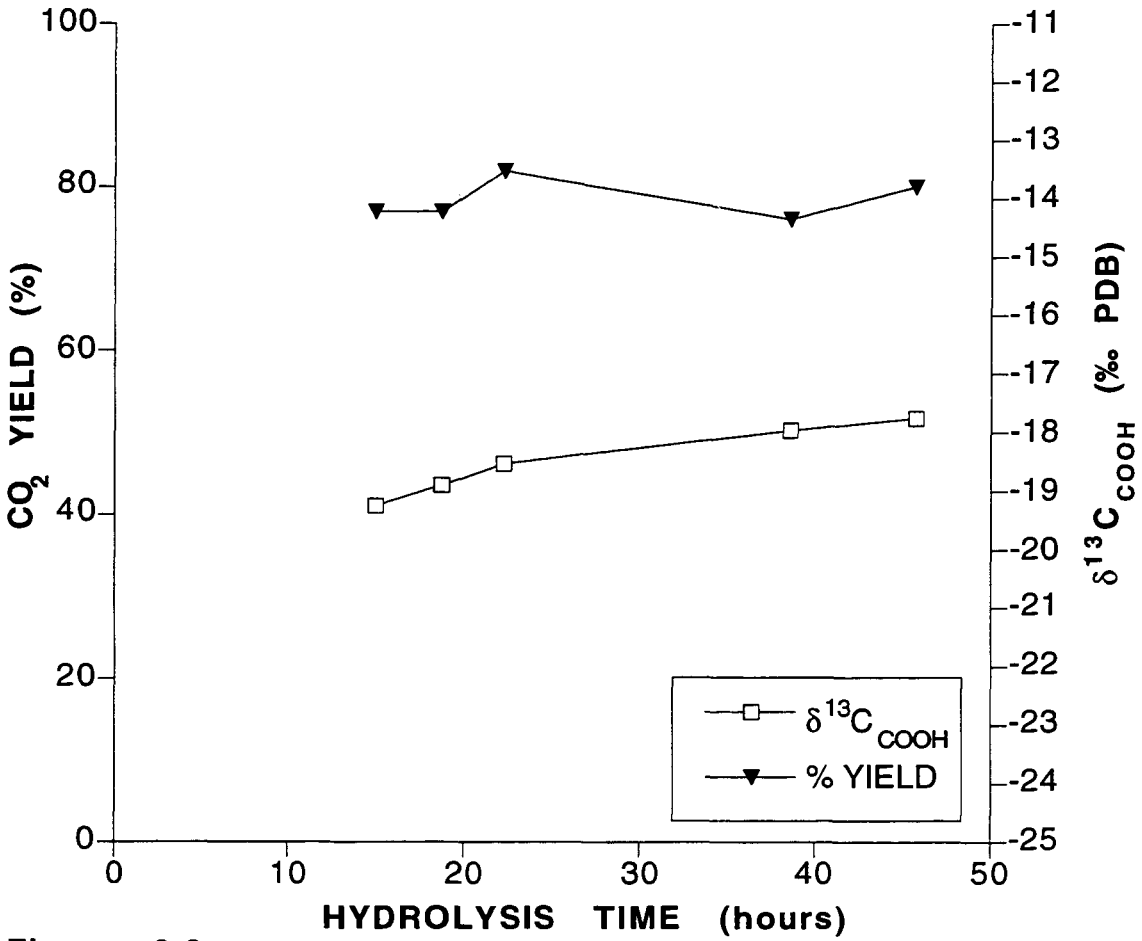


Figure 2.3:
Isotopic fractionation during acid hydrolysis of CBB-1 collagen. For comparison, the scales of this graph are the same as Figure 2.4. Each point represents one measurement.

hydrolyzed (32). A compromise of hydrolysis for 24 hours was used for all samples.

The amino acid composition of the hydrolyzed collagen extracts were similar for bone collagen samples of different species and archaeological age (Figure 2.5, Table 2.1). All collagen samples showed consistent compositions for the most abundant amino acids in collagen except for hydroxyproline, which tailed badly on the DB-23 column. Amino acid analysis confirmed that the method of bone collagen extraction yielded a fairly consistent protein fraction

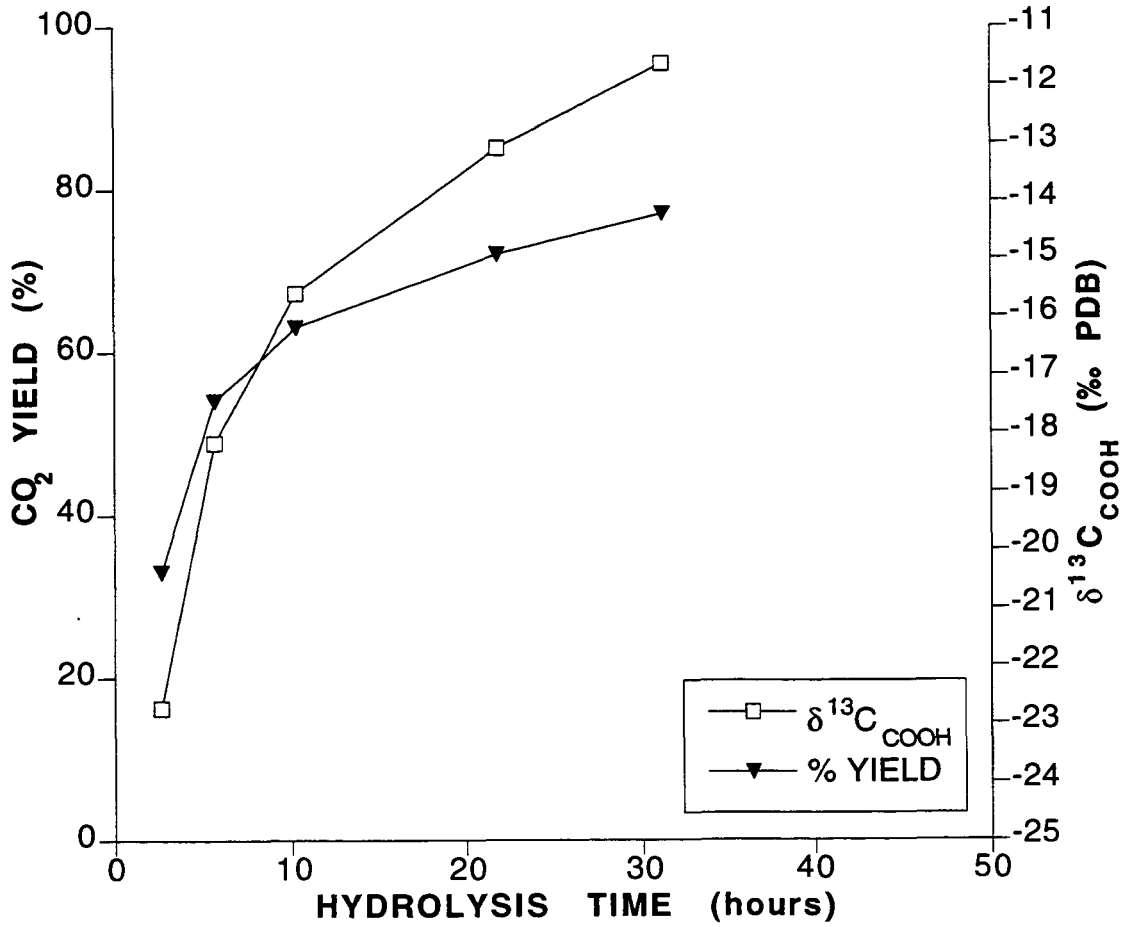


Figure 2.4:
Isotopic fractionation during acid hydrolysis of T-636 collagen. For comparison, the scales of this graph are the same as Figure 2.3. Each point represents one measurement.

irrespective of species or archaeological age (and thus possible diagenesis) of sample.

Table 2.1:
Information on collagen samples used for amino acid analysis shown in Figure 2.5. Ages are approximate years before present (B.P.) based on radiocarbon dating or sample site context.

Sample ID	Species	Age (years B.P.)
Bower Bird-4	wallaby	modern
Cooinda-3	water buffalo	modern
PRB	bison	10,000
Test-1	bison	10,000
Test-2	domestic pig	modern
Test-3	human	4,000
Test-4	cave bear	48,000
Test-5	bison	9,500
Test-6	human	1,700
Test-7	coyote	modern
Test-8	harp seal	3,000
Test-9	human	2,000
Test-10	human	4,000
Test-11	fox	modern

For both proline and PRB collagen hydrolysate, CO_2 evolution was complete after 30 minutes of ninhydrin reaction at 105°C (Figures 2.6 and 2.7). The $\delta^{13}C$ of evolved CO_2 approached steady values (within experimental error) at about 30 minutes. Isotopic fractionation was evident in the reaction of ninhydrin with proline. The CO_2 evolved after 5 minutes of reaction was over 1‰ lighter than that after 30 minutes. This suggests that the decarboxylation of a ^{12}C carboxyl carbon was slightly faster than a ^{13}C carbon. For PRB hydrolysate, the CO_2 evolved after 5 minutes of reaction was over 5‰ lighter

than the CO_2 evolved after 30 minutes. This larger isotopic fractionation compared to the fractionation for proline probably resulted from the different reaction rates of amino acids with ninhydrin. As measured previously (23), some amino acids react with ninhydrin faster than others and would thus bias the $\delta^{13}C_{COOH}$ of CO_2 evolved early in the reaction. Allowing the reaction to proceed for 30 minutes prevents this bias in $\delta^{13}C_{COOH}$.

