Amino Acid Racemization Dating in New Zealand: An overview and Bibliography

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Contents

1 Introduction **2**

2 Biochemical Background **4**

2.1 Sources of amino acids 5

2.2 Racemization 8

2.2.1 Racemization of free amino acids 12

2.2.2 Racemization of bound amino acids 16

2.3 Racemate ratio measurement 23

2.3.1 Sample preparation 23

2.3.2 Analysis of the hydrolysate 24

3 Applications **27**

3.1 General 27

3.1.1 AAR and Bone 27

3.1.2 AAR and Teeth 29

3.1.3 AAR and Molluscs 30

3.1.4 AAR and Eggshells 31

3.1.5 AAR and Palaeotemperature estimations 32

3.2 Applications to New Zealand 32

3.2.1 Conclusion 33

Chapter 1

Introduction

Amino acid racemization (AAR) is used to determine relative dates of biological materials such as bone, shell and teeth and has been used in an archaeological context for over 30 years. During this time a number of significant results have been generated but many have been questioned and the technique remains controversial. In spite of this the possibility of reliable AAR dating is attractive. The technique potentially serves as an independent method for dating faunal material, which is useful in the context of providing support for chronometric information produced by other methods. Further to this, AAR allows the possibility of being able to provide information on palaeotemperature histories through analysis of cross-dated specimens.

Amino acids are the building blocks of protein and most can exist in two different forms levorotatory (L) and dextrorotatory (D), these forms are called enantiomers. In living organisms the amino acids in protein are almost exclusively L and the D/L ratio approaches zero. After death proteins break down and the D and L forms start to inter convert. This process is called racemization. In time the D/L ratio approaches one. If the D/L ratio is less than one it may be possible to use it to estimate age.

The presence of proteins in archaeological remains has been known for some time. Nearly fifty years ago Abelson (1954) separated amino acids from sub-fossil shell. He suggested the possibility of using the kinetics of the degradation of amino acids as the basis for a dating method (Abelson 1955). In 1967 Hare and Abelson measured the extent of racemization of amino acids extracted from modern and sub-fossil Mercenaria mercenaria shells (edible clam). They found that the total amount of amino acids present in shell decreased with the age of the shell. The amino acids in recent shell were all in the L configuration and over time the amount of D configuration amino acid increased (Hare and Abelson 1967). However, even after 35 years this method of dating is still subject

to vigorous debate, with the application of AAR to date bone being particularly controversial (Bada 1990; Marshall 1990). Major reviews of AAR include: (Johnson and Miller 1997), (Hare, von Endt, and Kokis 1997), (Rutter and Blackwell 1995), (Murray-Wallace 1993), (Bada 1991) and (Schroeder and Bada 1976).

Racemization is a chemical reaction and a number of factors influence its rate (Rutter and Blackwell 1995). These include; amino acid structure, the sequence of amino acids in peptides, pH, buffering effects, metallic cations, the presence of water and temperature. To establish a dating method the kinetics and mechanisms of the racemization (and epimerization) reaction of free and peptide bound amino acids need to be established. To this end various workers in the late 1960s and the 1970s studied free amino acids in solution and carried out laboratory simulations of post mortem changes in the amino acids in bone (Bada 1972) and shell (Hare and Abelson 1967; Hare and Mitterer 1969). Attempts have also been made to relate the kinetics of free amino acids, with those in short polypeptides and the proteins in various archaeological samples (Bada 1982; Smith and Evans 1980). In the following we provide a summary of the biochemical basis to AAR analysis, a review of archaeological applications of AAR and an assesment of the potential for AAR analysis as a chronometric method in New Zealand archaeological research.

Chapter 2

Biochemical Background

There are approximately twenty different amino acids and these comprise the building blocks of proteins. See Lehninger (1982) Mathews (1996) and Stryer (1995) for general reference and an overview. Amino acids consist of an alpha carbon atom (C_α) which has four different groups bonded to it. These are an amino group ($-NH_2$), a carboxyl group (-COOH), a hydrogen atom (-H) and a side chain often called an R group (-R) see figure 2.1.

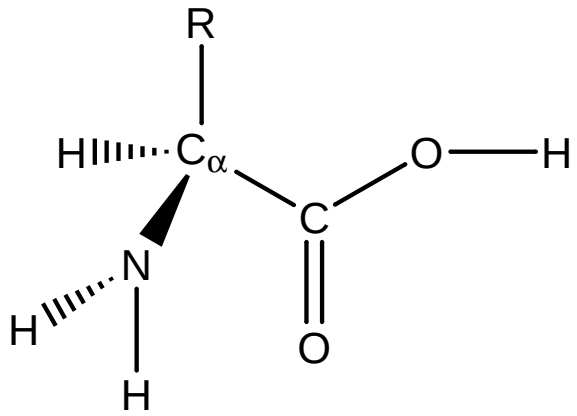
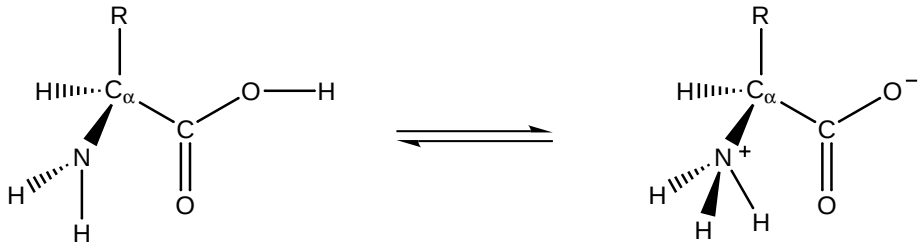


Figure 2.1: The structure of a generalised amino acid.

The form of an amino acid depends on the pH of its environment and in living cells the pH is close to neutral. The ionic form, which predominates at neutral pH, is called a zwitterion. The two forms of a generalised amino acid (either of which can be seen in papers and texts) are shown in figure 2.2 on the next page.

Amino acids are distinguished from one another by differences in their side chains. The twenty amino acids coded for in the genes of living organisms and incorporated directly into protein are shown in figure 2.3 on page 7.



nonionic form zwitterion form

Figure 2.2: A generalised amino acid forming a zwitterion at neutral pH.

Some amino acids can be modified after they have been incorporated into protein, for example proline can become hydroxy proline (figure 2.4 on page 8) as occurs in collagen (a protein found in bone and connective tissues).

When amino acids combine an amide linkage called a peptide bond is formed and a molecule of water is released. Two amino acids form a di peptide, three amino acids form a tri peptide and many amino acids form a polypeptide. The peptide bond forms between the amino end of one amino acid and the carboxyl end of another. An example of this type of reaction is shown in figure 2.5 on page 9.

Most polypeptides retain an unreacted amino group at one end (called the amino or N terminus) and an unreacted carboxyl group at the other end (called the carboxyl or C terminus). When writing the amino acid sequence of a polypeptide the convention is to write the N terminus to the left and the C terminus to the right. Either the standard three letter abbreviations or the one letter abbreviations for the amino acid residues can be used (for these see table 2.1 on the next page).

All proteins are polymers in which the monomers (amino acids) are linked to one another through peptide bonds. Thus all proteins are polypeptides. When proteins are broken down their peptide bonds are cleaved and the process is called hydrolysis.

2.1 Sources of amino acids

Amino acids are found primarily in the proteins of living or decaying organisms (Lehninger 1982, p.60). Proteins constitute 50% or more of the dry weight of living cells (Lehninger 1982, p.95). They make up a large part of the structural framework of cells and tissues, some carry out the transport and storage of small molecules, some are antibodies and some are enzymes (Stryer 1995)

Table 2.1: The names, codes and number of chiral centres of the twenty amino acids encoded in DNA.

Name	Three letter code	One letter code	Number of chiral centres
Alanine	Ala	A	1
Arginine	Arg	R	1
Asparagine	Asn	N	1
Aspartic acid	Asp	D	1
Cysteine	Cys	C	1
Glutamine	Gln	Q	1
Glutamic acid	Glu	E	1
Glycine	Gly	G	0
Histidine	His	H	1
Isoleucine	Ile	I	2
Leucine	Leu	L	1
Lysine	Lys	K	1
Methionine	Met	M	1
Phenylalanine	Phe	F	1
Proline	Pro	P	1
Serine	Ser	S	1
Threonine	Thr	T	2
Tryptophan	Trp	W	1
Tyrosine	Tyr	Y	1
Valine	Val	V	1

