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Predicting protein decomposition: the case of aspartic-acid racemization kinetics

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The increase in proportion of the non-biological (D-) isomer of aspartic acid (Asp) relative to the L-isomer has been widely used in archaeology and geochemistry as a tool for dating. The method has proved controversial, particularly when used for bones. The non-linear kinetics of Asp racemization have prompted a number of suggestions as to the underlying mechanism(s) and have led to the use of mathematical transformations which linearize the increase in D-Asp with respect to time. Using one example, a suggestion that the initial rapid phase of Asp racemization is due to a contribution from asparagine (Asn), we demonstrate how a simple model of the degradation and racemization of Asn can be used to predict the observed kinetics. A more complex model of peptide bound Asx (Asn + Asp) racemization, which occurs via the formation of a cyclic succinimide (Asu), can be used to correctly predict Asx racemization kinetics in proteins at high temperatures (95–140 °C). The model fails to predict racemization kinetics in dentine collagen at 37 °C. The reason for this is that Asu formation is highly conformation dependent and is predicted to occur extremely slowly in triple helical collagen. As conformation strongly influences the rate of Asu formation and hence Asx racemization, the use of extrapolation from high temperatures to estimate racemization kinetics of Asx in proteins below their denaturation temperature is called into question.

In the case of archaeological bone, we argue that the D:L ratio of Asx reflects the proportion of non-helical to helical collagen, overlain by the effects of leaching of more soluble (and conformationally unconstrained) peptides. Thus, racemization kinetics in bone are potentially unpredictable, and the proposed use of Asx racemization to estimate the extent of DNA depurination in archaeological bones is challenged.

Keywords: aspartic-acid racemization; deamidation; kinetics; dating; bone; collagen

1. INTRODUCTION

How predictable is protein preservation in the fossil record? The question remains largely unanswered despite its pertinence to current research and potential applications. Ancient DNA studies have mirrored research into ancient proteins—practitioners initially prospected for the oldest or most spectacular samples rather than systematically investigating the processes of long-term survival. Despite the 25-year head-start that ancient protein research has had over that of DNA, the failure to address the issue of diagenesis means that we still have not successfully moved beyond this prospecting phase.

One area of ancient protein research where much greater investment has been made into predictive models of diagenesis, is that of amino-acid racemization. The gradual increase in non-biological isomeric forms of the constituent amino acids within proteins has been used as both a dating tool and a method of palaeothermometry. Amino acids with one or more chiral carbon centres undergo isomerization (termed racemization); the rate of inter-conversion is governed by time and temperature. Thus, if temperature remains constant, the increase in the D-isomer can be used chronometrically (e.g. Goodfriend 1991); conversely if the age is known information can be obtained on palaeotemperature (e.g. Miller *et al.* 1997).

The most common approach has been to use kinetic models based upon isomerization of free amino acids at high temperatures to estimate racemization rates at burial temperatures. The problem with this approach (recognized early in the investigations, e.g. Bada 1972; Bender 1974) is that the rate of racemization in fossil proteins is governed not by the simple, predictable kinetics of free solution, but by the local environment of the bound residue, which changes during protein diagenesis and can be influenced by the burial environment. The challenge is to identify those key factors which influence racemization in the burial environment.

2. ASPARTIC-ACID RACEMIZATION

Of all the amino acids used for racemization analysis, it is undoubtedly aspartic acid (Asp) which has the most chequered history as a dating tool. Measurable increases in D-Asp accrue over years (Goodfriend 1992) to tens of millennia (Goodfriend 1991). In the early 1970s the method excited interest as a means of dating archaeological bone

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because it required less material than early radiocarbon dating techniques (e.g. Bada & Protsch 1973). Unfortunately problems were encountered with the method. Upper Pleistocene racemization dates for Palaeoindian remains in California, which supported an early colonization of North America, became a cause célèbre (e.g. Pollard & Heron 1996) when AMS ^{14}C dates proved that the bones were only 5100 ± 500 BP (Bada 1985). In retrospect, part of the error arose from inaccurate radiocarbon dating of the calibration material, but racemization dating in the archaeological community was near-fatally mauled. Slowly, however, the method is re-establishing itself in the 'geoarchaeological' community (e.g. Brooks *et al*. 1990; Goodfriend 1991 1992; Johnson & Miller 1997), and has found a valuable niche in the forensic sciences for estimating age at death (Ritz & Kaatsch 1996). Furthermore, Asp racemization is becoming more widely used as a screening method for bones prior to DNA analysis (Poinar *et al*. 1996; Krings *et al*. 1997).

3. PROBLEMS WITH THE KINETICS OF ASPARTIC ACID

The very strength of 'Asp' racemization, its unusually rapid initial rate (Goodfriend 1992), is also a drawback, in that the kinetics are atypical. The rapid initial rate of 'Asp' can be illustrated by plotting relative rates of racemization for different amino acids measured in the same system (figure 1). Where amino acids display comparable kinetics, their rates will correlate. This is not the case for 'Asp' which has both a 'fast' and 'slow' component (figure 1). The atypical pattern of 'Asp' racemization has prompted a number of explanations. Smith & Evans (1980) have suggested that the change to a slower rate in heated collagen was due to the increasing proportion of more slowly racemizing free amino acids as a result of hydrolysis of peptide bonds over time. Goodfriend (1991) notes that the signal measured as Asp is actually the combined responses of Asp + asparagine (Asn), the latter decomposing to Asp upon acid hydrolysis; to avoid confusion this combined signal is hereafter referred to as Asx. Goodfriend (1991) suggested that the pattern of Asx racemization may be due to differences in the rate of succinimide formation (and concomitant racemization) from Asn and Asp. Brinton & Bada (1995) argued that the effect was due to differences in the rate of racemization and the direct decomposition of Asn to Asp by hydrolysis of the amide group. Using pure Asn they were able to obtain kinetic patterns for Asx similar to those reported by Goodfriend *et al*. (1992) (figure 2, inset).

The kinetics of Asx racemization have proved more difficult to describe mathematically than those of most other amino acids. In order to produce a proportional increase in D-Asx with age, Goodfriend & Hare (1995) and Goodfriend *et al*. (1996) adopted power-function transformations of the D:L ratio for both mollusc and ostrich shells (e.g. figure 2). Such transformations are useful in calibrated investigations to assess age over a broad range of D:L ratios. Applying the same power transformation to the data from Brinton & Bada (1995) also produces a strong correlation with time (figure 2). However, the transformation does not offer an explanation

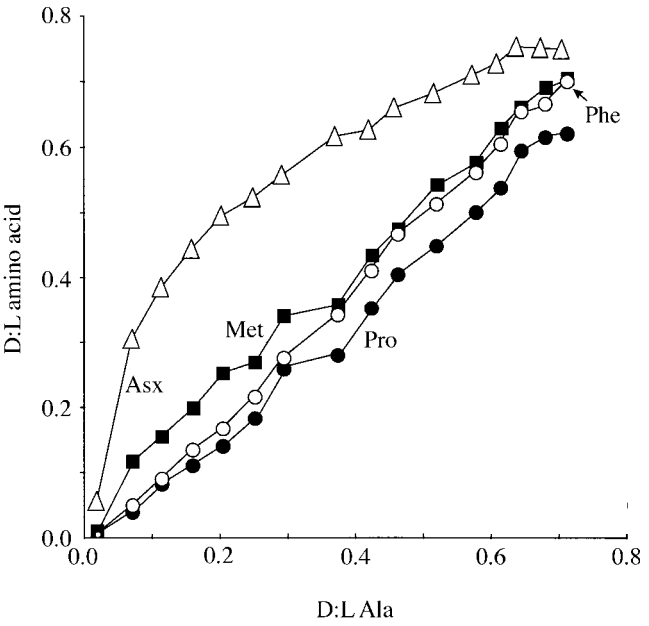

Figure 1. Comparative rates of racemization for different amino acids from mollusc shells during artificial diagenesis plotted against D:L ratio of alanine (Ala). Note that unlike methionine (Met, solid squares), proline (Pro, solid circles) and phenylalanine (Phe, open circles), the rate of increase of Asx (Δ) is not linear with respect to Ala, but is distorted by a rapid initial phase (data from Goodfriend & Meyer (1991)).

of the observed kinetics. Different power-functions are used for different taxa—in the case of the mollusc *Mya*, no transformation of the D:L ratio satisfactorily linearizes the data over the full range of values (Goodfriend *et al*. 1996).

