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Chapter 2

Application of the Amino Acid Racemisation Reaction in Quaternary Science

2.1 INTRODUCTION

Racemisation is one of many chemical reactions that amino acids undergo during diagenesis. The reaction slowly interconverts L-amino acids of fossil protein residues to D-amino acids. Because the temperature dependency of racemisation rates may be modelled, the reaction has versatile applications in the study of Quaternary environments. With an understanding of the thermal history of a specimen, the extent of the reaction may be interpreted in terms of time to determine the age of an amino acid-bearing fossil. Alternatively, the integral of the temperatures to which a fossil has been exposed can be determined with independent numeric age control. A variety of variables influence the rate and extent of amino acid racemisation in natural systems, dominantly temperature but also sample type, stratigraphic context and amino acid, and are important in both geochronological and palaeothermometry applications.

This chapter reviews the application of the amino acid racemisation reaction in the Quaternary sciences with particular emphasis on the factors that influence the apparent rate and extent of the reaction. Early reviews are provided by Schroeder & Bada (1976) and Williams & Smith (1977). More recent reviews include Miller & Brigham-Grette (1989), Kaufman & Miller (1992), Murray-Wallace (1993) and Wehmiller & Miller (2000). These reviews illustrate the strengths and weaknesses of the application, stress the importance of appropriate field and subsampling protocols, and tend to emphasise

the extraction of chronological information from the extent of amino acid racemisation, rather than information on palaeotemperatures. Two volumes edited by Hare *et al*. (1980) and Goodfriend *et al*. (2000) are collations dedicated to the chemistry of amino acids in geological environments.

The first section of this chapter begins by outlining the origin of the application of amino acid racemisation to Quaternary studies, the stereochemistry of biologically active amino acids and the preservation of these molecules over geological time. The factors that control the rate and extent of amino acid racemisation are then reviewed. These factors include the amino acid R-group, temperature, the position of amino acids in polypeptide chains, and the preservation state of protein residues. Finally, the methods used to determine the age and the temperature to which protein-bearing fossils have been exposed are explained.

In the latter sections of this chapter the knowledge of amino acid racemisation in avian eggshells is reviewed critically. The factors that differentiate the study of amino acid racemisation in eggshells from other sample types in which the reaction is commonly investigated are identified and evidence for the ability of eggshells to preserve their indigenous amino acids is examined. The methods used to estimate numeric ages and palaeotemperatures from the extent of isoleucine epimerisation in avian eggshells is then reviewed.

2.2 AMINO ACIDS IN THE GEOLOGICAL RECORD

A protein is a polypeptide chain, or interacting polypeptide chains, each consisting of a precise sequence of amino acids. Each amino acid consists of an α-carbon atom with an

amino (NH_3^+) and carboxyl (COO^-) group attached, along with a hydrogen atom and a characteristic side chain, termed the R-group. There are twenty common protein amino acids, each distinguished by its R-group. These amino acids and their respective abbreviations are listed in Table 2.1 (the three letter abbreviations are used herein). The four different groups attached to the α-carbon of an amino acid can be arranged to form one of two stereochemical, or mirror image forms. With few exceptions, life is stereoselective with the majority of biologically produced amino acids being left-handed, that is, they rotate polarised light to the left. Amino acids produced *in vitro* are racemic, consisting of equal concentrations of the right (*dextro*, or D-amino acids) and left-handed (*laevo*, or L-amino acids) stereoisomers (Miller, 1955). Similarly,

Table 2.1 The twenty common protein amino acids (Budavari *et al.*, 1996).

[a]Upon hydrolysis, asparagine (Asn) and glutamine (Gln) residues are converted to Asp and Glu. The abbreviations shown in brackets are used to signify combined Asp plus Asn, and Glu plus Gln residues, respectively. The formulae and molecular weights shown in brackets refer to Asn and Gln.

extraterrestrial amino acids detected in meteorites are predicted to be racemic therefore a prevalence of left-handed amino acids is a means of identifying terrestrial contamination (Cronin & Pizzarello, 2000).

As well as influencing protein function, the characteristic R-group of each amino acid is an important determinant of protein structure because the R-group determines the rotational flexibility between two adjoining amino acids in a polypeptide (Mandelkern, 1983). When sequences of amino acids are repeated, polypeptides assume ordered three-dimensional structures such as the α-helix and β-sheet. By necessity, all the amino acids in an α-helix must be left-handed. The inclusion of D-amino acids disrupts the rotation of the polypeptide and the spacing of amino acid residues, inhibiting correct folding of the protein. The results of Kim *et al.* (1997) indicate that the absence of an enzyme known to initiate the conversion of D-Asp to its left-handed form *in vivo*, thereby stereochemically 'repairing' the amino acid, retards growth and produces fatal seizures in laboratory mice, dramatically illustrating the necessity of L-amino acids for normal protein function.

Despite the predominance of L-amino acids of biological origin, varying concentrations of D-amino acids have been found in a large number of organisms. For example, D-amino acids are synthesised by many gram-positive bacteria for inclusion in cell wall proteins (Friedman, 1999). Extreme examples cited by Nagata (1999) include the marine macroalgae *Hizikia fusiformis* which contains more D-Asp than L-Asp, and D-Ala is ten times more abundant than L-Ala in the foot tissues of clams. In humans, D-Asx and D-Ser residues accumulate in metabolically inactive tissues including eye lenses (Masters *et al.*, 1977), enamel (Helfman & Bada, 1975) and dentine (Helfman &

Bada, 1976). The accumulation of D-Asx in human teeth may be used to determine age at death due to the rapid and predictable racemisation of Asx in teeth over the course of a human lifetime. Majer *et al*. (1999) have implicated the racemisation of Asp and Ser residues of long-lived proteins in the formation of amyloid plaques, aggregations of ‘misfolded’ proteins that result from diseases such as Alzheimer’s.

The study of amino acids in fossils originated from research on the preservation of organic compounds in geological environments. Philip Abelson of the Carnegie Institution of Washington conducted the first survey of amino acids in fossils in 1953. Abelson (1954) reported that the amino acids most stable at high temperatures in the laboratory were also present in fossils, the oldest of which was a 360 million year old Devonian fish. Using cation-exchange columns, Hare & Abelson (1967) were able to separate L-isoleucine from its epimer D-alloisoleucine because these two molecules differ in their hydrophobicity. Analysing fossil molluscs, these authors observed a correlation between the ratio of these epimers and time. Further research by Hare & Mitterer (1968) demonstrated that changes in the amino acid composition of mollusc shells over geological time could be induced in the laboratory by heating at high temperatures, implying that the diagenesis of amino acids took place in a regular manner, without intolerable overprints produced by processes such as contamination or amino acid loss. Supported by this observation, these authors were the first to estimate an age for a fossil based upon the stereochemistry of amino acids.

Amino acids are preserved in the geological record if they fail to enter the biotic nitrogen cycle. The nitrogen of polypeptides represents the major pool of organic nitrogen readily utilised in the biosphere (Bada, 1998). Pools of proteins that are

isolated from the biosphere and undergo limited biodegradation are of great interest to biogeochemists. A common environment for the preservation of amino acids is the carbonate skeleton of aquatic invertebrates such as molluscs and foraminifera. The amino acids that once comprised the proteins that precipitated the carbonate and gave it strength are isolated from biotic decomposition for thousands of years. Under exceptional circumstances amino acids may fail to be incorporated into the nitrogen cycle for millions of years. Amino acids, along with fragments of DNA, have been detected in dinosaur bones and amber-entombed insects (Bada *et al.*, 1994). Because the activation energy of aspartic acid racemisation is similar to that of DNA depurination, Asx D/L ratios provide a criterion for assessing DNA preservation (Bada *et al.*, 1994, Poinar *et al.*, 1996).

For amino acids, 'diagenesis' refers to the sum of the processes influencing these molecules during their residence in geological environments following the death of the host organism (e.g. bone collagen) or its disuse of the molecules (e.g. avian eggshell proteins), ultimately degrading complex proteins into simple hydrocarbons. The organic residues present in fossils are not proteins in a biochemical sense. Proteins are organic polymers with specific amino acid sequences and precisely defined three-dimensional configurations. The amino acids in fossils are subject to a variety of degradative reactions including racemisation, decarboxylation, deamination and condensation, and are located in polypeptides of various molecular weights distinct from their precursor proteins. It is due to these diagenetic reactions that antibodies raised against modern protein complexes exhibit reduced immunological reactivity in ancient residues (Muyzer *et al.*, 1988).

2.3 AMINO ACID RACEMISATION

2.3.1 Mechanism

Although reporting the extent of racemisation as %D [= D/(D + L)] or as a concentration (D-amino acid/mg) is equally valid, the convention in the geological sciences is to report D/L values or ratios. Although, technically R and S should replace D and L, respectively (Child *et al.*, 1993), the use of the latter terms persists in the geological literature. Racemisation is the chemical reaction that interconverts a stereoisomer of an asymmetric molecule, such as an amino acid, into its alternative enantiomeric, or mirror image, form (Fig 2.1). For amino acids that feature two chiral carbon atoms, such as isoleucine and threonine, the reaction may take place at either α or β carbon centres. However, for isoleucine at least, epimerisation takes place almost exclusively around the α-carbon under conditions found on earth (Bada *et al.*, 1986). In these multiasymmetric amino acids the observed reaction is termed epimerisation.

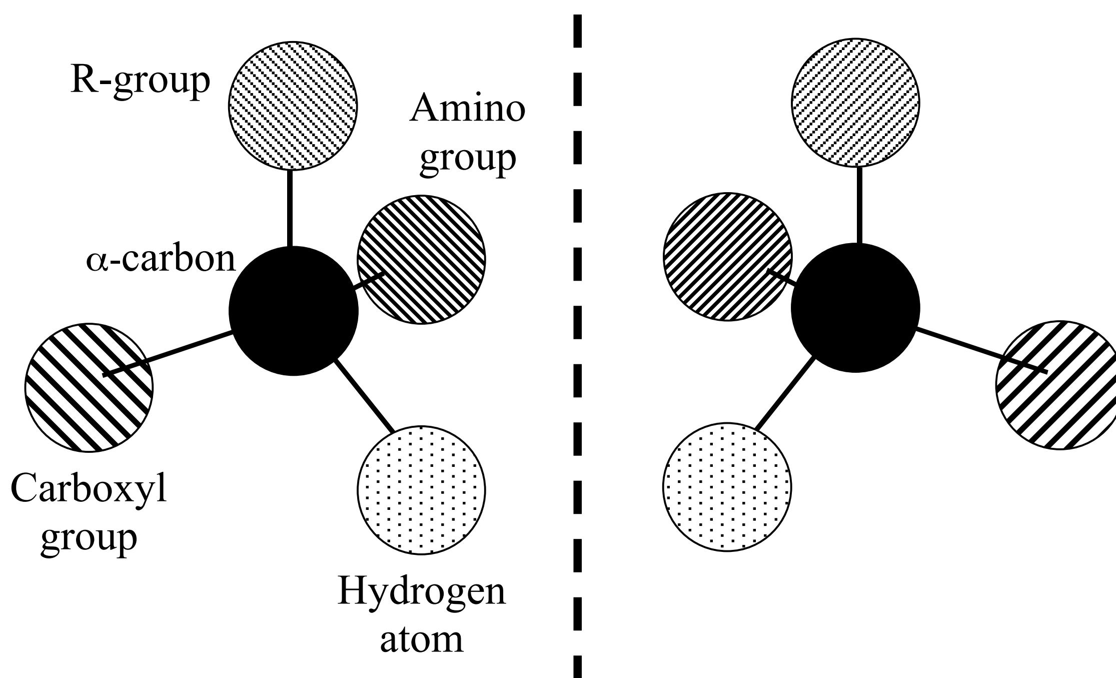

Figure 2.1 Stylised depiction of amino acid enantiomers. An L-enantiomer is shown on the left and D-enantiomer on the right. The two forms cannot be superimposed in three-dimensional space.

