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Pleistocene geochronology and palaeothermometry from protein diagenesis in ostrich eggshells: implications for the evolution of modern humans

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SUMMARY

Proteinaceous residues incorporated within the crystal structure of ostrich eggshells (OES) are retained without loss over geological time exceeding 10 million years. Degradation of the polypeptides, including hydrolysis to smaller peptide fragments and eventual release of free amino acids, decomposition, and racemization and epimerization occur at regular, predictable rates dependent on ambient temperature. The extent of isoleucine epimerization (aIle/Ile ratio) in OES follows linear first-order reversible kinetics in controlled-temperature laboratory simulations of time up to an aIle/Ile ratio in excess of 1.0. The hydrolysis of leucine also follows a predictable pattern, but deviates from first-order kinetics. A non-linear mathematical model has been developed that adequately describes the pattern of leucine hydrolysis through a wide temperature range. Arrhenius parameters were derived from laboratory experiments combined with rate constant values found for ^{14}C-dated OES from stratified caves in southern Africa. These parameters for isoleucine epimerization and leucine hydrolysis differ by *ca.* 10%, allowing the simultaneous solution of the two equations for temperature, independent of sample age. Although the uncertainty of the simultaneous temperature is relatively high ($\pm 10°C$), it provides an effective means of identifying burned samples. If sample age is known, palaeotemperatures (the integrated thermal history experienced by an eggshell as opposed to an 'instantaneous' temperature) can be calculated with a precision of better than $\pm 1°C$.

The ages of levels at Border Cave, South Africa, from which anatomically modern human skeletal remains have been recovered, are dated by the extent of isoleucine epimerization in associated OES. The reaction is calibrated in the upper levels by a series of concordant radiocarbon dates on charcoal at 38 ka before present (BP). The amino acid dates on deeper levels indicate that the Howiesons Poort stratum at Border Cave is more than 70 ka old, and that anatomically modern humans occupied the site as early as 100 ka ago.

1. INTRODUCTION

As the dates on the first appearance of anatomically modern humans have receded steadily backward in time to beyond the limits of the radiocarbon method, the need for additional techniques that can provide chronological information in the time range between about 30 ka and 200 ka has become increasingly apparent. Despite more than two decades of effort, and the rapid advances in conventional and accelerator mass spectrometry, no single technique has emerged that can reliably fill this temporal gap for most sites. For the foreseeable future, the best dating strategies are going to be those that combine a variety of independent methods, the results of which can be cross-checked at key stratigraphic horizons. The best results will be from those techniques that require sufficiently small samples that individual specimens can be dated.

One technique that has gained increasing attention in recent years is based on the epimerization of the protein amino acid isoleucine preserved within the calcite crystals of ostrich eggshells (OES). The integrity of the eggshell and the precision of the epimerization measurements have been addressed previously (Brooks *et al.* 1990). In this paper, we present new data that better define the temperature sensitivity of the epimerization reaction, derive the average temperature

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independent of sample age by simultaneous solution of the isoleucine epimerization and leucine hydrolysis reactions, and use this as an objective basis for identifying burnt eggshell. We conclude with a discussion of the implications of isoleucine epimerization ratios in OES recovered from archaeological excavations at Border Cave, South Africa.

2. MATERIALS AND METHODS

Ostrich eggshells were cleaned for amino acid analyses by physically removing the outer layers, followed by the removal of one-third of the sample with 2 N HCl. The naturally hydrolyzed fraction (free fraction) was prepared by digestion in 1 ml 7 N HCl per 50 mg of eggshell, followed by desiccation under vacuum and rehydration in weak HCl (pH 1.8). The total fraction (naturally occurring free amino acids plus those still peptide-bound) was prepared by digestion in 1 ml 7 N HCl per 50 mg of eggshell, followed by hydrolysis under N_2 at 110°C for 22 h in a forced-convection oven. After hydrolysis, excess HCl was removed under vacuum and the sample rehydrated with dilute (pH 1.8) HCl. In both free and total fractions the 7 N HCl was spiked with the non-protein amino acid norleucine (1.25 nmol mg^{-1} OES) to enable quantitative recovery of amino acids. Amino acid separation was by automated ion-exchange liquid chromatography followed by post-column derivitization with *o*-phthalaldehyde and fluorescence detection integrated electronically. Each analysis requires 0.2 mg of eggshell. All preparations were analysed at least twice, many were analysed three or more times. AIle/Ile ratios are based on the average ratio of peak heights as computed by the integrators. The aIle/Ile ratio for modern OES is 0.018. Leucine hydrolysis is based on peak areas normalized to the norleucine calibration spike. A standard amino acid protein hydrolysate, to which a known amount of D-alloisoleucine had been added, was included with each batch of 15 samples and used to judge instrumental errors. High-temperature simulations were carried out in forced-convection ovens continuously monitored by a precision thermometer.

Eggshell was prepared for radiocarbon dating by mechanical cleaning followed by removal of 50 to 90% of the remaining eggshell in 2 N HCl to minimize contamination by exchange with younger carbon. Cleaned OES were submitted to the Arizona facility where they were digested in phosphoric acid (85%); the released CO_2 was purified and then reduced to graphite. Details of the Arizona laboratory procedures are given by Linick *et al.* (1986) and Donahue (1992).