In addition to the difficulties in obtaining a function to describe the complex kinetics of Asx, other problems with the application of the method include the following:

(i) in free solution serine and threonine racemize more rapidly than Asp (Wonnacott (1979) cited in Smith & Evans (1980)), but in proteins Asx residues have the highest rate;
(ii) an 1800-year age for a living deep-water anemone *Gerardia* determined by ^{14}C (Druffel *et al*. 1995) is apparently contradicted by an Asx date of only 250 years estimated by extrapolation from high temperature experiments (Goodfriend 1997);
(iii) despite the fact that Asx racemization in dentine is predictable (Ritz & Kaatsch 1996), the results can be significantly influenced by preparation method (Collins & Galley 1998), and Asx in rat dentine appears to racemize ten times faster than human dentine (Ohtani *et al*. 1995);
(iv) in historical material the D-Asx concentration in dentine collagen all tends towards a consensus value, independent of age (Gillard *et al*. 1991; Carolan *et al*. 1997).

4. AN ALTERNATIVE APPROACH TO ASX KINETICS

As problems with the method begin to emerge, and in the absence of a satisfactory mathematical description of the kinetics, we propose an alternative approach. Instead of relying on ever more complex mathematical

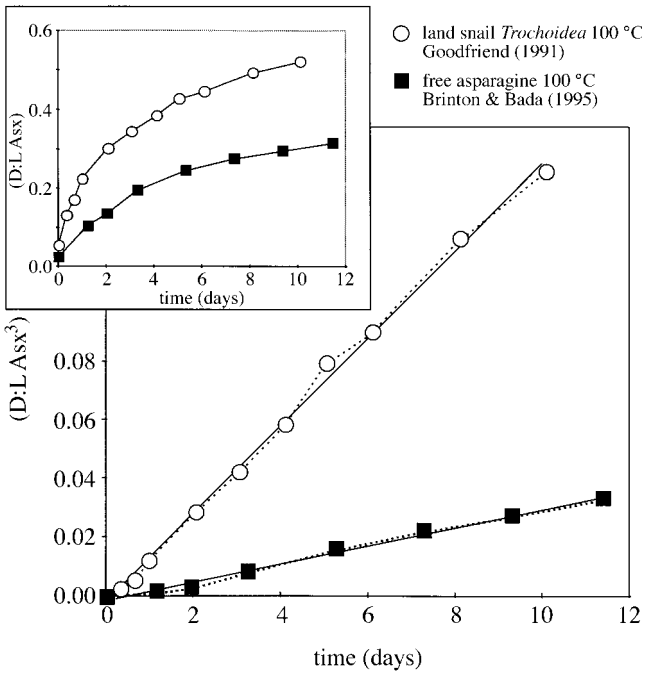

Figure 2. The use of a cubic transformation of the D:L ratio for data from Goodfriend (1992) and Brinton & Bada (1995) to linearize the original kinetics (inset) and derive a slope which is proportional to age.

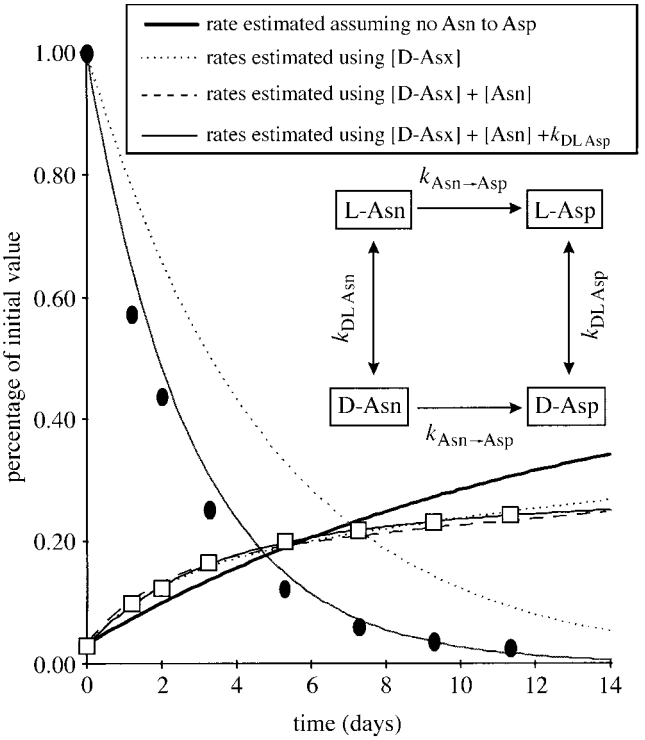

Figure 3. Illustration of the application of the modelling approach to explain the observed kinetics of increase in D-Asx (open squares) caused by the simultaneous racemization of Asn, Asp and the decomposition of Asn (closed circles) to Asp (data from Brinton & Bada (1995)). Lines represent output from the model when optimized using different data sets.

transformations we use simple mathematical models to describe the kinetics of a system, and then estimate unknown rates by optimization. The models describe the system under investigation as accurately as is practicably possible; the number of unknown parameters is kept to a bare minimum to reduce the number of unique solutions. Our approach can be illustrated by reference to the previously cited study of Brinton & Bada (1995).

Most racemization studies assume that the system conforms to simple first-order reversible kinetics. However, figure 3 shows that, when only the L-Asn to D-Asn reaction is taken into account in the model, the initial fast racemization of D-Asx cannot be reproduced as the predicted initial reaction rate is too slow. Asn is unstable and over the course of the experiment will degrade to Asp. When the potential for decomposition of Asn to Asp is added to the model a much better fit with the experimental data is obtained, faster racemizing Asn decomposing rapidly to slower racemizing Asp. The rate of Asn decomposition predicted solely from the racemization kinetics is in reasonable agreement with the measured rate for this experiment, although these data were not used in the optimization (figure 3). If these data are used in the optimization, more accurate estimates of the remaining unknown reaction rates are obtained.

The simple model illustrated in figure 3 was appropriate for degradation of pure Asn *in vitro*, but is clearly too simple for studies of Asx racemization in proteins. Here, the most probable pathway of racemization of Asx is via an aminosuccinyl residue (Asu; Geiger & Clarke 1987). *Ab initio* calculations have been used to illustrate that racemization in the Asu residue can be five orders of magnitude faster than free Asp (Radkiewicz *et al.* 1996). Aspartic acid and Asn form Asu by nucleophilic attack on the β-carbonyl group by the NH-group of the downstream (C-terminal) peptide bond, resulting in the formation of D-Asp isomers and isoaspartyl (iAsp) residues. The rate of formation of Asu is dependent upon factors which increase the deprotonation of the peptide nitrogen. These factors include high pH, (Capasso *et al.* 1992, 1993), high ionic strength (Capasso *et al.* 1991; Tyler-Cross & Schirch 1991), high dielectric constant (Brennan & Clarke 1993) and conformational flexibility within the residues (Kossiakoff 1988; Lura & Schirch 1988; Bongers *et al.* 1992; Stevenson *et al.* 1993; Tomizawa *et al.* 1995).

5. A MODEL OF PEPTIDE-BOUND ASX KINETICS

Most investigations of Asx racemization have used collagen rich tissues (bone or dentine; Appendix A). Thus, collagen would seem a suitable protein in which to model Asx racemization and test our approach; the most satisfactory study for our purposes is de Sol (1978) who investigated the rate of Asx racemization in collagen at pH 8 between 95 and 140 °C. We have developed a model of Asx racemization based upon data given in Geiger & Clarke (1987). The original model of Geiger & Clarke (1987) was developed to describe Asn decomposition and Asx racemization in a hexapeptide from adrenocorticotropic hormone and requires some modification for use with collagen. The modifications take into account (i) the impact of residues adjacent to Asn and Asp, (ii) additional information on the rate of deamidation of Asn, and (iii) an observed equilibrium D:L ratio of 1 observed by de Sol (1978) in collagen.