The rate at which racemisation proceeds varies between amino acids according to the ability of R-groups to stabilise the carbanion intermediate. The reaction is rapid in amino acids that feature an R-group highly effective at stabilising the carbanion intermediate. The carbanion intermediate is the molecule formed when the hydrogen side chain is abstracted from an amino acid. In an ideal system, upon re-addition of the hydrogen atom to the carbanion intermediate there is an equal probability of L- and D-amino acid formation, accordingly the forward and backward reaction rates are equal. Under these conditions the D/L value progresses from values near to zero in modern tissues until equilibrium is reached at unity. Although an artefact of these conditions is predicted to be slowing of the racemisation rate as the extent of racemisation increases, the characteristic curvilinear trend produced when D/L ratios are plotted against time always deviates from the results expected of an ideal system (Wehmiller & Miller, 2000). Examples from high temperature experiments of the typical monotonic, convex trend of D/L versus time are presented in Figure 2.2. In some amino acids the forward and backward reaction rates are not equal and accordingly D/L values do not approach unity. For example, the equilibrium ratio of D-alloisoleucine to L-isoleucine of approximately 1.30 indicates the backward reaction (D-aIle formation) is 30 % more likely than the forward reaction (L-Ile formation) (Williams & Smith, 1977). To emphasise this distinction between the extent of isoleucine epimerisation and amino acid racemisation, the extent of the former reaction is traditionally referred to as an A/I value or ratio.

Figure 2.2 The characteristic curvilinear trend in D/L values with respect to time. Although more complex patterns are known, these monotonic, convex curves illustrate the typical pattern. From top-left to bottom right, the data sets shown are from the high temperature experiments of Kaufman (2000), Manley *et al*. (2000), Goodfriend & Meyer (1991), and Miller *et al*. (2000). Amino acid, sample type and isotherm are indicated.

2.3.2 Temperature

The Arrhenius equation is a mathematical description of the temperature sensitivity of chemical reactions. Reaction rate (k_1) is described by two parameters, the Arrhenius factor (A) and activation energy (E_a) (Schroeder & Bada, 1976):

$$\ln (k_1) = \ln (A) - E_a/RT \quad \text{Eqn. 2.1}$$

where R is Boltzmann's constant (= $1.9872*10^{-3}$) and T is temperature measured in Kelvin. The equation is named after Svante August Arrhenius, a Swedish physical chemist and winner of the Nobel Prize for chemistry in 1903, who is credited with being the first to assert that Equation 2.1 is applicable to all reactions (Logan, 1982). The Arrhenius parameters rarely have any physical significance. Rather, the equation is an empirical best fit to the observed change in reaction rates with respect to temperature (Logan, 1982). Determination of A and E_a have traditionally involved the extrapolation from the rate of amino acid racemisation (k) observed at various temperatures between 200 ºC and temperatures characteristic of the natural environment. The rate constant is related to time (t, measured in years) by the integrated rate equation (Bada & Schroeder, 1972):

$$\ln [(1 + D/L)/(1 - K'D/L)] = (1 + K')\, k_1\, t \qquad \text{Eqn. 2.2}$$

where $K' = k_1/k_2$. For most amino acids the forward and reverse rate constants, k_1 and k_2, are equal, therefore $K' = 1$. For the interconversion of L-Ile to D-aIle $k_1/k_2 = 1.0/1.3 = 0.77$. An additional term is commonly introduced to account for laboratory-induced racemisation:

$$\ln [(1 + D/L)/(1 - K'D/L)] = (1 + K')\, k_1\, t + c \qquad \text{Eqn. 2.3}$$

where $c = \ln [(1 + D/L_{t=0})/(1 - K'D/L_{t=0})]$ and $D/L_{t=0}$ represents the extent of racemisation measured in a modern specimen (e.g. live at time of collection). Isoleucine epimerisation Arrhenius parameters extracted from the literature reveal that values for A and E_a are conservative (Table 2.2). Using a representative E_a of 29 kcal

mol^{-1}, a 5 °C increase in temperature will increase k_1 observed at 20°C by 130 %, and a 5 °C drop will decrease k_1 by about 60 %. From this example it is apparent that warm temperatures have a disproportionately larger effect on the rate of racemisation than cooler temperatures. It is for this reason that an important distinction is made between

Table 2.2 Arrhenius parameters reported for isoleucine epimerisation in the total acid hydrolysate of proteinaceous fossils.

the temperature over which racemisation takes place and average temperature, such as mean annual temperature. For example, the highs in the sinusoidal variation in ambient temperature observed at diurnal and seasonal time scales have a disproportionately large influence on the rate of racemisation such that the average temperature is less than the effective diagenetic temperature (EDT) (Miller & Brigham-Grette, 1989). The EDT is the integral of all temperatures experienced over the course of the reaction.

2.3.3 Position in polypeptide

The negative correlation between the molecular weight of polypeptides and the extent of racemisation amongst their amino acids (Kaufman & Miller, 1995, Kaufman & Sejrup, 1995) is a product of the relationship between the racemisation rate of an amino acid and its location within the polypeptide. This phenomenon is illustrated in Figure 2.3 where the extent of isoleucine epimerisation observed by Kaufman & Miller in various molecular fractions of three *Genyornis* eggshells is presented. The highest molecular weight fraction consistently features the lowest A/I values and the low molecular weight and free amino acid fractions feature the highest values. Laboratory experiments (Kriausakul & Mitterer, 1978, Gaines & Bada, 1988) indicate that amino acids located at interior positions within peptide chains feature slow rates of racemisation, whereas amino acids located at terminal positions and incorporated in diketopiperazines racemise most rapidly. The D/L ratio of free amino acids is commonly high but the reaction rate is slow when the molecules exist in this state. This potential incongruity is explained by the preferential release of amino acids from terminal positions into the free pool. Relatively intact polypeptides feature a higher ratio of interior to terminal amino acids than smaller polypeptides, consequently high molecular weight separations feature a lower extent of racemisation.

Figure 2.3 Data of Kaufman & Miller (1995) on variations in the extent of isoleucine epimerisation according to the molecular weight of polypeptides in three *Genyornis* eggshells. The HMW, MMW and LMW fractions are enriched in molecules approximately > 15,000, 15,000-2000 and 2000-200 Daltons, respectively. The FREE fraction consists of naturally-hydrolysed amino acids. Also shown is the extent of isoleucine epimerisation in the total acid hydrolysate (TOTAL).

The differences in racemisation rates according to position within the polypeptide present the geochronologist with two pools of amino acids that are readily separated. Normally, all peptide bonds are hydrolysed for amino acid analysis by heating the acidic residue at high temperatures (e.g. 150 ºC for 15 min., Blackwell *et al.*, 1990, 6 or 22 hrs at 110 ºC, Kaufman & Manley, 1998). However, elimination of this step results in a solution dominated by naturally-hydrolysed amino acids. Due to the *apparently* rapid rate of racemisation in this pool of free amino acids the D/L values in this unhydrolysed solution can provide a more effective means of separating intervals of

time than is attainable with D/L values observed for the acid hydrolysate (e.g. Nelson, 1982).

2.3.4 Tertiary structure

The three-dimensional configuration of a protein also influences the rate of racemisation. However, in terms of diagenesis, only Asx racemisation in the three-dimensional structure of collagen has been considered in detail (van Duin & Collins, 1998). Collagen is a Gly, Ala and Pro-rich protein that plays an important role as a connective fibre in tissues such as skin and bones. It is the most abundant protein in fresh bone (Hare, 1980) and is therefore important when studying the racemisation of amino acids in ancient skeletal remains. However, collagen is not resistant to diagenetic alteration. Its triple helical structure is rapidly denatured and removed by groundwater from the vesicular structure of bones. When the three-dimensional structure is degraded, the decomposition of Asn into rapidly epimerising succinimide residues is enhanced. The succinimide residues convert into molecules of Asp (and isoaspartyl), increasing the apparent rate of Asx racemisation (van Duin & Collins, 1998). Consequently, Asx D/L ratios in bones vary according to the preservation state of collagen as well as time and temperature, making their interpretation highly problematic. The most striking example of this problem was the use of Asx D/L ratios to determine numeric ages of North American palaeoindian skeletal remains (Bada *et al.*, 1974, Bada & Helfman, 1975, Bada *et al.*, 1979). The extent of racemisation was used to derive Late Pleistocene ages for the presence of people in North America, necessitating a revision of American prehistory. However, the bones were later demonstrated to be Holocene in age with AMS radiocarbon analyses (Taylor, 1983, Bada *et al.*, 1984, Stafford *et al.*, 1984, Bada, 1985, Ennis *et al.*, 1986). The erroneous

racemisation-based age estimates were due in part to inaccurate calibration of the reaction rate in the field, but were chiefly due to the failure to account for the influence of preservation state on amino acid racemisation.

2.3.5 Primary structure

Amino acid sequences are genetically determined. Consequently, the same protein in different species may feature minor but significant differences in their primary sequence due to the evolution of mutations in the base pairs encoding the polymer (Doolittle, 1985). The amino acid sequence affects racemisation because neighbouring residues influence diagenetic reactions that in turn influence the rate of hydrogen abstraction. *In vitro* experiments using small polypeptides provide one source of evidence for neighbouring residue effects. For example, Smith *et al*. (1986) observed that Ala racemised faster when located in the dipeptide Gly-Ala than Ala-Gly. Hydrolysis experiments provide evidence that peptide bonds at Asp residues are readily cleaved, preferentially placing Asp and neighbouring residues at fast-racemising terminal positions (Partridge & Davis, 1950, Inglis, 1983, Kaufman & Manley, 1998).