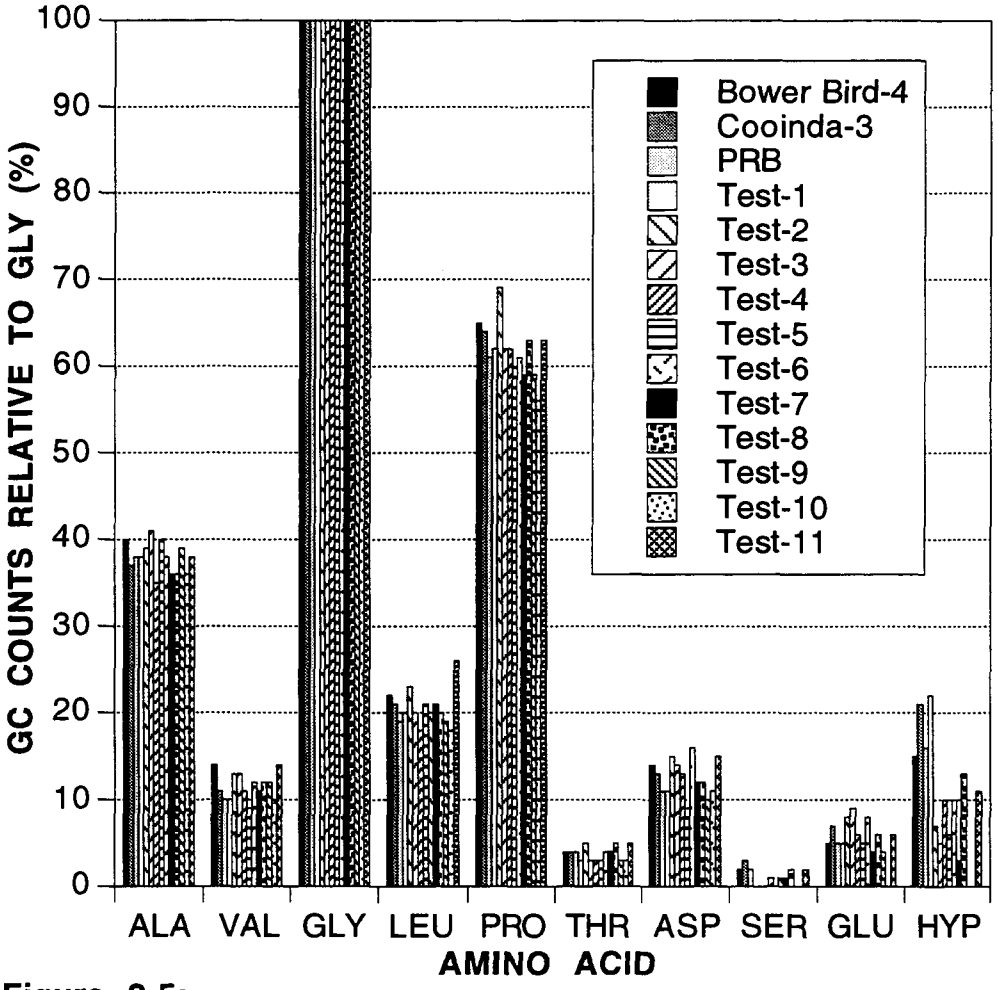


Figure 2.5:
Amino acid analysis of bone collagen hydrolysates. Samples are described in Table 2.1. Only a few of the major amino acids in collagen are shown. Bar heights are relative to GLY and do not represent percent composition.

The transfer between the reactor and trap P was rapid and complete in two minutes (Figure 2.8). Transferring for longer only filled up the trap with water. The isotopic fractionation during this step was minimal. Tests with different quantities of CO_2 indicated that 2 minutes was appropriate for the range of sample sizes encountered in this procedure.

The "warming trap transfer" was continued for 10 minutes. This was performed for longer than actually necessary because a transfer of this type could easily result in isotopic fractionation if not complete (Figure 2.9). In this

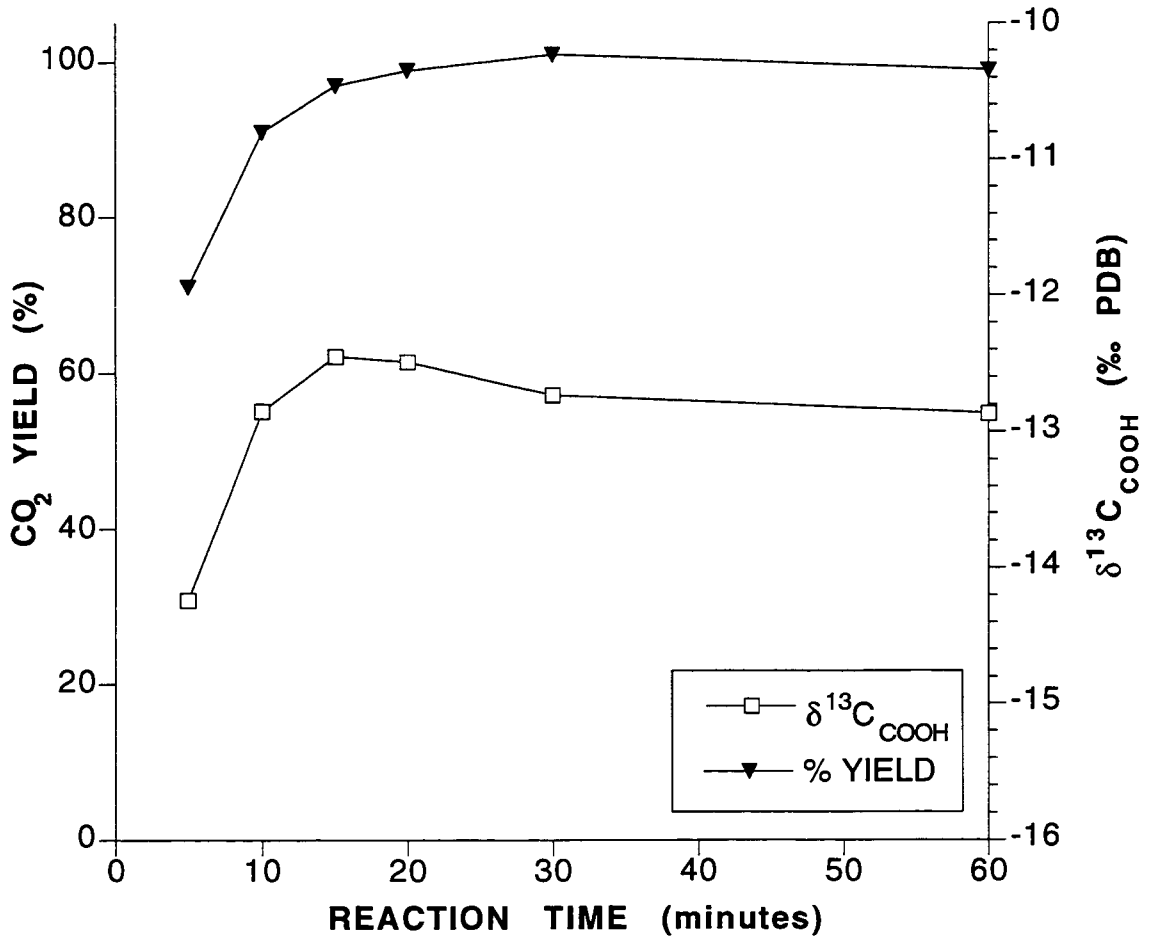


Figure 2.6:
Isotopic fractionation during ninhydrin reaction with proline. For comparison, the $\delta^{13}C_{COOH}$ scale of this figure is the same as Figures 2.7-2.10. Each point represents one measurement.

case however, very minimal isotopic fractionation occurred during the transfer.

The $\delta^{13}C_{COOH}$ and overall yield of CO_2 appeared independent of sample size over the range measured (see Figure 2.10). The overall yield of evolved CO_2 was reproducible. For individual amino acids, yields were quantitative and standard deviations of less than 1% were typical (n=5). For collagen samples, standard deviations were typically less than 3% (n=5). The larger errors associated with the collagen samples most likely arose from the greater manipulation required for these samples. Yields for different collagen

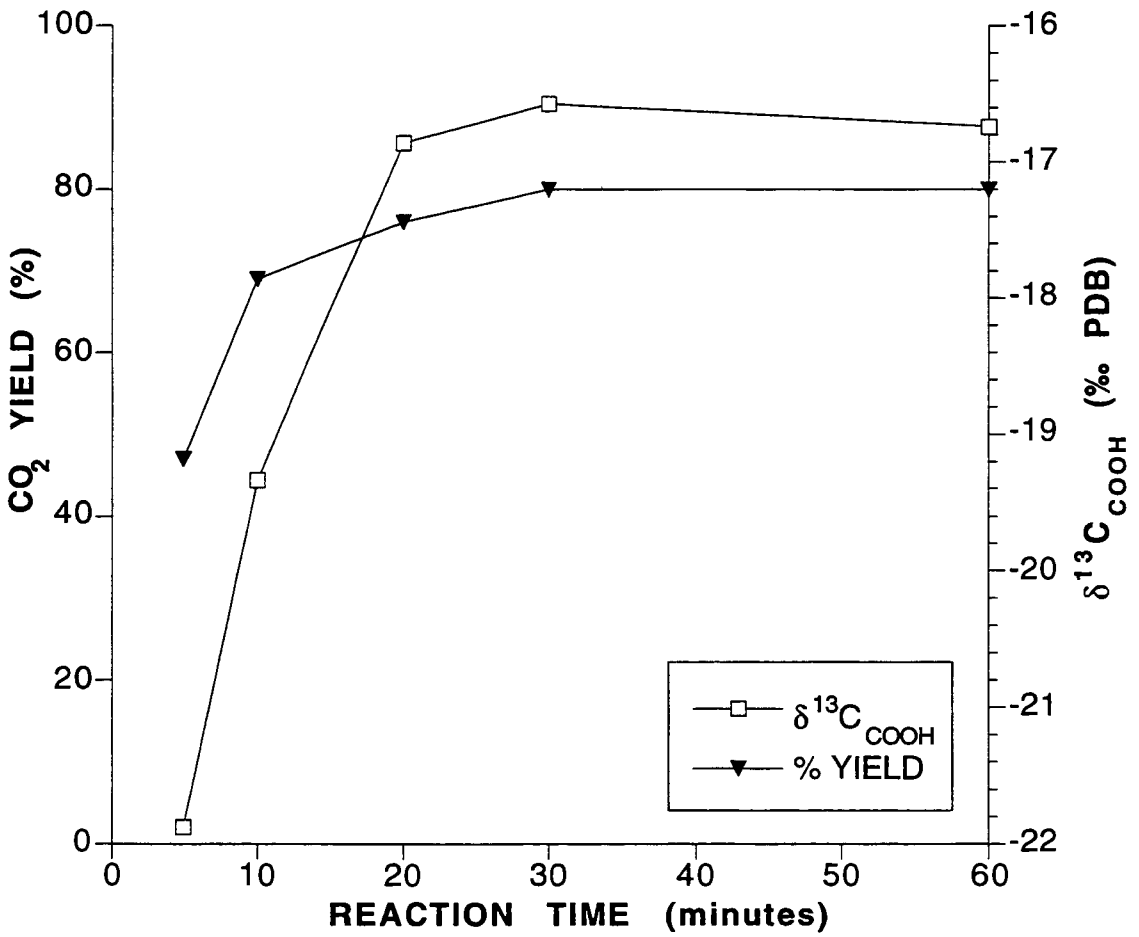


Figure 2.7:
Isotopic fractionation during ninhydrin reaction with PRB collagen hydrolysate. For comparison, the $\delta^{13}C_{COOH}$ scale of this figure is the same as Figures 2.6-2.10. Each point represents one measurement.

samples ranged from 43 to 84% but were concentrated around 75% (see chapter 3).

For individual amino acids, standard deviations of less than 0.20‰ in $\delta^{13}C_{COOH}$ were typical (n=4). This was a definite improvement over the standard deviation of over 1‰ reported previously (15). For collagen samples, standard deviations were often less that 0.50‰ (n=5) (see chapter 3 for more information).