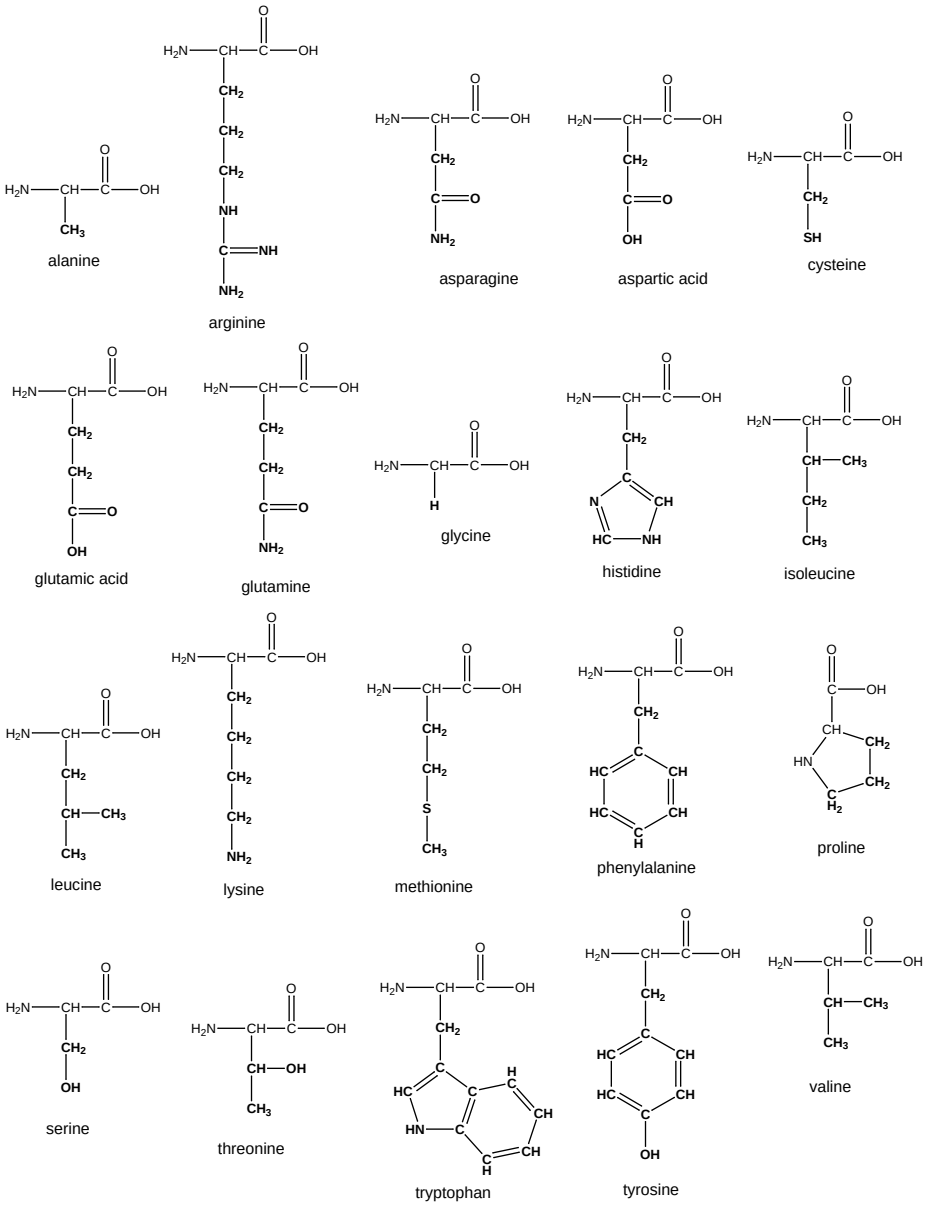


Figure 2.3: The structure of the twenty amino acids most commonly incorporated into protein.

chapter 2. Protein is present in soft tissue and also in bone, shell, feathers and teeth (Voet and Voet 1995, p.156). Not all the amino acids found in cells are incorporated into protein. There are others that occur in cells and they play important roles in various biochemical processes including synthesis, metabolism and neurotransmission, (Mathews and van Holde 1996, p.134).

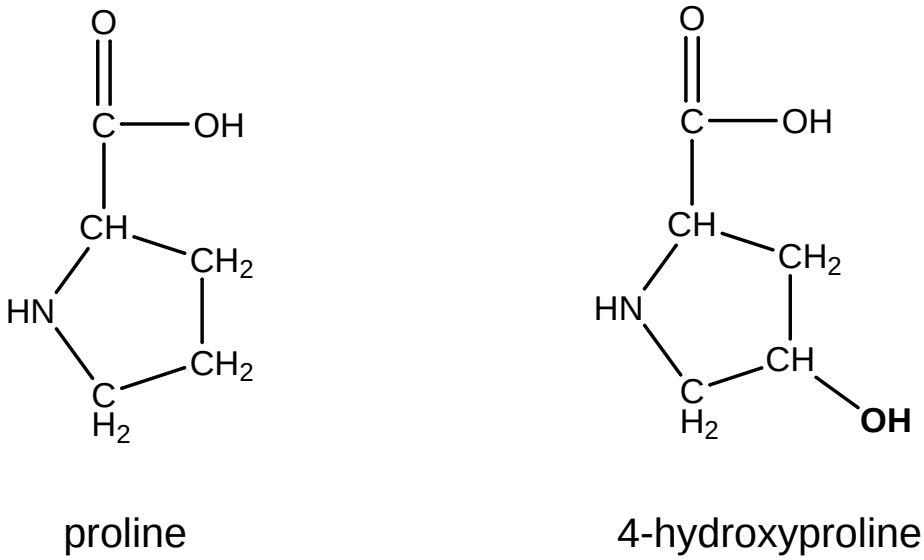


Figure 2.4: The structure of proline and its derivative hydroxyproline.

2.2 Racemization

In some cases molecules of the same compound can have their atoms arranged differently in space. These different arrangements are called stereoisomers. The amino acids with the exception of glycine, exhibit a particular form of stereoisomerism called optical isomerism. They can exist in mirror image forms, called enantiomers and these forms cannot be superimposed on one another. The relationship is rather like that of our hands; the left hand is not superimposable on its mirror image, the right hand. A compound with all of its molecules in the same mirror image form is described as optically active. When a beam of plane (also called linearly) polarized light, is shone through a solution of a pure enantiomer the light will be rotated to the left or to the right. See Clark (1999) for a concise and colourful explanation of the polarization of light and the handedness of molecules. The isomers are designated either L (levorotatory, or“left-handed") or D (dextrorotatory, or “right-handed"). The designations are now based on chemical structure but they originated from the way solutions rotated polarized light. It is difficult by most techniques to differentiate either physically or chemically between the D- and L- isomers of a compound.

A molecule that contains a carbon atom with four different substituents (atoms or groups) bonded to it is said to have an asymmetric carbon atom. Such carbon atoms can also be called asymmetric centres, chiral atoms or chiral centres. The carbon atom in figure 2.6 on page 10 is a chiral centre. Molecules with chiral centres exhibit optical isomerism and their mirror image pairs are called enantiomers.

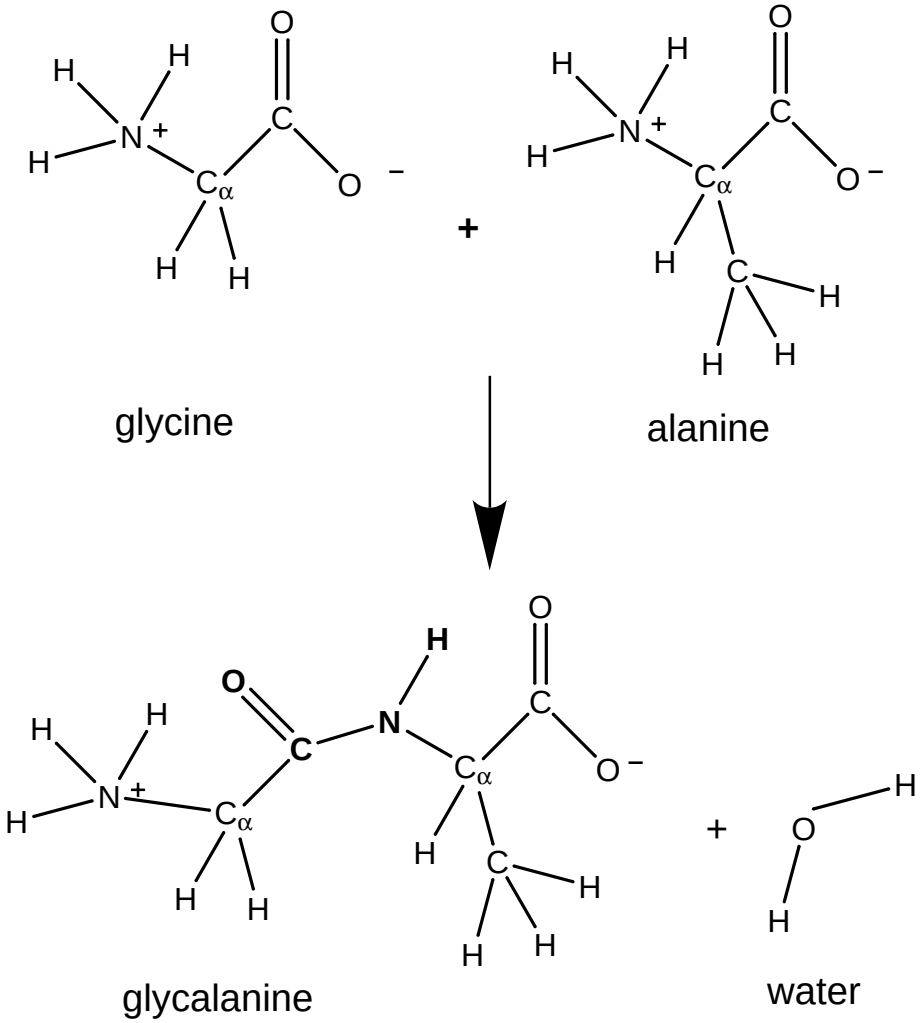


Figure 2.5: The formation of the dipeptide glycalanine from the amino acids glycine and alanine. The peptide bond is shown in bold type and encircled.

Of the twenty amino acids in figure 2.3 on page 7 and table 2.1 on page 6 only glycine does not have a chiral centre. This is because it has three and not the required four, different substituents bonded to the α-carbon. When there are only three different substituents bonded to an α-carbon the mirror images are superimposable and the molecules are the same (figure 2.7 on the next page).

Seventeen of the common amino acids have only one chiral centre situated at the α-carbon. When a compound has two or more chiral centres it has 2^n possible stereoisomers. Isoleucine has two chiral centres so it has four possible stereoisomers comprising two pairs of enantiomers (figure 2.8 on page 11).