2.3.6 Taxonomic effects

Differences in racemisation rates according to taxonomy have been noted amongst molluscs (LaJoie *et al.*, 1980), foraminifera (King & Hare, 1972) and avian eggshells (Magee & Miller, 1998, Miller *et al.*, 1999b). Amongst molluscs there are significant differences between genera but broadly comparable reaction rates amongst species of the same genus. The reason for the taxonomic-dependency is not precisely known but because they are accompanied by differences in amino acid composition, likely explanations are either differences in the amino acid composition of the precursor

proteins or in the relative abundance of the numerous proteins involved in the secretion of these carbonate structures. For example, Goodfriend *et al*. (1992) observed high concentrations and very rapid racemisation of Asx residues in the coral *Porites australiensis*. Because peptide bonds at Asx sites are prone to hydrolysis the authors interpreted these observations as cause and effect. Where multiple species are recovered in association inter-generic differences in racemisation rates provide an internal check on the reliability of D/L values (Wehmiller, 1984b). Similarly, because amino acids feature characteristic rates of racemisation, a comparison of the D/L values of different amino acids within a sample provides an additional means of assessing the veracity of data.

2.3.7 Chemical degradation

The formation of a peptide bond results in the release of a water molecule and, as the reaction is reversible, the presence of water may result in cleavage of the bond. Progressive hydrolysis results in the formation of increasingly small polypeptides, ultimately producing a pool of free amino acids. Deamination and decarboxylation result in the loss of the amino and carboxyl groups attached to the chiral carbon molecule. Condensation reactions between carbohydrates and protein residues result in the formation of characteristically brown, partly insoluble melanoidins (Collins *et al.*, 1992). This formation has been demonstrated to retard racemisation rates (Rafalksa *et al.*, 1991). In 'open' systems, the degradation of polypeptides may be enzymatically catalysed by the activities of microorganisms (Child *et al.*, 1993).

The interpretation of amino acid D/L ratios and concentrations are complicated by the chemical decomposition of complex amino acids into more simple ones (Table 2.3).

For example, as well as racemising rapidly, Ser residues are relatively unstable. The decomposition of Ser, along with Thr and Met, may produce the lower molecular weight amino acids Ala and Gly. Due to complications arising from decompositional genesis, the D/L value and concentration of these low molecular weight amino acids, along with Pro residues as they can be formed from Arg, should be interpreted cautiously (Table 2.3).

Table 2.3 Products formed by the decomposition of amino acids (Vallentyne, 1964, Bada *et al.*, 1978).

2.3.8 Leaching

The physical transport of amino acids out of mineral matrices by an aqueous phase, commonly water, is termed ‘leaching’ (Mitterer, 1993). The rate of diffusion (leaching) is controlled by the size of the mobile molecules and their sorption to the fossil matrix, the diameter and complexity of the diffusion pathway, and temperature (Collins &

Riley, 2000). Molecules that are least susceptible to leaching are those enveloped by the crystals of the biomineral. Amino acids in these positions are termed 'intracrystalline', and those located within the fossil but not completely isolated from the external environment are termed 'intercrystalline' (Sykes *et al.*, 1995). Intracrystalline amino acids are preferred for analysis because in this environment the residues exist in a closed system free from the complicating influence of extrinsic factors such as microbial decomposition. Molecules most susceptible to leaching are the low molecular weight solutes (i.e. free amino acids, dipeptides, diketopiperazines) of poorly preserved biominerals in which the loss of amino acids is well underway, located in a depositional environment periodically subjected to inundation. Because small peptides feature a characteristically high extent of racemisation, leaching tends to decrease the extent of racemisation determined for the total acid hydrolysate.

2.3.9 Contamination

Contamination is the introduction of non-indigenous amino acids to the fossil matrix. Contaminants are commonly L-enantiomers, however, bacterial cell walls represent a potential source of contaminant D-amino acids (Friedman, 1999). Relatively high concentrations of the thermally unstable amino acids Ser and Thr or abnormal D/L ratios may identify contamination. Laboratory preparatory techniques aim to isolate indigenous amino acids residues and eliminate potential contaminants. The most common method involves mechanical abrasion to remove external surfaces followed by acid dissolution. More intensive techniques for the isolation or verification of indigenous polypeptides include molecular weight 'seiving' (Kaufman & Sejrup, 1995), isotopic analysis of enantiomer pairs (Engel *et al.*, 1994), extraction of intracrystalline amino acids (Sykes *et al.*, 1995), and confirmation that the relative pattern of carbon

isotope ratios amongst individual amino acids in modern specimens are replicated in the fossil record (Johnson, 1995, Johnson *et al.*, 1998).

2.4 GEOCHRONOLOGICAL APPLICATIONS

Amino acid racemisation has potential application in the Quaternary sciences wherever amino acid residues are preserved over geological time. Marine shells (e.g. Wehmiller *et al.*, 1988, Wehmiller *et al.*, 1995), land snail shells (e.g. Goodfriend, 1987, 1991) and eggshells (e.g. Magee & Miller, 1998, Miller *et al.*, 1999b) are commonly used because they are abundant and preserve amino acids well over geologically significant timescales. Other materials that have been analysed include fish otoliths (Rosewater, 1995), wood-rat middens (Petit, 1974), wood (Rutter & Vlahos, 1988), bones and teeth (Blackwell *et al.*, 1990), as well as corals (Goodfriend *et al.*, 1992, Nyberg *et al.*, 2001), foraminifera (Murray-Wallace & Belperio, 1994), ostracodes (Kaufman, 2000), and charophyte gyrogonites (DeVogel, 2003). Applications are most confident when it can be demonstrated that amino acids exist in a closed system during diagenesis. It is also important that the amino acids are derived from the same biological source. Interestingly, studies of the reaction in biogenic carbonate breccia (e.g. 'whole rock' aeolinites) (Hearty, 1998, Hearty *et al.*, 1999, Murray-Wallace *et al.*, 2001, Brooke *et al.*, 2003), speleothems (Lauritzen *et al.*, 1994) and paleosols (Kimber *et al.*, 1994) have proven successful. The latter sample type has proven successful because amino acids bind to silica minerals such that they are unavailable to biodegradative processes. In a novel extension of these observations Kimber & Griffin (2000) demonstrated that the ratio of biologically useful L-amino acid enantiomers to D-enantiomers in agricultural soils is a possible means of assessing pasture health. Whole-rock amino acid analyses have proven reliable because the results integrate the D/L values of amino acids from

fragments of different taxa that racemise at broadly comparable rates. Further work is necessary to demonstrate the reliability of speleothem analyses such as determination of the source of the calcite-entrapped amino acids.

The period that D/L values are effective at resolving time is determined by the rate of racemisation. Two examples from tropical settings are presented in Figure 2.4. It is seen that the rapid racemisation of Asx residues provide excellent age resolution amongst young samples, whereas the slower epimerisation of isoleucine is effective at distinguishing events that are thousands of years apart. The use of a suite of amino acid D/L values enables age resolution over an extensive portion of the Quaternary timescale.

Figure 2.4 Examples illustrating the range of timescales over which D/L values are capable of providing age resolution. The D/L values of Asx residues in submodern *Porites australiensis* coral observed by Goodfriend *et al*. (1992) are presented in (a). The A/I values reported by Hearty & Aharon (1988) for *Tridacna* shells from the Huon Peninsula sequence are presented in (b). Note the timescales differ by over two orders of magnitude. Curves fit to the data are in the format $y = mx^n + b$ and are provided to accentuate the trends.

The numeric age of a fossil can be determined using the integrated rate equation (Eqn 2.3) if k_1 is known, providing the reaction obeys reversible first-order kinetics over the range of D/L ratios of interest. An appropriate value for k_1 can be determined from heating experiments if the thermal history of the specimen is accurately known. However, for many deposits the thermal history is poorly constrained, rendering this approach inappropriate. Bada & Protsch (1973) presented an alternative approach in which an appropriate *in situ* value for k_1 is determined from the D/L ratio of a specimen of known age. The known age is used to solve Equation 2.3 for k_1 then this 'calibrated' rate constant is used to determine the numeric age of analogous specimens from their D/L value, assuming they have experienced a comparable thermal history and both D/L values are within the same kinetic phase of the reaction (usually the initial phase of rapid racemisation).

One of the earliest attempts to use the integrated rate equation to estimate the age of fossils was reported by Bada et al. (1970). These authors used a simplified form of Eqn. 2.3 to estimate the accumulation rate of carbonate-rich deep-sea sediments. However, it was soon noted (Wehmiller & Hare 1971) that the reaction conformed to reversible first-order kinetics in foraminifera up to an A/I value of about 0.25, after which the reaction rate decreases abruptly. Wehmiller & Hare observed that the extent of isoleucine epimerisation in naturally-hydrolysed amino acids initially increased at a greater rate than the reaction in the total hydrolysate, indicating that the reaction system differed between bound and free amino acids, and was likely to deviate from reversible first-order kinetics during diagenesis in response to changes in the relative size of these pools. Failure to observe the D/L values of numerically dated fossils conforming to reversible first-order kinetics due to slowing of the reaction rate has led researchers to

use various functions to transform D/L values so that they are linear with respect to time. Kaufman (2000) and Manley *et al.* (2000) raised a modified version of the integrated rate equation to the power n to model Asx D/L values. Mitterer & Kriausakul (1989) and others have noted that the transformation $(D/L)^2$ improves the linear correlation between D/L and time and also increases the range of D/L ratios that can be converted into numeric ages (Goodfriend *et al.*, 1992, Murray-Wallace & Kimber, 1993). Similarly, Goodfriend *et al.* (1995) observed that a cubic transformation of serial D/L Asx values in shells of long-lived clams approximated a linear relationship to time.

Due to the sensitivity of amino acid racemisation to environmental parameters, the strength of the technique as a chronometer is in relative age assessments, rather than numeric ages (Mitterer & Kriausakul, 1989, Kaufman & Miller, 1992, Wehmiller, 1993). The technique is highly suited to refining the chronology of episodic events such as sea-level highstands (e.g. Hearty, 1998). The highstands correspond with interglacials or interstadials whose timing is precisely known from the astronomically-tuned marine oxygen isotope chronology or independent methods of dating. Within this context, with verification from independent dating methods, D/L values can be used to rapidly develop a chronological framework for stratigraphic successions. The D/L values of these discrete events may be used to define aminozones. An aminozone is an chemostratigraphic unit applied to a well-defined geological deposit based on clustering in the extent of amino acid racemisation in replicate fossils (Nelson, 1982). Aminozones are the fundamental units of ‘aminostratigraphy’, the arrangement of geological deposits into a relative sequence based on the D/L values of associated fossils (Miller & Hare, 1980).