(a) *Modification 1—the influence of flanking residues*

Studies on the rate of Asn deamidation in synthetic peptides have revealed that the rate of Asu formation (and hence deamidation) can vary by almost three orders

Table 1. *Relative rate of Asu formation in relationship to residues carboxyl and amino to Asn*

(Comparison of the enthalpy (H kJ mol^{-1}) and entropy (S J mol^{-1}N K) of energy-minimized structures in gas phase using molecular mechanics (Burkert & Allinger 1982). Rates of deamidation are from McKerrow (1973; pH 7.4, 0.02 M phosphate, 37 °C). The gas-phase energies are of limited value as they do not take into account any interactions with water, but they reveal that in general the ease of Asu formation decreases with increasing bulkiness of the downstream residue but are much less sensitive to the upstream flanking residue.)

sequence	H(Asn)	S(Asn)	H(Asu)	S(Asu)	k (s^{-1})	ΔH	ΔG_{310}
Gly-Gly-Asn-Gly-Gly	−330.74	789.36	−112.41	714.22	—	−218.33	−241.63
Gly-Gly-Asn-Ala-Gly	−305.38	811.85	−64.27	717.22	9.20×10^{-8}	−241.11	−270.45
Gly-Ala-Asn-Leu-Gly	−376.79	910.10	−139.49	819.12	2.90×10^{-8}	−237.29	−265.50
Gly-Ala-Asn-Ala-Gly	−280.02	831.72	−44.20	735.56	8.40×10^{-8}	−235.82	−265.62
Gly-Ile-Asn-Gly-Gly	−322.00	887.86	−130.01	776.72	—	−191.99	−226.44
Gly-Ile-Asn-Val-Gly	−381.06	961.36	−160.79	891.80	—	−220.26	−241.83
Gly-Ile-Asn-Leu-Gly	−393.86	988.69	−166.29	881.38	—	−227.57	−260.84
Gly-Ile-Asn-Ala-Gly	−299.47	910.35	−70.10	799.09	1.60×10^{-8}	−229.37	−263.86
Gly-Ile-Asn-Ile-Gly	−313.71	989.80	−74.82	916.14	—	−238.89	−261.73
Gly-Leu-Asn-Ala-Gly	−378.39	909.86	−154.23	802.17	3.70×10^{-8}	−224.16	−257.54

Table 2. *The distribution of residues carboxyl to Asn and Asp in bovine type I collagen, and the effect of residues carboxyl to Asn on the rate of deamidation relative to Asn-Gly*

	Asn-X			Asp-X			rate relative to Gly[a]		
residue X	α-1	α-2	total	α-1	α-2	total	x	s.d.	n
Gly	6	10	22	15	13	43	1	0.8	5
Ala	2	1	5	6	1	13	80.3	86.8	21
Arg	0	1	1	5	1	11	31.2	—	1
Pro[b]	2	5	9	0	0	0	73.9	13.7	4
Asp	4	1	9	1	0	2	23.8	—	1
Lys	2	2	6	2	2	6	79.7	0.4	2
Val	2	1	5	0	0	0	117.3	69.9	4
Leu	0	3	3	2	0	4	86.9	102.7	4
Ser	2	0	4	0	2	2	8.9	5.6	3
Tyr	0	0	0	1	0	2	—	—	
Thr	0	0	0	1	0	2	23.8	—	1
Gln	0	0	0	0	3	3	—	—	
total	20	24	64	33	22	88			

[a] Data from McKerrow (1973), Brennan & Clarke (1995), Tyler-Cross & Schirch (1991), Patel & Bouchardt (1990*a*).
[b] Includes Hyp.

depending upon the primary sequence (e.g. Cleland *et al.* 1993). Although the rate is influenced by both flanking residues, the greatest influence is exerted by the downstream (i.e. carboxyl to Asn) residue which takes part in Asu formation; generally the bulkier the side chain of the downstream residue the less readily Asu formation occurs (Brennan & Clarke 1993; Oliyai & Borchardt 1994). We have observed the potential impact of bulky residues on the rate of Asu formation in molecular mechanics calculations of energy minimized structures (table 1).

Bovine type I collagen contains a total of 88 Asx residues. The downstream residues found in collagen are listed in table 2, along with average deamidation (and hence Asu formation) rate relative to an Asn-Gly sequence; almost half of the Asn and Asp residues are adjacent to Gly, (the most reactive combination). The large standard deviations for each of the residues reflects the effect of other factors, such as inter-study variations in peptide length, buffer concentration, pH and adjacent primary structure, (see source references for further details). The data in table 2 are used to derive estimates of the reduction in rate of Asu formation caused by downstream residues. In the model the difference between labile (i.e. Asx-Gly) and bulky residues carboxyl to the Asx residue are accounted for by fast and slow pools (figure 4).

(b) *Modification 2—estimate of deamidation rate*

Deamidation to form L-Asu is the first step in the decomposition of Asn residues. We believe that the activation energy (E_a) for deamidation estimated by Geiger & Clarke (1987; $E_a = 88.7$ kJ mol^{-1}) is too low. We have derived a consensus kinetic parameter for $k_{deamidation}$ using the high temperature data of Sinex (1960; pH 7.35) for collagen and the rates of Bongers *et al.* (1992; pH 8.0) and Geiger & Clarke (1987; pH 7.4) for Asn-Gly residues in oligo- and polypeptides. The temperature dependence in the three studies is remarkably consistent (figure 5) and yields a consensus E_a of 93.6 kJ mol^{-1} (cf.

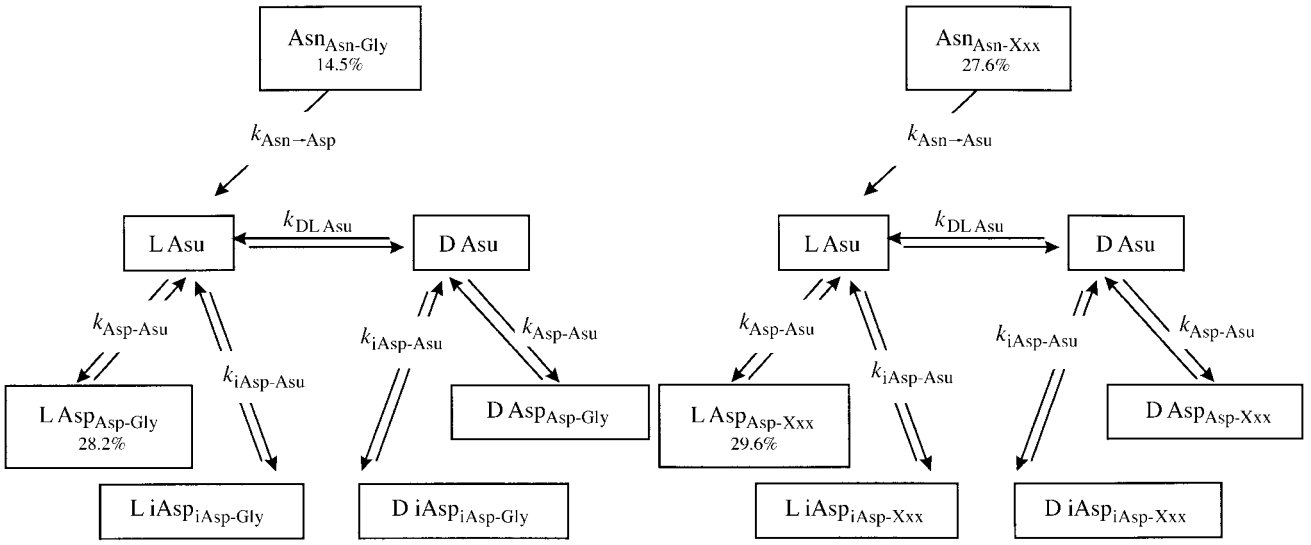

Figure 4. Model for racemization of Asx and decomposition of Asn as peptide-bound residues based upon Geiger & Clarke (1987). The model is divided into two components, the left-hand side represents fast racemizing Asx-Gly residues, which account for almost half of the residues in collagen. The right-hand side combines all the other residues in a 'slow' fraction (see table 2). Kinetic parameters for deriving rate constants are given in table 3. Note that the model does not include hydrolysis of peptide bonds which will become a significant feature of Asx decomposition over archaeological time-scales.

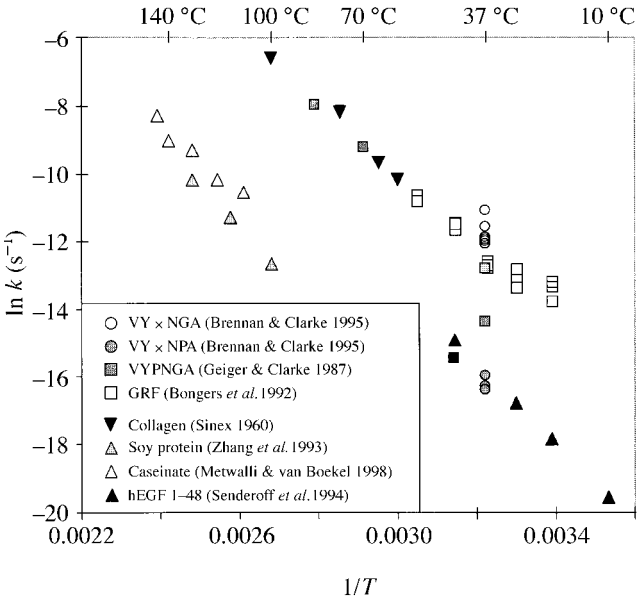

Figure 5. Rate of deamidation of Asn in proteins (triangles) and peptides (squares and circles). Note the very similar rates of deamidation observed in collagen and peptides. In the case of collagen, soy protein and caseinate rates are determined from the release of ammonia and thus will include a contribution from glutamate. Note the influence of flanking residues, the rate of deamidation of Asn-Gly is almost two orders of magnitude faster than Asn-Pro in Val-Tyr-Xxx-Asn-Xxx-Ala peptides.