3. PROTEIN DIAGENESIS IN OSTRICH EGGSHELL

Dating methods must satisfy two broad prerequisites: a process is required that is dependent on time (radioactive decay, chemical reaction), and a medium must be available in which the initial conditions are known, and no subsequent uptake or loss of interfering molecules occurs. In this study we rely on two well documented reactions: (i) the racemization, or in the case of isoleucine, epimerization, reaction, by which the original protein L-amino acids invert to a mixture of D- and L-configurations; and (ii) the hydrolysis reaction, by which the bonds linking amino acids into peptide chains are cleaved, releasing free amino acids. The medium in this study is the ostrich eggshell, which satisfies the prerequisite for closed system behaviour with respect to peptides and amino acids to a degree not previously found in any other biomineralized remains (e.g. Brooks *et al.* 1990).

(*a*) *Isoleucine epimerization*

Although most amino acids can occur in two configurations, designated D- (right-handed) or L- (left-handed), only L-amino acids occur in most polypeptides, including those within the OES. Isoleucine, a particularly stable protein amino acid, has two chiral carbon centres, and although racemization of L-isoleucine (L-Ile) could form the D-enantiomer (D-Ile), thermodynamics favours racemization about the alpha carbon only, creating D-alloisoleucine (D-aIle). The different physical and chemical properties of the diasteriomers allow rapid separation and precise quantification of the proportion of D-alloisoleucine to L-isoleucine (aIle/Ile) by high-pressure liquid chromatography; analytical uncertainty is generally better than ± 1%. The aIle/Ile ratio increases from near zero in a modern eggshell to an equilibrium ratio of 1.30, by which time the inversion of L- to D-forms is balanced by the reverse reaction.

The extent of isoleucine epimerization in fossil eggshell is a function of sample age and the integrated thermal history experienced by the sample. Thus, although it is possible to use the aIle/Ile ratio directly as a measure of relative age for nearby sites for which the average temperatures are similar (less than 1°C difference), conversion of the epimerization ratio to absolute age requires an assessment of thermal history.

The dependency of the rate constant for isoleucine epimerization (k_{Ile}) on temperature is described by the Arrhenius equation:

$$k_{\mathrm{Ile}} = A\mathrm{e}^{(-E_a/RT)}, \qquad (1)$$

where A is a constant, E_a is the energy of activation, R is the universal gas constant, and T is the effective temperature in Kelvin.

The Arrhenius parameters ($\ln(A)$ and E_a) can be derived empirically from high-temperature experiments where reaction rates in modern OES are monitored over specific time and temperature increments and from independently dated sites at ambient temperatures. In practice, laboratory simulations can be undertaken at temperatures between about 100 and 170°C, and natural samples no older than 12 ka are used to avoid uncertainties in the glacial–interglacial temperature change. We undertook laboratory simulations using modern OES at 110, 142.5 and 161°C, and have numerous radiocarbon-dated early Holocene samples with current mean annual temperatures ranging from 16 to 29°C (table 1).

Isoleucine epimerization obeys first-order reversible kinetics in modern OES heated at 142.5°C up to an

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Table 1. *Calibration samples from which the Arrhenius parameters for isoleucine epimerization and initial leucine hydrolysis were derived*

(Ages of samples heated in the laboratory were directly measured; radiocarbon dates on all other samples were obtained by AMS ^{14}C dating of a fragment that was also analysed for the extent of protein diagenesis. Temperatures were monitored by precision thermometer for the heating experiments, and are based on the period of instrumentation for the nearest weather station for the archaeological sites unless otherwise indicated.)

site name	dated samples	Temp. (°C)	k_{Ile}	$\ln(k_{\mathrm{Ile}})$ (avg)	$k_{\mathrm{Leu\ Hyd}}$	$\ln(k_{\mathrm{Leu\ Hyd}})$
lab. simulation	0.0006 to 0.007 years	161	2.11 e+2	5.35	1.112 e+2	4.71
lab. simulation	0.001 to 0.06 years	142.5	4.06 e+1	3.70	3.343 e+1	3.51
lab. simulation	0.02 to 0.8 years	110	2.05	0.718	2.363	0.86
Bir Tarfawi[a]	7500 ± 80 years BP (AA-3292A)	29.5			7.667 e−5	−9.48
Heuningsneskrans	9675 ± 75 years BP (AA-6449)	21	1.25 e−5	−11.23	3.591 e−5	−10.23
	12 030 ± 139 years BP (AA-5829)	21	1.34 e−5			
	14 450 ± 105 years BP (AA-6450)	21	1.40 e−5			
Apollo 11 Cave	9290 ± 70 years BP (AA-6448)	20	1.35 e−5	−11.21	3.916 e−5	−10.15
	9150 ± 70 years BP (AA-5824)	20				
Elands Bay Cave	8110 ± 90 years BP (AA-5832)	18	6.83 e−6	−11.89	2.656 e−5	−10.50
	10 840 ± 70 years BP (AA-5833)	18				
	11 415 ± 80 years BP (AA-5834)	18				
Boomplass Cave	10 430 ± 80 years BP (AA-6958)	16.5	4.93 e−6	−12.22	no data	no data
Equus Cave[b]	11 870 ± 105 years BP (AA-5826)	16	5.34 e−6	−12.14	2.176 e−5	−10.74
	27 730 ± 340 years BP (AA-5827)					

[a] Owing to shallow depth of burial, this site has an effective temperature significantly higher than the current mean annual temperature (26.5°C). The effective temperature was calculated from the D/L ratio and radiocarbon age.