Racemization is the interconversion of the L and D enantiomers of a compound with one chiral centre. The racemization of aspartic acid (figure 2.9 on the next

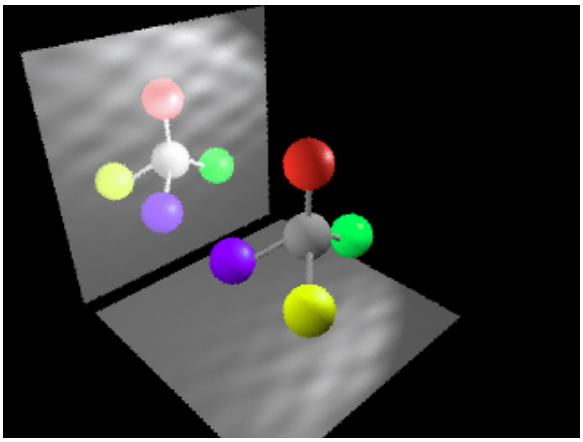


Figure 2.6: The central grey atom represents carbon. The molecule, even when it is rotated, cannot be superimposed on its mirror image. (Graphic by George Baxter)

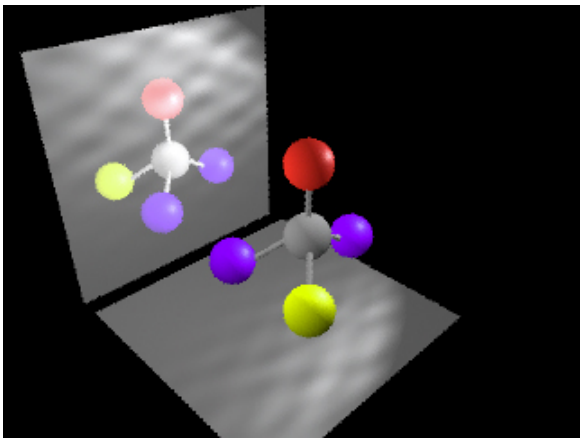
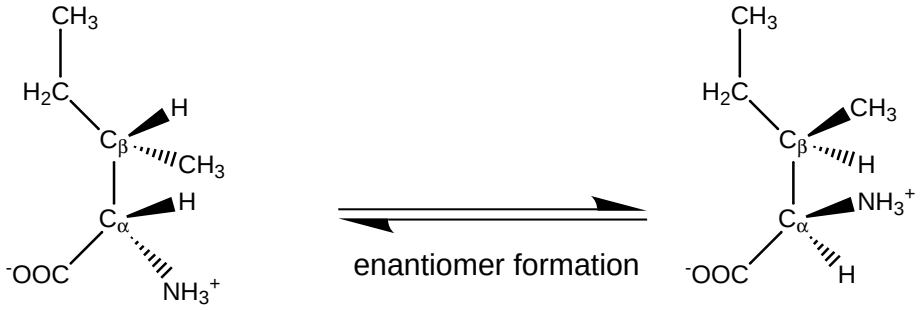


Figure 2.7: This molecule, when rotated, is superimposable on its mirror image. (Graphic by George Baxter)

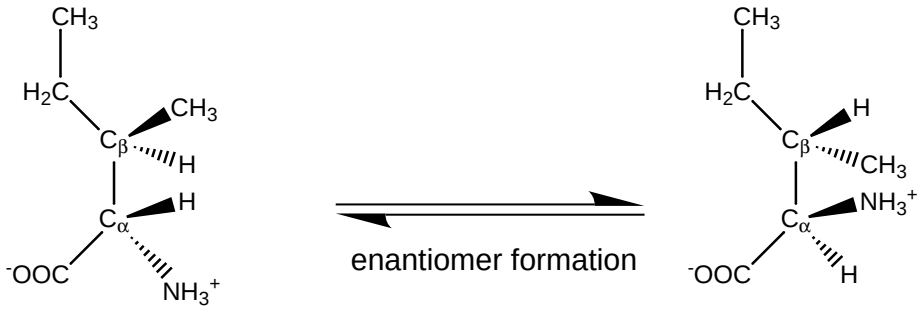
page) is an example of this.

In the case of interconversion at the α-carbon of an amino acid with two chiral centres such as isoleucine the term epimerization is used (figure 2.10 on page 12).

The terms racemization and epimerization are sometimes used interchangeably in the AAR literature with the racemization of aspartic acid and the epimerization of isoleucine being the two reactions most widely used in AAR dating studies (Johnson and Miller 1997).

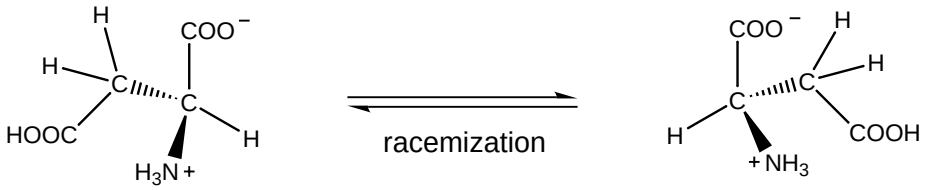


L-isoleucine D-isoleucine



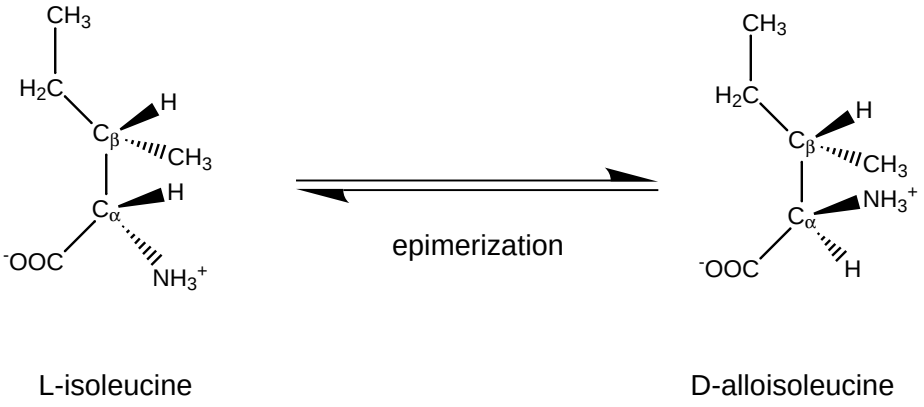
L-alloisoleucine D-alloisoleucine

Figure 2.8: The two pairs of enantiomers of isoleucine. The mirror image pairs are L and D-isoleucine and L and D-alloisoleucine.



L-aspartic acid D-aspartic acid

Figure 2.9: The racemization of aspartic acid. These two molecules are mirror images of one another.



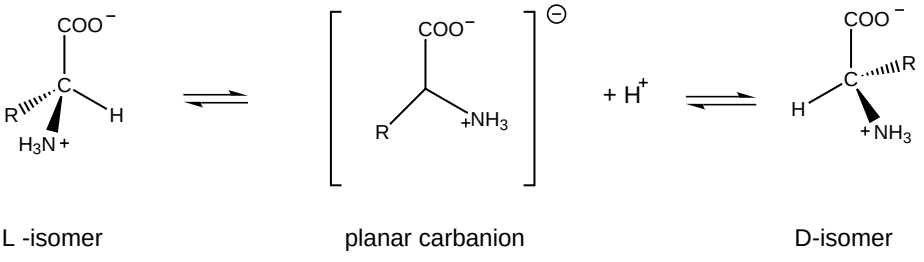
L-isoleucine D-alloisoleucine

Figure 2.10: The epimerization of isoleucine to alloisoleucine. These two molecules are not mirror images of one another.

2.2.1 Racemization of free amino acids

In aqueous solutions, racemization rates of amino acids are influenced by; molecular structure, pH, ionic strength, buffer concentration and temperature (Bada and Schroeder 1975; Rutter and Blackwell 1995; Smith and Evans 1980).

The generally accepted mechanism for racemization of amino acids in aqueous solutions invokes a carbanion intermediate (Ebbers et al. 1997). This was first postulated by Neuberger (1948) and the reaction mechanism involves the removal of the hydrogen bonded to the α-carbon by a base such as an hydroxide ion or a water molecule. Subsequently a negatively charged planar carbanion intermediate is formed. The last step is the readdition of a hydrogen ion to the carbanion and there is an equal probability of either the D or the L enantiomer being formed. figure 2.11 illustrates this mechanism. Kinetic studies carried out on L-isoleucine in deuterated water (Bada and Schroeder 1975) is one of many studies that supports the carbanion mechanism.



L -isomer planar carbanion D-isomer

Figure 2.11: The mechanism for the racemization of a generalised amino acid. After Bada (1984)

Molecular Structure

The groups bonded to the α-carbon of a free amino acid influence the rate at which racemization occurs. Groups that stabilise the carbanion charge accelerate racemization and groups that destabilise this charge retard racemization (Smith and Evans 1980). In the zwitterionic form the -COO^- group of an amino acid will destabilise the carbanion because of the proximity of two negative charges. The -NH_3^+ group on the other hand will stabilise the carbanion because of the proximity of opposite charges. Since all free amino acids have a -COO^- group and an -NH_3^+ group the characteristics of the different R groups are the most important in determining the rate of racemization. The greater the electron withdrawing capacity of the R group of an amino acid the more stable the carbanion will be and the faster the racemization rate will be (Bada and Shou 1980; Bada 1982; Liardon and Ledermann 1986). Smith and Evans (1980) added that the overall effect of an R group depends on the combination of its electron withdrawing or donating characteristics (electronic factors) and its bulk and shape (steric factors). Bulk can hinder proton removal and thus slow the rate. Based on earlier studies Smith et al., (1978) and (Wonnacott 1979) as cited by (Smith and Evans 1980), reported the following order for the rate of racemization of free amino acids in aqueous solution.