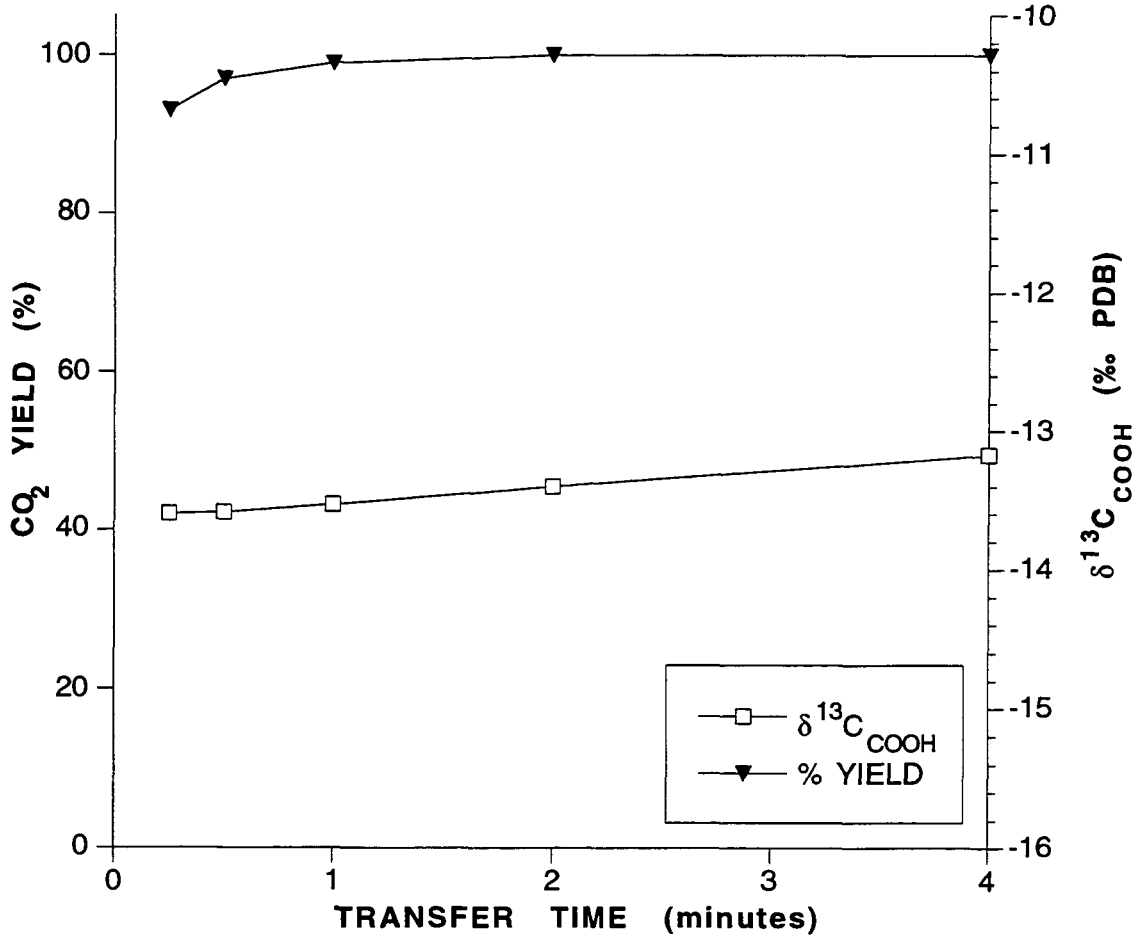


Figure 2.8:
Isotopic fractionation during the collection of CO_2 (from proline) from the reaction tube. Each point represents one measurement.
For comparison, the $\delta^{13}C_{COOH}$ scale of this figure is the same as Figures 2.6-2.10.

2.6 *Discussion of Method*

The Brown *et al.* method of collagen extraction puts very stringent conditions on what is retained. Both acid soluble and acid insoluble contaminants are removed. Only substances, like collagen, that are insoluble in the stronger acid but soluble, with heating, in the weaker acid are kept. Prior to ultrafiltration, the soluble portion contains collagen fragments of various molecular weights. Keeping the molecular weight fraction larger than 30,000

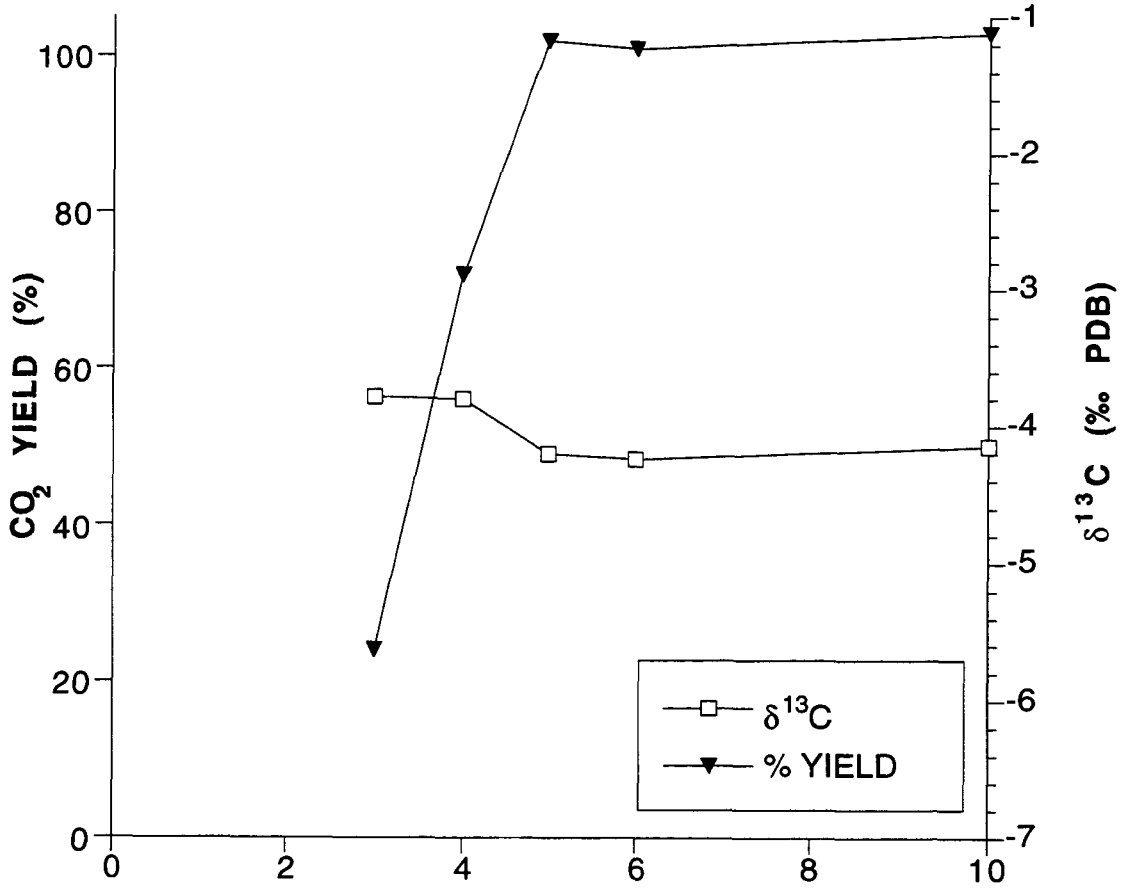


Figure 2.9:
Isotopic fractionation during the warming trap transfer. CO_2 was derived from $CaCO_3$ ($\delta^{13}C$=-4.1‰) by reacting with 85% H_3PO_4 under vacuum. For comparison, the $\delta^{13}C_{COOH}$ scale of this figure is the same as Figures 2.6-2.10 and the time scale is the same as Figure 2.2. Each point represents one measurement.

Daltons removes low molecular weight contaminants and retains the least-degraded collagen chains. By convention, this fraction will still be called *collagen* in this thesis although it is not collagen in its intact, native form. This method has been used successfully for several years for both radiocarbon dating and stable isotope analysis.

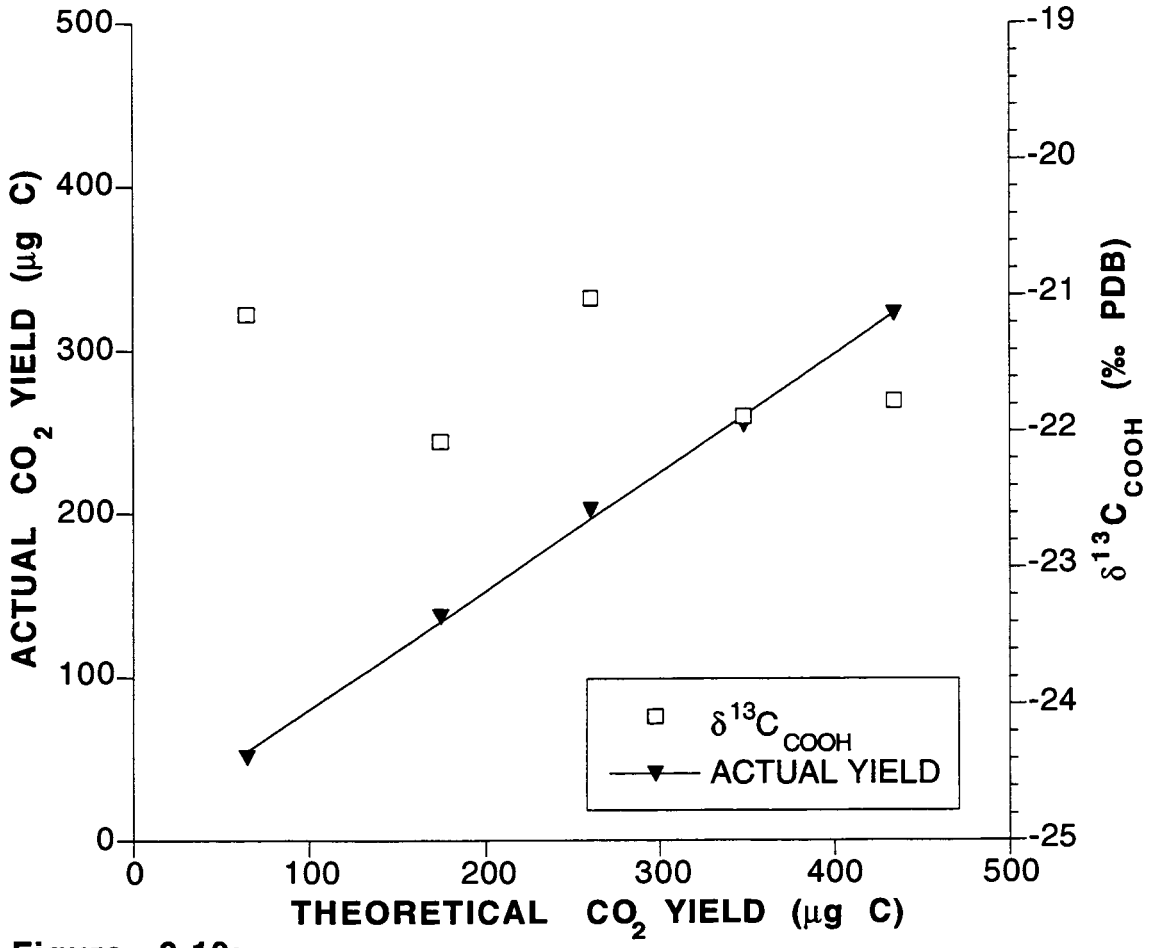


Figure 2.10:

Effect of collagen sample size on yield and $\delta^{13}C_{COOH}$. Each point represents one measurement. For comparison, the $\delta^{13}C_{COOH}$ scale of this figure is the same as Figures 2.6-2.9.
Actual yield=0.79 + 0.72*Theoretical yield (R^2>0.99).