Patel & Borchardt 1990*b*: 90.8 kJ mol^{-1}; Senderoff *et al.* 1994: 98.9 kJ mol^{-1}). The rate of deamidation in collagen is much higher than reported rates for other proteins, reflecting the abundance of Asn-Gly residues in collagen sequences (figure 5).

(c) *Modification 3—equilibrium D:L ratio*

Deviations from the expected 1:1 D:L ratio are anticipated in peptide bound residues if the local environment within the protein structure favours the D-Asx residue over its L-counterpart. Fujii *et al.* (1994) have reported the phenomenon of 'stereoinversion', in which certain sites appear to rapidly accumulate D-Asp, such that the D:L ratio at one site in αA-crystallin was 5.7 in iAsp residues of an 11-month-old human lens. In the Val-Tyr-Pro-Asn-Gly-Ala peptide used by Geiger & Clarke (1987) the equilibrium D:L ratio for Asp was estimated to be 0.38 at 37 °C. It is much more surprising to find non-unity equilibrium ratios in short peptides than in proteins; we believe that this phenomenon requires further investigation as it is never reported in the geochemical literature. It may be that non-unity equilibrium ratios are common at individual Asx sites but that in a polypeptide they broadly even out. A more likely and probably more significant cause in geochemical studies is that investigations extend over longer time-scales of protein degradation ultimately leading to the release of free amino acids (whose equilibrium D:L ratio is 1). As equilibrium ratios were unity in the modelled data sets we have chosen to use the L-Asx kinetic parameters for both L- and D-residues (table 3). The E_a of reactions involving D-Asx residues (illustrated in fig. 4c of Geiger & Clarke (1987)) are markedly higher than

Table 3. *Values for activation energy (E_a) and the pre-exponential factor (A) used in collagen racemization model, where Xxx is any residue other than Gly*

reaction			Asx-Gly	Asx-Xxx
from	to	E_a kJ mol^{-1}	A	A
L-Asn	imide	93.6	1.99×10^{10}	4.94×10^{8}
Imide	isopeptide	93.7	3.96×10^{11}	9.83×10^{9}
isopeptide	imide	90.8	3.23×10^{8}	8.01×10^{6}
Imide	peptide	94.1	1.28×10^{11}	3.18×10^{9}

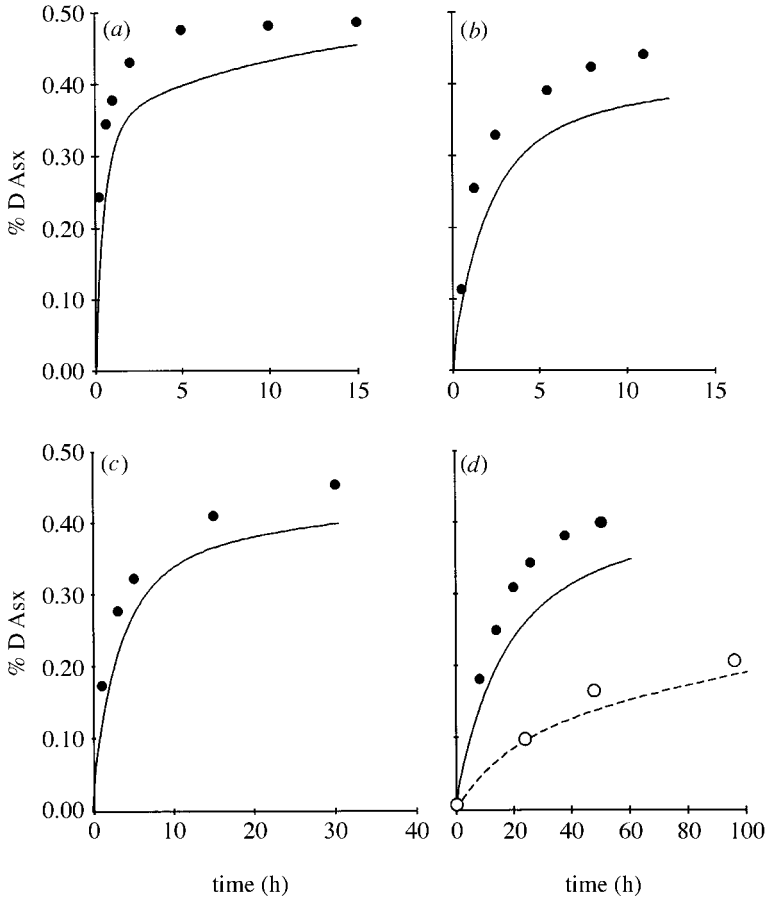

Figure 6. Results of applying the model illustrated in figure 4 to the data on Asx racemization in collagen (closed circles; data from de Sol (1978)) and α-crystallin at 96 °C (open circles; data from Masters (1985)). The parameter settings for the proportions of Asn to Asp in collagen are 14.5% Asn-Gly, 27.6% Asn-Xxx, 28.2% Asp-Gly, 29.6% Asp-Xxx.; for α-crystallin (assuming a ratio of α_A- to α_B- crystallin of 3) 1.6% Asn-Gly, 12.4% Asn-Xxx, 7% Asp-Gly, 79% Asp-Xxx. The estimated dampening effect of flanking residues on Asu formation for collagen are Asn-Xxx 63-fold, Asp-Gly 52-fold, and for α-crystallin, Asn-Xxx 61-fold, Asp-Gly 58-fold. (*a*) 140.5 °C; (*b*) 121.5 °C; (*c*) 114.6 °C; (*d*) 95.5 °C.

those for L-Asx residues, in the Val-Tyr-Pro-Asn-Gly-Ala peptide (Geiger & Clarke 1987), favouring D-Asx at high temperatures and L-Asx at low temperatures (e.g D:L ratio 2.05 at 140 °C but 0.24 at 11 °C). Our modification ensures that equilibrium ratios for our model are always unity, but highlights the lack of sufficient data on the kinetics of D-Asx residues.

6. TESTING THE MODEL

Having adapted the model of Geiger & Clarke (1987) for collagen, its predictions can be compared with the observed racemization rates reported by de Sol (1978). Despite (or perhaps because of) the fact that additional information, such as the rate of deamidation or release of free amino acids, is not available and therefore cannot be tested, the shapes of the curves are remarkably similar (figure 6).

The observed break in slope, observed by de Sol (1978), for collagen is predicted by the model and derives from the different rates of Asu formation from Asn and Asp, and also the influence of fast versus slow Asx-Xxx residues. Brinton & Bada (1995) are correct in believing that the initial racemization rate of Asx can be explained by the contribution and decomposition of Asn. However this is not due to the accelerated rate of racemization of free Asn, but rather the greater propensity of some residues, notably (i) Asn-Xxx, and (ii) Asx-Gly, to form Asu than Asp-Xxx residues. The model also predicts that the break in slope occurs at lower D-Asx values at lower temperatures; this temperature dependence can be clearly seen if the model is run assuming 100% Asn-Gly at extremes of temperature (figure 7).

If the model works for collagen, can it work for other proteins? If the primary structure of a protein is known, the model should be able to make a reasonable prediction of its racemization rate at high temperature. Changes must be made in the model to reflect the different proportions of Asx-Gly to Asx-Xxx, Asn to Asp and also the dampening effect of residues downstream of the Asx. In the case of α-crystallin the model successfully predicts a slower racemization rate than for collagen, which is due to the lower concentrations of (i) Asn and (ii) Gly carboxyl to Asx (figure 6).

Despite the success of the model in predicting racemization rates from high temperature experiments, it fails to predict rates in mineralized collagen. Figure 8 illustrates data on racemization of Asx in total human dentine and dentine collagen. The rates are transformed to yield straight lines with a slope $2k$ assuming reversible first order kinetics. Correlations between the increase in %D-Asx with time are high for total dentine. However the observed rate is 350 times slower than the prediction of the model.