Independent of numeric age control, D/L values can provide detailed insights into the depositional history of a sequence, especially if it is rich in fossils. For example, several authors have used the distribution of D/L values in taphonomic studies to identify mixed-age assemblages (Murray-Wallace & Belperio, 1994, Wehmiller *et al.*, 1995, Kowalewski *et al.*, 1998). Ellis *et al*. (1996) provide numerous examples of the down-profile patterns in D/L values expected of stratigraphic events. These authors emphasise that the within-unit variability in D/L values is expected to be highest in sites featuring low sedimentation rates. This trend is the expected influence of shallow burial on D/L values. Near the Earth's surface specimens are exposed to the sinusoidal daily and seasonal variations in temperature. Due to the exponential relationship between reaction rate and temperature, this shallow burial promotes variable exposure to temperatures higher than the mean annual temperature. It is for this reason that samples for amino acid analysis are preferably rapidly buried after deposition and remain more than one metre from the surface during diagenesis (Miller & Brigham-Grette, 1989).

As a geochronological technique based on a chemical reaction, the racemisation of amino acids has several advantages when compared to geochronometers based on the decay of radioactive elements. Alternative Quaternary geochronological techniques that can directly determine the age of organic remains are based on the decay of radiocarbon, electron spin resonance and uranium-series disequilibrium. Of these techniques the most commonly applied is radiocarbon dating but this has an effective upper limit of approximately 50,000 years B.P (depending on the integrity of the geological material and the background radiocarbon content of the detection system) and is problematic over the last 500 years due to fluctuations in atmospheric levels of ^{14}C. If the rate of racemisation can be accurately modelled, D/L values can provide

excellent chronological control beyond the 'radiocarbon barrier' (e.g. Miller *et al.*, 1999b) and the D/L of rapidly racemising amino acids (e.g. Asx and Ser) can be used to resolve the age of very young specimens (e.g. Rosen & Goodfriend, 1993). In some instances, D/L values can be particularly useful because samples are unsuitable for radiocarbon dating. For example, the consumption of limestone by land snails may yield anomalously old radiocarbon ages (Goodfriend & Stipp, 1983) but will not affect D/L values.

Racemisation age estimates remain dependent on other geochronological techniques for field calibration despite investigations by P.E. Hare formerly of the Carnegie Institution of Washington and colleagues into 'protein diagenesis dating' (e.g. Kokis, 1988). Hare noted that different diagenetic reactions such as hydrolysis and racemisation have different activation energies. For a given specimen it should therefore be possible to establish analytically the extent of two or more reactions and with knowledge of their Arrhenius parameters determined experimentally, simultaneously determine both time and temperature. Although Miller *et al.* (1992) used this concept to identify *Struthio* eggshells exposed to high temperatures it is yet to achieve widespread application.

2.5 PALAEOTEMPERATURE APPLICATIONS

Abelson (1954) demonstrated that the degradation of Ala is temperature dependent and therefore may be described by Arrhenius parameters. He concluded that the system could be used as a 'geological thermometer'. Since these initial insights, the diagenetic alteration of amino acids by racemisation, not decomposition, has received interest as a palaeothermometer. The temperature-sensitivity of the racemisation reaction has been traditionally modelled by measuring the extent of racemisation in modern samples

exposed to high temperature isotherms (e.g. Hare & Mitterer, 1968, Miller *et al.*, 1983, Sejrup & Haugen, 1992, Oches *et al.*, 1996, Kaufman, 2003). Because the reaction exponentially increases with temperature, high isotherms (> 80 ºC) enable the racemisation reaction to reach an extent over a period of days or weeks that takes thousands of years to reach at environmental temperatures. Heating samples at different high temperatures enables the Arrhenius parameters that define the temperature sensitivity of the reaction to be determined.

To determine the Arrhenius parameters, activation energy (E_a) and Arrhenius constant (A), modern samples are heated for intervals at isotherms between 80 and 200 °C. Because uncertainty associated with the calculation of Arrhenius parameters represents the largest error in amino acid palaeothermometry (McCoy, 1987) it is beneficial to obtain a calibration sample exposed to environmental temperatures. The most appropriate samples are those whose EDT is known or can be confidently assumed, such as Late Holocene specimens from sites that have experienced a temperature regime equivalent to that defined by meteorological records (an environmental isotherm). Typically, this results in anchoring of the activation energy at a point over six orders of magnitude removed from results of the high temperature isotherms. The calculation of Arrhenius parameters from reaction rates observed at high temperatures combined with field calibration is illustrated in Figure 2.5 using the data of Haugen & Sejrup (1992) on isoleucine epimerisation in the outer shell of *Arctica islandica*. For each isotherm, the natural logarithm of the rate constant ($\ln k_1$) for the initial range over which the reaction obeys reversible first-order kinetics is plotted against the inverse of the isotherm temperature ($1/T$), measured in Kelvins. The natural logarithm of the y-intercept of this

Figure 2.5 Arrhenius plot for isoleucine epimerisation in the outer shell of *Arctica islandica* from the high temperature experiments of Haugen & Sejrup (1992). Results of the linear regression and the calculations used to estimate the Arrhenius parameters are indicated.

line ($y = mx + b$) is equivalent to A, and the slope of the line is proportional to E_a ($m = R \times E_a$). Once A and E_a are determined, the D/L value and age of a proteinaceous fossil can be used to calculate the EDT to which that sample has been exposed

providing the D/L value is within the range the reaction is known to obey first-order kinetics. The D/L value and age are used to solve Equation 2.3 for k_1. This *in situ* rate constant is then used with A and E_a to solve Equation 2.1 for T.

Because EDT is a technique-specific record of thermal history it is desirable to convert such values into estimates that are more readily compared to palaeotemperature records derived from other proxies. One such conversion is the use of EDT to estimate the difference in temperature between two different intervals of time, such as the LGM relative to the Holocene. This is achieved by determining the rate of racemisation during each interval (e.g. 0-10 ka and 10-25 ka) and converting the reaction rate into an estimate of EDT. The difference in EDT between the two intervals is equivalent to the degree of temperature depression in degrees Celsius. Although the EDTs estimated for the two intervals in this manner are potentially erroneous, uncertainty associated with the *difference* in EDT between the two intervals is likely to be small (McCoy, 1987).

In this manner the use of D/L values for estimating palaeotemperatures is more statistically reliable than its use as a numeric geochronometer (McCoy, 1987). An additional advantage is the integrated nature of racemisation-based palaeotemperature estimates. Unlike instantaneous climatic signals such as pollen or invertebrate assemblages or stable isotope ratios, the extent of racemisation is the product of the reaction rate ‘averaged’ over time. At low sample densities the instantaneous records are susceptible to bias resulting from short-term fluctuations. Conversely, at typical sampling densities the integrated nature of the amino acid temperature record makes the technique unsuitable for identifying intermittent climatic excursions.

The key assumption concerning the use of high temperatures to model diagenetic reactions that take place at environmental temperatures is that all reactions that influence the rate of racemisation respond uniformly to changes in temperature. If for example, the hydrolysis of peptide bonds, an important determinant of D/L values, does not accelerate in response to high temperatures at a rate equivalent to that of the racemisation reaction, then the experimental data will fail to simulate the reaction at environmental temperatures.

2.6 AMINO ACID RACEMISATION IN AVIAN EGGSHELLS

As sample types for amino acid analysis, eggshells differ from other commonly studied sample types in terms of amino acid concentration and ability to retain these molecules. According to Miller *et al.* (Brooks *et al.*, 1990, Miller *et al.*, 1991, Miller *et al.*, 2000) amino acids are 50 times more abundant in avian eggshells than mollusc shells. In contrast to the intra-shell trends observed in mollusc shells, it appears that there are no differences in amino acid composition or racemisation kinetics between separate fragments of the same eggshell. This accords with the lateral homogeneity of the eggshell structure. There are, however, intra-eggshell differences in amino acid composition according to structural phase (Miller *et al.*, 2000). This accords with the variation in the localisation of eggshell proteins between the mammillary and squamatic zones (Hincke *et al.*, 1999).

Like mollusc shells, taxonomic effects on the rate of racemisation have been observed amongst avian eggshells. Miller *et al*. (2000) noted that on average A/I values in *Dromaius* eggshells were 1.16 times higher than in *Genyornis* eggshells recovered from the same stratigraphic unit, an observation confirmed in laboratory heating experiments.

Differences in the A/I values of *Genyornis* and non-ratite (presumably water bird) eggshells were observed by Magee & Miller (1998) in Lake Eyre beach ridges. Where A/I < 1.0 the extent of isoleucine epimerisation was between 1.10 and 1.15 times greater in non-ratite eggshells than *Genyornis* eggshells, and where A/I > 1.0 the values in *Genyornis* eggshells were slightly higher.

The investigation of amino acid racemisation in avian eggshells has been almost exclusively restricted to the extent of isoleucine epimerisation. The one exception is a survey of *Struthio* eggshell Asx D/L values reported by Goodfriend *et al*. (1991). These authors observed significant racemisation in submodern eggshells (up to *c*. 300 years old) suggesting the reaction may be useful for resolving time on a decadal timescale, at least amongst very young specimens.

2.7 EVIDENCE FOR RETENTION OF AMINO ACIDS IN EGGSHELLS

Eggshells have been promoted as a sample type for biogeochemical analyses because they provide an excellent environment for the preservation of indigenous residues (Brooks *et al.*, 1990, Miller *et al.*, 2000). Here the lines of evidence used to support this assertion with respect to amino acid racemisation are reviewed.