Ser > Thr > Asp > Phe > Ala > Glu > Leu >Ile > Val

Effect of pH and buffers

Bada and colleagues (Bada 1972; Bada and Shou 1980; Bada 1982) studied racemization as a function of pH in various buffered aqueous solutions for eight amino acids at elevated temperatures (142 ^oC). For monocarboxylic amino acids, racemization rate was independent of pH in two regions, between pH 3 - 6.5 and between pH 9 - 12. Evidence was presented that the first plateau from pH 3 - 6.5 would widen at lower temperatures and that at 25 ^oC it would extend from pH 3 to pH 9. The pH range of most natural environments is 5 - 8. Thus it was predicted that at the lower temperatures commonly found in nature, racemization would be effectively independent of pH.

Smith and colleagues (Smith, Williams, and Wonnacott 1978) in a study of phosphate buffered solutions of alanine found an increased racemization rate with increasing pH in the range of 6.5 - 8.5, at 125^oC. These results are in agreement with those of Bada and Shou (1980).

Smith et al., (1978) also studied the effect of buffer concentration on the rate of racemization of alanine at constant pH. The racemization rate increased with increasing buffer concentration. They also noted that unbuffered solutions did not maintain a constant pH when heated.

Both groups thought that in biominerals with either a calcareous (shell) or hydroxylapatite (bone) matrix pH effects on the proteins would be minimised due to the buffering effect of the matrices.

Temperature

Temperature influences reaction rates and in general the higher the temperature the faster a reaction will proceed (Pilling and Seakins 1995). Rate constants are temperature specific and are experimentally determined. Reactions are carried out at constant temperature and reactant concentration is measured against time. A first order kinetic relationship will yield a straight line if the natural log of reactant concentration is plotted against time (Pilling and Seakins 1995, p.9). A first order rate implies that for a given temperature a reaction will proceed at a constant logarithmic rate until the reactant is consumed. This linear relationship can be used to predict how long it will take for an observed chemical change to occur. Knowing the rate of a reaction allows it to be used to predict time to completion.

Free amino acid racemization follows reversible first order kinetics (Bada 1972; Bada and Schroeder 1975). In a review paper Schroeder and Bada (1976) summarised their kinetic studies of the previous few years.

The general amino acid racemization equation can be written as:

$$\text{L} - \text{amino acid} \underset{k_D}{\overset{k_L}{\rightleftharpoons}} \text{D} - \text{amino acid} \tag{2.1}$$

Where k_L and k_D are the first order rate constants for inversion at the a-carbon of the L and D isomers. For amino acids with only one chiral centre k_L/k_D is exactly 1, but for those with more than one chiral centre k_L/k_D differs slightly from 1.

The differential kinetic expression for first order reversible racemization as given in equation (2.1) is:

$$-\frac{d[L]}{dt} = k_l[l] - k_D[D] \tag{2.2}$$

The general solution as a function of time is:

$$\ln\left[\frac{1+\left(\frac{D}{L}\right)}{1-K'\left(\frac{D}{L}\right)}\right]_t - \ln\left[\frac{1+\left(\frac{D}{L}\right)}{1-K'\left(\frac{D}{L}\right)}\right]_{t=0} = (1+K')kt \tag{2.3}$$

$t = 0$ allows for the small amount of racemization that takes place during sample preparation. This is particularly applicable to archaeological samples that are prepared by acid hydrolysis.

$K' = \frac{1}{K_{eq}}$ where K_{eq} is the ratio of $\frac{D}{L}$ for a particular amino acid at equilibrium. $K_{eq} = 1$ for amino acids with one chiral centre but for isoleucine, which has two chiral centres $K_{eq} = 1.38$ (Bada 1972).

The full derivation of equation (2.3) on the page before is described in Bada and Schroeder (1972).

In the case of amino acids with one chiral centre eq. 3 can be simplified to:

$$\ln\left[\frac{1+\left(\frac{D}{L}\right)}{1-\left(\frac{D}{L}\right)}\right]_t - \ln\left[\frac{1+\left(\frac{D}{L}\right)}{1-\left(\frac{D}{L}\right)}\right]_{t=0} = 2kt \tag{2.4}$$

It is found experimentally that the majority of reactions have rate constants that follow an Arehhnius type relationship (Laidler 1984):

$$k = A_e^{\frac{E}{RT}} \tag{2.5}$$

Where A_e is the pre-exponential or A factor
E is the gas activation energy
T is the temperature in degrees Kelvin
R is the universal gas constant (8.14 J Mol^{-1} K^{-1})

A_e and E can be determined from experimental data by plotting $\ln(k)$ against $1/T$.

$$\ln(k) = \ln(A_e) - \frac{E}{RT} \tag{2.6}$$

It must be emphasised that the Arrhenius equation is an experimental observation that is followed approximately over a finite temperature range (Pilling and Seakins 1995, p. 21). It is widely accepted that, even though over a limited temperature range plots of $ln(k)$ against $1/T$ are acceptably linear, and may be used for interpolation purposes, unique constants A_e and E do not exist for each reaction even in the case of elementary chemical reactions (Logan 1982). Also, according to Logan, in many cases the gas activation energy (E) is a quantity with no physical significance and should always be differentiated from the activation energy (E_a) of a reaction profile which at best it might approximate. E_a is the energy required for reactants to achieve the transition state.

Bada and Schroeder (1975) used equation (2.6) to predict low temperature rates for a number of amino acids by extrapolation from high temperature rate data.

They reported Arrhenius *E* values as 31.2 kcalmol-1 for several amino acids and a value of 28.6 kcalmol-1 for phenylalanine. Smith et al. (1978) report Arrhenius parameters for racemization of free amino acids from a number of sources including their own work. They report on aspartic acid, alanine, valine, leucine, isoleucine and phenylalanine. The range of Arrhenius E a values given is 24.0 - 31.4 kcalmol-1. They emphasise the importance of accurate parameters since a 1% error in Arrhenius E a will give a corresponding 20% error in the calculated age. They urge caution in applying racemization kinetics of even bound amino acids to geochronology.

Collins and Riley (in press) say that racemization in free amino acids has, unfortunately, little bearing on observed racemization of archaeological biominerals.

2.2.2 Racemization of bound amino acids

Studies have been carried out on a wide range of materials, for example: dipeptides (Gaines and Bada 1988); tripeptides (Capasso, Mazzarella, and Zagari 1991); tetrapeptides (Lura and Schirch 1988); hexapeptides (Brennan and Clarke 1995; Geiger and Clarke 1987); collagen from bone (Elster, Gil-Av, and Weiner 1991); bone (Bada 1985; Prior et al. 1986); teeth (Bada 1987; Helfman and Bada 1976; Masters 1987); mollusc shell (Goodfriend and Meyer 1991; Miller et al. 1992; Wehmiller 1984); eggshell (Brooks et al. 1990; Miller et al. 1992). Rutter and Blackwell (1995) present in table 3 a comprehensive list of references to AAR analyses of a variety of sample types with relevance to Quaternary deposit dating.

The simple mechanism of a planar carbanion does not explain the observed rapid rate of racemization of bound aspartic acid compared with bound serine and threonine (Radkiewicz et al. 1996). Geiger and Clarke (1987) showed that in short peptides the racemization of both aspartic acid and asparagine involved a succinimide intermediate called an amino succinyl residue (Asu). This intermediate can be formed from either L-aspartic acid or L-asparagine. Figure 11 illustrates this process. In the case of L-asparagine a deamidation step (loss of the amino group) occurs before the intermediate forms.

It is now generally assumed that the major pathway for aspartic acid and asparagine racemization in polypeptides and proteins is via a succinimide intermediate (Radkiewicz et al. 1996; van Duin and Collins 1998). It is important to note that both L-aspartic acid and L-asparagine can form D-aspartic acid.

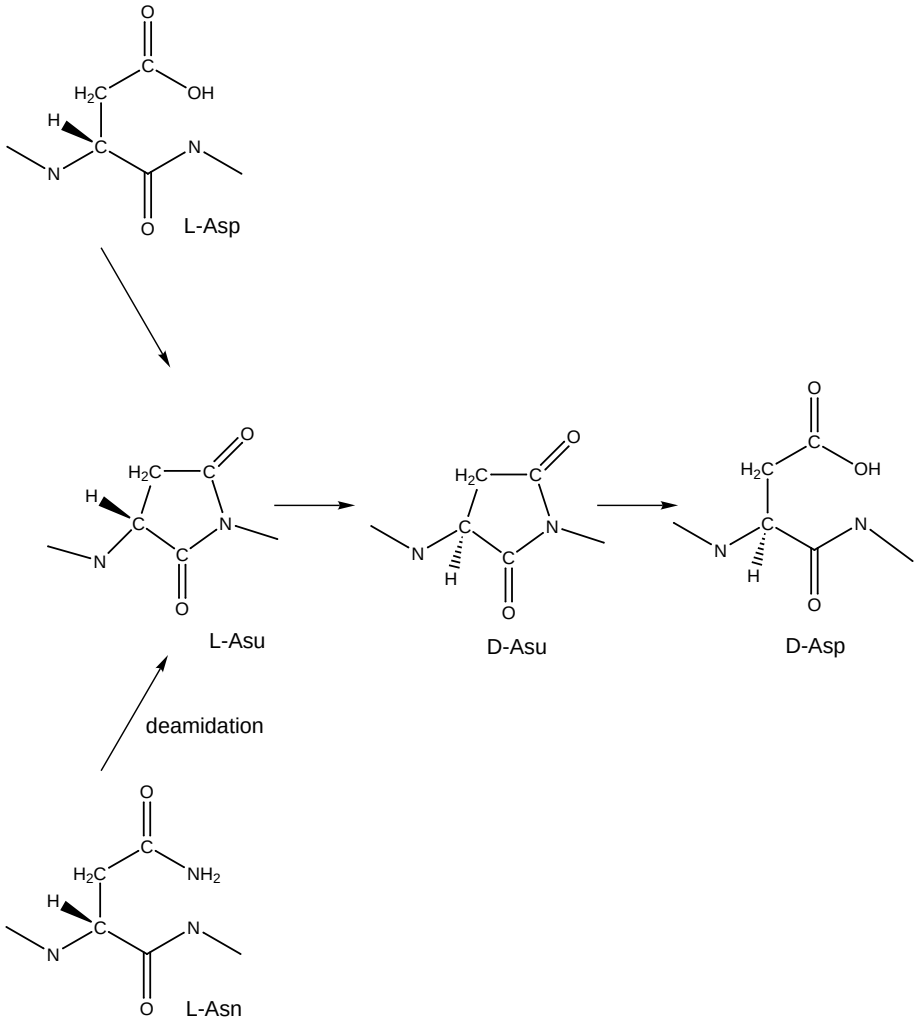


Figure 2.12: Racemization of L-aspartic acid (L-Asp) and deamidation of L-asparagine (L-Asn) via a succinimide intermediate (Asu) to form D-aspartic acid (D-Asp). (After van Duin and Collins (1998)).