[b] The older sample has experienced glacial-age temperature reduction. However, the effective temperature, calculated from the D/L ratio and radiocarbon age, is not significantly different from the current site temperature.

aIle/Ile ratio close to 1.2 (figure 1), whereas it deviates from the linear kinetic model much earlier in similar experiments conducted on molluscan fossils (see, for example, Mitterer (1989), Kaufman (1992)). By plotting the rate constants derived from the high-temperature simulations and dated ambient temperature samples on an Arrhenius diagram (figure 2), the temperature sensitivity of the epimerization reaction is defined. From these parameters a single expression can be derived relating aIle/Ile (D/L) ratio to time (t) and temperature (T):

$$\ln\left(\frac{1+\mathrm{D/L}}{1-0.77\,\mathrm{D/L}}\right) = 1.77\,t\left(\mathrm{e}^{(40.23-\frac{15152}{T})}\right) + 0.032. \quad (2)$$

Arrhenius parameters have been reported previously for isoleucine epimerization in OES; Brooks *et al.* (1989) derived values of 30.01 kcal mol^{-1} (E_a) and 40.37 ($\ln(A)$), whereas Miller (1992) reported values of 30.33 and 40.83, respectively. The current derivation (30.11, 40.23, respectively) incorporates the most complete set of dated control sites, and differs only slightly from the earlier determinations.

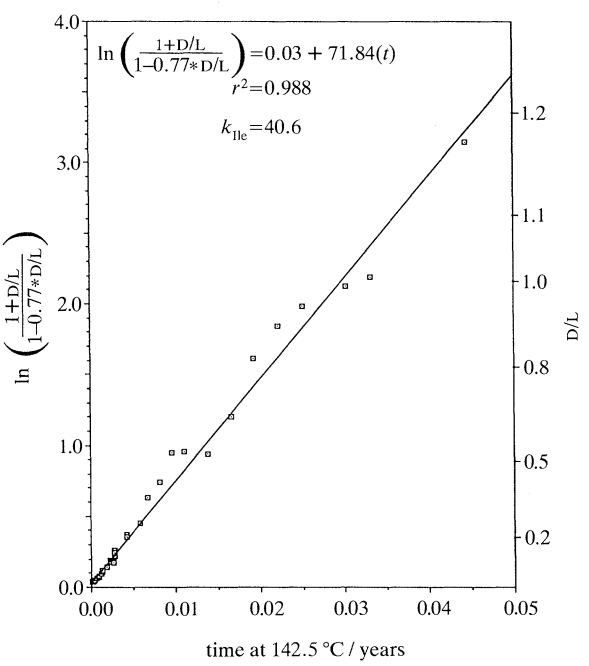

Figure 1. Measured aIle/Ile ratio in modern OES heated for specific time intervals at 142.5°C. Points are the mean of at least two different eggshell fragments at each temperature. Linear kinetics are approximated up to an aIle/Ile ratio of at least 1.2 (scale on right).

(*b*) *Leucine hydrolysis and decomposition*

Leucine, a protein amino acid only slightly less stable than isoleucine, hydrolyses more rapidly and thus offers better resolution in the early stages of diagenesis. The general pattern of leucine hydrolysis and decomposition in OES is shown in figure 3. Because the eggshell acts as a closed system, the decrease in total leucine over time is due strictly to decomposition, whereas the concentration of free leucine reflects the competition between the hydrolysis reaction, which releases free leucine from polypeptides, and the decomposition reaction. Initially, hydrolysis exceeds

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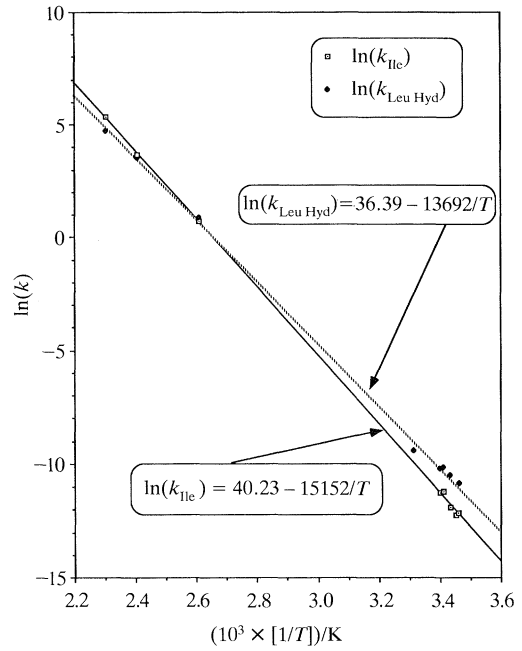

Figure 2. Arrhenius plot of isoleucine epimerization (k_{Ile}) and initial leucine hydrolysis ($k_{Leu\ Hyd}$) based on high temperature simulations (e.g. figure 1) and radiocarbon-dated samples for which the current site temperature can be used as the long-term average (table 1). The difference in slope for the two reactions allows the simultaneous solution for temperature independent of time (figure 5).