2.7.1 Retention of amino acids

As the abundance of amino acids in a fossil is a product of both their diffusion and decomposition, the most thermally stable molecules are preferable for assessing closed system behaviour. From the *Bos* bone (Hare, 1980) and *Struthio* eggshell (Kokis, 1988) presented in Figure 2.6, it is seen that after more than 60 hours of simulated diagenesis

Figure 2.6 Loss of amino acids from modern *Struthio* eggshell and *Bos* bone under similar laboratory conditions. Bone data from Hare (1980) on bone chips (2 – 3 mm across) heated at 105 ºC with excess water. Eggshell data from Kokis (1988) on *Struthio* eggshell fragments heated at 106 ºC with excess water.

at *c*. 105 ºC under conditions of excess water these biominerals retain about 20 % and 99 % of their indigenous amino acids, respectively. Thus, it appears that eggshells provide a more favourable environment for retaining amino acids. Ernst (1987) conducted a similar experiment designed to assess the retention of indigenous amino acids in *Struthio* eggshells. A subset of the results of this study for the thermally stable amino acids Glx, Val, Ile + aIle and Leu are presented in Figure 2.7. There is general agreement between the control experiment and the 'leached' eggshells. Over 14 days of

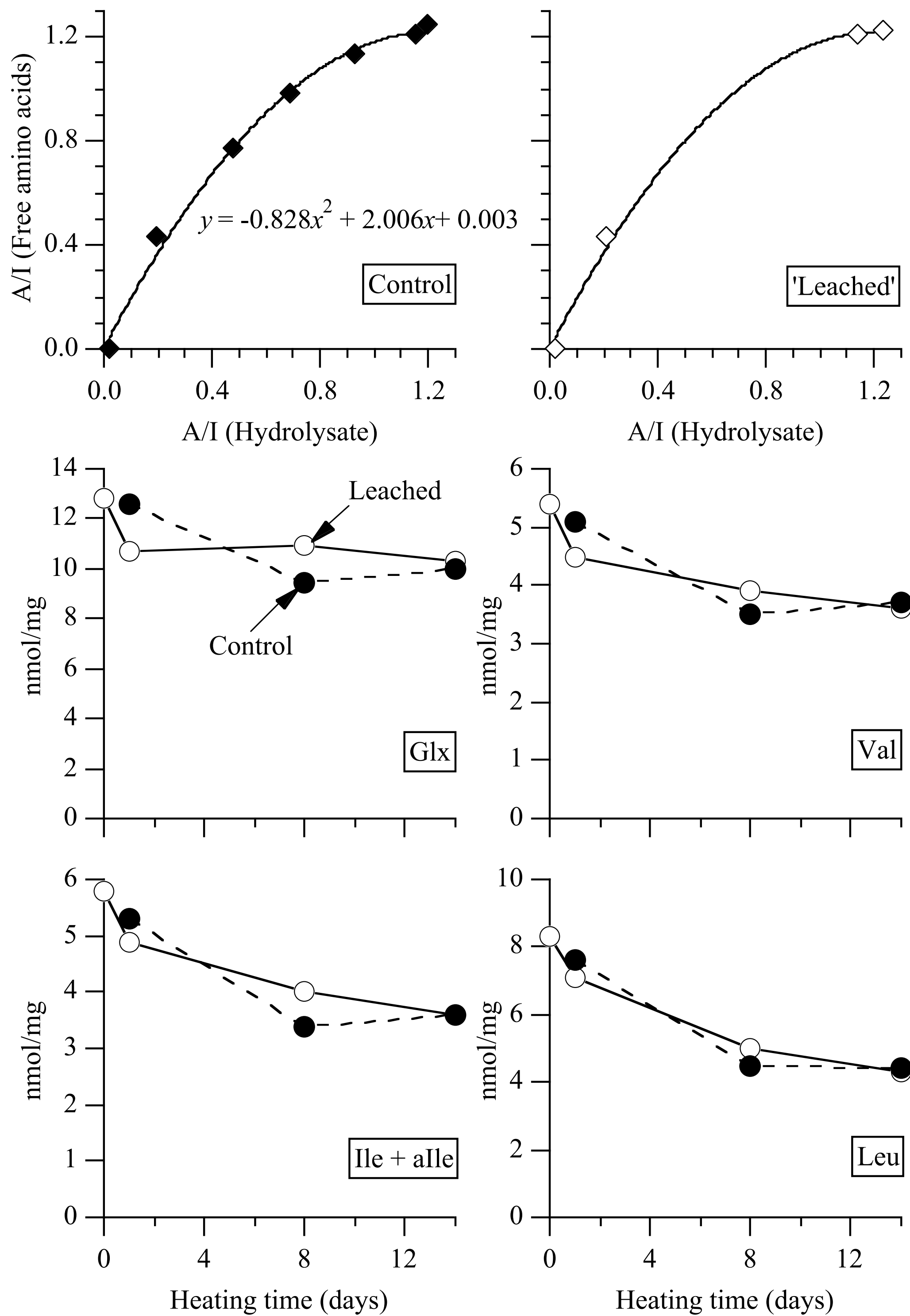

Figure 2.7 Isoleucine epimerisation and amino acid concentrations in *Struthio* eggshells heated at 144 ºC. The third-order polynomial fit to the hydrolysate and free amino acid A/I values of the control experiment is repeated in the plot of A/I values in 'leached' eggshells. Black and white symbols indicate A/I values and amino acid concentrations in the control and 'leached' experiments, respectively.

heating at 144 ºC declines in the abundance of these amino acids ranged from about 20 % (Glx) to 50 % (Leu). To determine whether the declines were due to decomposition or diffusion, the concentration of amino acids in the water in which the 'leached' eggshells were submersed was analysed. After one day of heating the concentration of amino acids in the water was about 10 nmol/ml and remained at this concentration over the remainder of the experiment. Although the presence of amino acids in the water could be evidence of diffusion from the eggshell there are two factors that complicate the relevance of the water results. Firstly, the norleucine spike used to calculate amino acid concentration in the water relates to the volume of water and not to the weight of the eggshell. Therefore, the significance of the observations in terms of amino acids lost from the eggshell is unclear. Secondly, prior to the experimental treatment, the exterior 30 % of the eggshell was removed with acid. This was necessary to avoid contaminating the water with contaminants on the surface of the eggshell. The pretreatment is problematic when trying to model closed system behaviour because the etch will increase the exposure of amino acids to the environment. Hence, the water in which the specimen was immersed is likely to contain an unrealistically high concentration of amino acids.

Reporting of amino acid concentrations in fossil eggshells is not routine. Miller *et al.* (2000) reported Glx concentrations of 7.7 ± 1.4 nmol/mg in a set of more than 400 Quaternary *Dromaius* eggshells. This concentration is similar to the abundance of Glx in submodern *Dromaius* eggshell (*c*. 9 nmol/mg). Bada *et al.* (1999) observed that the abundance of amino acids in two *Struthio* eggshells with ages in excess of 20,000 years were about 0.75 %, and this is close to the abundance they observed in modern eggshell (1.2 %).

Kaufman & Miller (1995) reported amino acid concentrations in several different molecular weight fractions of *Genyornis* eggshells. Amongst four eggshells with A/I values of *c*. 0.3, 0.6, 0.9 and 1.0 Glx concentrations in the total acid hydrolysate ranged from about 5.6 – 8.0 nmol/mg. Concentrations of Val, Ile + aIle and Leu were typically *c*. 3.0, 2.0 and 3.5 nmol/mg, respectively, exhibiting little variation with changing A/I. In terms of relative amino acid composition, the abundance of all amino acids in the *Genyornis* eggshells remained constant with the exception of a decline in Thr + Ser and, to a lesser degree, Asx.

Johnson (1995) reported the amino acid concentration of *Struthio* eggshells to test the yields obtained under different protocols for the isolation of the organic residue. Changes in the concentrations of the more stable amino acids using the traditional protocol are presented in Figure 2.8. The amino acid concentrations in the *Struthio* fossil eggshells are about 60 % of the values observed in modern eggshell (A/I = 0.02), although one specimen with A/I = 0.4 featured a yield of about 70 % relative to the modern specimen. This atypical preservation is unlikely to be an artefact of laboratory procedures because the specimen also exhibited an abundance of amino acids using the experimental protocol.

2.7.2 Pattern of isoleucine epimerisation independent of environmental conditions

The diagenetic environment potentially produces variations in the amino acid composition, concentration and racemisation of a fossil through processes such as leaching, contamination and microbial degradation. The ability to successfully simulate

Figure 2.8 Abundance of stable amino acids in *Struthio* eggshells. The eggshells range in age from modern (A/I = 0.025) to approximately 8,000 years BP (A/I > 0.5). Data from Johnson (1995).

patterns of amino acid diagenesis at ambient temperatures in a laboratory setting potentially provides evidence for the existence of amino acid residues that are isolated from extrinsic processes.

Miller *et al*. (2000) cited two forms of evidence suggesting that laboratory-induced diagenesis in *Dromaius* eggshells accurately portrayed these processes at ambient temperatures. Firstly, two fossil eggshells (A/I = 0.4 and 0.9) were heated at 143 ºC and their A/I values were compared to those obtained on modern eggshell under the same laboratory conditions. If diagenesis at environmental temperatures proceeded in a fashion distinct from that induced at high temperatures, the pattern of isoleucine epimerisation at high temperatures would have differed between the modern and fossil eggshells. However, despite differences in the thermal history of the specimens (e.g.

thousands of years at ambient temperature) the kinetic curves matched well, with the cessation and rate of epimerisation of first-order kinetics at 143 ºC in the fossil eggshells being indistinguishable from that observed in modern eggshell. The second form of evidence cited was the pattern of A/I values in the free and total acid hydrolysate amino acid fractions. Again, the pattern of isoleucine epimerisation observed at high temperatures was in accordance with the pattern observed amongst fossil *Dromaius* eggshells (n = 48), although there was a tendency for more scatter in the A/I values of the latter dataset produced by slightly high A/I values in the hydrolysate or low values in the free amino acid fraction.

Kokis (1988) sought to identify the importance of water in governing isoleucine epimerisation in *Struthio* eggshells at high temperatures, thereby providing insight into the sensitivity of A/I values to water in the depositional environment. The A/I values in both free and total acid hydrolysate amino acids in modern *Struthio* eggshell heated at 105, 146 and 157 ºC are presented in Figure 2.9. Kokis devised a treatment in which eggshells were immersed in water that was regularly changed and compared these A/I values to those observed in eggshells exposed to water vapour only. Despite the contrasting treatments, the pattern of isoleucine epimerisation matched well. The only systematic deviation appeared to be where A/I > 1.10. Kokis did not consider these deviations significant because they were explicable in terms of routine analytical error. During these latter stages of the experiment there was a tendency for A/I values in both the free and acid hydrolysate fractions of eggshells exposed to excess water to be slightly lower than A/I values in the control. The slightly lower A/I values in the hydrolysate are explicable in terms of the loss of small polypeptides and free amino acids with high A/I values. The tendency in the free amino acids does not have a

conventional explanation but may reflect an acceleration of hydrolysis relative to isoleucine epimerisation in the eggshell exposed to excess water.

Further support for the retention of indigenous amino acids and the exclusion of extraneous amino acids in eggshells was provided by Bada *et al.* (1999). These authors observed D/L values for Asx, Ala and Val close to equilibrium (D/L > 0.9) in three *Struthio* eggshells with ages in the ranges 20 – 40 ka, 400 – 600 ka and 800 – 1000 years. The attainment of D/L values close to unity is promoted by the retention of highly racemised amino acids in low molecular weight fractions and the isolation of the amino acid pool from contaminants with low D/L values. Hence, the results are consistent with the excellent preservation of eggshell amino acids.

Figure 2.9 Extent of isoleucine epimerisation in *Struthio* eggshells under controlled conditions. Data are from Kokis (1988) and transformed using the natural logarithm term of the integrated rate equation.