The isotopic fractionation during the acid hydrolysis of collagen was significant. Silfer *et al.* (34), looking at total carbon and nitrogen isotope ratios (not carboxyl carbons), have completed experiments on the hydrolysis of the dipeptide glycylglycine. They found the isotopic fractionation of nitrogen followed theoretical expectations; that is, the glycine released early in hydrolysis had a nitrogen isotope ratio lighter than later in hydrolysis. In carbon however, the isotopic ratio became lighter as the hydrolysis proceeded. This was contrary to both theoretical expectations and the results found in this thesis for the carboxyl carbons. They could not explain this apparent anomaly.

Isotopic fractionation during hydrolysis is not very well understood. Since hydrolysis apparently plays a fundamental role in diagenesis (34), an understanding of the isotopic fractionation during this process is necessary for all analyses of ancient bone and is not limited to methods that use acid hydrolysis in isotope analysis methodologies. The isotopic fractionation observed during collagen hydrolysis in this thesis might provide an indication of the isotopic fractionation that occurs in the carboxyl carbons during the diagenesis of ancient collagen.

The theoretical yield of CO_2 produced during the reaction of collagen hydrolysate with ninhydrin was calculated from the amino acid composition of human bone collagen (9) to be 137.4 μg C/mg collagen (503 μg CO_2/mg collagen). The theoretical yield assumed i) the amino acid composition of the extracted collagen was independent of species, ii) water was not present in the lyophilized sample, and iii) the sample as prepared contained only protein. The first assumption is realistic because there is minimal collagen variation among species (9). The second assumption is not; collagen binds about 20% water by weight at 50% relative humidity and several percent under vacuum (35). The

extracted collagen was stored in a vacuum desiccator after lyophilization to reduce the absorption of water. The third assumption is not completely accurate. Bone collagen is a glycoprotein; however, very few of its hydroxylysine residues are glycosylated (and only 0.9% of the residues in bone collagen are hydroxylysine). When glycosylated, the carbohydrate units are no longer than a disaccharide (9). Thus, glycosylation should not contribute much to the mass of extracted collagen (less than 1%). The percent carbon, percent nitrogen, and C/N ratio of the extract would be particularly sensitive to contamination. Due to its high glycine content, collagen has a C/N ratio (by weight) of about 2.7, much lower than most proteins (about 4.3). A significant contamination of non-collagenous protein would change this ratio noticeably. The degradation of collagen during diagenesis of ancient samples might contribute non-proteinaceous material to the collagen fraction. Comparing CO_2 yields among modern and ancient samples might indicate if such diagenesis products were present in the collagen fractions. A recent study (36) has suggested that lipids should also not contribute much mass to the extracted collagen fraction of both ancient and modern samples. Since lipids contain no nitrogen, any contamination by lipids (or any other non-proteinaceous, organic substance) would also dramatically alter the C/N ratio. Finally, because lyophilized collagen is not very dense (approximately 0.01 g/cm^3), and is sensitive to static, errors could be made during weighing. Weighing lyophilized collagen on aluminium foil or directly into glassware was more reliable than using weighing paper.

Removal of the water vapour and aldehyde byproducts from the desired CO_2 evolved was necessary to measure $\delta^{13}C_{COOH}$ on very clean CO_2 (see

Appendix B for a discussion on the importance of gas purity). Traps cooled to –78°C (dry ice/2-propanol) were effective in removing water vapour and most aldehydes. Unfortunately, a very small quantity of acetaldehyde (produced in the reaction of alanine and aspartic acid with ninhydrin) was able to pass through the traps at this temperature. Because acetaldehyde is isobaric with CO_2 (m/z 44), contamination with this species could dramatically change the $\delta^{13}C_{COOH}$ measured. A 1% contamination of acetaldehyde could theoretically change $\delta^{13}C$ by +10‰! The quantity of acetaldehyde in a CO_2 sample was approximated by observing the m/z 29 peak (the base peak of acetaldehyde) in the mass spectrum (using the HP 5985 GC/MS). Contamination of several percent was observed when –78°C traps were used. Of the several traps tested, the coldest and most effective was a slush of methylcyclohexane (–126°C), but even this trap proved ineffective at removing all detectable traces of acetaldehyde. The use of solid sodium bisulfite to absorb the acetaldehyde also proved ineffective in this situation although it has been used previously (37).

Mass spectrometry also revealed that the CO_2 gas samples were contaminated with a small quantity of carbon disulfide. This contaminant was traced to the purified water used in the ninhydrin reaction. Because fragmentation of carbon disulfide in the isotope ratio mass spectrometer leads to $[CS]^+$ (m/z 44), removal of this contaminant was also necessary.

A trap at less than -126°C was desired to obtain a superior, nonspecific purification. Because the use of very low temperature slushes involves additional cost, attention, and safety considerations, the "warming trap transfer" described above was developed to purify the CO_2 after passing through the

–78°C traps. Mass spectrometry with both the HP 5985 GC/MS and the VG Isotech Prism IRMS has indicated that this method of purification was effective in removing both acetaldehyde and carbon disulfide for the $\delta^{13}C_{COOH}$ measurement of collagen samples. Many of the CO_2 samples prepared from bones described in chapter 3 were checked for contamination on the VG Isotech Prism IRMS at the same time $\delta^{13}C_{COOH}$ was measured and no contamination was detected.

3 APPLICATION OF METHOD

3.1 *Choice of Samples*

Fifty five samples from many species, ecosystems, trophic levels, and time periods were analysed to survey the magnitude and range of $\Delta\delta^{13}C$. They were not collected especially for this study but were chosen from samples already available from previous studies. Of these samples,13 were modern. Cooinda-3 and Bower Bird-4 were collected in Northern Australia. CBB-1 was obtained locally (Canada Safeway Ltd). The remaining modern samples were obtained from the Zooarchaeology Collection of the Department of Archaeology at Simon Fraser University and were collected throughout British Columbia with the exception of 10068 (Kentucky, USA). The 42 remaining samples ranged in age between 100 and 48,000 years before present. Samples PB-95.01 through PB-95.10 originated from the Bluefish Cave site in the Northern Yukon. The European cave bear (*Ursus spelaeus*, extinct) samples originated from the Divje Babe archaeological site in Northwestern Slovenia. They were chosen for this study as Nelson *et al.* (38) have shown a biological age effect in $\delta^{13}C_{TOTAL}$. DeRt-2-2 and DgRr-1-1 originated from South Pender Island and Crescent Beach, Boundary Bay, British Columbia respectively. PRB was collected from the Peace River, British Columbia. The Naukan humans originated from a Siberian coastal community along the Bering Strait (39). The Oxford humans all originated in the general area of Oxford, United Kingdom (40). The PAR humans originated from a mortuary cave in the Sierra de San Francisco, Baja California Sur, Mexico (41). The Viking samples were collected in Greenland (42). Additional sample information is summarised in Appendix C.

3.2 *Collagen Extraction and Overall CO_2 Yield*

Table 3.1 describes the samples measured and the yields obtained. Extraction yield is the quantity of collagen extracted as a percentage of the total bone sample weight. For comparison, fresh, modern bone contains about 22% collagen by weight (7). With the exception of Oxford-B218 and Oxford-B387, the collagen extracts were obtained in satisfactory yields for all samples and were not considered problematic.

Overall yield is the quantity of CO_2 collected after ninhydrin reaction with the collagen hydrolysate and purification as a percentage of the theoretical yield calculated from the weight of collagen hydrolyzed. The samples gave reasonably consistent overall yields, which suggested that the collagen extraction procedure did indeed provide a consistent protein fraction irrespective of archaeological age and diagenesis for non-problematic bone samples. The samples found to be problematic during collagen extraction (Oxford-B218 and Oxford-B387) also yielded significantly lower CO_2. With the exception of these two samples, there was no correlation between extraction yield and overall yield.

Table 3.1:
Sample information and yields obtained during sample preparation. Age is approximate years before present (B.P.) based on radiocarbon dating or sample site context. Extraction yield is the quantity of collagen extracted as a percentage of the total sample weight. Overall yield is the quantity of CO_2 collected after ninhydrin reaction with the collagen hydrolysate and purification as a percentage of the theoretical yield calculated from the weight of collagen hydrolyzed. All values are individual measurements unless otherwise noted with ±SD (n replicates). Replicates are individually hydrolyzed and reacted from a common collagen extract. Yields marked with an asterisk (*) might be inaccurate due to weighing the collagen on weighing paper before hydrolysis.

Sample ID	Species/Info	Age (Years B.P.)	Yields (%) Extraction	Overall
		Cats		
T-53	mountain lion	modern	14.6	71
10027	lynx	modern	11.7	78
		Sea Mammals		
T-636	California sea lion	modern	12.8	79
10114	harbour seal	modern	9.8	81
Grey Whale	grey whale	modern	8.9	79
		Modern Bears		
T-33	grizzly bear (adult)	modern	12.9	70
T-313	black bear (adult)	modern	14.7	79
10048	black bear (immature)	modern	15.3	74
		European Cave Bears		
B1:188	neonate/fetus	48,000	3.7	80
B1:193	neonate/fetus	48,000	5.1	106 *
B2:173-2	cub	48,000	4.2	82
B2:182	cub	48,000	5.8	110 *
B3:181-2	yearling	48,000	4.1	77
B3:188-1	yearling	48,000	6.0	84
A1	juvenile	48,000	5.6	83
A3	juvenile	48,000	7.9	92 *
B5:188-1	sub adult	48,000	5.3	80
B5:188-2	sub adult	48,000	6.8	84 ±3 (5)
B7:183	adult	48,000	7.1	84
B8:181	adult	48,000	6.8	84

		Ungulates		
Cooinda-3	water buffalo	modern	10.9	84
DeRt-2-2	deer	100	4.3	79
DgRr-1-1	deer	100	2.0	74
PB-95.01	wapiti	11,000	8.8	100 *
PB-95.06	wapiti	>10,000	11.6	78 *
PB-95.09	wapiti	>10,000	10.7	77
PB-95.02	caribou	13,000	7.0	66 *
PB-95.10	caribou	14,000	6.2	76 ±2 (5)
PB-95.03	bison	>10,000	7.9	85 *
PB-95.04	bison	32,000	9.3	93 *
PB-95.07	bison	24,000	13.6	81
PRB	bison	10,000	13.1	80 ±2 (5)
PB-95.08	moose	12,000	8.3	83
CBB-1	domestic cow	modern	12.0	78 ±2 (5)
		Humans		
Naukan-3	female	100	7.2	70
Naukan-5	female	100	6.9	74
Naukan-6	male	100	8.9	72
Naukan-24	female	100	5.7	66
Naukan-27	male	100	7.5	70
Oxford-B2		600	2.1	64
Oxford-B178		1,400	12.3	80
Oxford-B216		1,700	1.5	68
Oxford-B218		1,700	0.9	47
Oxford-B387		1,500	0.9	43
PAR-4		3,000	3.7	68
PAR-5		3,000	5.9	77
PAR-16		3,000	6.8	78
Viking-13		500	7.8	77
Viking-14		500	3.5	71
Viking-15		500	10.4	74
Viking-16		900	4.9	76
		Other		
10062	snowshoe hare	modern	5.3	73
10068	Eastern cottontail	modern	5.9	74
Bower Bird-4	wallaby	modern	10.3	72
PB-95.05	mammoth	23,000	7.2	81 *

Elemental analyses (C and N) were completed on the Oxford samples and others to find the source of the low overall yields with the two problematic samples. Although they all had C/N ratios consistent with collagen (i.e., around 2.7), the percent carbon and nitrogen were lower than would be expected for theoretically pure collagen, especially the problematic samples (see Table 3.2).