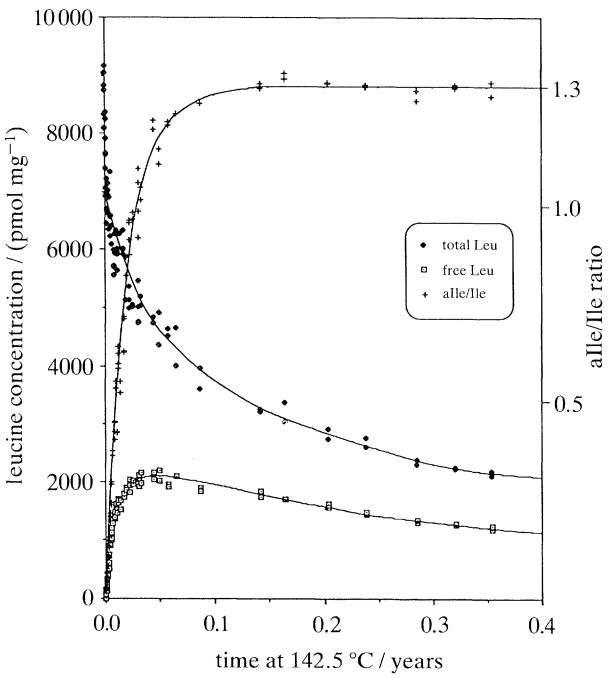

Figure 3. The observed decomposition (total Leu), leucine hydrolysis (expressed as the concentration of free leucine), and isoleucine epimerization (scale on right axis) in modern OES heated at 142.5°C. Each point represents a different eggshell fragment. The isoleucine epimerization reaction reaches equilibrium after about 0.15 years, whereas leucine hydrolysis continues, albeit at reduced rates, throughout the experiment.

decomposition and the concentration of free leucine increases, but in the later stages of diagenesis (aIle/Ile > 1.2), decomposition exceeds hydrolysis and the concentration of free leucine decreases steadily, although the proportion of free to total leucine continuously increases.

The rate of leucine hydrolysis initially follows linear irreversible first-order kinetics of this form:

$$\ln(\mathrm{Leu_B}/\mathrm{Leu_T}) = -k_{\mathrm{Leu}}t, \quad (3)$$

where $\mathrm{Leu_B}$ and $\mathrm{Leu_T}$ are the concentration of peptide-bound and total leucine respectively, k_{Leu} is the rate constant leucine hydrolysis and t is the time in years.

Based on the same series of experimental and natural samples as for epimerization (table 1), the Arrhenius parameters for the initial linear portion of leucine hydrolysis (figure 2) are 27.21 kcal mol^{-1} (E_a) and 36.39 ($\ln(A)$).

After the initial linear period the rate of leucine hydrolysis decreases. This deviation is due to the variable bonding strength between leucine and other amino acids in the original polypeptide chains. The observed hydrolysis rate (figure 4) is a composite of nearly 20 different rate constants, modulated by decomposition reactions. A nonlinear mathematical model (Appendix) defines an effective rate constant (k_{eff}) from which the observed nonlinear pattern of leucine hydrolysis for any temperature is given by:

$$\ln(\mathrm{Leu_B}/\mathrm{Leu_T}) = -k_{\mathrm{eff}}t. \quad (4)$$

In figure 4, the predicted pattern of leucine hydrolysis (from equation (4)) is superimposed on observed leucine hydrolysis in modern OES heated for specific intervals at 142.5°C.

(*c*) *Simultaneous temperatures*

Because the activation energies for leucine hydrolysis (27.2 kcal mol^{-1} in both linear and extended

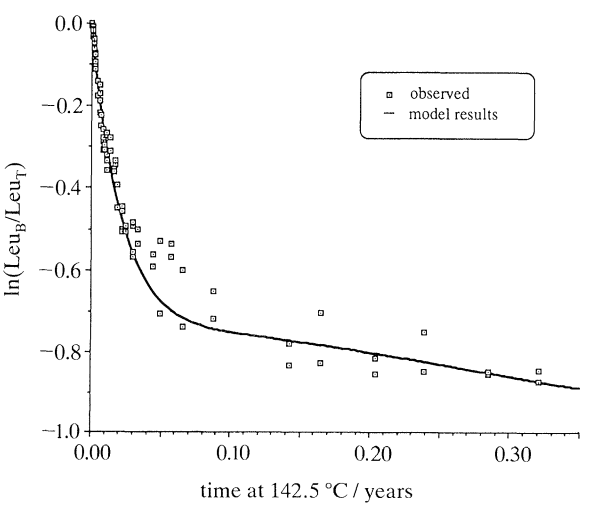

Figure 4. Leucine hydrolysis in modern OES heated at 142.5°C, expressed as the log of the peptide-bound leucine ($\mathrm{Leu_B}$) divided by total leucine ($\mathrm{Leu_T}$) plotted against heating time. The deviation from linear kinetics is due to the variable bonding strengths between leucine and adjacent amino acids in the peptide chain, modulated by decomposition reactions. The model results are based on equation (4).

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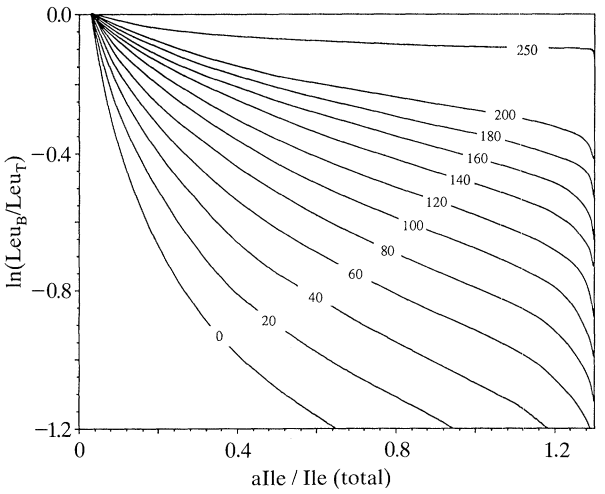

Figure 5. Isotherms derived from the simultaneous solution of the isoleucine epimerization and leucine hydrolysis reactions as described in the Appendix. Isotherms are labelled in °C.

models) and isoleucine epimerization (30.1 kcal mol^{-1}) differ by almost 10%, it is possible to solve the equations simultaneously and estimate the effective sample temperature directly from the extent of the two reactions, without knowledge of sample age (Appendix). To distinguish this temperature from that derived from the extent of epimerization or hydrolysis alone in a sample of known age, we define it as the simultaneous temperature. In figure 5, isotherms of simultaneous temperature are shown for a spectrum of possible aIle/Ile and ln(Leu$_B$/Leu$_T$) values.