2.7.3 Range of A/I values over which first-order kinetics are observed

Although the range of A/I values over which reversible first-order kinetics are observed does not provide direct evidence for closed system behaviour, it may be indicative of the underlying processes that control the extent of isoleucine epimerisation (Collins & Riley, 2000). Under controlled conditions isoleucine epimerisation in avian eggshells is commonly observed to obey first-order kinetics to an A/I value in excess of 0.9 (Kokis, 1988, Ernst, 1989, Brooks *et al.*, 1990, Miller *et al.*, 1991, Miller *et al.*, 1992, Miller *et al.*, 1997, Miller *et al.*, 2000). This is a much larger range than that commonly observed for isoleucine epimerisation in mollusc shells (Kriausakul & Mitterer, 1980, Haugen & Sejrup, 1992) (but see Mitterer, 1975) or foraminifer tests (Müller, 1984).

The larger range of A/I values over which isoleucine epimerisation approximates first-order kinetics in eggshells is consistent with the suggested high integrity of this sample type. The preferential loss of low molecular weight fractions should reduce the apparent extent and rate of isoleucine epimerisation because this pool is typified by amino acids high A/I values. This loss is expected to operate in concert with the diagenetic processes that produce the deviation from reversible first-order kinetics to enhance the characteristic decline in the rate of isoleucine epimerisation with increasing A/I and thereby shorten the length of the initial phase of rapid epimerisation and first-order kinetics. In a closed system, molecules of all sizes are retained, from high molecular weight polypeptides to free amino acids, and the apparent reaction rate is not constrained by the loss of fractions with high A/I values. The extended phase of first-order kinetics observed in *Struthio* and *Dromaius* eggshells is therefore explicable in terms of the retention of indigenous amino acid residues in a range of molecular weight fractions.

2.7.4 A labile fraction during early diagenesis

Miller *et al*. (2000) provided evidence for a labile population of amino acids in *Dromaius* eggshells that is rapidly lost during the early stages of diagenesis. These authors observed the concentration of the thermally stable Glx residue drop from an initial concentration of about 14 nmol/mg to a plateau at about 12 nmol/mg after four days of laboratory diagenesis at 143 ºC. This same decline in Glx was not observed in sub-modern or fossil *Dromaius* eggshells subjected to the same treatment. Furthermore, the loss was suggested to be insensitive to temperature because the plateau in the sub-modern and fossil eggshells centred on approximately 9 and 8 nmol/mg, respectively. Based upon these observations, and accompanied by supporting data on the concentrations of moderately stable Asx and unstable Ser residues, the authors suggested that there was a pool of amino acids amounting to approximately 20 – 30 % of the residues in fresh eggshells that were lost within the first few decades of diagenesis.

2.7.5 Intracrystalline amino acids

The ability to isolate intracrystalline organic residues in mollusc shells (Walton *et al.*, 1993, Walton, 1998b, 1998a) and bones (DeNiro & Weiner, 1988) indicates that organic matter protected from the external environment might be found in a range of biominerals. Using a scanning electron microscope, Miller *et al.* (2000) observed 'sheaths' of organic matter in an acid-etched *Struthio* eggshell. Their suggestion that the sheaths reside within rather than between the calcite is the only direct support for the intracrystalline residence of amino acids in eggshells. The presence of intracrystalline amino acids in avian eggshells could be verified by exposing specimens to oxidising reagents such as sodium hypochlorite (NaOCl) (Sykes *et al.*, 1995).

2.8 AGE ESTIMATION IN ARCHAEOLOGICAL CONTEXTS

Aside from a recent publication stemming from this research (Pasveer *et al.*, 2002), application of the extent of isoleucine epimerisation in avian eggshells for geochronological purposes in archaeological contexts has been restricted to *Struthio* eggshells from the African continent. The volume of research is extensive (Brooks & Smith, 1987, Kokis, 1988, Brooks *et al.*, 1990, Miller *et al.*, 1991, Henry & Miller, 1992, Miller *et al.*, 1992, Johnson *et al.*, 1997, Kokis *et al.*, 1998, Miller *et al.*, 1999a) and brief reviews are provided by Hare *et al.* (1997) and Johnson & Miller (1997). Here the methods used to estimate ages from eggshell A/I values in archaeological contexts are examined. A distinction is made between relative and numeric ages, and particular focus is given to ages derived for the ≠Gi and Border Cave sequences.

2.8.1 Examples of relative ages

The research on isoleucine epimerisation in *Struthio* eggshells demonstrates the usefulness of the reaction for assessing relative chronologies in stratified archaeological sites. The A/I values may be useful for identifying both well-stratified deposits and assemblages subject to extensive mixing. At Mumba Shelter (Tanzania), the A/I values obtained by Kokis (1988) fall into two groups, one in which A/I < 0.2 and another in which 0.3 < A/I < 0.9 (Fig 2.10). The low A/I values occur only in the upper 40 cm of the sequence that features Holocene radiocarbon ages. Thus, the values appear to separate young eggshells from older (probably Pleistocene) eggshells. Kokis also observed a similar but more striking contrast in A/I values in the stratified sequence of ≠Gi, Botswana (Fig 2.10). Above 85 cm depth A/I values are less than 0.55, and above this point they occupy the range 0.90 < A/I < 1.10. Again, the impression is a

Figure 2.10 Examples of relative trends in the extent of isoleucine epimerisation in *Struthio* eggshells from archeological sites. Mumba Shelter and ≠Gi data from Kokis (1988), and Equus Cave data from Johnson *et al*. (1997).

population of younger eggshells immediately overlying a series of older specimens. The contrast in A/I values at ≠Gi is more pronounced because the difference in the

extent of isoleucine epimerisation (ΔA/I = 0.35) is much larger between the young and old groups at the site relative to Mumba Shelter (ΔA/I = 0.25). As expected of a sample type in which the diagenesis of amino acids takes place systematically, the relative trends with respect to depth in hydrolysate A/I values at both Mumba Shelter and ≠Gi were replicated in paired analyses of naturally hydrolysed amino acids (Kokis, 1988).

The antipode of these simple chemostratigraphic trends was observed at Equus Cave (Fig 2.10). At this site no trend in A/I values with respect to depth was observed (Johnson *et al.*, 1997). Instead, values expected of modern *Struthio* eggshells were observed throughout the sequence and a radiocarbon age indicated that an eggshell with a relatively high A/I value recovered close to the surface was first deposited during the Pleistocene. The occurrence of eggshells with modern A/I values throughout the profile is indicative of downward transport, and the paired radiocarbon/amino acid results indicate upward transport has also taken place within the profile.

2.8.2 Examples of numeric age estimates

From the extent of isoleucine epimerisation, Henry & Miller (1992) determined that *Struthio* eggshells from two Levantine Mousterian rock shelters in southern Jordan were of equivalent ages after adjusting the A/I values for expected inter-site differences in temperature. Assuming an EDT between 2 and 3 ºC cooler than the current mean annual temperature of 16.7 ºC an age of 69 ± 6 ka was estimated for the eggshells. Hare *et al*. (1993) presented age estimates of *c*. 80 and 125 ka for depths bracketing remains of anatomically modern human fossils at Klasies River Mouth based on the extent of isoleucine epimerisation in *Struthio* eggshells. However, no supporting information on A/I values or number of analyses was presented so no further comments on the research

can be made. Similarly, Kokis *et al*. (1998) reported obtaining A/I values on a large number of *Struthio* eggshells from White Paintings Shelter (Botswana) but further details are yet to be reported. Brooks & Smith (1987) observed an A/I value of 0.43 in a single piece of *Struthio* eggshell recovered from Ishango (Zaire). By comparing this value to the extent of isoleucine epimerisation in Pleistocene eggshells from ≠Gi (*c*. 200 km south) the authors judged that the specimen was more than 9000 years old. Assuming this is the same piece of eggshell radiocarbon-dated by Brooks *et al*. (1990) then their judgement was corroborated by an age of 25,570 ± 350 BP (AA-3300) on the Ishango specimen.

2.8.3 Numeric ages for ≠Gi

Age estimates derived from the extent of isoleucine epimerisation in *Struthio* eggshells from the archaeological sequence of ≠Gi provide an interesting case study. Two different approaches to the interpretation of *Struthio* eggshell A/I values in terms of age have been employed at the site. One of these is based largely on extrapolation from Arrhenius parameters generated from high temperature experiments. The other relies upon field calibration of the reaction rate.

Kokis (1988) used an Arrhenius parameter-based approach to interpret the A/I values of *Struthio* eggshells from ≠Gi in terms of age. This approach depends on a reliable estimation of EDT from modern meteorological data often because no eggshell of known age is available to calibrate the reaction in the field. Other researchers who have adopted this approach include Henry & Miller (1992) and Johnson *et al.* (1997) . From Arrhenius parameters calculated by Hare from the work of both Kokis (1988) and Ernst (1987), the expected relationship between time and *Struthio* eggshell A/I was

determined for various temperatures (Kokis, 1988). These linear calibration curves were then used to interpret ≠Gi eggshell A/I values in terms of age assuming that the modern mean annual temperature (21 ºC) provided a reliable estimate of EDT. Unfortunately, the linear calibration curves were erroneous. Consequently, the ages presented by Kokis were grossly in error: the ages were typically 100 % greater than the expected ages. However, recalculation of the ages using the Arrhenius parameters of Brooks *et al*. (1990) (ln (A) = 40.37, E_a = 30.01 kcal/mol) generates ages in agreement with radiocarbon and thermoluminescence dates for the sequence (Fig 2.11).

LSA = Late Stone Age; MSA = Middle Stone Age.
[b]Ages not calculated by Brooks *et al*. but estimated here using their field calibration, $k_1 = 1.591 \times 10^{-5}$ yr^{-1}.
[c]Calibration sample.

Figure 2.11 Summary of the lithostratigraphy, archaeology, radiocarbon, thermoluminescence and *Struthio* eggshell amino acid results for ≠Gi. Two sets of amino acid ages are presented: (1) the original results of Kokis (1988), (2) a revised set of results using the EDT assumed by Kokis and the Arrhenius parameters of Brooks *et al*. (1990), and (3) results of Brooks *et al*. (1990). Data from Kokis (1988) and Brooks *et al*. (1990).

Brooks *et al*. (1990) used a radiocarbon-dated eggshell with an A/I value of 0.534 to calibrate the rate of isoleucine epimerisation at ≠Gi. The quoted EDT of 20.5 ºC was calculated by inserting the A/I value and radiocarbon date (34,010 ± 100 BP, AA-3302) of an eggshell into the integrated rate equation and interpreting this value in terms of temperature using their estimates of E_a and A. Then, employing a linear calibration curve in the same manner as Kokis, aminoages close to the expected ages were generated for the ≠Gi sequence. These ages are also presented in Figure 2.11.