For the samples tested, overall yield was correlated with both percent carbon (R^2= 0.89) and percent nitrogen (R^2= 0.92). Since the C/N ratio remained collagen-like, the collagen extracts were probably contaminated with inorganic material. The identity of this inorganic material has not been established; but, it was most likely water in the lyophilized collagen. Although inorganic material would not have affected the measurement of either $\delta^{13}C_{TOTAL}$ or $\delta^{13}C_{COOH}$, it did lower the overall yields of CO_2. By extrapolation, the overall yield of an extract containing purely collagen should be around 87%. As the steps in the procedure after hydrolysis are quantitative for individual amino acids, losses with collagen samples likely occurred prior to these steps. Thus, total losses due to sample handling, incomplete hydrolysis or destruction during hydrolysis were about 13%.

Table 3.2:
Elemental analysis of collagen extracts (by weight). Data for theoretically "Pure collagen" were calculated from the amino acid composition of human bone collagen (9). (n/r - not recorded)

Sample ID	C/N Ratio	% Carbon	% Nitrogen
Pure Collagen	2.7	50	18
Oxford-B2	2.7	36	13
Oxford-B178	2.7	43	15
Oxford-B216	2.8	38	13
Oxford-B218	2.8	29	10
Oxford-B387	2.7	24	8
PAR-4	2.9	45	15
PAR-5	2.8	45	16
PAR-16	2.9	45	15
Naukan-3	2.8	n/r	n/r
Naukan-5	2.8	n/r	n/r
Naukan-6	2.8	n/r	n/r
Naukan-24	2.8	n/r	n/r
Naukan-27	2.7	n/r	n/r
DgRr-1-1	2.7	43	16
Grey Whale	2.7	44	17
T-53	2.7	44	16
T-313	2.7	45	17
T-636	2.6	44	17
10027	2.8	46	16
10062	2.8	44	16
PB-95.01	2.7	n/r	n/r
PB-95.02	2.7	n/r	n/r
PB-95.03	2.7	n/r	n/r
PB-95.04	2.7	n/r	n/r
PB-95.05	2.7	n/r	n/r
PB-95.06	2.7	n/r	n/r
PB-95.07	2.6	45	17
PB-95.08	2.7	n/r	n/r
PB-95.09	2.7	45	17
PB-95.10	2.7	44	16

3.3 *Isotopic Analysis*

The isotopic data obtained for these samples are summarised in Table 3.3. This table provides the largest data set ever reported for the $\Delta\delta^{13}C$ of bone collagen. $\delta^{13}C_{TOTAL}$ and $\delta^{13}C_{COOH}$ are plotted against one another in Figure 3.1, and a summary of $\Delta\delta^{13}C$ values is shown in Figure 3.2. For the samples measured, $\Delta\delta^{13}C$ ranged between -0.9 and 4.0‰. Although very few data have previously been reported for bone collagen, the magnitude and range of $\Delta\delta^{13}C$

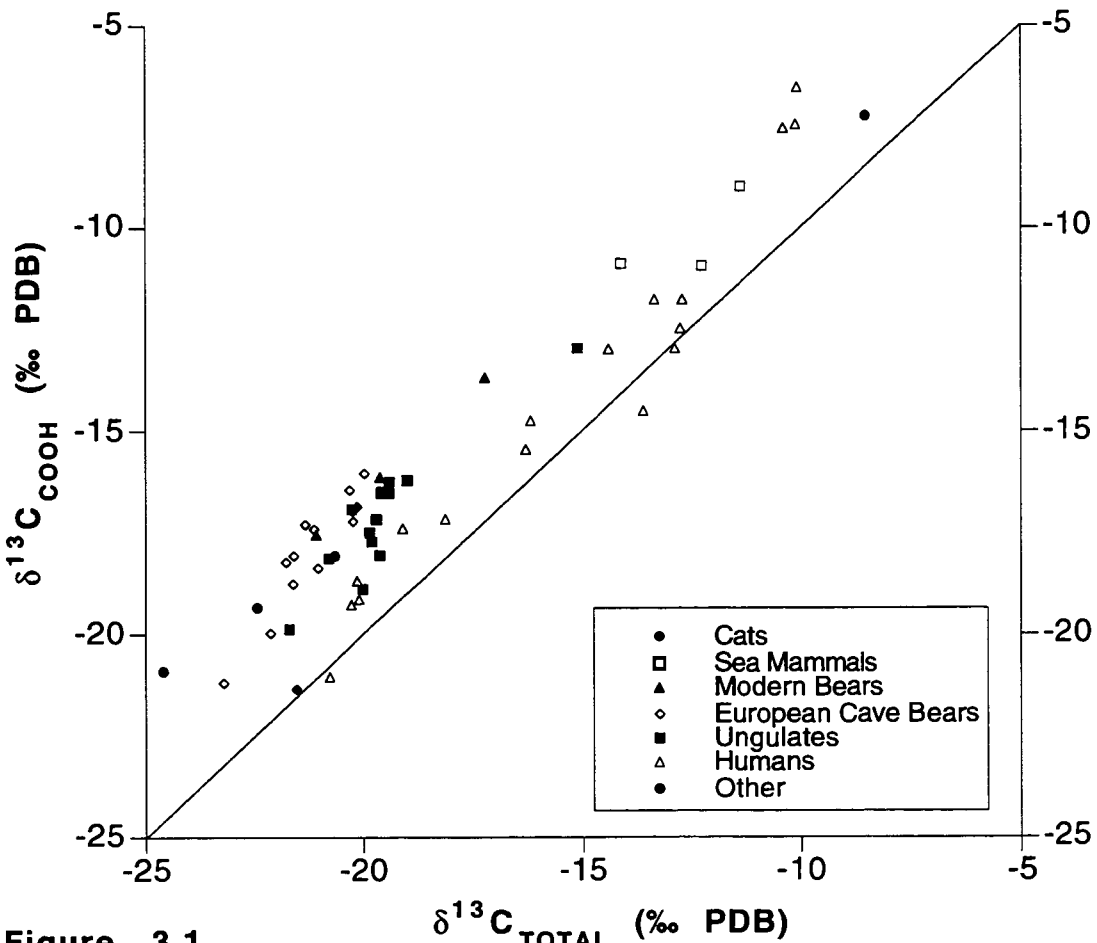


Figure 3.1

Relationship between $\delta^{13}C_{TOTAL}$ and $\delta^{13}C_{COOH}$ for bone collagen samples. Diagonal line indicates $\delta^{13}C_{TOTAL} = \delta^{13}C_{COOH}$.

were smaller than expected from previous studies. Nelson (26) found much larger $\Delta\delta^{13}C$ values for the three samples he measured. In his study, the $\Delta\delta^{13}C$ of PRB collagen was 7.6‰. The $\Delta\delta^{13}C$ of this same sample was measured in the present study to be 2.9‰. Nelson's measurements were most likely completed on CO_2 containing considerable quantities of acetaldehyde; the present method was modified from Nelson's method due to this contamination. Abelson and Hoering (15) found even larger values for the $\Delta\delta^{13}C$ of algae. These samples were not bone collagen and it is not known if they can be compared directly. However, in their limited set of measurements, Bada *et al.* (29) found $\Delta\delta^{13}C$ values of modern moose and wolf collagen (from dentin) to be very similar in magnitude to the present study.

Both $\delta^{13}C_{TOTAL}$ and $\delta^{13}C_{COOH}$ were affected by whether the sample's diet originated in a marine, C_3-, or C_4-plant terrestrial environment (see Figure 3.1). $\delta^{13}C_{TOTAL}$ of the sea mammals were typical of a marine environment. The grizzly bear (T-33) appeared to have a partial marine diet (e.g. salmon). The water buffalo (Cooinda-3) and particularly the wallaby (Bower Bird-4) appeared to be making use of C_4 plants in their diet, as expected for Northern Australian herbivores. The humans sampled represented several different environments. Based on $\delta^{13}C_{TOTAL}$ measurements, the Naukans appear to have relied heavily on the sea for food, the Vikings less so (especially Viking–16). The PAR humans might have consumed a mixture of marine, CAM, C_3- and C_4-based foods, and the Oxford humans, a C_3-based diet.

However, as expected, whether the sample's diet originated in a marine, CAM, C_3-, or C_4-plant terrestrial environment did not appear to affect $\Delta\delta^{13}C$. These differences in biochemical pathways occur during photosynthesis. The resulting glucose is believed to be isotopically homogeneous within the molecule (22), and thus, should not contribute to $\Delta\delta^{13}C$ in any way.

Table 3.3:
Isotopic results of modern and archaeological bone collagen from several species. Both $\delta^{13}C_{TOTAL}$ and $\delta^{13}C_{COOH}$ are relative to PDB (in ‰). $\Delta\delta^{13}C = \delta^{13}C_{COOH} - \delta^{13}C_{TOTAL}$. All values are individual measurements unless otherwise noted with ±SD (n replicates). Replicates are individually hydrolyzed and reacted from a common collagen extract.