Molecular structure

It is generally observed that bound amino acids racemize faster than free amino acids (Liardon and Ledermann 1986; Smith and Evans 1980). Kriausakul and Mitterer (1978) studied the epimerization of isoleucine at 152^oC in various peptides and polypeptides. The relative order of racemization was found to be

N-terminal > C-terminal » interior >free

This order is considered to be an exception rather than a rule (Smith and Evans 1980) but it serves to emphasise the importance of position on reaction rate.

In peptides, bound asparagine racemizes much faster than bound aspartic acid and the influence of neighbouring amino acid residues is considerable (Brennan and Clarke 1993, 1995). In short peptides, replacement of the asparagine residue with aspartic acid resulted in a 34 fold decrease in the rate of succinimide (Asu) formation. In the position carboxyl to asparagine in the peptide the replacement of glycine with a bulky amino acid such as proline or leucine resulted in a 33-50 fold decrease in the rate of deamidation (Geiger and Clarke 1987). The flexibility of the short peptides allows them to assume the angles necessary for Asu formation. The constraints of tertiary structure (folding and coiling) will restrict Asu formation and subsequently racemization in proteins (Collins, Waite, and van Duin 1999).

The sequence of amino acids has very important effects on hydrolysis rates and subsequently racemization rates in particular proteins. Hydrolysis is the break down of a peptide bond in a protein chain and over time shorter and shorter peptide chains form until eventually only free amino acids remain (Mathews and van Holde 1996, p. 141-142). The hydrolysis rate depends on the strength of the individual peptide bonds, which in turn is determined by the characteristics of the amino acid on either side of the bond, the presence of water and the temperature (Collins, Walton, and King 1998; Kriausakul and Mitterer 1978). Aspartic acid forms a relatively weak peptide bond which probably accounts for its rapid hydrolysis and racemization rate (Goodfriend, Hare, and Druffel 1992).

Measurements of aspartic acid from archaeological samples actually include asparagine. During the complete acid hydrolysis of protein, which is a step used in sample preparation, all asparagine moieties decompose into aspartic acid (Mathews and van Holde 1996, p. 157). This combined signal of aspartic acid plus asparagine (Asp +Asn) is referred to as Asx. (Collins, Waite, and van Duin 1999).

Goodfriend (1991) studied patterns of racemization and epimerization of amino acids in a series of radiocarbon dated land snail shells. The radiocarbon series of dates span the last 10,000 years. The D/L ratios of each of the amino acids showed a strong correlation with radiocarbon age but for the faster racemizing amino acids, the relationship was nonlinear. The transformation of the radiocarbon ages to the square root of the age (parabolic kinetics) improved the correlation and linearized the data for several of the amino acids. Aspartic acid, however, still showed a rapid initial rate of racemization against time followed by a much slower rate and Goodfriend suggested that the fast rate might reflect predominantly asparagine racemization while the slower rate reflects predominantly aspartic acid racemization.

Later Goodfriend et al., (1992) reported a similar pattern of aspartic acid racemization against time in a living coral colony. This coral, *Porites australiensis*, produces distinct annual growth bands so the age of the samples was independently determined and 33 samples spanning a 350-year time period were analysed. Although the kinetic pattern was similar in both land snails and coral, coral

showed a very much higher rate of aspartic acid racemization than land snails. The snails, in turn, showed a much higher rate than that in either bones or teeth (Goodfriend 1992). Total amino acid composition of coral was measured and found to be unusually rich in aspartic acid (approximately 50 mol%). This lead to the suggestion that since the aspartic acid peptide bonds are more labile than most other peptide bonds, a high rate of hydrolysis and subsequently a high rate of racemization would occur in proteins with a high proportion of aspartic acid. The change in total aspartic acid racemization rates could reflect different racemization rates among the range of aspartic acid moieties within the degrading protein (e.g. free aspartic acid, C terminally bound aspartic acid, and interior aspartic acid). Over time total aspartic acid racemization would be dominated by the slowest racemizing moiety. Neither the structure of the proteins in snail shells and coral nor the proportion of aspartic acid to asparagine within them is known. Thus, if much of the aspartic acid were actually asparagine even faster initial racemization rates would occur. Commenting on these ideas Brinton and Bada (1995) added experimental support to the suggestion that the initial rapid rate of racemization of Asx observed in land snail shells and coral could be due to the racemization of asparagine in their respective proteins. They heated pure L-asparagine in a buffered solution of pH 7.0 at 100^oC for several days. Aliquots of the mixture were analysed at various intervals, first for the degree of conversion of asparagine to aspartic acid and then for the extent of Asx racemization. The early aliquots were dominated by asparagine and showed a fast racemization rate. Over time the rate slowed and aspartic acid dominated the aliquots. The observed kinetic pattern for Asx racemization was similar to those reported by Goodfriend. Brinton and Bada (1995) suggest that “The observation of an initial period of rapid racemization of Asx followed by a declining racemization rate indicates that a significant fraction of the Asx in these biogenic carbonates may be asparagine."

Goodfriend and Hare (1995) responded to Brinton and Bada (1995) and also reported another example of a “bent kinetic pattern" for aspartic acid racemization, in this case ostrich eggshell heated at 80^oC in the presence of water. Goodfriend and Hare also favour the hypothesis that Asx "racemization actually consists of a rapidly racemizing initial portion dominated by the racemization of asparagine and a slower, later portion dominated by aspartic acid racemization." In addition, Goodfriend and Hare (1995) pointed out the difficulties in estimating the amount of asparagine in proteins and reminded researchers of the dangers of extrapolating from the behaviour of pure Asn in solution at high temperature, to the behaviour of Asn in proteins associated with biominerals at ambient temperature. Work by Collins et al., (1999) supports the idea of Asn contributing to the initial high racemization rate of Asx but they assert that it is the ability of bound asparagine to form Asu that determines the rate rather than racemization of free asparagine. While their model was successful at predicting Asx racemization rates for collagen at high temperature it was not successful at

predicting rates in mineralized collagen. They believe that the model works for small peptides and denatured proteins because the peptide backbones of these are conformationally unrestrained (i.e. free to move and fold). The model does not work for mineralized collagen because it retains secondary structure that is highly sterically restrained and thus the molecule cannot fold sufficiently to allow Asu formation to occur. Asx because of its rapid racemization rate is of great interest to those wishing to date recent samples but results are difficult to interpret because of the complexities of the system.

Collagen is the best-characterised protein that has been used for AAR studies and will serve to demonstrate further some of the complexities of the system. See Voet (1995, p.156-151) for an overview of collagen chemistry and Ramachandran (1976) for a much more detailed account. Collagen is the major protein in bone and teeth. Vertebrate collagens among species are very similar structurally and consist of multiples of three intertwined helical chains of polypeptides. Every third amino acid residue in the primary sequence of collagen is glycine. Proline together with hydroxyproline makes up approximately 25% of the residues. The order of residues can be represented as (Gly - X - Y)$_n$. Hydroxyproline occurs only in position Y but hydroxyproline can occur in position X or Y. Each of these amino acids has a nitrogen containing five membered ring present in its structure (figure 3). When the nitrogen from this ring is part of a peptide bond the bond cannot rotate freely. This gives considerable rigidity to the collagen chain. Alanine makes up approximately 10% of the residues and the remaining approximately 30% is made up of arginine, lysine, aspartic acid and glutamic acid. These latter amino acids are important in the linkages that occur between the chains. Hydroxyproline also contributes to the stability of collagen by hydrogen bonding to water molecules, which in turn create bridges to other helices.

In bovine type I collagen almost half the asparagine and aspartic acid residues occur adjacent to glycine which is the most reactive amino acid combination leading to Asu formation (Collins, Waite, and van Duin 1999). The rate of deamidation in collagen is faster than in other proteins reflecting the abundance of Asn - Gly residues in collagen. Denatured collagen (uncoiled) is expected to show high rates of racemization whereas that bound in triple helix will racemize very slowly. Racemization is unlikely to occur in triple helical collagen below its denaturation temperature (T_m); T_m (demineralized collagen) = 68^oC and T_m (mineralized collagen) = 150^oC. Collins (1999) presents a range of evidence supporting this. The non-helical (i.e. telopeptide) regions of collagen are not coiled and do contain aspartic acid residues which are subject to racemization. Complete racemization of the telopeptide aspartic acid would yield a D/L ratio of 0.09 which, would be achieved in about 500 years at 11^oC (typical U K burial temperature). As collagen degrades over time the triple helix will uncoil and D-Asx will accumulate at frayed ends. Hydrolysis is thought to target flexible regions so chain scission will preferentially release racemized residues, which then can be lost through leaching. Using a simplified model of 'racemization

kinetics' of bone collagen over geological time Collins shows that almost any value of D-Asx can be obtained by varying the rates of collagen denaturation and leaching of the denatured product. In an earlier paper Collins et al., (1995) present a more detailed description of the degradation of collagen.