In practice, of the two methods used to calculate ages for the ≠Gi sequence, that based on field calibration of the rate of isoleucine epimerisation is more statistically robust than the Arrhenius-based approach in which it is necessary to estimate EDT from the modern thermal regime. This is because small uncertainties in EDT (e.g. ± 0.5 %) are magnified by the Arrhenius equation and combine with the uncertainties in A and E_a (e.g. ± 35 %) to produce age estimates with large errors (e.g. ± 40 – 50 %) (McCoy, 1987). An important source of uncertainty in the field calibration approach is contamination of the radiocarbon-dated eggshell by young radiocarbon. For example, Miller *et al*. (1999a) considered an eggshell radiocarbon age of 35 ka or greater to be a minimum age. If the radiocarbon age of *c*. 34 ka used by Brooks *et al*. (1990) is anomalously young then the epimerisation age estimates will be too old. Another source of error that concerns both sets of aminoages for the ≠Gi sequence relates to the duration of first-order kinetics. Brooks *et al*. noted that laboratory-induced isoleucine epimerisation in *Struthio* eggshells follows first-order kinetics where A/I < 1.0. Therefore, the upper limit of both sets of ages assigned to the Middle Stone Age horizon must be considered minimum estimates because these are based on A/I values above A/I = 1.0. Furthermore, Miller *et al*. (1991) cautioned that the duration of linear

kinetics at ambient temperature may be shorter than that observed at high temperatures. This was based on their 110 ºC experiment in which the phase of linear kinetics extended only as far as 0.9. However, these authors cited but provided no details on unpublished results that suggested the linear phase extends until A/I = 0.6 at 17 ºC.

2.8.4 Numeric ages at Border Cave

The recent publication of revised ESR and radiocarbon ages for the archaeological sequence of Border Cave, South Africa, provides an opportunity to assess the age estimates Miller *et al.* (1999a) obtained from *Struthio* eggshell A/I values. The reliability of A/I values in this study, which includes results from Apollo 11 Cave and Boomplaas Cave, is increased by screening eggshells against burning according to the extent of Leu hydrolysis (Miller *et al.*, 1992). As at ≠Gi, only one eggshell in the Border Cave sequence featured both radiocarbon and amino acid analyses, and like the former site, the radiocarbon age (36,100 ± 900 BP, AA-4254) is close to the limit of the technique. Instead of generating a potentially erroneous field calibration from these paired analyses the A/I values from all of lithostratigraphic unit 1 were pooled (A/I = 0.266 ± 0.020, n = 34) and their age was assumed to be 38.5 ka based on a 'large number' of radiocarbon ages averaging between 38 and 39 ka. The reaction rate calculated in this manner corresponds with an EDT of 17.5 ºC (NB: Miller *et al.* recorded the EDT between 14 and 38.5 ka to be 14.2 ºC which appears to be incorrect. The correct EDT should be 16.0 ºC, which translates to Pleistocene temperatures 3.5 ºC cooler than the post-glacial period. This correction has no influence on isoleucine epimerisation ages). The field calibration of the rate of isoleucine epimerisation was used to estimate the age of *Struthio* eggshells with A/I values that cluster about 0.33,

0.39, 0.47, 0.87 and 0.97. These ages are compared with radiocarbon and ESR dates in Figure 2.12.

With two anomalous ages removed from the Border Cave radiocarbon chronology of Bird *et al.* (2003), there is excellent agreement between the aminoages and revised ^{14}C

[a]ELSA = Early Late Stone Age; MSA = Middle Stone Age.
[b]Calibration sample.

Figure 2.12 Summary of the lithostratigraphy, archaeology, electron spin resonance, radiocarbon, and *Struthio* eggshell amino acid results for Border Cave. Electron spin resonance (ESR) and radiocarbon (^{14}C) from Grün & Beaumont (2001) and Bird *et al.* (2003), respectively. Amino acid (AAR) results from Miller *et al.* (1999a). Ages shown are mean values for their respective lithostratigraphic units.

chronology for the site. Four radiocarbon ages between 42 and 48 ka were obtained for a unit with an isoleucine epimerisation age of approximately 47 ka. Another two radiocarbon ages of *c*. 55 and 58 ka were obtained for a unit with an isoleucine epimerisation age of about 56 ka, and a further age of about 69 ka was obtained from a unit featuring five radiocarbon ages between about 55 and 58 ka and another with a minimum age of 58 ka. There is similar excellent agreement between the Border Cave isoleucine epimerisation ages and recently revised ESR chronology of Grün & Beaumont (2001). There is, however, a tendency for the ESR ages to fall slightly below those of the radiocarbon and amino acid chronologies.

2.9 AGE ESTIMATION IN GEOLOGICAL CONTEXTS

There have been two localities in which the extent of isoleucine epimerisation in avian eggshells has been used to generate chronological control for geological sequences. These localities are the lacustrine sequences of Bir Tarfawi in the Sahara (which are of archaeological significance) (Miller *et al.*, 1991) and Lake Eyre, South Australia (Magee *et al.*, 1995, Magee & Miller, 1998). At the latter locality the technique has been used for determining the timing of lacustrine and aeolian events, and in a separate study it has been used to estimate the timing of the late-survival of an extinct member of the Australian megafauna (Miller *et al.*, 1999b). Here the methods used to estimate numeric ages from the extent of isoleucine epimerisation in these contexts are examined.

2.9.1 Timing of Saharan lacustrine sequences

Miller *et al.* (1991) used the extent of isoleucine epimerisation to estimate the age of *Struthio* eggshells from lacustrine sequences in the Sahara, northeast Africa. The mean A/I value of eggshells from the base of the sequence was 1.016 and had an age of 130 ka estimated by independent means. The A/I values of eggshells in the overlying horizons were similar, about 0.98, and have an estimated age of 125 ka. The inferred rate of isoleucine epimerisation in an Early Holocene eggshell about 7500 years old with an A/I value of 0.45 was used to calculate the extent of isoleucine epimerisation expected in a terminal Pleistocene eggshell (A/I = 0.59). These paired A/I values and independent ages were then used to construct a simple model for the interpretation of *Struthio* eggshells of unknown age (Fig 2.13). Interpolating between the terminal Pleistocene and 125 ka calibration points, the A/I value (0.914) of the eggshells of unknown age was estimated to be 104 +10/-20 ka. The asymmetrical error terms reflect uncertainties in thermal history. While this framework is potentially erroneous because it assumes that the relationship between A/I and time is linear between the calibration points (e.g. long-term temperature change would readily confound this assumption), the proximity of the A/I values of the eggshell of unknown age and those of known age increases confidence in the inferred age.

2.9.2 Age of Lake Eyre sequences

Cliff and beach ridge sequences surrounding Lake Eyre, central Australia, comprise one of the most intensely dated terrestrial Quaternary sequences on the continent (Magee *et al.*, 1995, Magee & Miller, 1998). The sequence is rich in the eggshells of waterbirds (perhaps pelican) and *Genyornis* eggshells. The sequence spans about 120 ka and features radiocarbon, thermo-luminescence (TL), optically-stimulated luminescence

(OSL) and uranium series numeric ages, as well as non-eggshell fossils suitable for amino acid analyses. Thus, the setting provides an excellent setting to assess numeric ages derived from eggshell A/I values.

To interpret eggshell A/I values in terms of age, the reaction was calibrated in *Dromaius* eggshells with radiocarbon dates (Miller *et al.*, 1997) and OSL and uranium-thorium disequilibrium dates for older samples. This *Dromaius* eggshell calibration was then used to cross-calibrate the reaction in stratigraphically associated *Genyornis* and non-

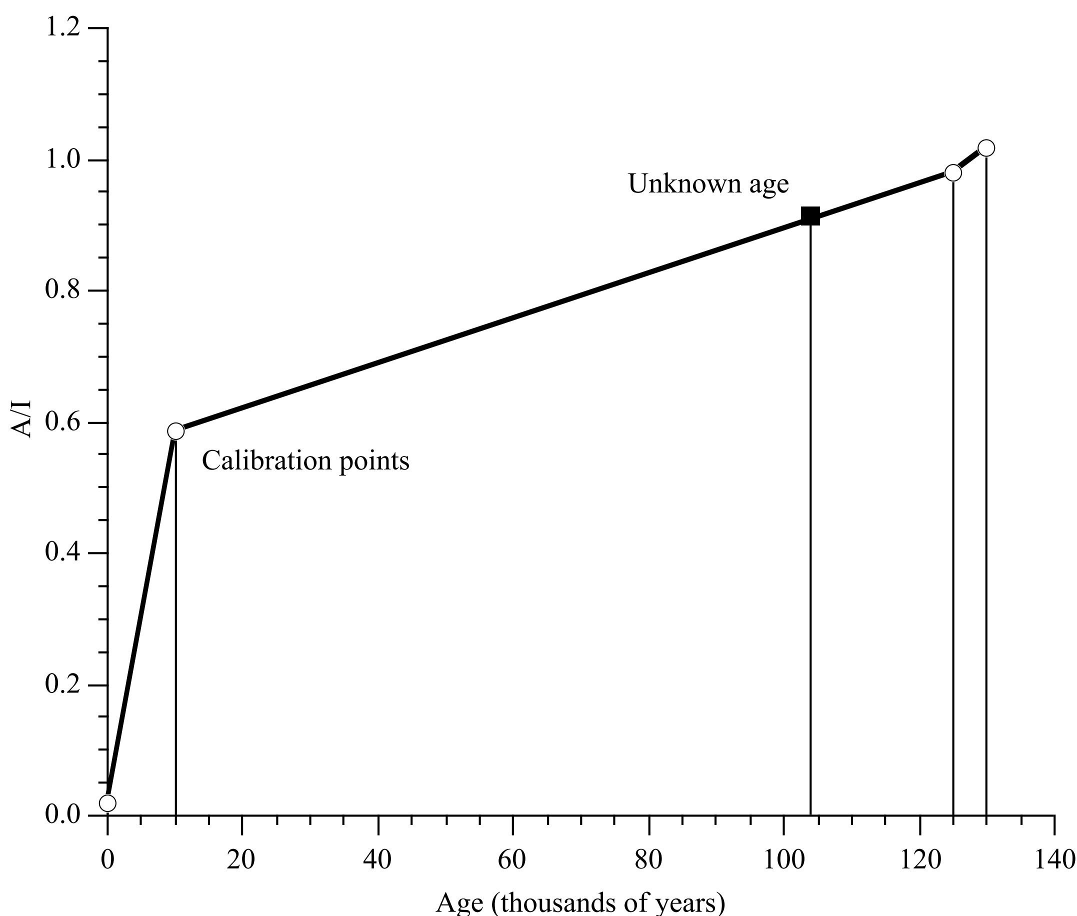

Figure 2.13 Interpolation of *Struthio* eggshell ages from the extent of isoleucine epimerisation at Bir Tarfawi. The calibration points (white circles) were used to estimate the age of eggshells of unknown age (black square) with A/I values of 0.914. The inferred age is 104,000 years.