Sample ID	Species/Info	$\delta^{13}C_{TOTAL}$	$\delta^{13}C_{COOH}$	$\Delta\delta^{13}C$
		Cats		
T-53	mountain lion	-21.54	-21.35±0.70 (6)[a]	0.2
10027	lynx	-20.13	-16.85	3.3
		Sea Mammals		
T-636	California sea lion	-12.23	-10.93	1.3
10114	harbour seal	-11.38	-8.98	2.4
Grey Whale	grey whale	-14.12	-10.88	3.2
		Modern Bears		
T-33	grizzly bear (adult)	-17.24	-13.69	3.5
T-313	black bear (adult)	-19.61	-16.14	3.5
10048	black bear (immature)	-21.09	-17.54	3.5
		European Cave Bears		
B1:188	neonate/fetus	-23.22	-21.24, -21.17	2.0
B1:193	neonate/fetus	-22.14	-19.97	2.2
B2:173-2	cub	-21.79	-18.23	3.6
B2:182	cub	-21.62	-18.76	2.9
B3:181-2	yearling	-20.23	-17.22	3.0
B3:188-1	yearling	-21.59	-18.06	3.5
A1	juvenile	-21.04	-18.36	2.7
A3	juvenile	-21.33±0.02 (5)	-17.29	4.0
B5:188-1	sub adult	-19.96	-16.05	3.9
B5:188-2	sub adult	-20.24	-16.97±0.81 (5)[b]	3.3
B7:183	adult	-21.14	-17.42	3.7
B8:181	adult	-20.30	-16.44	3.9
		Ungulates		
Cooinda-3	water buffalo	-15.13	-12.96	2.2
DeRt-2-2	deer	-20.80	-18.14	2.7
DgRr-1-1	deer	-21.71	-19.26, -20.49	1.8
PB-95.01	wapiti	-19.39	-16.24	3.1
PB-95.06	wapiti	-19.58	-16.53	3.1
PB-95.09	wapiti	-20.26	-16.93	3.3

PB-95.02	caribou	-18.99	-16.21	2.8
PB-95.10	caribou	-19.60	-18.05±0.34 (6)	1.5
PB-95.03	bison	-19.80	-17.72	2.1
PB-95.04	bison	-19.56	-16.50	3.1
PB-95.07	bison	-19.84	-17.49	2.3
PRB	bison	-19.40	-16.53±0.29 (5)	2.9
PB-95.08	moose	-19.69	-17.16	2.5
CBB-1	domestic cow	-20.00	-18.88±0.05 (4)	1.1
		Humans		
Naukan-3	female	-13.60	-14.51	-0.9
Naukan-5	female	-12.69	-11.77	0.9
Naukan-6	male	-12.86	-12.97	-0.1
Naukan-24	female	-13.33	-11.77	1.6
Naukan-27	male	-12.73	-12.48	0.3
Oxford-B2		-18.14	-17.17	1.0
Oxford-B178		-20.13	-18.67	1.5
Oxford-B216		-20.09	-19.14	1.0
Oxford-B218		-20.28	-19.26	1.0
Oxford-B387		-20.78	-21.04	-0.3
PAR-4		-10.12	-7.44	2.7
PAR-5		-10.40	-7.53	2.9
PAR-16		-10.10	-6.54	3.6
Viking-13		-14.40	-12.98	1.4
Viking-14		-16.20	-14.75	1.5
Viking-15		-16.30	-15.46	0.8
Viking-16		-19.10	-17.40	1.7
		Other		
10062	snowshoe hare	-24.61	-20.91	3.7
10068	Eastern Cottontail	-22.45	-19.35	3.1
Bower Bird-4	wallaby	-8.50	-7.27, -7.21	1.3
PB-95.05	mammoth	-20.65	-18.07	2.6

Notes:

a. This set includes 1 questionable measurement that cannot be rejected with 90% confidence using the Q-test. If rejected, the mean would be –21.58‰ and the SD would be 0.47‰.

b. This set includes 1 questionable measurement that cannot be rejected with 90% confidence using the Q-test. If rejected, the mean would be –17.30‰ and the SD would be 0.41‰.

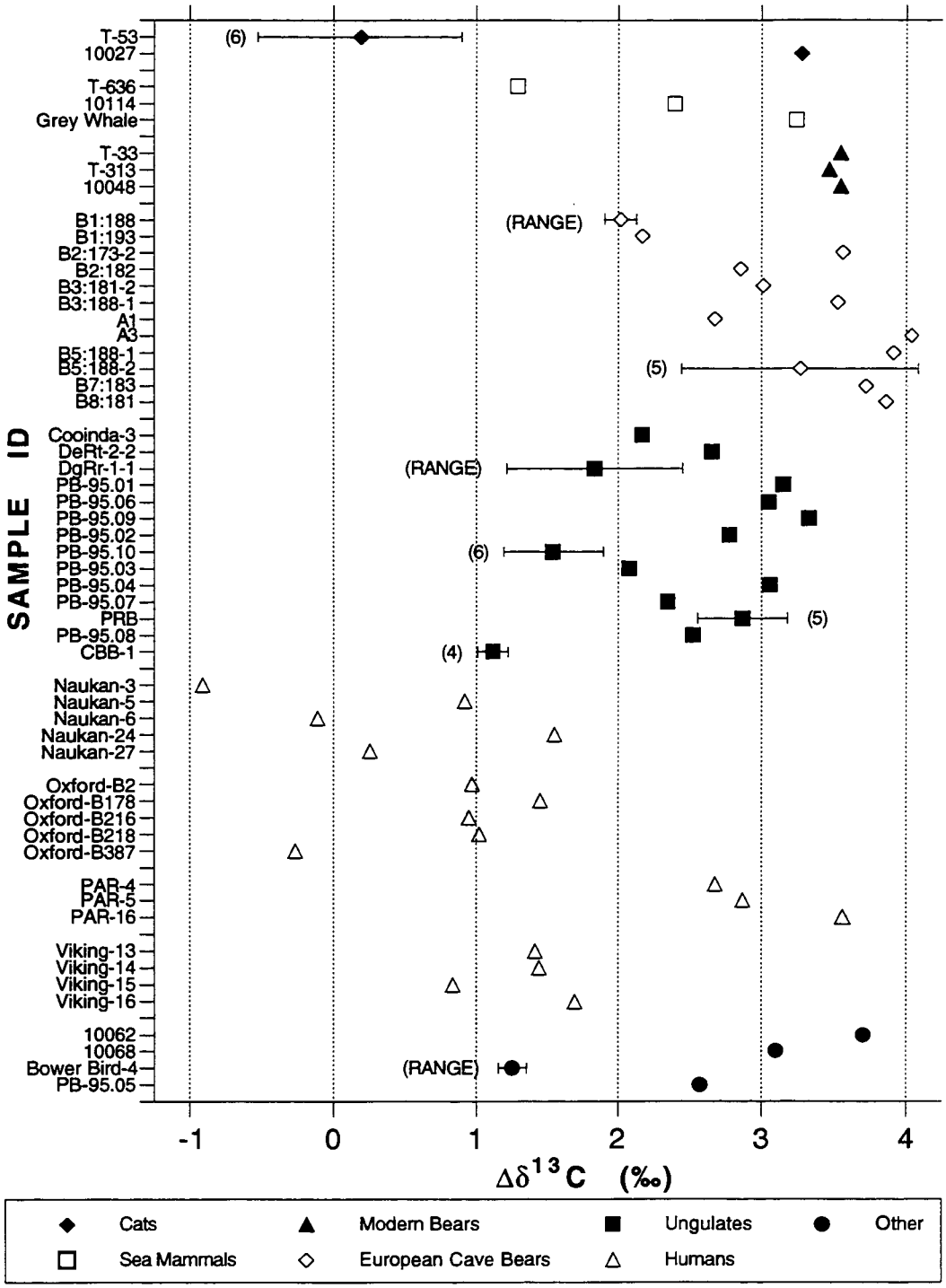


Figure 3.2:

$\Delta\,\delta^{13}C$ for bone collagens. $\Delta\,\delta^{13}C = \delta^{13}C_{COOH} - \delta^{13}C_{TOTAL}$. Error bars show ± SD (n measurements) or range (2 measurements). SD in $\delta^{13}C_{TOTAL}$ assumed to be 0.1‰.

The biological age effect in $\delta^{13}C_{TOTAL}$ described previously by Nelson *et al.* (38) was reproduced with the set of cave bears measured in this study. A more pronounced age effect was observed in $\delta^{13}C_{COOH}$. Both of these trends are shown in Figure 3.3.

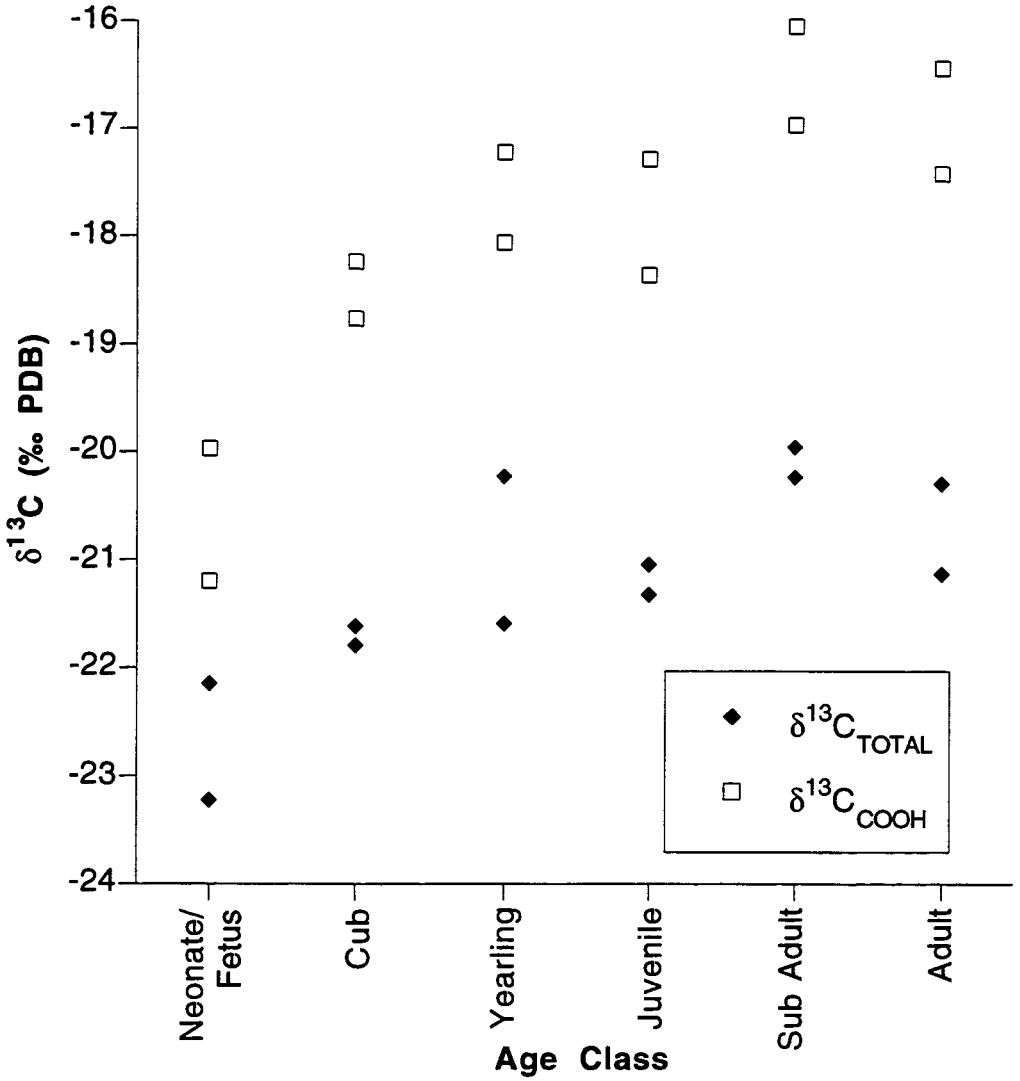


Figure 3.3:
Changes in $\delta^{13}C_{TOTAL}$ and $\delta^{13}C_{COOH}$ with age in the European cave bear. Two bears were sampled from each age class. Each point represents one measurement.