Effect of pH and buffers

Peptide bonds hydrolyse at pH values of 3.0 or less and acid hydrolysis is commonly used to break down proteins to their constituent amino acids. Complete hydrolysis of all peptide bonds can be achieved by heating the protein in 5 or 6 molar hydrochloric acid (Mathews and van Holde 1996, p. 142).

Bada and Shou (1980) reported that a study of racemization rates of aspartic acid in modern bone fragments were independent of pH in the range 3-9 at a temperature of 96^oC. They suggest that the bone matrix itself acts as a buffer that stabilises the pH of the system. Thus they believe that environmental pH is not important with respect to archaeological bones. Acidic soils and microbial fermentation, however, will contribute to the dissolution of the mineral phase of bone and shell (Collins et al. 1995).

Water

Protein degrades through the hydrolysis of peptide bonds and this process requires water. Bones are porous and the pore size is such that water is retained even in quite arid conditions. (Collins et al. 1995). Hare (1980) noted that if bone is dried at 110^oC and subsequently kept dry it will not undergo hydrolysis and racemization. In contrast when samples of bone were heated in frequently changed water 95% of the amino acids were leached into the water. The D/L ratio of the amino acids in the water increased while it stayed low in the bone samples. The movement of water through archaeological material not only removes amino acids, but also may introduce contaminants. Either situation would lead to spurious D/L ratios.

Although mollusc shells are less porous than bone, leaching is still a possibility and should be considered. Ratite eggshell thus far appears to be a closed system with no evidence of leaching (Collins and Riley in press).

Contamination

Deterioration of both the protein and the mineral components of archaeological bone and teeth is due in part to the action of micro- organisms (Hanson and Buikstra 1987; Nicholson 1996; White and Hannus 1983). Invasion by microorganisms allows contaminants to be introduced into bone and solubilization of bone mineral by microbial metabolites facilitates the leaching of endogenous material from bone (Grupe and Piepenbrink 1988; Piepenbrink 1986).

A study of archaeological teeth from 18^{th} and 19^{th} century burials showed unexpectedly high levels of aspartic acid racemization and the authors suggested

that either biological or chemical degradation of the tooth collagen may have caused these results (Gillard et al. 1990).In two subsequent studies researchers investigated the types of micro organisms that grew in cultures inoculated with soil samples or human source material such as faeces, skin and mouth swabs. They isolated a number of micro-organisms which produced either a collagenase (an enzyme that catalyses the break down of collagen) or a racemase (an enzyme that catalyses the racemization of an amino acid) and pointed out that such enzymes could degrade the collagen in dead teeth or bone (Child, Gillard, and Pollard 1993; Child and Pollard 1991). Some of these micro-organisms were shown to produce collagenases when they were grown at 10^oC, a low temperature typical of many burial sites. Archaeological bone ,and associated soils were used as sources of micro-organisms in a later study by Child (1995). Several species of fungi and bacteria that produced collagenases at low temperatures and that could utilise collagen as a sole nitrogen source were isolated from these sources. Child concluded that while organisms capable of modifying collagen in the burial environment had been found their precise role in the diagenesis of bone was still uncertain. Thus the question of whether or not micro-organisms contribute directly to anomalous racemization rates remains unanswered.

Temperature

As with free amino acids the racemization rate of amino acids in biominerals increases with increasing temperature. Bada (1972), and Bada et al., (1973) carried out rate studies on modern samples heated, in sealed containers, for varying times and at various temperatures. Based on these high temperature studies Bada and his co-workers considered that aspartic acid, alanine, glutamic acid, leucine and isoleucine racemization in bone followed reversible first order kinetics. They extrapolated using Arrhenius plots to estimate rates for low temperatures. Bada (1972) noted a major limitation of using the racemization reaction to date bone was that even small uncertainties in the temperature history of the bone would give rise to large errors in age estimation.

To overcome the problem of inherent uncertainty in the temperature history of sub-fossil bone Bada and colleagues (Bada and Protsch 1973; Bada et al. 1974) developed a “calibration" method for dating bones using aspartic acid racemization. A bone from a site was chosen as a “calibration" sample and both a radiocarbon date and a D/L aspartic acid ratio were determined. These values were substituted into equation (2.4) on page 15 and it was solved for k. The result was an *in situ* k_{asp} value for the site. After substituting in this k_{asp} value equation (2.4) on page 15 was used to determine the age of other samples from the site for which only D/L aspartic acid values had been determined. The major assumption required with this approach is that the average temperature experienced by the “calibration" sample is representative of the average temperature experienced by other samples from the deposit.

More recently researchers have developed calibration curves using a number of

age estimations by independent dating methods (e. g. radiocarbon) and considerable effort has gone into transforming data in various ways to achieve linearity in the relationship between D/L ratio and age. Goodfriend et al., (1992) used a cubic transformation of D/L data for aspartic acid to achieve linearity. Later, power function transformations were used for D/L ratios in both mollusc and ostrich shells (Goodfriend and Hare 1995; Goodfriend 1996). Such transformations allow a strong correlation with time but do not explain the observed kinetics.

2.3 Racemate ratio measurement

2.3.1 Sample preparation

The methods of preparing bone, shell, or eggshell involve at least three steps; the cleaning of the surface of the sample, the removal of the biomineral matrix and the hydrolysis of the protein into individual amino acids for analysis.

One of the simpler methods is described by Bada et al., (1999). Pieces of mammalian bone and pieces of ostrich eggshell were used. Samples weighing about 100 mg were manually cleaned, then washed for a few minutes with dilute hydrochloric acid and rinsed twice with doubly distilled water. The washing and rinsing steps were repeated two or three times. Samples were then dissolved in 6 molar hydrochloric acid and hydrolysed for 24 hours at 100^oC. The dissolution step breaks down the biomineral and the hydrolysis step cleaves the peptide bonds in the proteins thus releasing the individual amino acids. The samples were evaporated to dryness, redissolved in doubly distilled water and desalted using cation exchange chromatography. Miller et al., (1992) used a slightly more involved method for ostrich eggshell preparation.

More complex methods developed to obtain a range of molecular weight fractions from bone collagen are described by Kimber and Hare (1992). The bone was ground in a liquid nitrogen cooled mill and the powder was dissolved in 1 molar hydrochloric acid with stirring at 4^oC overnight. Ultrafiltration through various membranes was used to remove salts and to collect different molecular weight fractions from the samples.

Van Klinken and Mook (1990) give a detailed procedure for the preparation of collagen from bone which includes a gelatinization step but does not use ultrafiltration.

Collins and Galley (1998) compared different methods of bone preparation and concluded that demineralisation should be carried out at low pH (using hydrochloric acid) low temperature (4^oC) and that excessive grinding should be

avoided. They found that an alternative demineralisation method using ethylenediaminetetraacetic acid (EDTA) at a pH of 8 damaged the collagen more than the mineral acid extraction.

Goodfriend (1991) described the preparation of land snail shells. These were mechanically cleaned using a combination of dental tips and ultrasonication and were ground in a mortar and pestle after etching with dilute hydrochloric acid. The powdered shell was hydrolysed in 6 molar hydrochloric acid, under nitrogen, at 100^oC. Desalting was achieved by the addition of hydrofluoric acid and the subsequent precipitation of calcium fluoride.

A method for the analyses of marine molluscs is described by Murray-Wallace (1993). Shell samples were not ground and hydrolysis was carried out in 8 molar hydrochloric acid, at 110^oC.

2.3.2 Analysis of the hydrolysate

Two common methods for separating and quantifying amino acids are gas chromatography (GC) (Blau 1981) and high pressure liquid chromatography (HPLC) (Annan 1981). The latter technique is sometimes called high performance liquid chromatography.

Chromatography is a general term applied to a wide range of related techniques used to separate mixtures into their components. For a short overview, aimed at the non-specialist, see Pollard and Heron (1996, p. 66-72). Each chromatographic system has a stationary phase (either a solid or a liquid) and a mobile phase (either a liquid or a gas) into which the mixture to be separated is incorporated. The mobile phase, called the solvent, carries the mixture, called the solute, through the system. At any one time solute molecules are distributed between the mobile and the stationary phases and the amount of time spent in each depends on the physical properties of the molecule and its affinity for the stationary phase. The temperature of the system, the length of the column and the flow rate of the mobile phase also influence how long molecules stay on the column. Separation is effected by the careful manipulation of these variables. Samples need to be injected onto a column and a detection system is needed at the end of the column. Data are usually presented as a chart where peak height is a function of time and the area under each peak is proportional to the amount of each component in the mixture. Detailed accounts of chromatography can be found in Smith (1988) and Braithwaite and Smith (1996).

In a non chiral environment optical isomers exhibit identical physical and chemical properties (Frank, Nicholson, and Bayer 1978). Originally GC and HPLC systems were non chiral and could not separate optical isomers (Allenmark 1988, p. 35). The reaction of D and L enantiomers with an optically pure reagent produces

diastereomeric derivatives. Since diastereomers are not optical isomers and have different properties they can be separated by common chromatographic techniques (Allenmark 1988, p. 51). The development of a range of chiral phases has increased the available separation options (Barrett 1985).

