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Age determination based on amino acid racemization: A new possibility

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Summary. A method has been developed to determine the age of fossil bone samples based on amino acid racemization (AAR). Approximately one hundred fossil bone samples of known age from Hungary were collected and analysed for D- and L-amino acids. As the racemization of amino acids is affected by temperature, pH, metal content of the soil, and time passed since death, these factors were eliminated by comparing the estimated age to age determined by the radiocarbon method. Determining the D- and L-amino acid contents in samples of known age, determining the half life of racemization and plotting the D/L ratio as a function of time, calibration curves were obtained. These curves can be used for the age estimation of samples after determining their D- and L-amino acid content. The D/L ratio for 2 to 3 amino acids was determined for each sample and the mean value of estimated ages based on calibration curves was considered to estimate age of the fossil samples.

Keywords: Amino acid racemization – D-amino acids – Age determination – D-Ala – D-Asp – D-allo-Ile – D-Glu – D-Phe – D-Val

Introduction

Amino acid contents in fossil shell, bone and tooth samples from early ages were reported first by Abelson in 1954. In 1967, Hare and Abelson reported that D-amino acids in fossils resulted from conversion of L-amino acids of protein. It was found that the older the fossil the higher the D/L ratio and, after a certain age, amino acids occurred in racemic form. The ratio of D-allo isoleucine and L-isoleucine content in a fossilised shell sample was found to be 0.32 and the

fossil was estimated to be 70000 years old, as reported by Hare and Mitterer, in 1968. It is considered as the first application of amino acid racemization (AAR) – or rather epimerization – in geochronology.

Subsequently, racemization of amino acids was used for age determination of various materials containing protein. Isoleucine and aspartic acid were given special attention because L-isoleucine can be easily separated from D-allo isoleucine by an amino acid analyser and aspartic acid, being the most acidic of amino acids, is the first to come off of the ion exchange column. However, some errors of age determination based on AAR were reported by Williams and Smith in 1977. Temperature, pH, soil composition and various contaminants should also be considered when estimating the age of fossil bone samples. Recently Marshall (1990) established that the bones are not reliable materials for AAR testing, particularly if they come from a warm environment. The statement was based on differences observed between the age of the California bones determined by C^{14} accelerator mass spectrometry (5000–6000 years) and by AAR (50000–60000 years). Milford Wolpoff, paleoanthropologist, expressed the opinion (cited by Marshall, 1990) that many people currently regard AAR as "some kind of joke".

Since various changes in temperature during the past and other conditions influencing dead biological organisms are not well known, the reaction temperature of racemization can only be estimated and not accurately determined. This is the reason that – in this study – contents of D- and L-amino acids and their ratio were determined in samples of known age (as determined by the radiocarbon method). These data were then compared with data obtained from the analysis of amino acids in samples of unknown age. To make the comparison more accurate, the antecedents of samples of known age when analysed were the same as or similar to those of unknown age. Therefore, 100 fossil bone samples previously analysed by the radiocarbon method were collected from various Hungarian museums, and their D- and L-amino acid contents were determined. The D/L ratio was calculated and plotted against time which produced a calibration curve. This curve can be used for age estimation of samples of unknown age after their D- and L-amino acid contents have been determined. The D/L ratio for 2 to 3 various amino acids was determined for each sample and the mean value of ages estimated from calibration curves was considered the true age of the fossil sample.

Materials and methods

Sample preparation

The samples were washed in running- and distilled water, dried in a vacuum drying oven and ground to produce powder material as fine as flour. Apolar contaminants were removed with petroleum ether in a Soxhlet extractor for 3 hours at 40 °C. The free amino acids were extracted by 0.1 M HCl solution for 16 hours. The nitrogen content of the residue was determined by Kjel-Foss nitrogen analyser. Sample size (200–2000 mg residual material containing app. 10–20 mg protein) was dependent on nitrogen content. Samples were weighed and hydrolysed with 6 M HCl at 110 °C for 24 h. HCl was removed by lyophilization, the residue was dissolved in water, and the precipitated silicate compounds were separated from the liquid containing free amino acids using a centrifuge. The solution was

Table 1. D/L ratios for various amino acids determined by ion exchange column chromatography (IEC) and by high performance liquid chromatography (HPLC)

Number and age of samples	The D/L ratios for various amino acids				
	Phe	Asp	Ala	Ile	Val (year)
Analytical method					
1. 15600					
IEC	0.568	0.367	0.153	—	—
HPLC	0.553	0.389	0.163	—	—
2. 38450					
IEC	—	—	0.395	0.123	—
HPLC	—	—	0.401	0.121	—
3. 46900					
IEC	—	—	0.487	0.146	—
HPLC	—	—	0.492	0.149	—

alkalised to pH = 9 for a moment and precipitated metal hydroxides were filtered. The hydrolysed solution was neutralised and evaporated to dryness by lyophilization.