Calculating the extent of leucine hydrolysis is inherently less precise than for isoleucine epimerization because it requires measuring naturally hydrolysed leucine (free fraction) in one portion of the eggshell, and the total amount of leucine in a different part of the same fragment. Reproducibility is ± 7%, significantly less than for isoleucine epimerization (± 1%). Consequently, the uncertainty in the simultaneous temperature solution has a coefficient of variation of about ± 3% in the Kelvin temperature, or ± 10°C for most sites. The simultaneous temperatures from OES in our high-temperature experiments and from OES in archaeological sites compare favorably to their known temperatures (table 2).

4. APPLICATIONS TO ARCHAEOLOGY

(*a*) *Resolving the dilemma of burnt eggshell*

Ostrich eggshell is commonly associated with archaeological sites primarily because the egg was a food source, and to a lesser degree the eggshell was utilized for artistic and practical purposes. It is to be expected that the eggs were cooked, and after consumption of the egg, the eggshell may have been discarded near or into the hearth area. At many sites obvious examples of burnt OES occur, for which the temperatures were so high that polypeptides were reduced to elemental carbon. Because the rates of racemization, hydrolysis and decomposition are dependent on temperature, heating of the eggshell by humans will accelerate the extent of protein degradation, artificially 'ageing' the eggshell. In contrast, the act of cooking, even if the eggshell is placed directly in a fire, is not of sufficient duration or intensity to cause significant epimerization or hydrolysis (temperatures rarely exceed 100°C, and cooking time is less than one hour).

Although extreme heating is obvious from visual inspection, more subtly heated samples cannot be recognized by visible characteristics; the simultaneous temperature offers an objective criterion by which such samples can be identified. From the isotherms in figure 5 it can be seen that the aIle/Ile ratio increases more rapidly relative to the extent of hydrolysis at higher temperatures than at lower temperatures. Consequently, epimerization should be more advanced than hydrolysis within eggshell discarded in a campfire and subjected to brief periods of relatively high temperature. Such fragments will have simultaneous temperatures well above the site temperature.

For example, four well-preserved eggshell fragments collected from each of two superimposed early Late Stone Age (ELSA) horizons (strata 2a and 3b) at Heuningsneskrans, South Africa, showed no visual indication of heating. The aIle/Ile ratios measured in

Table 2. *Comparison between simultaneous temperatures and known temperatures for high-temperature simulations and unburnt radiocarbon-dated late Quarternary ostrich eggshell*

site	experimental or site temperature (°C)	Ln(Leu$_B$/Leu$_T$)	aIle/Ile ratio(s)	number of samples	simultaneous temperature (°C)
lab. simulation	161	−0.075 to −0.547	0.11 to 1.09	20	149 ± 13
lab. simulation	142.5	−0.017 to −0.594	0.03 to 1.18	62	137 ± 8.5
lab. simulation	110	−0.014 to −0.782	0.04 to 1.14	32	98 ± 12
Apollo 11 Cave	20	−0.361 ± 0.026	0.144 ± 0.001	3	26 ± 5
Heuningsneskrans	21	−0.364 ± 0.003	0.141 ± 0.003	2	22 ± 1
	21	−0.427 ± 0.055	0.181 ± 0.003	2	28 ± 5
Elands Bay Cave	18	−0.233 ± 0.012	0.082 ± 0.009	3	17 ± 5
	18	−0.370 ± 0.001	0.115 ± 0.013	2	10 ± 7
Equus Cave	16	−0.292 ± 0.032	0.091 ± 0.005	4	11 ± 3

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Table 3. *Isoleucine epimerization, leucine hydrolysis and derived simultaneous temperatures for* OES *from stratified sites in southern Africa, ordered by increasing simultaneous temperature within each stratum.* AMS ^{14}C *dates are on a portion of the same fragment for which amino acid analyses were completed*

T_{Simul} = site temperature (°C) as defined by the solution to the simultaneous temperature equation (see figure 5). T_{Site} = current mean annual site temperature (°C) based on the arithmetic mean annual temperature extrapolated from the nearest weather station.