The failure of our model to successfully predict successfully the rate of Asx racemization *in vivo* illustrates the key problem with the racemization method. The model works for short peptides (Geiger & Clarke 1987) and proteins at high temperatures (de Sol 1978; Masters 1982) for one key reason—in small peptides and denatured proteins the peptide backbone is conformationally unconstrained. The formation of the Asu places considerable strain on the surrounding peptide backbone and thus secondary and higher order structure severely dampen the rate of deamidation (e.g. Kossiakoff 1988; Stevenson

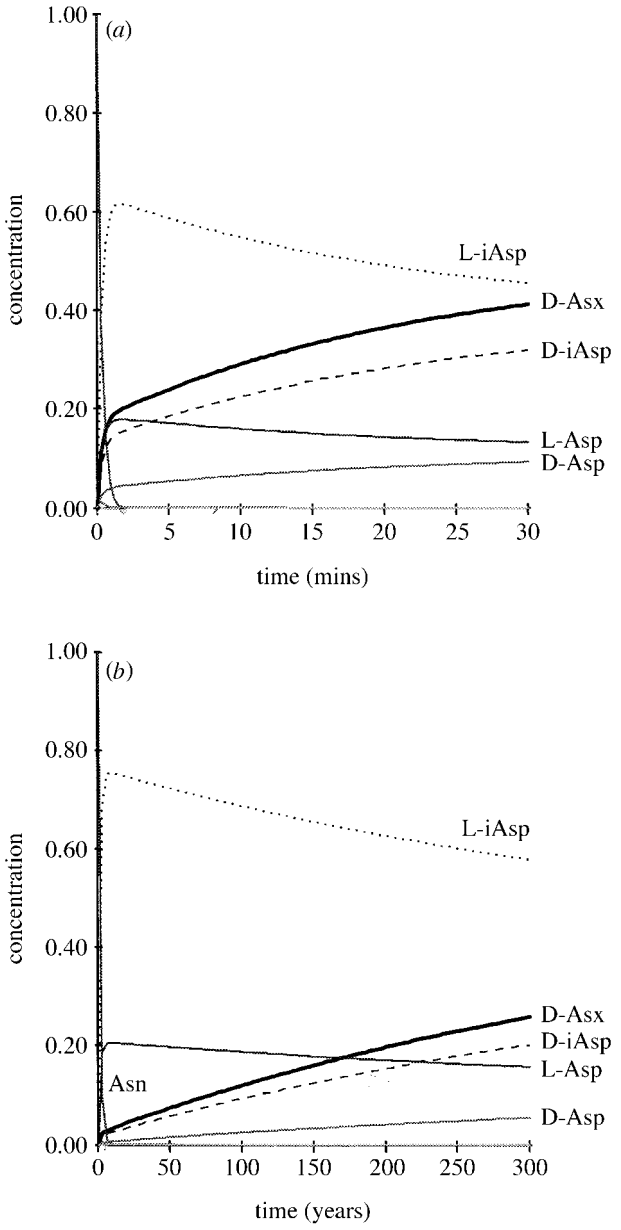

Figure 7. Predicted racemization kinetics of Asn-Gly rich protein at high and low temperatures. Compare the position of the break in slope, due to the lower activation energy of deamidation. This is a much more significant process in high temperature experimental diagenesis than it is at geochemically significant temperatures. (*a*) 150 °C; (*b*) 0 °C.

et al. 1993) and racemization. Collagen, although the protein most commonly analysed in archaeological studies of Asx racemization (Appendix A), offers one of the most extreme examples of this effect. Glycine is carboxyl to almost half of the Asn and Asp residues (table 2), thus denatured collagen is anticipated to display high rates of deamidation and racemization. The rates of deamidation of collagen at high temperatures (Sinex 1960), are similar to those for Asn-Gly residues in oligo- and polypeptides (figure 5). Within the extreme conformational constraints imposed by the collagen triple helix rates are anticipated to be much slower; Sinex (1960) reported that below the shrinkage (i.e. denaturation) temperature of collagen, the deamidation rate was too slow to be estimated accurately.

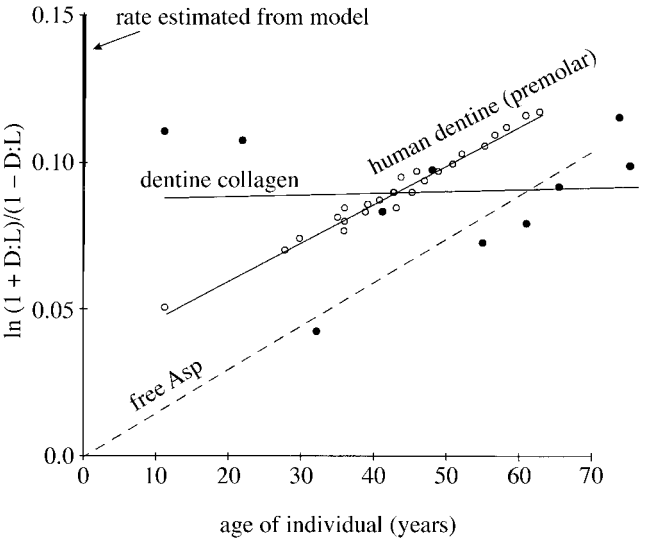

Figure 8. Illustration of the use of the model to predict the rate of racemization of Asx in total human dentine (Ohtani & Yamamoto 1991; $k_{\mathrm{DL\ Asx}} = 0.65 \times 10^{-3}$, $R^2 = 0.9773$) and dentine collagen (Cloos 1995; $k_{\mathrm{DL\ Asx}} = 3 \times 10^{-5}$, $R^2 = 0.0037$). The high levels of D-Asx for the CNBr digests of dentine collagen are caused by the method of isolation. An estimate based upon the rate of racemization of free Asp (Bada 1971) is more similar to the rate of human dentine than the model, although this is a coincidence.

Using molecular dynamics (MD) we have estimated that the rate of Asn deamidation by Asu formation at 37 °C would be some 10 000-fold lower in an extended α-chain than in random coil gelatin (van Duin & Collins 1998). So slow do we predict the rate of Asu formation to be, that deamidation of Asn in native collagen will probably occur by direct hydrolysis. This suggestion is of relevance to the leather industry. It is common practice to lime hides, as the resulting deamidation of Asn to Asp improves the efficiency of chrome tanning (used to stabilize the helix). Introduction of iAsp residues into the α-chain, which results from deamidation via Asu, would lead to local disruption of the triple helix; if deamidation conditions are chosen to prevent significant gelatinization this problem should not occur.

7. ASX RACEMIZATION IN ARCHAEOLOGICAL BONE AND DENTINE

The mean D:L ratio from an insoluble fraction of a 20 000-year-old bone from Taishaku Konondo Cave Site, Japan (estimated burial temperature 19 °C) is only 0.084 (Matsu'ura & Ueta 1980). The same amount of racemization which was measured over 20 000 years in the 'collagen' extract is estimated (from our model) to accumulate in gelatin in less than a year. What relevance does our model have to Asx racemization in archaeological bone?

On the evidence of our MD calculations we believe that racemization is unlikely to occur in triple helical collagen below its denaturation temperature (T_{m}); T_{m} (demineralized collagen) = 68 °C, T_{m} (mineralized collagen) = 150 °C (M. J. Collins, unpublished data). This conclusion is supported by a number of lines of evidence: (i) rates of Asx racemization reported by