Determination of amino acids

An aliquot of hydrolysed material was dissolved in a citrate buffer solution of pH = 2.2 and isoleucine and D-allo isoleucine were determined by LKB 4101 type amino acid analyser as described by Csapó et al. (1986). The other D- and L-amino acids were separated in the form of alanyl- (Csapó et al., 1991) and 2-sulphonylic acid alanyl diastereomerisomer dipeptides (Csapó et al., 1990) by ion exchange column chromatography and by the method of Einarsson et al. (1987) with reversed-phase HPLC using precolumn derivatization with the chiral reagent O-phthalaldehyde/2,3,4,6,-tetra-O-acetyl-1-thio-β-glucopyranoside.

Prior to conducting analyses of all samples by HPLC, the D- and L-amino acids of three samples were determined by both HPLC and ion exchange column chromatography (IEC). The results are in Table 1, and the D/L ratios determined by the two methods were in excellent agreement.

Results and discussion

The analyses data on 24 fossil bone samples from various Hungarian museums of known age are summarised in Table 2. Six amino acids (His = histidine, Phe = phenylalanine, Asp = aspartic acid, Ala = alanine, Ile = isoleucine, Val = valine) are presented. These may be considered as being the most suitable for age determination because some of them show very fast racemization (His, Phe, Asp), while others show very slow racemization (Ile, Val). Analytical data for other analysed amino acids are not presented in Table 2 in order to make it more synoptic. None of the ratios lower than 0.1 or higher than 0.7 are presented in Table 2 because, in these cases, the accuracy of age determination was doubtful. Calibration curves of phenylalanine, aspartic acid, alanine and isoleucine plotted on the basis of the data in Table 1 can be seen in Figs. 1, 2, 3 and

Table 2. D/L ratios for various amino acids concerning ages of fossil samples determined by the radiocarbon method

Age of samples determined by the ^{14}C corrected method (year)	The D/L ratios for various amino acids					
	His	Phe	Asp	Ala	Ile	Val
2200	0.138	—	—	—	—	—
2800	0.162	0.101	—	—	—	—
3110	0.181	0.109	—	—	—	—
3240	0.199	0.128	—	—	—	—
4630	0.253	0.179	0.109	—	—	—
5460	0.312	0.225	0.128	—	—	—
6850	0.419	0.252	0.171	—	—	—
11200	0.618	0.442	0.271	0.112	—	—
12400	0.682	0.473	0.289	0.131	—	—
15600	—	0.561	0.378	0.158	—	—
18600	—	0.654	0.432	0.192	—	—
20200	—	0.689	0.491	0.209	—	—
22600	—	—	0.543	0.228	—	—
25400	—	—	0.580	0.246	—	—
28600	—	—	0.621	0.289	—	—
30400	—	—	0.643	0.321	—	—
32500	—	—	0.702	0.343	0.099	—
36900	—	—	—	0.381	0.118	—
44600	—	—	—	0.465	0.134	—
46800	—	—	—	0.483	0.142	—
54300	—	—	—	0.510	0.169	0.100
62200	—	—	—	0.586	0.188	0.115
65000	—	—	—	0.613	0.199	0.119
72400	—	—	—	0.652	0.221	0.136