depth	stratum	laboratory identification	aIle/Ile	$\ln(B/T)_{Leu}$	T_{Simul}	T_{Site}	AMS ^{14}C age BP
Heuningsneskrans (Honey Nest Cave), N.E. South Africa (P. B. Beaumont, unpublished data)							
69–122 cm	2a	AAL-6012C	0.144	−0.387	21	21	
69–122 cm	2a	AAL-6012D	0.138	−0.362	23	21	9675 ± 75 (AA-6649)
69–122 cm	2a	AAL-6012A	0.261	−0.428	52	21	
69–122 cm	2a	AAL-6012B	0.567	−0.467	100	21	9935 ± 75 (AA-8563)
221–259 cm	3b	AAL-6014A	0.184	−0.463	23	21	
221–259 cm	3b	AAL-6014C	0.178	−0.390	34	21	12 030 ± 130 (AA-5829)
221–259 cm	3b	AAL-6014D	0.851	−0.779	69	21	
221–259 cm	3b	AAL-6014B	0.529	−0.517	84	21	12 405 ± 90 (AA-8564)
373–434 cm	3e	AAL-6014B	0.254	−0.563	26	21	21 940 ± 230 (AA-6451)
373–434 cm	3e	AAL-6014C	0.388	−0.664	38	21	24 700 ± 250 (AA-8565)
Apollo 11 Cave, Namibia (Wendt 1972, 1975*a*,*b*)							
78–82 cm	D	AAL-5911C	0.145	−0.384	22	21	
78–82 cm	D	AAL-5911B	0.144	−0.367	25	21	
78–82 cm	D	AAL-5911A	0.144	−0.337	31	21	9290 ± 70 (AA-6448)
78–82 cm	D	AAL-5911D	0.365	−0.668	33	21	34 955 ± 775 (AA-8562)
87–93 cm	D	AAL-5912D	0.343	−0.660	30	21	
87–93 cm	D	AAL-5912A	0.346	−0.655	31	21	
87–93 cm	D	AAL-5912B	0.344	−0.590	41	21	41 200 ± 1650 (AA-5825)
87–93 cm	D	AAL-5912C	0.367	−0.576	47	21	

two of the fragments from each level were in close agreement and an accelerator mass spectrometry (AMS) ^{14}C date on one of these fragments at each level (table 3) is consistent with the associated artifacts. In contrast, the other two fragments from each level gave inconsistent and much higher ratios; they may have been subtly heated or reworked from older levels. The simultaneous temperatures for each pair of fragments with low aIle/Ile ratios are similar to the site temperature, whereas the two other fragments have significantly higher simultaneous temperatures (table 3), implying that they experienced short-term heating. To test this conclusion, we subsequently submitted one of the 'heated' fragments from each level for AMS ^{14}C dating. The dates (table 3) confirm that the 'heated' samples are indeed of the same age as the OES with lower, concordant aIle/Ile ratios. A more subtle degree of heating is identifiable in stratum 3e within the same excavation where two eggshells had very different aIle/Ile ratios. The sample with lower aIle/Ile ratio has a simultaneous temperature similar to the current site temperature and an AMS ^{14}C of about 22 ka BP, whereas the sample with higher ratio has a simultaneous temperature more than 10°C higher, but a similar radiocarbon age (table 3). Apparently this sample has been slightly heated, causing the epimerization ratio to be more advanced than expected.

A different conclusion can be drawn from a series of OES excavated at the mouth of Apollo 11 Cave in Namibia (Wendt 1972, 1975*a*,*b*; figure 6), in which the different fragments from an ELSA level gave essentially identical aIle/Ile ratios, one of which has an AMS ^{14}C age of 9290 ± 70 years BP, but a fourth fragment had a much higher ratio (table 3). In this instance, all samples produced similar simultaneous temperatures, implying that the higher ratio cannot be explained by heating, and that it must have been reworked. Four OES from only slightly deeper in same excavation have aIle/Ile ratios and, with one exception, simultaneous temperatures similar to that of the outlier from the younger horizon; one fragment in this collection gave an AMS ^{14}C date of 41 200 ± 1650 years BP. To test whether the anomalously high aIle/Ile ratio in the ELSA horizon was reworked as indicated by its simultaneous temperature, rather than burnt, a piece of the same fragment was subsequently dated by AMS ^{14}C; its age, *ca.* 35 ka BP (table 3), confirms reworking. Note that the average simultaneous temperature for eggshell with aIle/Ile ratio at *ca.* 0.35 is 36 ± 7°C, suggesting that all five shells may have been slightly heated, possibly when more recent occupants built fires on top of the older deposits.

By deriving the simultaneous temperature from isoleucine epimerization and leucine hydrolysis it is possible to evaluate whether samples of mixed apparent age are caused by heating. As long as the heating was due to a brief interval of high temperature, the simultaneous temperature will be significantly above the mean annual site temperature.

(*b*) *Geochronology*

(*i*) *Chronostratigraphy*

The extent of isoleucine epimerization can be used

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Lithostratigraphy			Industry	^{14}C age	alle/Ile ratio	amino age
1	1BS	UP				
		LR.A				
		LR.B	ELSA	38 ± 2	0.271 ± 0.018 (25)	*
		LR.C				
	1WA			38-40	0.255 ± 0.022 (9)	*
2	2BS	UP	MSA 3b	>49	0.328 ± 0.022 (2)	47
		LR.A				
		LR.B				
		LR.C	MSA 3a		0.388 ± 0.012 (18)	56
	2WA				0.468 ± 0.018 (4)	69
3	3BS		MSA2 (H.P.)			
	3WA					
4	4BS/4WA		MSA1		0.87 ± 0.11 (4)	145[1]
5	5BS				0.87 ± 0.07 (7)	106[2]
	5WA					
6	6BS					

Figure 6. Summary of the stratigraphy at Border Cave, showing the mean aIle/Ile ratio in unburnt OES (number of analyses at each level given in parentheses), available radiocarbon dates, and the amino acid dates derived from the calibration in stratum 1. The two options for the age of stratum 4/5 is based on a maximum age model (1) and a minimum age model (2) as discussed in the text.

directly to provide a relative chronostratigraphic framework for a specific site, or for sites that are sufficiently close goegraphically that they can be considered to have experienced a similar thermal history (within 1°C). It is important to note in this regard, however, that depth of burial in open-air sites can influence the effective temperature of a sample. If the sample has been close to the surface, within the zone of high-amplitude annual temperature cycles (range greater than 12°C), there can be a significant acceleration of the diagenetic reactions (McCoy 1987; Miller 1992). Stratified cave sites, where seasonal and diurnal temperature fluctuations are highly attenuated, provide optimal samples for amino acid geochronology.