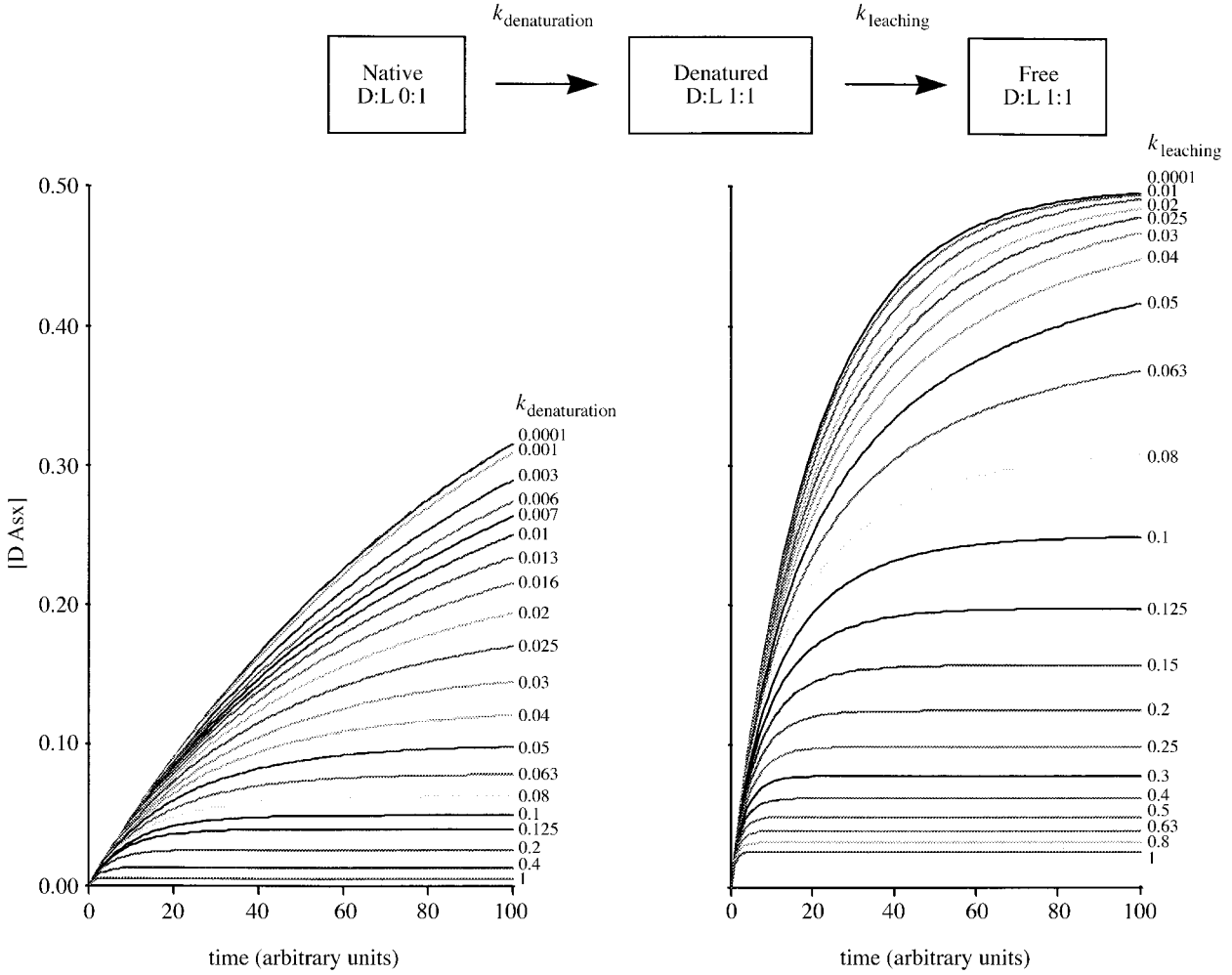

Figure 9. A simplified model of 'racemization kinetics' of bone collagen over archaeological time. Almost any value of D-Asx can be obtained by differences in the rate of collagen denaturation ($k_{denaturation}$) and leaching of the denatured product ($k_{leaching}$).

Ohtani & Yamomoto (1992) for dentine at 140 °C are 1000 times slower than gelatinized collagen at the same temperature (de Sol 1978); (ii) Julg *et al.* (1987) failed to measure racemization below the melt temperature of collagen in the laboratory; (iii) Cloos (1995) failed to observe any increase in %D-Asx in dentine collagen isolated by CNBr digests of human teeth (figure 8); (iv) Weiner *et al.* (1980) observed a correlation between the proportion of gelatin in parchment and the amount of D-Asx; some of the D:L ratios in triple helical rich regions of the parchments were of the same order as modern collagen.

If there is no racemization in the collagen triple helix, what does the increase in D-Asx in archaeological bones actually represent? Only 90% of the protein in bone (Triffit 1980) and dentine (Linde 1989) is collagen. Some of the so-called non-collagenous proteins (NCPs), notably phosphophoryns in dentine, have sequences rich in Asp-Ser (Ritchie & Wang 1996; Hirst *et al.* 1997) which should be prone to rapid racemization (Masters 1985). Many studies of dentine proteins have observed a much faster rate of racemization in the soluble protein fraction (e.g. Ohtani & Yamomoto 1991; Ritz *et al.* 1993), the observed rate being dependent upon the extent of contamination with collagen (Collins & Galley 1998). These soluble proteins contribute to the increase in D-Asx seen in dentine with time, but risk being leached from bones and teeth in the burial environment; no systematic studies have investigated their survival in archaeological bone.

The insoluble fraction of dentine and bone is not composed solely of collagen. Some NCPs are very tightly associated with collagen and are not removed upon extraction (e.g. Stetler-Stephenson & Veis 1986; Fujisawa *et al.* 1994). Even if pure collagen is isolated we would anticipate racemization in the non-helical (i.e. telopeptide) regions—iAsp residues have been observed in telopeptides *in vivo* (Garnero *et al.* 1997). Complete racemization of the telopeptides would yield a D:L ratio of 0.09, which would be achieved in about 500 years at 11 °C (i.e. typical UK burial temperatures). We envisage that in the absence of any microbial influence (Child *et al.* 1993) the loss of faster racemizing soluble proteins and the complete racemization of Asp in the telopeptides will result in a trend towards a consensus value for all mineralized collagen over periods of decades to centuries. Both Gillard *et al.* (1991) and Carolan *et al.* (1997) observed such trends in historical material.

As collagen degrades over time and the triple helix denatures, the proportion of D-Asx will increase. D-Asx

will accumulate at flexible frayed ends of the helix following chain scission (Collins *et al.* 1995) or in gelatinized regions following wholesale denaturation (e.g. cooking). Further scission will tend to preferentially release these racemized residues if, as evidence suggests, hydrolysis targets the flexible regions of a peptide backbone (e.g. Hensel *et al.* 1992; Müller & Heidemann 1993). Thus racemization analysis of the insoluble fraction of bone will indicate the relative proportion of denatured to triple helical collagen, itself a partly time–temperature dependent phenomenon. So called 'racemization kinetics' observed in bone in burial environments can be simplified to the model illustrated in figure 9. Almost any value of D-Asx can be produced by varying the rate of generation of denatured collagen $k_{\text{denaturation}}$ or the rate of loss of the denatured (and racemized) residues k_{leaching}. The analysis of insoluble (or high molecular weight) extracts, which is being promoted as a refinement to the Asx dating in bone, may hold some promise (e.g. Julg *et al.* 1987; Elster *et al.* 1991; El Mansouri *et al.* 1996). However the method would be readily compromised by processes (such as condensation reactions; Collins *et al.* 1992) which retain denatured peptides within the insoluble residue.

8. ASX RACEMIZATION IN BONE AS A PROXY FOR DNA DEPURINATION

Bada *et al.* (1994, p. 3131) noted that 'the rate of (DNA) depurination and aspartic acid racemization at neutral pH are nearly identical'. Subsequently, Poinar *et al.* (1996) tested the use of Asx racemization to screen a small sample of ancient tissues for amplifiable DNA. Despite the success reported in their study we believe that there cannot be more than a weak correlation between Asx racemization in bone and DNA depurination. This is because (i) the mechanism of racemization of free Asp *in vitro* is not equivalent to that of peptide-bound Asx, (ii) the correspondence between the rate of racemization of free Asp and Asx in human dentine is mere coincidence (e.g. the reported rate in rat dentine is tenfold higher; Ohtani *et al.* 1995), (iii) racemization of Asx is highly conformation dependent but the impact of conformation on DNA depurination is unknown, (iv) the measured extent of racemization of Asx in bone is dependent upon the fraction analysed and the extent of leaching of the soluble fraction (e.g. figure 9). Thus, where two bones of identical age have experienced the same extent of collagen degradation, the more heavily leached sample will have the lower D:L ratio and will appear to be the best for DNA amplification.