ratite eggshells (Magee & Miller, 1998). Using this calibration, *Genyornis* eggshells from the aeolian unit in the upper reaches of the Williams Point cliff sequence were assigned aminoages of 56 to 69 ka with error terms of 10 ka. Radiocarbon ages close to 40,000 BP on *Genyornis* eggshells and *Coxiellada* shells from the unit are considered minimum ages. The isoleucine epimerisation ages are in general agreement with U/Th (64.3 ± 1.6 ka) and TL (49.1 ± 3.3 ka) ages. This aeolian unit is capped by the Shelly Island unit and this features *Dromaius* eggshell radiocarbon ages (n = 11) ranging from 12,000 to 30,000 BP, stratigraphically consistent with the isoleucine epimerisation ages. Amongst the beach ridges surrounding Lake Eyre there is a positive relationship between A/I values in *Coxiella*/*Coxiellada* shells, the eggshells of non-ratites (n = 26) and *Genyornis* (n = 53) versus the height of the ridges above sea level, reflecting different lake filling events (Fig 2.14) (Magee & Miller, 1998). The eggshell A/I values successfully separate one beach ridge from the next but the separation is not as well defined for the *Coxiella*/*Coxiellada* shells. Due to the larger number of mollusc shells analysed (n = 169) this observation may be an artefact of sample size. The eggshell and mollusc shell amino acid results provide a framework of relative ages supporting independent ages for the sequential deposition of the beach ridges.

2.9.3 Timing of *Genyornis* extinction

Building upon the extensive series of paired amino acid analyses and radiocarbon ages obtained on *Dromaius* eggshells by Miller *et al.* (1997), Miller *et al.* (1999b) constructed a model spanning the last 120 ka for interpreting the age of *Dromaius* and

Figure 2.14 Extent of isoleucine epimerisation in fossils from Lake Eyre beach ridges (Magee & Miller, 1998).

Genyornis eggshells from Lake Eyre according to their A/I values. The aim of the model was to provide a means of assigning a numeric age to the chemostratigraphically youngest *Genyornis* eggshells, thereby constraining the timing of the extinction of the taxa. The age model (Fig 2.15) is based upon 17 radiocarbon ages (some ages appear to have been rejected for reasons not stated because although they are listed in the supplementary material they were not used to construct the age model), eight OSL ages and five U-series ages. This number of calibration points is far in excess of the sample size typically used to constrain isoleucine epimerisation in avian eggshells at a given locality. The calibration curve (Fig 2.15) features four distinct segments reflecting changes in the thermal regime over glacial-interglacial timescales as well as the kinetics

Figure 2.15 Numeric calibration of the extent of isoleucine epimerisation in *Dromaius* and *Genyornis* eggshells from Lake Eyre, central Australia according to Miller *et al*. (1999b). A/I values are plotted as *Genyornis* eggshell equivalents (A/I values in *Dromaius* eggshells are 1.18 times higher than those of *Genyornis* eggshells). *Genyornis* extinction is estimated to have taken place at 50 ± 5 ka, therefore the radiocarbon ages on eggshells of this taxon represent minimum ages. The rate of isoleucine epimerisation is most rapid during warm periods as represented by more negative $\delta^{18}O$ values in b) the ice core oxygen isotope record from Vostok, Antarctica (Petit *et al.*, 1999, 2001).

of isoleucine epimerisation (i.e. initially rapid and slowly progressively). Unfortunately, the lowest *Genyornis* eggshell A/I values (0.46 < A/I < 0.50) coincided with the period of least rapid isoleucine epimerisation and this reduces the age-resolving power of the reaction, increasing uncertainty in the inferred timing of *Genyornis* extinction. To circumvent this problem the authors relied upon independent chronological control (an OSL age on sediments enclosing the youngest fragment, U-series on eggshells, and minimum radiocarbon dates on eggshell calcite) to constrain the timing of *Genyornis* extinction. These dates placed the extinction between *c*. 46 and 65 ka: the authors favoured an estimate of approximately 50 ± 5 ka.

2.9.4 Palaeotemperatures from amino acids in eggshells

Although palaeotemperatures have been inferred from the rate of isoleucine epimerisation in *Struthio* eggshells in several studies (Brooks *et al.*, 1990, Miller *et al.*, 1991, Miller *et al.*, 1992, Johnson *et al.*, 1997, Miller *et al.*, 1999a), there has been only one study in which the primary aim was to estimate the temperature at which the reaction has proceeded in order to provide insights into palaeoenvironmental conditions (Miller *et al.*, 1997). However, Miller *et al.* (1992) generated what they termed 'simultaneous temperature' estimates in order to detect *Struthio* eggshells with anomalously high thermal histories. Supported by evidence suggesting the retention of the products of amino acid diagenesis in avian eggshells, these simultaneous temperatures were estimated from paired analyses of the extent of both isoleucine epimerisation and Leu hydrolysis in *Struthio* eggshells. The temperature sensitivity of both reactions was described in terms of Arrhenius parameters by conducting high temperature experiments with ambient temperature controls. Then A/I values and the ratio of bound Leu to Leu in the total acid hydrolysate were used to solve a single

expression for the 'simulatenous temperature'. These 'simultaneous temperatures' ascribed relatively high effective temperatures to eggshells with high A/I values (i.e. presumably 'burnt') relative to the extent of the reaction in specimens from the same horizon. Hence, although the palaeotemperature estimates may be prone to large errors (± 10 ºC) the approach seems highly suited to detecting *Struthio* eggshells with anomalous thermal histories, at least where the degree of the anomaly is pronounced. The large uncertainty is an artefact of the analytical uncertainty associated with the determination of the extent of Leu hydrolysis. The extent of Leu hydrolysis is inherently more uncertain than the calculation of A/I values because it is dependent on accurately determining sample weight and the relative abundance of Leu relative to a laboratory spike for two separate eggshell preparations. By comparison, A/I is simply the relative abundance of D-aIle and L-Ile in one eggshell and is independent of sample weight.

Miller *et al.* (1997) estimated LGM temperature depression in central Australia from the extent of isoleucine epimerisation in *Dromaius* eggshells from Madigan Gulf, Lake Eyre. The rate of isoleucine epimerisation in eggshells younger than 16 ka was *c*. 37 times more rapid in eggshells aged between 16 and 50 ka, corresponding to a temperature depression of *c*. 20 ºC during the Pleistocene. However, Miller *et al.* considered this dramatic cooling an artefact of shallow burial during the LGM. It was argued that shallow burial accelerated the rate of isoleucine epimerisation in eggshells deposited between 15 and 25 ka, thus minimising the difference between A/I values in eggshells deposited during this period and those thousands of years older. To account for the influence of shallow burial the authors corrected the A/I values using asymmetric errors bars. With these corrected values the post-16 ka reaction rate was 4.9

times faster than the Pleistocene rate, corresponding with temperature depression of approximately 9 ºC. Supporting the temporal trend in isoleucine epimerisation at Lake Eyre were similar trends in A/I values with respect to time at other sites.

While statistically and analytically sound, this estimate of Pleistocene temperature depression in central Australia nonetheless appears anomalously large. However, an independent evaluation of the estimate of temperature depression is confounded by a lack of alternative palaeothermometers. Vegetation records for the region are sensitive to changes in effective moisture (Singh & Luly, 1991, McCarthy *et al.*, 1996, Johnson *et al.*, 1999), not temperature, as are other palaeoclimatic archives, such as fluvial, aeolian and lacustrine sequences (Nanson *et al.*, 1992). Estimates of temperature depression from climate models suggest less dramatic cooling 18,000 years ago than that interpreted from the eggshell A/I values (e.g. 4 ºC, Hubbard, 1995). From the altitude of evidence for Pleistocene periglacial activity (*c*. 1000 m) in the southeastern highlands, Galloway (1965) estimated Last Glacial Maximum temperature depression of at least 9 ºC. Barrows *et al.* (2004) obtained cosmogenic exposure ages of 17 – 22 ka for periglacial deposits at elevations of 1100 – 1200 m above sea level, confirming that the palaeotemperature estimate of Galloway are applicable to the most recent glacial advance. If the temperature depression inferred from *Dromaius* eggshell A/I values is anomalously large then the Arrhenius parameters must be overestimating the temperature sensitivity of the reaction at ambient temperatures. This would require that the reaction rate observed during simulated diagenesis at high temperatures be too high, ascribing an erroneously steep slope to the Arrhenius equation.

2.10 CONCLUSIONS

The racemisation of amino acids is one of the many reactions that these molecules undergo over geological time. The racemisation reaction is best suited to geochronological applications where fossils are deposited in discrete episodic events because the factors that influence the extent and rate of racemisation serve to reduce the accuracy of the technique for numeric age estimation. These same factors have a lesser impact on the use of D/L values for assigning relative ages, an application to which the technique is highly suited. Palaeotemperature applications necessitate both numeric age control on field specimens and mathematical description of the temperature sensitivity of the reaction. Statistically, the use of D/L values for investigating temperature change is more robust than their use for numeric age estimation. Both palaeotemperature and geochronological applications would benefit from further investigation into the applicability of observations of amino acid racemisation at high temperatures to environmental temperatures.

The prime advantage of eggshells as a sample type for amino acid racemisation geochronology and palaeothermometry is their ability to retain indigenous amino acids. That eggshells preserve amino acids well is supported by the relatively long range over which first-order kinetics are observed (at least at high temperatures), the suggestion that eggshell organics are locked amongst the calcite crystals, the insensitivity of isoleucine epimerisation in the total acid hydrolysate to the abundance of water under controlled conditions, and the ability for results of experiments at high temperatures to be replicated in both modern and ancient eggshells. The best evidence for the excellent retention of eggshell amino acids are steady concentrations of thermally stable amino acids in fossil eggshells during diagenesis. However, it is important to note that the

research of Miller *et al*. (2000) indicates that eggshells fail to approximate a closed system during early diagenesis (*c*. 100 years). Given that the loss of these labile amino acids is rapid, this process will not convolute the long-term diagenesis of eggshell amino acids in the field. However, the implications of this geologically-ephemeral open system behaviour for extrapolations from laboratory experiments conducted on modern eggshells to field conditions is not clear. Providing the amino acids lost during early diagenesis are similar in their biogeochemistry to those that are retained, scope remains for relevant extrapolations from observations made under controlled laboratory conditions to the amino acids residues of ancient eggshells.