Although the exact same bones were sampled as with Nelson *et al.*'s study, it became apparent in remeasuring the cave bears' $\delta^{13}C_{TOTAL}$ for this study that the $\delta^{13}C_{TOTAL}$ measured sometimes differed between the two studies. This was especially apparent for the very young cave bears. This might be explained as follows. With the older bears, the bones were so large that sampling adjacent to the previous sampling sites was easy. For the very young bears, the bones were so small that the first samples required destroying a large portion of each bone. The samples taken for this study were therefore taken on the remaining, completely different section of each bone. The differences between these measurements suggest that the bones were not isotopically homogeneous. These intra-bone differences might be due to bone remodelling or changes in diet as the cave bears developed. Bocherens *et al.* (43) have reported that the dentin collagen in adult cave bears had a $\delta^{13}C$ about 1‰ more negative than bone collagen. They suggested that this indicates a high lipid diet during tooth formation, which occurred very early in the young bear's life. Additionally, Sealy *et al.* (44) have recently described differences between the different calcified tissues (teeth and bone) of ancient humans. A deeper understanding of the inter-, and intra-bone differences in $\delta^{13}C$ is warranted as certain tissues might reflect diet during specific times in an animal's life instead of an average over many years (44).

The human samples measured were particularly interesting. As a species, their $\Delta\delta^{13}C$ had the largest range. With the exception of the PAR samples, all humans measured had smaller $\Delta\delta^{13}C$ values than most other animals. These values are discussed in greater detail in chapter 4.

4 DISCUSSION

4.1 *Method*

In general, the procedure was relatively free from isotopic fractionation provided every step was allowed to go to completion. Analyte CO_2 was free from detectable contaminants that would effect $\delta^{13}C_{COOH}$. The overall uncertainty in measuring the $\delta^{13}C_{COOH}$ of bone collagen averaged about 0.4‰ (1 SD). Since the uncertainty in measuring $\delta^{13}C_{TOTAL}$ was small (less than 0.1‰), the uncertainty in $\Delta\delta^{13}C$ was less than 0.5‰ (1 SD).

The largest isotopic fractionation occurred during the acid hydrolysis of collagen. Errors in $\delta^{13}C_{COOH}$ would be considerable if hydrolysis was not complete. Unfortunately, the time for complete hydrolysis was not consistent for the collagen samples tested. It was surprising to find that the most routine technique in this procedure, acid hydrolysis, produced the largest uncertainty. This study has revealed that great care is needed to avoid isotopic fractionation when stable isotope methodologies involve protein hydrolysis. Further experiments are warranted to identify the best conditions for complete hydrolysis of all collagen samples. The other steps were predominantly free from isotopic fractionation if the procedure described was carefully followed.

The overall yields of CO_2 from the ninhydrin reaction with individual amino acids were quantitative, whereas those from lyophilized collagen extracts were dependent upon the quantity of protein in each extract. These extracts contained significant quantities of inorganic material (likely water). To correct for this, percent carbon and percent nitrogen analysis should be completed on

the lyophilized collagen samples to normalise yields to the actual quantity of protein in the sample. With these corrections, overall yields of CO_2 were about 87% for collagen samples. In summary, the method was tested in enough detail to be confident in its use for this preliminary survey of the $\Delta\delta^{13}C$ of animal bone collagen.

4.2 *Survey of $\Delta\delta^{13}C$*

This study has provided the most comprehensive set of $\Delta\delta^{13}C$ values for both modern and ancient bone collagen. Although not as large as expected from the limited data previously published (26), there were measurable differences between the $\delta^{13}C_{TOTAL}$ and $\delta^{13}C_{COOH}$ of bone collagen. In addition, $\Delta\delta^{13}C$ differed among bone collagen samples, ranging from -0.9 to 4.0‰. $\Delta\delta^{13}C$ seemed to be independent of the method of photosynthesis in the food web, the sample's archaeological age and collagen preservation. As discussed by Bada *et al.* (45), diagenesis can affect the isotopic composition of the residual collagen, making carboxyl carbons heavier. This effect did not appear significant here as the $\Delta\delta^{13}C$ values of the cave bears (the oldest samples) were very similar to modern bears. This effect might have been circumvented by the method of collagen extraction (only higher molecular weight collagen fragments were kept).

However, there appeared to be changes in $\Delta\delta^{13}C$ with animal or trophic level. $\Delta\delta^{13}C$ centred around 2.5‰ for ungulates and other herbivores. Animals at higher trophic levels tended to have smaller $\Delta\delta^{13}C$ values. The marine

mammals at higher trophic levels (i.e., California sea lion (T-636) and harbour seal (10114)) had smaller $\Delta\delta^{13}C$ values than the grey whale (Grey Whale), a lower trophic level baleen whale. However, there were apparent exceptions in the trophic level trend. For example, the lynx (10027), which would have eaten primarily snowshoe hare, had a $\Delta\delta^{13}C$ similar to the snowshoe hare (10062), 3.3 and 3.7‰ respectively. The population of lynxes fluctuates widely with hare population (46). Thus, lynxes often face starvation which might conceivably affect $\Delta\delta^{13}C$ as the routing of carbon changes in the animal. This is very different from mountain lions which have more diverse diets. The mountain lion measured (T-53) had a $\Delta\delta^{13}C$ of 0.2‰.

Bears produced some of the largest $\Delta\delta^{13}C$ values of all species analysed. Modern bears were remarkably consistent at about 3.5‰. The European cave bears were more variable in $\Delta\delta^{13}C$, ranging between 2.0 and 4.0‰. The changes in $\delta^{13}C$ with cave bear age were more pronounced in the carboxyl carbons than the total carbon. Mass balance calculations suggested the carboxyl carbons were fractionated over twice as much with age as the remaining carbons in collagen. Whether this indicated an isotopic fractionation during the routing of amino acids into collagen, a change in the carbon source of amino acids with age, or some other effect was not clear and merits further investigation.

Bears are unique in that gestation and birth take place during winter hibernation. At this time, the mother maintains her normal body temperature without eating, defecating, or urinating. All metabolic processes depend on the mother's stored fat and protein for carbon. Nelson *et al.* (38) have postulated

that the mother's proteins are being extensively recycled during hibernation and that isotopically light lipids from the hibernating mother might have also been used as a source of carbon for the biosynthesis of (non-essential) amino acids in fetal collagen. Fetal collagen may therefore incorporate amino acids that have been highly recycled. It is reasonable to assume that carboxyl carbons are more sensitive to isotopic fractionation during protein recycling than total carbon. This suggestion is consistent with observations in this study. Also, if the isotopically light carbon from lipids were used in the biosynthesis of amino acids, fetal and neonate cave bears would be expected to have the most negative $\delta^{13}C_{TOTAL}$. As the young bears stopped feeding off their mothers and began eating other food, their $\delta^{13}C_{TOTAL}$ would be expected to become more positive and eventually reach adult-like values. This change was observed in the present study. It is not known how using lipids for amino acid biosynthesis will affect $\Delta\delta^{13}C$. As discussed below for the Naukan humans, there is evidence that the intramolecular distribution of $\delta^{13}C$ is not homogeneous in fatty acids.

Although they were a third larger than grizzly bears, adult European cave bears were almost exclusively herbivores (43, 47). But, neonate cave bears were carnivores as they fed off their hibernating mothers. Thus, a significant change in trophic level took place when the young cave bear left its winter den. This change in trophic level is not unique to bears. What makes bears unique is their gestation and birth during hibernation. Investigating possible changes in $\delta^{13}C_{COOH}$ with age for modern bears and other species could provide further insight.

Of all species analysed, humans had the most diverse diets and the largest range in $\Delta\delta^{13}C$. The Naukans had the lowest $\Delta\delta^{13}C$ values, averaging 0.3‰. With their Eskimo-like existence, these humans were thought to be largely carnivorous and probably consumed sea mammals which were not primary consumers (39). Therefore, these humans were very high up the food chain, eating a high protein and high fat diet. High protein diets can stimulate endogenous acid production and increase the rate of bone turnover (2). This has been observed in humans on controlled diets (48). The effect of increased bone turnover on $\Delta\delta^{13}C$ or $\delta^{13}C$ is not yet known. Without further study, it is premature to suggest that increased bone turnover due to a high protein intake caused low $\Delta\delta^{13}C$ values in the Naukan humans. The effect of a high fat diet on $\Delta\delta^{13}C$ is also unknown. The Naukans had enough protein in their diet to expect that little amino acid biosynthesis occurred. However, if fat was used for amino acid biosynthesis (either in the Naukans themselves, or their prey), the resulting amino acids could reflect the intramolecular isotopic distribution of metabolised fat. Monson *et al.* (22) have reported that the fatty acids in *Escherichia coli* have an alternating pattern of $\delta^{13}C$ along the hydrocarbon chain. One carbon is 6.4‰ more negative than the other. If this pattern occurs with higher organisms, the metabolism of fat and its incorporation into protein could be reflected by a change in $\Delta\delta^{13}C$.

At the other end of the range, the PAR humans had $\Delta\delta^{13}C$ values averaging 3.1‰. Living on an arid peninsula, these people probably consumed C_3, C_4, CAM, and marine dietary components. Establishing a diet from stable isotopes in this situation is difficult without an extensive survey of the possible

dietary components. Although this survey is not yet complete (41), these people are known to have led a very meagre existence as hunter-gatherers in an arid terrestrial environment with some marine dietary inputs.

The Viking and Oxford humans had mid-range values of $\Delta\delta^{13}C$, averaging 1.4 and 0.8‰ respectively. Both of these groups are not believed to have had diets as extreme as either the Naukan or the PAR humans (40, 42).

It is encouraging to find that the largest range in $\Delta\delta^{13}C$ was in humans, who also had the largest range in diet. Thus, the origin of $\Delta\delta^{13}C$ might be related to trophic level, or to the quantity of protein or fat consumed in the diet.

4.3 *Future Directions*

This preliminary survey has provided evidence that additional dietary information might be gained by measuring the $\Delta\delta^{13}C$ of archaeological bone collagen samples, particularly humans. A more comprehensive study is warranted to clearly identify the source(s) of differences in $\Delta\delta^{13}C$. In addition to the suggestions above, other studies are recommended:

(1) Experiments with laboratory animals on controlled diets would more clearly identify how the different dietary components affect the $\Delta\delta^{13}C$ of bone collagen.