9. ASX RACEMIZATION IN SHELLS

We have illustrated the problem of using Asx racemization in bones, where the presence of flanking residues in gelatin has the effect of accelerating racemization but this is set against the severe conformational constraint of the intact collagen triple helix. What of racemization in other calcified tissues? One problem with application to systems such as mollusc shells or ratite eggshells, is that at present the sequence of most proteins is unknown. Indeed in all cases Asx analyses have been conducted on a mixture of proteins. As can be seen from table 2, differences in

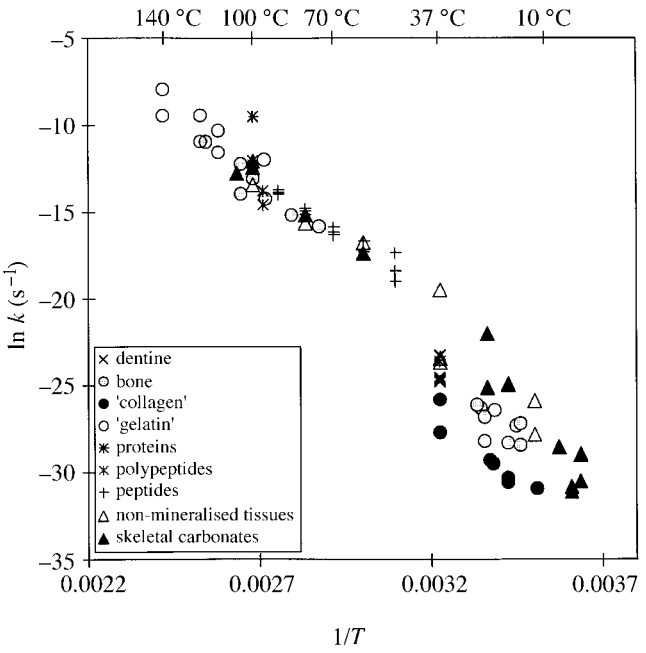

Figure 10. An Arrhenius plot of published kinetics of Asx racemization (data in Appendix A). Note that all experiments yield broadly similar rates and apparent activation energies at high temperatures but that (due to conformational constraints) the rates are slower at lower temperatures ($<40\,^{\circ}\text{C}$). This is particularly true of collagen in which Asu formation is severely restricted in the triple helix.

primary sequence can alter the rate of deamidation and hence Asu formation by two orders of magnitude.

If the rate of Asx racemization in proteins is strongly dependent upon conformation, what does this mean for attempts to derive rate constants by extrapolation from high temperatures? Investigations of thermophilic bacteria reveal the importance of conformation in the rate of deamidation and succinimide formation (Daniel *et al.* 1996). Since the denaturation temperatures of the proteins in biogenic carbonates are unknown, it would seem impossible to use extrapolations to predict low temperature racemization rates. If the rates of initial Asx racemization in high and low temperature studies are compared on an Arrhenius plot, it can be seen that the spread of rates broadens dramatically at lower temperatures, and is slowest for conformationally constrained proteins such as collagen (figure 10).

Because conformation will have a greater effect at lower temperatures extrapolations from high temperature studies will tend to overestimate low temperature rates (and thus underestimate ages derived from Asx racemization at low temperatures). The Asx estimated age of 250 years for the deep-sea anemone *Gerardia* made by Goodfriend (1997) using an extrapolation approach is probably of this type as it conflicts with the radiocarbon age of 1800 years (Druffel *et al.* 1995). Correspondingly, if rates of racemization from low temperatures are incorporated in estimates of activation energies they will tend to overestimate the E_a as they do not take account of the influence of conformation at lower temperatures. This explains the high E_a calculated by Goodfriend & Meyer (1991) from heating modern shells ($121.4\,\text{kJ mol}^{-1}$; cf. Fujii *et al.* (1996), average value of $109.2\,\text{kJ mol}^{-1}$). The

importance of conformational constraints on Asx racemization will diminish as the protein degrades and as the proportion of free amino acids increases. A lower E_a was obtained by Goodfriend & Meyer (1991) when the kinetic parameters were derived from heated fossil material (110.2 kJ mol^{-1}).

10. HOW PREDICTABLE IS PROTEIN PRESERVATION?

Asx racemization neatly illustrates the difficulties of predicting the long-term rates and patterns of diagenesis. Despite the large number of experiments which have been conducted to derive kinetic parameters for Asx racemization, when applied to fossil material the pattern and rates are unpredictable. However greater appreciation of the underlying mechanisms which contribute to the increase in D-Asx help identify many of these 'unusual' features. The use of simple models which describe the key reactions can enable comparison between apparently contradictory rates derived from different proteins and between high and low temperature experiments. In the light of this study we would conclude the following:

(i) estimating rates of Asx racemization by extrapolation from high temperatures is to be avoided. At low temperatures, Asx racemization will remain unpredictable due to the overwhelming influence of protein conformation of the rates of bound residues;
(ii) rates estimated from (degraded) fossil material may produce more accurate estimates of E_a as the conformational effect will be reduced;
(iii) although they are the most widely used archaeological materials for Asx dating, bones and teeth are probably the worst materials to choose, due to the extreme conformational effect of the collagen triple helix;
(iv) leaching distorts the kinetics by removing the peptides and free amino acids, which racemize more predictably. If the intra-crystalline fraction is used (Sykes *et al*. 1995), the effect of leaching can be avoided; unfortunately this approach is not applicable to bone and dentine (see Hare 1980).

11. SUMMARY

(i) Mathematical models can be used to derive unknown rate constants in a simple system if the chemistry of the reactions is known.
(ii) A mathematical model of Asx (Asn + Asp) racemization via Asu formation of Geiger & Clarke (1987) was modified for use with collagen. The modifications included the influence of flanking adjacent residues, deamidation rate of collagen and an observed D:L equilibrium ratio of 1. The model successfully predicted racemization rates for collagen between 95–140 °C but not at 37 °C.
(iii) Asx racemization in collagen at low temperatures is not predicted by the model due to the influence of conformation on rate of Asu formation.
(iv) The D:L ratio in bones reflects the ratio of non-helical (high proportion of D-Asx) to triple helical (L-Asx) collagen. Initially the D:L ratio will trend towards a value of 0.09, reflecting complete racemization of the non-helical telopeptides.
(v) Over archaeological time the D:L ratio will increase due to the decreasing proportion of triple helical collagen. The D:L ratio itself will reflect the rate of loss of helix (dependent on time and temperature) and leaching of soluble material (dependent on burial environment).
(vi) Asx racemization is not a reliable proxy for the extent of DNA depurination in bones.
(vii) Asx racemization rates cannot be extrapolated from high temperatures to those below the denaturation temperature of the protein under investigation.

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APPENDIX A

Table A1. *Data on racemization rates used in figure 10*

(Where the rates are at variance with the published rate, we have recalculated the rate from the available data to estimate the initial rapid phase of Asx racemization. Some temperatures were not given and these have been estimated by us from data given in Meeson *et al*. (1995), Sellers *et al*. (1995) (indicated by *) or The National Oceanographic Data Center Oceanographic Profile Database (http://www.nodc.noaa.gov, July 1998, indicated by †).)

sample	details	°C	rate (s^{-1})	environment	author
dentine	human total	37	2.06×10^{-11}	*in vivo* dentine	Ohtani & Yamomoto (1991)
dentine	human total	37	1.92×10^{-11}	*in vivo* dentine	Shimoyama & Harada (1984)
dentine	human vertical section	37	2.31×10^{-11}	*in vivo* dentine	Saleh *et al*. (1993)
dentine	human horizontal section	37	1.75×10^{-11}	*in vivo* dentine	Saleh *et al*. (1993)
dentine	rat	37	7.92×10^{-11}	*in vivo* dentine	Ohtani *et al*. (1995)
bone	boiled	100	2.22×10^{-6}	in distilled water	Skelton (1983)
bone	boiled	120	1.85×10^{-5}	in distilled water	Von Endt (1980)
bone	heated in water	75	1.44×10^{-7}	in distilled water	M. J. Collins, unpublished data
bone	heated in water	85	2.74×10^{-7}	in distilled water	M. J. Collins, unpublished data
bone	heated in water	95	6.93×10^{-7}	in distilled water	M. J. Collins, unpublished data
bone	heated in water	105	5.11×10^{-6}	in distilled water	Hare (1980)