GC is described in general terms in Pollard and Heron (1996, p. 68-69). For in depth coverage of GC see Braithwaite and Smith (1996, p. 165-257) and Baugh (1993). In gas chromatography the mobile phase is a carrier gas such as helium or nitrogen. The stationary phase is either a column packed with a fine grained inert support material coated with a liquid stationary phase or a much smaller diameter capillary column with a liquid stationary phase bonded to its internal walls. To be successfully separated by GC compounds must be thermally stable and able to be volatilised. The making of chemical derivatives of the original compounds can increase the volatility of some solutes. The column is housed in an oven and by increasing the temperature incrementally the components in the mixture can be eluted (washed out) from the column, one at a time. A flame ionization detector (FID) is a commonly used detector in GC analysis.

Engel and Hare (1985) described many aspects of the separation of amino acids by GC. As amino acids are insufficiently volatile they are converted to esters by reaction with an alcohol such as isopropanol. The esters are then acylated by the addition of an anhydride such as pentafluoropropionic anhydride. The resultant acylamino acid esters are dissolved in a suitable solvent for injection into a GC column. Diastereomeric derivatives can be prepared by esterification with an optically pure alcohol such as D-butan-2-ol. Optically pure alcohols are, unfortunately, expensive to buy and very time consuming to make. Engel and Hare considered the major breakthrough in the use of GC to separate enantiomers was the development of optically active (chiral) stationary phases. An important commercial column (Chirasil-Val) was developed by Frank et al. (1977). They were able to resolve the enantiomers of the twenty amino acids found in protein. Details of the development and chemistry of chiral stationary phases for GC are covered in Allenmark (1988, p. 75-89). Murray-Wallace et al., (1996) used a Chirasil-L-Val column to analyse N-pentafluoropropionyl D, L-amino acid 2-propyl esters derived from amino acids extracted from mollusc shells. They reported racemate ratios for six different amino acids; alanine, aspartic acid, glutamic acid, phenylalanine, valine and leucine.

There is a description of HPLC for the non-specialist in Pollard and Heron (1996, p. 69-71). For more detail see Braithwaite and Smith (1996) pages 258-365, Smith (1988) and Lim (1986). The stationary phase is a column containing either a solid packing, or a solid packing coated with a stationary liquid phase. The mobile phase (the eluant) is a liquid, forced under pressure through the column. There are usually two solvent reservoirs and the eluant composition is determined by the proportion of each solvent delivered to the column by a high pressure pump. The sample mixture is applied to the top of the column and

carried through it by the eluant. It is possible to have a mobile phase of either constant composition (isocratic elution) or changing composition (gradient elution). Changing the composition of the eluant in a controlled manner provides a powerful tool for more difficult separations. Compounds that absorb light at a particular wavelength can be detected by an ultraviolet/visible spectrophotometer. Compounds that absorb light of a particular wavelength and re-emit it at a longer wavelength can be detected by a spectrofluorometer. The re-emission of light is known as fluorescence. Fluorescent techniques are generally more sensitive than absorption ones (Hare, St John, and Engel 1985).

There are several different modes of separation used in HPLC and these are summarised in Lim (1986) and Braithwaite and Smith (1996) pages 260-266. Ion exchange columns are used to separate amino acids (Hare, St John, and Engel 1985).

Amino acids have to be derivatized for detection at analytical levels and it is possible to derivatize before or after separation on the column. A discussion of the various types of derivatives, derivatization techniques and the resolution of D and L amino acid enantiomers can be found in Perrett (1985). Commonly for amino acids a post column reaction with the fluorescent compound ortho-phthaldialdehyde (OPA) is used. L-isoleucine and D-alloisoleucine are separated well in this system. Since they are diastereoisomers and not enantiomers their physical properties are slightly different from one another. To resolve D and L enantiomers of amino acids other strategies must be employed. Hare and Gil-Av (1979) used a chiral copper-proline complex in the mobile phase and were able to separate a number of non-derivatized amino acid enantiomers. After separation the amino acids were reacted with OPA and measured with a fluorometer.

Reverse-phase HPLC uses a nonpolar organic stationary phase such as octadecyl-silane. Aswad (1984) produced fluorescent diastereomeric derivatives of aspartic acid which were then separated on a reverse phase HPLC column. The fluorogen used is an adduct of ortho-phthaldialdehyde with an optically active thiol N-acetyl-L-cysteine.

Marfey's reagent (1,5-difluoro-2,4-dinitrobenzene)has also been reacted with amino acids to produce diastereoisomers that can be separated on a reverse phase HPLC column and detected by ultraviolet absorption spectrophotometry (O'Connell, Hedges, and van Klinken 1997; Szókán, Mezö, and Hudecz 1988).

Kaufman and Manley (1998) compare a number of methods and describe a new method using reverse-phase HPLC and pre-column derivatization of amino acids with OPA and the chiral thiol N-isobutyryl-L-cysteine (IBLC). They report good resolution of the D and L enantiomers of nine amino acids and their results compare favourably with a number of GC results. Their instrumentation is fully automated and they use commercially available reagents and equipment.

Chapter 3

Applications

3.1 General

AAR is used to estimate the age of various types of archaeological material. The epimerization of isoleucine and the racemization of aspartic acid are the most commonly used amino acids. Isoleucine epimerizes slowly and is suited to studies where the age of the artefact is expected to be 20,000 to 200,000 years old at temperatures of 18-24^oC (Brooks et al. 1990). Where ambient temperatures are lower the range shifts and isoleucine is suitable for even older samples

Aspartic acid racemizes quickly and is the amino acid most researchers have used for younger samples a few thousand years old to 80,000 years depending on temperatures (Bada 1985). Goodfriend and colleagues (Goodfriend, Hare, and Druffel 1992; Goodfriend 1992) show it can be suitable for samples as recent as 300 years old but only in conjunction with reliable alternative dating methods as a comparison. Due to the complexities of the kinetics and diagenesis of aspartic acid and asparagine there is still considerable debate as to its reliability (Bada 1990; Collins, Waite, and van Duin 1999; Marshall 1990).

3.1.1 AAR and Bone

Much of the initial controversy over AAR resulted from estimations of the age of human remains in North America. A detailed review and analysis of the evidence can be found in Pollard and Heron (1996, chapter 5). Pollard and Heron, quoting Meltzer (1989) described a “late school" of archaeologists who believed that the first people came to America between 14,000 and 12,000 uncalibrated radiocarbon years before present (BP) and an “early school" of archaeologists who believed that the first people came before 15,000 and possibly as early as

35,000 uncalibrated radiocarbon years BP.

Bada et al., (1974) stunned the archaeological community with their analysis of Paleo-Indian skeletal material from Del Mar, California. They reported aspartic acid racemization dates as old as 48,000 years BP. The Laguna skull with an estimated radiocarbon age of 17,150 ± 1,470 years was used as the “calibration" sample. A subsequent publication (Bada and Helfman 1975) reported more samples from California with ages in the 35,000 to 48,000 year range together with the even more startling claim of a 70,000 year old skeleton from Sunnyvale. These results put human inhabitation of the Americas 35,000 years earlier than anybody had suggested previously. Controversy regarding these claims continued for some time.

In the 1980s evidence mounted that there were serious problems with the AAR dates. The Sunnyvale skeleton and a Del Mar tibia were re-dated using uranium series dating (Bischoff and Rosenbauer 1981). This resulted in dates of 8,000 to 9,000 years BP for Sunnyvale and 11,000 to 11,500 for Del Mar. Conventional plus accelerator mass spectrometry (AMS) radiocarbon dating (Taylor et al. 1983) was carried out on the Sunnyvale skeleton and results of between 3,600 and 4,850 years BP were obtained. The original amino acid extractions from the racemization studies of the Paleo-Indian remains were independently dated by the AMS radiocarbon method at the Oxford Radiocarbon Accelerator Unit of Oxford University and the NSF Accelerator Facility for Radioisotope Analysis, University of Arizona. Bada et al., (1984) published the Oxford results and Taylor et al., (1985) published a paper combining the results from both laboratories. The Oxford dates were all between 4,500 and 8,500 years BP and the Arizona dates were between 3,000 and 6,600 years BP. Bada et al., (1984) stated that the Oxford AMS results “reveal no clear relationship between the radiocarbon ages of the various skeletons and the extent of the aspartic acid racemization" page 443. They did note that there appeared to be a direct relationship between the extent of racemization and the level of preservation of collagen in the bones. Those samples with the most racemization had the lowest amino acid content and this poor preservation of protein would contribute to anomalous AAR results.

Later, based on AMS radiocarbon dates, Bada (1985) calculated a new value for k_{asp} for the Californian samples. He used the Laguna skull and the Los Angeles Man skeleton as “calibration" samples for this. Using the revised value for k_{asp} he recalculated the AAR dates of the other Paleo-Indian samples. They all fell within the Holocene but had much larger error estimates than those of the AMS values. Although Bada claimed consistency between AAR and AMS dates others (Pollard and Heron 1996, p. 228) argue that the dates only appear to be consistent with one another because of the unacceptably large error range associated with the AAR dates. Pollard and Heron also point out that there is poor concordance between the conventional and the AMS radiocarbon dates and there is no concordance between the uranium series dates and any of the other dates either.

At best three of the four methods put the bones in the Holocene.

Stafford et al., (1991) discussed AMS radiocarbon dating in bone at the molecular level. They dated a number of fractions (ranging from insoluble collagen to individual amino acids) from each of a selection of differentially preserved mammoth and human bone. Age estimates from the fractions within a bone were consistent if it was well preserved. They concluded “that a poorly preserved Pleistocene-age fossil >11,000 years in age would go unrecognised because it would yield a Holocene 14C date" page 54. Thus the final irony is that the poorly preserved Californian Paleo-Indian bones would return Holocene 14C dates even if they were actually Pleistocene. The state of preservation of the bone appears to be as important an issue for radiocarbon dating as it is for AAR dating.