Isoleucine epimerization ratios in a series of OES from excavations at Border Cave, South Africa (figure 6) define a relative chronostratigraphy. Conclusions that can be drawn from these data, without evaluating temperature variations due to climate change, are (i) that there is no significant age difference between strata 1BS and 1WA, (ii) that stratum 2BS is significantly older than either 1BS or 1WA and (iii) that a long time interval separates stratum 4 from stratum 2, but that stratum 5 may not be much older than stratum 4.

(*ii*) *Absolute dating*

Converting the aIle/Ile ratios to absolute age requires a suitable calibration or evaluation of the thermal history. Unless there is a series of samples within the range of radiocarbon dating, from which the glacial/interglacial temperature changes can be assessed, it is preferable to use the calibration technique. The conversion of aIle/Ile ratios to age at Border Cave is described in § 5.

(*c*) *Palaeothermometry*

Although the simultaneous temperature provides an estimate of average site temperature, the lack of precision (± 10°C) limits its application beyond identification of heated eggshells. If the age of a sample is known, it is possible to compute the average, or effective temperature experienced by the sample with reasonable precision from the aIle/Ile ratio. This temperature differs from most other palaeotemperature estimates in that it is an integration of the entire thermal history since the egg was laid, as opposed to instantaneous temperatures derived from faunal or floral assemblages. Where several independently dated strata occur, it is possible to compute the average site temperature between dated horizons. If these temperatures are expressed as differences between intervals (ΔT), then a precision of ± 1°C is attainable, whereas the accuracy of absolute temperature estimate is somewhat less (± 3°C; McCoy 1987).

5. DATING THE EARLIEST OCCURRENCE OF MODERN HUMANS

Anatomically modern human skeletal material beyond the range of radiocarbon dating has been recovered in stratigraphic context from caves in southern Africa. The common association of OES with human occupation sites, and the potential of the isoleucine epimerization in OES to provide chronological information, offers the possibility of dating the first appearance of anatomically modern humans at these sites.

Excavations at Border Cave, South Africa, revealed alternating strata of powdery brown sandy silts (BS) and white-black ash (WA), with a cumulative thickness of about 4 m. Based on typological and morphometric studies three major phases of the Middle Stone Age (MSA 1, 2, 3) and a very early manifestation of the ELSA have been identified (Beaumont 1980). Partial human skeletons (individuals designated BC1–5) have been recovered during excavations at the cave. BC1, BC2, and various postcranial pieces were recovered out of stratigraphic context in 1940–1941 (Cooke *et al.* 1945), but matrix in the interstices of one skull (BC1) only matched the sediments at the base of stratum 4BS (Beaumont 1980). Subsequent excavation in 1941 produced a largely complete infant skeleton (BC3) associated with a perforated *Conus* shell, in a shallow grave cut from within the upper 4BS and certainly older than stratum 3 (Cooke *et al.* 1945; Beaumont 1973, 1980). BC5, a nearly complete adult mandible with four teeth, was found more recently at the base of stratum 3WA (Howiesons Poort level; Beaumont 1980; De Villiers 1976). Most researchers, but not all (e.g. van Vark *et al.* 1989), have attributed all of these

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remains to modern *Homo sapiens*, although the precise provenance of some bones remains contentious.

The aIle/Ile ratio was measured at least twice in 146 preparations of OES fragments excavated from Border Cave that did not appear to be burnt on visual inspection. Of these, 16 had been so severely heated that amino acids were absent, and a further eight had such low levels of amino acids that severe heating must have occurred. Simultaneous temperatures could be calculated for 51 fragments (only total amino acid analyses were completed for the remainder). Of these, 19 had simultaneous temperatures more than 15°C above the site temperature, and are consequently rejected as burnt. In all cases, these samples also contained aIle/Ile ratios higher than expected. The aIle/Ile ratios in unburnt, un-reworked OES show a clear increase with increasing stratigraphic age (figure 6). We were unable to locate any unburnt OES from stratum 3BS (MSA 2) containing hominid remains and lithic pieces of the Howiesons Poort industry. The underlying strata 4WA, 5BS and 5WA (MSA 1) contained occasional OES fragments, most of which were burnt. Insufficient unburnt fragments were available from the deeper strata (stratum 6 (MSA 1)) to provide chronological information.