bone	heated in water	105	9.17×10^{-7}	in distilled water	Hare (1980)
bone	Wadi Halfa, Sudan	26	3.83×10^{-12}	archaeological bone	King & Bada (1979)
bone	Mindano, Phillipines	27	4.69×10^{-12}	archaeological bone	King & Bada (1979)
bone	La Jolla, California	16	4.59×10^{-13}	archaeological bone	King & Bada (1979)
bone	Olduvai, Kenya	25	2.34×10^{-12}	archaeological bone	King & Bada (1979)
bone	Murray Springs, Arizona	17	1.43×10^{-12}	archaeological bone	King & Bada (1979)
bone	Nasera Rock Shelter	25	5.74×10^{-13}	archaeological bone	King & Bada (1979)
bone	Mt Carmel, Israel	19	5.20×10^{-13}	archaeological bone	King & Bada (1979)
bone	Hungarian collections	16*	1.58×10^{-12}	archaeological bone	Csapo *et al*. (1994)
collagen	CNBr collagen digest	37	9.51×10^{-13}	*in vivo* dentine	Cloos (1995)
'collagen'	acid insoluble fraction	37	6.34×10^{-12}	*in vivo* dentine	Gillard *et al*. (1991)
'collagen'	parchment scrolls	22.4	1.58×10^{-13}	Dead Sea Scrolls	Weiner *et al*. (1980)
'collagen'	German insoluble fraction	13*	3.80×10^{-14}	archaeological bone	Elster *et al*. (1991)
'collagen'	Levant insoluble fraction	19*	6.97×10^{-14}	archaeological bone	Elster *et al*. (1991)
'collagen'	Egyptian insoluble fraction	22*	1.90×10^{-13}	archaeological bone	Kimber & Hare (1992)
'collagen'	Japanese insoluble fraction	19*	5.39×10^{-14}	archaeological bone	Matsu'ura & Ueta (1980)
'gelatin'	parchment scrolls	22.4	3.42×10^{-12}	Dead Sea Scrolls	Weiner *et al*. (1980)
gelatin	heated pH 8.0 initial rate	95.5	6.63×10^{-6}	pH 8 0.05M	de Sol (1978)
gelatin	heated pH 8.0 slow rate	114.6	1.01×10^{-5}	phosphate buffer	de Sol (1978)
gelatin	heated pH 8.0 initial rate	114.6	3.58×10^{-5}	phosphate buffer	de Sol (1978)
gelatin	heated pH 8.0 slow rate	122.5	1.91×10^{-5}	phosphate buffer	de Sol (1978)
gelatin	heated pH 8.0 initial rate	122.5	8.47×10^{-5}	phosphate buffer	de Sol (1978)
gelatin	heated pH 8.0 slow rate	140.5	8.28×10^{-5}	phosphate buffer	de Sol (1978)
gelatin	heated pH 8.0 initial rate	140.5	3.72×10^{-4}	phosphate buffer	de Sol (1978)
dentine	human soluble fraction	37	8.40×10^{-11}	phosphate buffer	Masters (1985)
tissue	blood cells	37	3.47×10^{-9}	*in vivo*	Brunauer & Clarke (1986)
tissue	inter-vertebral disks	37	6.84×10^{-11}	*in vivo*	Ritz & Schutz (1993)
tissue	eye lens	37	5.45×10^{-11}	*in vivo*	Garner & Spector (1978)
protein	aggrecan, articular Cartilage	37	5.70×10^{-11}	*in vivo*	Maroudas *et al*. (1998)
protein	lysozyme, heated egg white	100	7.94×10^{-5}	pH 8	Zhao *et al*. (1989)
protein	lysozyme, heated egg white	100	6.31×10^{-6}	pH 6	Zhao *et al*. (1989)
protein	acrystallin, heated eye lens	96	1.12×10^{-6}	unknown	Masters (1982)
protein	gcrystallin, heated eye lens	96	4.94×10^{-7}	unknown	Masters (1982)
polypeptide	$[Asp]_n$ heated	100	4.40×10^{-6}	pH 7.7 phosphate buffer	Steinberg *et al*. (1984)
peptides	Leu-Asp-Ala	90	1.14×10^{-6}	pH 7 0.1M	Fujii *et al*. (1996)
peptides	Leu-Asp-Ala	80	3.99×10^{-7}	phosphate buffer	Fujii *et al*. (1996)
peptides	Leu-Asp-Ala	70	1.35×10^{-7}	phosphate buffer	Fujii *et al*. (1996)
peptides	Leu-Asp-Ala	60	6.02×10^{-8}	phosphate buffer	Fujii *et al*. (1996)
peptides	Leu-Asp-Ala	50	3.01×10^{-8}	phosphate buffer	Fujii *et al*. (1996)
peptides	Leu-Asp-Ser	90	9.97×10^{-7}	phosphate buffer	Fujii *et al*. (1996)
peptides	Leu-Asp-Ser	80	3.41×10^{-7}	phosphate buffer	Fujii *et al*. (1996)
peptides	Leu-Asp-Ser	70	8.68×10^{-8}	phosphate buffer	Fujii *et al*. (1996)
peptides	Leu-Asp-Ser	60	3.70×10^{-8}	phosphate buffer	Fujii *et al*. (1996)
peptides	Leu-Asp-Ser	50	1.04×10^{-8}	phosphate buffer	Fujii *et al*. (1996)
peptides	Glu-Asp-Leu	90	9.10×10^{-7}	phosphate buffer	Fujii *et al*. (1996)
peptides	Glu-Asp-Leu	80	2.79×10^{-7}	phosphate buffer	Fujii *et al*. (1996)
peptides	Glu-Asp-Leu	70	1.01×10^{-7}	phosphate buffer	Fujii *et al*. (1996)
peptides	Glu-Asp-Leu	60	3.24×10^{-8}	phosphate buffer	Fujii *et al*. (1996)
peptides	Glu-Asp-Leu	50	5.79×10^{-9}	phosphate buffer	Fujii *et al*. (1996)
anemone	*Gerardia* ^{14}C estimate	12.5	8.24×10^{-13}	seawater	Goodfriend (1997)
anemone	*Gerardia* kinetic estimate	12.5	5.96×10^{-12}	seawater	Goodfriend (1997)
anemone	*Gerardia* heated	60	5.67×10^{-8}	in distilled water	Goodfriend (1997)
anemone	*Gerardia* heated	80	1.69×10^{-7}	in distilled water	Goodfriend (1997)
anemone	*Gerardia* heated	100	1.60×10^{-6}	in distilled water	Goodfriend (1997)
eggshell	*Struthio*	80	2.68×10^{-7}	carbonate skeleton	Goodfriend & Hare (1995)
gastropod	*Triodopsis*	19	1.43×10^{-11}	carbonate skeleton	Goodfriend (1992)
gastropod	*Trochoidea* museum colltn.	19	1.58×10^{-11}	carbonate skeleton	Goodfriend (1991)
gastropod	*Trochoidea*	106.5	3.00×10^{-6}	carbonate skeleton	Goodfriend (1992)
gastropod	*Entemnotrochus*	60	2.95×10^{-8}	carbonate skeleton	Goodfriend *et al*. (1995)
bivalve	*Mya*	100	6.09×10^{-6}	carbonate skeleton	Goodfriend *et al*. (1996)
bivalve	*Hiatella*	100	4.27×10^{-6}	carbonate skeleton	Goodfriend *et al*. (1996)
coral	*Porites* slow rate	24.3	1.27×10^{-11}	carbonate skeleton	Goodfriend *et al*. (1992)
coral	*Porites* initial rate	24.3	2.88×10^{-10}	carbonate skeleton	Goodfriend *et al*. (1992)
foraminifera	*Bulimina*	7	4.21×10^{-13}	carbonate skeleton	Némethy (1995)
foraminifera	*Orbulina*	4	3.17×10^{-14}	carbonate skeleton	Némethy (1995)
foraminifera	*Globorotalia*	4	4.12×10^{-14}	carbonate skeleton	Némethy (1995)
foraminifera	*Pulleniatina*	1.8†	2.66×10^{-13}	carbonate skeleton	Harada *et al*. (1996)
foraminifera	*Pulleniatina*	1.8†	5.70×10^{-14}	carbonate skeleton	Harada *et al*. (1996)
foraminifera	*Pulleniatina*	2†	2.73×10^{-13}	carbonate skeleton	Harada *et al*. (1996)

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Discussion

G. Eglinton (*University of Bristol, UK*). What precisely do you see is the relationship between conformation and rate of reaction? Also, what controls the conformation? The greater stability you site in the case of dentine is a case in point.

M. J. Collins. In the case of proteins, confirmation is critical to function and this over the working temperature of the protein, conformation is governed by interplay of the amino acid residues with each other and the solvent; as the protein is heated and/or degrades residues with each other and the solvent; as the protein is heated and/or degrades conformational freedom is anticipated to increase. Conformation influences the rate of reaction by hindering the formation of the reactive intermediates, the impact on rate being an interplay between the extent of the restriction and the structural requirements of the intermediates. Thus, in a highly stretched chain deamidation via the formation of a cyclic Asu requires chain shortening and will be hampered, but this conformation will have little influence on deamidation by direct hydrolysis. In the case of aspartic and racemization of the dentine proteins, the observed rate is much slower than our model predicts because many of the aspartyl and asparingly residues are within the triple helix of collagen and will not contribute to the pool of D-isomers which are accumulating in other (degrading) dentine proteins.