The use of AAR for the dating of bone has remained controversial. “Now, we know better than to attempt to date bones and teeth with amino acids, but the exercise taught us much about the geochemistry of bones, teeth and proteins" (Rutter and Blackwell 1995, p. 147). Most of the kinetic work on bone is high temperature and essentially a closed system, as the hydrolysis is carried out in sealed vials under nitrogen. Archaeological bone on the other hand is an open system. The bone matrix is porous so amino acids can be leached out and contaminants can be introduced. Some workers, however, consider that if bones are well preserved, have a known temperature history and have not been exposed to leaching they can be used for relative dating (Johnson and Miller 1997). Many researchers consider that the best hope for bone is to analyse large molecular weight collagen fragments only (Elster, Gil-Av, and Weiner 1991). Even this approach is problematic as collagen with intact tertiary structure is not very susceptible to racemization as it is too sterically constrained (Collins, Waite, and van Duin 1999).

3.1.2 AAR and Teeth

Amino acid racemization in teeth can also be difficult to interpret. Although amino acids in dentine and enamel are sequestered from the environment contamination, particularly from bacterial sources, may be a problem (Child 1995). Racemization begins in a tooth immediately after it is fully formed and continues throughout the life of the host (Helfman and Bada 1976). Racemization continues after death but probably at a reduced rate due to the presumed reduction in ambient temperature (Carolan et al. 1997). Human body temperature is 37^oC and an average temperate burial environment is approximately 10^oC (Child 1995). It is assumed that as long as burial temperature is low and time since death reasonably short, post mortem racemization will be negligible. AAR of aspartic acid in teeth has been used to estimate age at death of some mammals for example humans (Gillard et al. 1990) and rats (Ohtani et al. 1995).

Rat molars were shown to have a 10 times faster aspartic acid racemization rate than human teeth (Ohtani et al. 1995). These authors suggest that rat dental collagen is similar to that in humans but the body temperature difference alone, 37^oC in humans compared to 38-39^oC in rats, is not large enough to account for the 10 fold racemization rate difference. They were unable to explain this rate difference.

Modern teeth of known age, obtained from dentists, showed increased aspartic acid racemization correlating with increasing age in both dentine and enamel (Bada 1984). Gillard et al., (1991) used aspartic acid AAR to age archaeological teeth from 18th century vaults at Christchurch, Spitalfields, in London England. The AAR estimates from the archaeological teeth showed ages of ± 24 years at the 95% confidence level compared to estimates of ± 4 years for modern samples.

A more recent analysis (Carolan et al. 1997) using calibration curves derived from modern tooth racemization data to estimate age at death in early 19^{th} century dental remains showed that the regression lines for ancient and modern samples were not parallel. Within the archaeological samples there was a tendency for young individuals to be estimated as too old and old individuals to be estimated as too young. This difference cannot be explained at present and appears to reflect a fundamental difference in the racemization behaviour of dental proteins in pre and post mortem environments. In light of this Carolan et al., (1997) warn against using modern calibration samples for archaeological material. While AAR analysis of teeth is of limited use in archaeology it is a useful tool in modern forensic research.

3.1.3 AAR and Molluscs

A considerable amount of research has focussed on AAR in mollusc shells. Leaching is less of a problem with the calcium carbonate based shells than with bones (Miller and Hare 1980). Attempts are usually made to extract amino acids from internal portions of shell from which, the outer layers have been dissolved away under controlled conditions in the laboratory (Johnson and Miller 1997). It has been found that racemization rates vary among genera and have to be established for each organism of interest (Lajoie, Wehmiller, and Kennedy 1980).

This has been achieved with varying degrees of success. For example Kimber and Griffin (1987) studied three molluscs, *Ostrea angasi, Anadara trapezia* and *Katelysia rhytiphora* and observed that racemization is varied among them. Of the three they preferred *Katelysia* for dating. Within these genera the amino acids D-valine, and D-alloisoleucine showed a steady increase with time which approached linearity and because of this were considered to be the most reliable amino acids to use for AAR. Aspartic acid racemization was much more variable

and caution was advised in its use particularly with respect to *Ostrea* shells. In contrast Goodfriend (1992) from a study of aspartic acid racemization in land snails concluded that it was possible to date specimens less than 300 years old.

In an earlier study of the racemization patterns of six amino acids Goodfriend (1991) concluded that isoleucine and glutamic acid racemization approximated first order kinetics and are useful for older samples. The faster racemizing amino acids with the exception of aspartic acid approximated parabolic kinetics i.e. when the D/L ratios were plotted against the square root of the radiocarbon age of the samples the relationship approximates a straight line. Goodfriend states that aspartic acid, which showed a very high initial rate of racemization, is particularly useful for young samples. Little is known about the amino acid sequences of proteins in the molluscs (Collins, Waite, and van Duin 1999) thus making explanations of unusual kinetics difficult. It is known that different genera have different proportions of the various amino acids in their proteins (Miller and Hare 1980) and it is assumed that this leads to differences in racemization rates (Johnson and Miller 1997). The kinetic model assumptions are based on empirical relationships and not founded in theory (Goodfriend 1991).

3.1.4 AAR and Eggshells

Ratite eggshells such as emu and ostrich appear to have excellent characteristics with respect to amino acid racemization. They are essentially closed systems (Johnson et al. 1992) as cited by Johnson and Miller (1997) which are not susceptible to leaching. Therefore in the shell there is neither loss of protein hydrolysis products nor contamination from the environment. Brooks et al., (1990) showed that the epimerization of L-isoleucine in ostrich shell at high temperatures follows first order reversible kinetics almost up to racemic equilibrium and used it to date sites beyond the range of radiocarbon dating. Similarly Miller et al., (1997) showed that isoleucine epimerization in emu eggshell follows first order reversible kinetics and thus can be used reliably for AAR dating. Since ratite shell is commonly found in African and Australian archaeological sites there is considerable potential for dating using isoleucine epimerization.

This potential was demonstrated by Miller et al., (1992), who used isoleucine epimerization in ostrich eggshell to date strata at Border Cave, South Africa. Excavated ostrich eggshell fragments were analysed from each stratum of the site. The shell showed increasing alloisoleucine/isoleucine ratios with increasing stratigraphic age. The reaction was calibrated in the upper levels with radiocarbon dates on associated charcoal. One AMS date of eggshell confirmed the age association of the charcoal and the shell fragments. Anatomically modern human skeletal material from a well controlled stratigraphic context was recovered from the cave. Based on its association with dated eggshell Miller et al., suggest

occupation of the site by anatomically modern humans as early as 100,000 years BP.

Racemization rates in the eggshells of New Zealand ratites (moa and kiwi) have not been studied, but are likely to provide similar results to African and Australian ratites. While isoleucine epimerization in ratite eggshells appears to be a reliable dating method, it is of course limited to dating material in the 20,000 to 200,000 year age range (Brooks et al. 1990). Unfortunately this rate is too slow for applying to issues in New Zealand prehistory, and to date, little is known about the faster racemizing aspartic acid in ratite egg shell.

3.1.5 AAR and Palaeotemperature estimations

If the racemization rate for a particular system in any given material is known then AAR can be used to estimate the thermal history of any cross-dated samples. However, good independent age controls such as 14C dated samples and a reliable kinetic model for racemization rates are needed (Johnson and Miller 1997).

Although aspartic acid racemization has been used for palaeotemperature estimation (Schroeder and Bada 1973), such estimates from bone are now considered suspect (Rutter and Blackwell 1995). Miller et al (1997) successfully used isoleucine/alloisoleucine epimerization ratios in emu eggshell together with ^{14}C dated eggshell to reconstruct temperature history for the for the 45,000 to 16,000 BP period in inland Australia. Until racemization rates of aspartic acid in ratite eggshell are studied AAR is of limited value for reconstructing temperature history of more recent periods.

3.2 Applications to New Zealand

From the above evidence it seems that AAR has limited application to New Zealand archaeology. Given that samples of interest would be 2000 years old at the most and many would be less than 800 years old, aspartic acid is the only amino acid likely to show measurable levels of racemization. Aspartic acid and asparagine exhibit complex kinetics leading to considerable difficulty in the interpretation of results.

As part of a study of settlement in Aotearoa/New Zealand it was thought that AAR might be successfully used to date bones from the rat *Rattus exulans*. Work such as that of Collins et al., (1999) who present convincing documentation that racemization of Asx in bones is inherently unpredictable because it depends on the rate of collagen denaturation combined with leaching of the denatured

product makes it difficult to justify pursuing this technique with bone.

It might be possible to develop AAR methods for New Zealand molluscs but a large investment of both time and money would be required to do even a pilot study and it could result in the species investigated giving no reliable trends anyway.

The most promising material in New Zealand to work with is ratite eggshell. If kiwi and moa eggshells behave similarly to those of emu and ostrich they could provide a tool for AAR dating. The time frame of the last 2000 years for dates of archaeological interest in New Zealand means that aspartic acid would be the appropriate amino acid to use. Very little is known about the protein structure in ratite eggshell and differences in primary sequence can alter the rate of Asu formation by two orders of magnitude (Collins, Waite, and van Duin 1999). Goodfriend and Hare (1995) show that Asx racemization in ostrich eggshell heated at 80^oC has complex kinetics, similar to that seen in land snails (Goodfriend 1992). The extrapolation of high temperature rates to low temperatures is known to be problematic (Collins, Waite, and van Duin 1999). A pilot study would be necessary and a reliable relationship between racemate ratio and time could remain elusive.

3.2.1 Conclusion

The evidence presented here indicates that AAR has limited potential for chronometric control in New Zealand archaeology. While some materials such as ratite shell or mollusc shell could possibly be dated via AAR it is already possible to provide reliable dates for this material through radiocarbon dating. Any future development of AAR in New Zealand for analytical purposes would require significant research investment.

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