Twelve new radiocarbon dates on wood charcoal from strata 1BS averaging 38 ± 2 ka BP (J. Vogel and P. Beaumont, unpublished data) calibrate the epimerization rate at Border Cave. The mean aIle/Ile based on 34 separate measurements of OES from these two units is 0.266 ± 0.020; the corresponding rate constant (k_{Ile}) is 6.438×10^{-6}. An AMS ^{14}C date on one of these eggshell fragments is 36.1 ± 0.9 ka BP (AA-4254), confirming that the eggshell is of the same age as the associated charcoal. Because this calibrated rate constant incorporates almost equal parts of the Holocene warm period (0 to 10 ka BP), the temperature depression of the last glacial maximum (*ca.* 13–25 ka BP) and intermediate temperatures of the intervening interstadials, we argue that it can be used to date samples between *ca.* 30 and 80 ka old, but for older levels the temperature may have deviated from the average of the last 40 ka, particularly for samples from the last interglacial. Using the calibrated rate constant and an uncertainty of $\pm 10\%$, the age of stratum 2BS is 47 ± 7 ka BP at the top and 56 ± 6 ka BP at its base, and stratum 2WA is 69 ± 7 ka BP.

For samples older than 80 ka, the prolonged warmth of the last interglacial (*sensu lato*; 130 to 75 ka ago), may yield a higher effective temperature than for younger samples. To address this uncertainty we propose two models, a minimum age model that assumes the average temperature between about 80 and 130 ka (isotope stage 5) was continuously the same as the Holocene (19.5°C), and a maximum age model in which we assume that the average rate constant for the last 40 ka is valid for the entire Pleistocene. It is almost certain that the actual thermal history lies between these two extremes. Stratum 3 (Howiesons Poort) is bracketed between 69 ± 7 ka BP and 106 ± 11 ka BP, the minimum age of underlying stratum 4. The MSA 1 levels at Border Cave were deposited at least 100 ka ago (minimum age model), and possibly predate the last interglacial (linear extrapolation from the ELSA calibration).

6. DISCUSSION AND CONCLUSIONS

The integrity of the ostrich eggshell, retaining indigenous organic molecules and excluding the immigration of secondary amino acids, results in a predictable pattern of protein diagenesis in which the extent of the various reactions is dependent on the age of the sample and the effective diagenetic temperature. The extent of isoleucine epimerization in OES can be used directly as relative age indices, and with suitable calibration, can be converted to absolute age with an accuracy of *ca.* $\pm 10\%$ back to at least 80 ka, and with less precision to 500 ka. The simultaneous solution for temperature from the isoleucine epimerization and leucine hydrolysis reactions provides an effective means of differentiating subtly burned OES from those that have been reworked from deeper levels. Burnt eggshell occurs at most sites, often amounting to one-third to one-half of all samples, but reworked pieces, from both younger and older levels, are also common, indicating that despite meticulous sampling procedures, some faunal elements in any horizon may not be in place.

The significance of the four hominid specimens recovered from the MSA levels at Border Cave has been much debated, at least in part due to uncertainties in the dating of the stratigraphic sequence. Recent datings by electron spin resonance (ESR) using tooth enamel have been reported for Border Cave (Grün *et al.* 1989). The ESR dates for Border Cave are consistently younger than the amino acid dates (except for units 4WA and 5BS where the two methods agree); for stratum 1, the ESR dates are about 30% younger than the radiocarbon dates. Because we calibrate the isoleucine epimerization rate using the radiocarbon dates from stratum 1, we cannot independently evaluate the discrepancy between the ESR and amino acid dates. Although paired ^{14}C and U-series dates are not yet available prior to 30 ka (Bard *et al.* 1991), the magnitude of the carbon reservoir is so large that it is improbable that the ^{14}C production rate could change in such a way that a radiocarbon age of 38 ka BP could have a calendar age of 28 ka as predicted by the ESR dates; we suggest that the ESR dates have a systematic error that underestimates the accumulated dose and/or overestimates the annual dose rate.

Based on the calibrated amino acid dates (figure 6) Border Cave skeleton BC5 is older than 70 ka, whereas BC3 and BC1 (if its association with level 4BS.LR is correct) are presumably at least 100 ka old, and probably date from the early to middle period in the last interglacial. These conclusions support the contention of an African origin for *Homo sapiens*.

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APPENDIX: SIMULTANEOUS TEMPERATURE EQUATION

A mathematical expression defining the changing rate constant for leucine hydrolysis over time was derived empirically from the 142.5°C experiment (figure 4), then adjusted for temperature by comparing predicted and observed rates of leucine hydrolysis at other temperatures. The equation below defines an effective rate constant (k_{eff}) that is applicable to all temperatures between *ca.* 0 and 170°C.

$$k_{\mathrm{eff}} = ac(0.685e^{(-1.9atb)} + 0.43e^{(-0.43atb)} + 0.145e^{(-0.06atb)}), \quad \text{(A1)}$$

where

$a = e^{(36.39 - 13692/T)}$, the rate of initial Leu hydrolysis from equation 3 and figure 2

$b = 2.29 + [(9.774 \times 10^{-4} \times T) - (9.74 \times 10^{-6} \times T^2)]$

$c = 1.34 - (8.16 \times 10^{-4} \times T)$

T = the effective diagenetic temperature (Kelvin)

t = time in years

and leucine hydrolysis is then defined by equation (4).

Equation 2 describes the relation between aIle/Ile ratio, time and temperature. Solving equation (2) for t yields:

$$t = \frac{\ln((1 + \mathrm{D/L})/(1 - 0.77 \times \mathrm{D/L}) - 0.032}{1.77 \times e^{(40.23 - (15152/T))}}. \quad \text{(A2)}$$

Substituting equation (A2) into equation (4) and solving for T results in a single expression in which the only variables are aIle/Ile and ($\mathrm{Leu_B/Leu_T}$), both of which may be measured, allowing a value for T to be determined (the simultaneous temperature) without knowledge of sample age.

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