(2) If $\Delta\delta^{13}C$ relates to trophic level, changes in $\Delta\delta^{13}C$ should be compared to changes in stable nitrogen isotope ratios since the use of stable nitrogen isotope ratios to estimate trophic level is well described in the literature (2). Such a comparison has already begun with the samples measured in this thesis.

(3) Collagen is usually the most abundant protein in archaeological samples. However, this method is applicable to all proteins. The $\Delta\delta^{13}C$ measurement of other proteins might prove useful to both archaeology and biochemistry.

(4) Because isotopic fractionation was so evident during acid hydrolysis, a modification of the method described might be particularly useful in measuring kinetic isotope effects at the carboxyl carbon during amide hydrolysis.

(5) Although the method described here has proven effective for a preliminary survey, it was not ideal and would be considered very tedious for routine analysis of many samples. If available, the use of a GC-IRMS with an improved method would be superior for future, more extensive, studies.

5 CONCLUSIONS

Ninhydrin can be used to release the carboxyl carbons of amino acids in bone collagen hydrolysates as CO_2 for the measurement of $\delta^{13}C$. The method described was able to detect a difference in $\delta^{13}C$ between the carboxyl and total carbons in bone collagen. This difference ($\Delta\delta^{13}C$) changed with organism and might provide additional dietary information in combination with the $\delta^{13}C$ of the total carbon in bone collagen. The origin of this effect, and its application should be studied further. The dependence of $\Delta\delta^{13}C$ on trophic level and the amount of dietary protein and fat was postulated.

APPENDIX A Stable Carbon Isotopes

A.1 *Stable Carbon Isotopes in the Biosphere*

The isotopic ratios of inorganic carbon are determined primarily by the isotopic exchange between atmospheric and oceanic carbon. Organic carbon is typically depleted in ^{13}C due to isotopic fractionation during photosynthesis and other biological processes.

Atmospheric CO_2 links the inorganic and organic carbon pools in both the terrestrial and marine environments as it is both a substrate for photosynthesis and a product of the oceanic carbonate system. Although there are small temporal and geographical variations in the $\delta^{13}C$ of atmospheric CO_2, the mean value is presently approximately -7.8‰ (3). There is evidence that the $\delta^{13}C$ of atmospheric CO_2 has changed during the past 50,000 years. For example, a shift has occurred due to the increased deforestation and burning of fossil fuels by humans. This source of lighter carbon has changed the $\delta^{13}C$ of atmospheric CO_2 by approximately -1.1‰ from the late 1800's to 1980 (49). Before comparing $\delta^{13}C$ values among samples, deciding which time period the samples originated and applying the appropriate corrections are usually necessary. These corrections were not done in this study because $\Delta\delta^{13}C$ (defined in section 1.4) should be independent of atmospheric $\delta^{13}C$.

In the terrestrial environment, the largest isotopic fractionation of carbon occurs during photosynthesis when ^{13}C is discriminated against in the diffusion and subsequent fixation of atmospheric CO_2 into plants. Plants use one of three pathways for CO_2 fixation: C_3, C_4, or CAM. Plants with the C_3 pathway reduce

CO_2 to a three-carbon compound, phosphoglycerate. They discriminate against $^{13}CO_2$ the most, resulting in a $\delta^{13}C$ range of -32 to -20‰ and a mean value of –27‰. Plants with the C_4 pathway reduce CO_2 to a four-carbon compound, oxaloacetic acid, and discriminate much less, having a $\delta^{13}C$ range of –17 to –9‰ and a mean value of -13‰. Plants with the CAM (Crassulacean Acid Metabolism) pathway are flexible between C_3 and C_4 in their method of fixation and thus $\delta^{13}C$ ranges from -28 to -10‰ (3, 5). Most terrestrial plants are C_3. C_4 plants (for example, maize and sugar cane) and CAM plants (for example, cacti and pineapple) exist mostly in warm, arid, or semiarid environments.

Photosynthesis in the marine environment occurs primarily by the C_3 pathway but stable carbon isotope ratios of marine plants are not similar to terrestrial plants due to the former's use of dissolved inorganic carbon ($\delta^{13}C$ = 0‰) as the source of carbon, the existence of mechanisms in aquatic plants to actively transport dissolved inorganic carbon into the cell, and to the limited diffusion of CO_2 in water. Phytoplankton have a mean $\delta^{13}C$ of about -22‰ but the values range from -30 to -18‰ (3).

A.2 *Stable Carbon Isotopes in Food Chains*

Plants, converting inorganic carbon into organic carbon, are thus the bottom of the food chain. When other organisms consume these plants, they incorporate the plants' isotopic signatures into their own tissues with a shift in $\delta^{13}C$ of about +5‰ (from diet to collagen) (8). When organisms further up the food chain are studied (i.e. carnivores), another (smaller) trophic level shift is observed but the original isotopic signatures of the plants are still traceable.

Thus, 'you are what you eat' isotopically (with some shifts). This statement is the basis for extensive research into the dietary reconstruction of ancient and modern organisms including humans. The differences in CO_2 fixation can allow the type of plants or the contribution of terrestrial versus marine sources of the diet to be determined from the $\delta^{13}C$ of animal tissue.

APPENDIX B Measurement of $\delta^{13}C$

The measurement of $\delta^{13}C$ requires the $^{13}C/^{12}C$ ratio of a sample to be measured with very high precision. These ratios can best be measured by mass spectrometry of CO_2 (although not exclusively (50)). A typical mass spectrometer used for structural elucidation cannot provide the necessary precision in the $^{13}C/^{12}C$ ratio. Instead, a specially designed Isotope Ratio Mass Spectrometer (IRMS) is required (1). An IRMS consists of a dual inlet that alternately introduces the sample and standard gases (at equal pressures) into the ion source of the mass spectrometer. To achieve high precision, three ion beams are simultaneously measured while the magnetic field and accelerating potential are carefully held constant. For CO_2, these ion beams correspond to m/z 44 ($^{12}C^{16}O^{16}O$), 45 ($^{13}C^{16}O^{16}O$ and $^{12}C^{17}O^{16}O$), and 46 ($^{12}C^{17}O^{17}O$, $^{12}C^{18}O^{16}O$ and $^{13}C^{17}O^{16}O$). The $\delta^{13}C$ value is calculated from the measured 45/44 and 46/44 ratios of sample and standard (51). Instrumental precision is typically 0.01‰ for $\delta^{13}C$.

For organic samples, the most commonly used technique for preparing CO_2 is combustion. Traditionally, the sample is combusted by heating in a sealed, evacuated quartz or Vycor tube containing CuO as the source of oxygen (1). More recently, samples are combusted on-line in an elemental analyser and the CO_2 is separated from other combustion gases by gas chromatography. The purified CO_2 is then introduced into the IRMS. Other methods, like the one described in this thesis, use chemical means to produce CO_2 from the sample of interest.

Clearly, the sample introduced into the mass spectrometer must be very pure CO_2. Contamination, especially contaminants with m/z of 44, 45 or 46, will significantly alter the $\delta^{13}C$ measured. Any procedure used to prepare CO_2 for isotopic analysis must be checked for contamination. If the contaminant is also CO_2, the intensities of the 44, 45, and 46 peaks will be similar to the sample and the measured $\delta^{13}C$ will be the weighted average of both $\delta^{13}C$ values. Since the $\delta^{13}C$ value of the contaminant CO_2 (usually atmospheric) is usually not too different from the sample (within about 20‰), a few percent contamination does not affect the measurement greatly. However, if the contamination is not CO_2, the intensities of the 44, 45, and 46 peaks will be different and a substantial error is possible.

APPENDIX C Additional Sample Information

Sample ID	Species (and sex or biological age) Collection site Bone sampled
	Cats
T-53	mountain lion (*Felis concolor*) Ahousat, Vancouver Island, B.C. right femur
10027	lynx (*Lynx canadensis*) Mile 86, Alaska Highway, B.C. right femur
	Sea Mammals
T-636	California sea lion (*Zalophus californianus*) Gulf Islands, B.C. right femur
10114	harbour seal (*Phoca vitulina*) Nootka Sound, Vancouver Island, B.C. right femur
Grey Whale	grey whale (*Eschrichtius robustus*) Nootka Island, Vancouver Island, B.C. humerus
	Modern Bears
T-33	grizzly bear (*Ursus horribilis*) (adult) Kwatna, B.C. right femur
T-313	black bear (*Ursus americanus*) (adult) Port Hardy, Vancouver Island, B.C. right femur
10048	black bear (*Ursus americanus*) (immature) Princeton, B.C. right femur
	European Cave Bears (*Ursus spelaeus*)
B1:188	neonate/fetus tibia
B1:193	neonate/fetus tibia
B2:173-2	cub tibia

B2:182	cub
	tibia
B3:181-2	yearling
	tibia
B3:188-1	yearling
	tibia
A1	juvenile
	cranium
A3	juvenile
	femur
B5:188-1	sub adult
	tibia
B5:188-2	sub adult
	tibia
B7:183	adult
	tibia
B8:181	adult
	tibia

Ungulates

Cooinda-3	water buffalo (likely *Bubalus bubalis*)
	cranium
DeRt-2-2	deer (*Odocoileus sp*)
DgRr-1-1	deer (*Odocoileus sp*)
PB-95.01	wapiti (*Cervus elaphus*)
PB-95.06	wapiti (*Cervus elaphus*)
PB-95.09	wapiti (*Cervus elaphus*)
PB-95.02	caribou (*Rangifer tarandus*)
PB-95.10	caribou (*Rangifer tarandus*)
PB-95.03	bison (likely *Bison priscus*)
PB-95.04	bison (*Bison priscus*)
PB-95.07	bison (*Bison priscus*)
PRB	bison (likely *Bison bison cf. occidentalis*)
PB-95.08	moose (*Alces sp*)
CBB-1	domestic cow (*Bos taurus*)

Humans

(*Homo sapiens*)

Naukan-3	female
Naukan-5	female
Naukan-6	male
Naukan-24	female
Naukan-27	male
Oxford-B2	Blackfriars site, Oxford, U.K.
	femur

Oxford-B178	Lechlade site, near Oxford, U.K. left foot bone
Oxford-B216	Dorchester site, near Oxford, U.K. left foot bone
Oxford-B218	Dorchester site, near Oxford, U.K. right foot bone
Oxford-B387	Bidwell site, near Exeter, U.K. femur
PAR-4	adult left tibia
PAR-5	adult left tibia
PAR-16	adult left tibia
Viking-13	adult
Viking-14	adult
Viking-15	adult
Viking-16	adult

Other

10062	snowshoe hare (*Lepus americanus macfarlani*) Farrell Creek B.C. right femur
10068	Eastern Cottontail (*Sylvilagus floridans*) (immature) Livingston County, Kentucky, USA ribs
Bower Bird-4	wallaby (Family **Macropodidae**) jaw
PB-95.05	mammoth (*Mammuthus primigenious*)

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