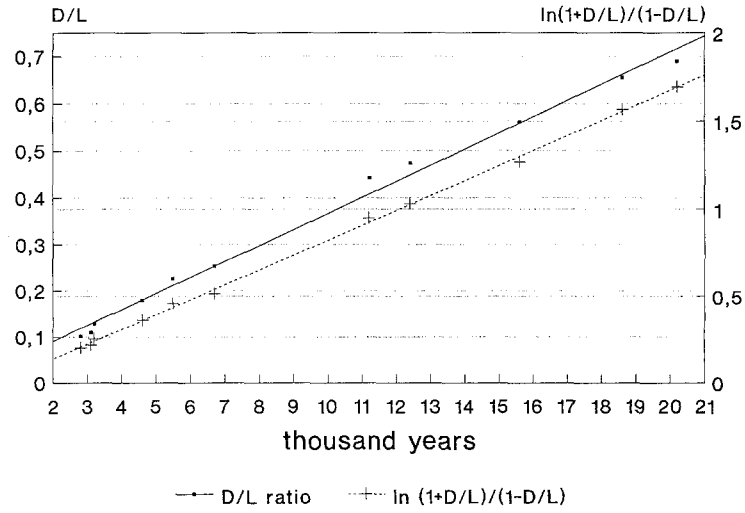

Fig. 1. Calibration curve of phenylalanine

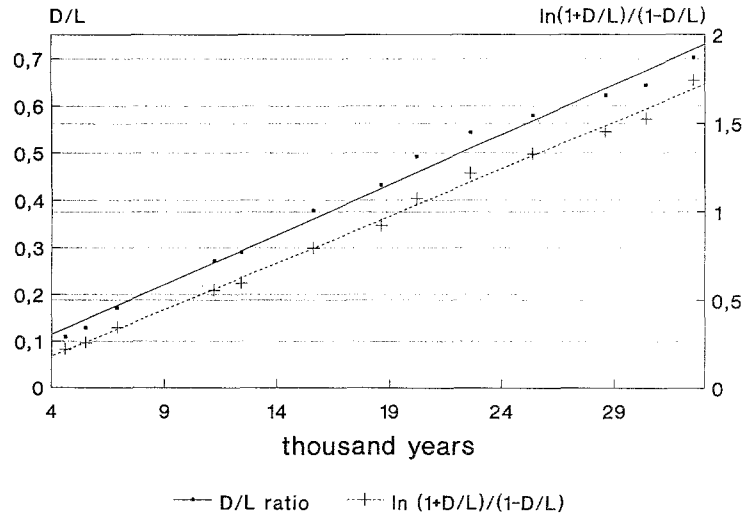

Fig. 2. Calibration curve of aspartic acid

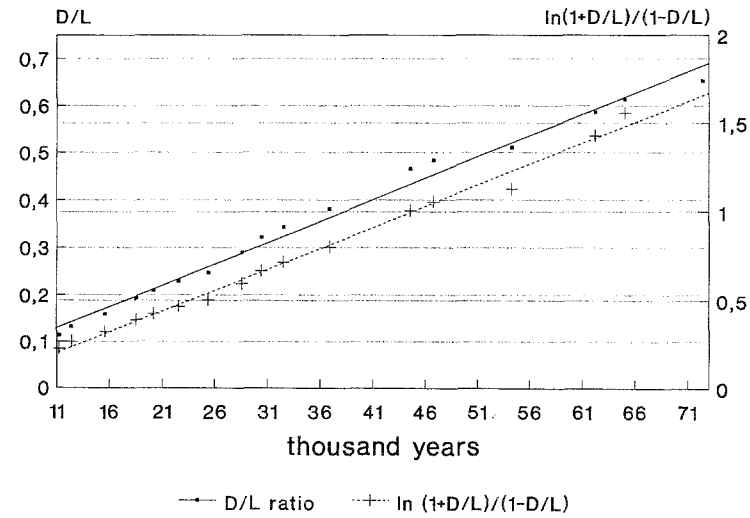

Fig. 3. Calibration curve of alanine

4, respectively. Half lives of AAR were also calculated from the data of Table 2 and are presented in Table 3.

From the data of Table 2, His, Phe, Asp and Ala contents can be used for the age determination of samples which are 2–12000, 3–20000, 5–35000 and 10–80000 years old, respectively. Age of samples older than 30000 and 50000 years can be determined on the basis of Ile and Val content, respectively. Data in Table 2 were corrected (reduced) with the D-amino acid content of a fresh pig bone to eliminate the errors of analysis. When fresh pig bone was hydrolysed with 6 M hydrochloric acid for 24 h at 110 °C, the D-forms of glutamic and aspartic acids, respectively, represented 1.9 and 1.3% of the totals due to racemization during processing. Concentrations of the D-form for the other amino

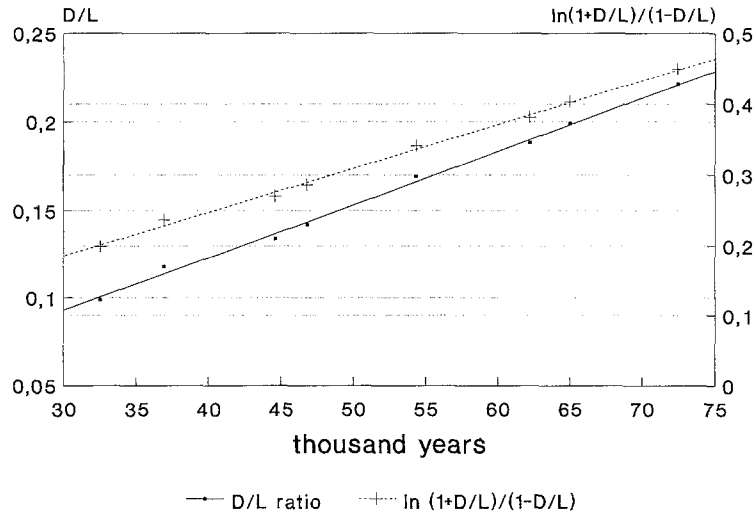

Fig. 4. Calibration curve of isoleucine

Table 3. Half lives of racemization and epimerization of various amino acids found in Hungarian fossil bone samples

Amino acids	Half life (year)
Histidine	5500
Phenylalanine	8500
Tyrosine	8600
Aspartic acid	13500
Serine	16500
Threonine	17000
Glutamic acid	28500
Alanine	32000
Isoleucine	110000
Leucine	140000
Valine	180000

acids were negligible. However, all analyses were corrected for the small concentrations present in fresh pig bone.

Studying the calibration curves, it can be concluded that, in the case of D/L ratio being lower than 0.1, the D-amino acid content is too low and age determination is uncertain. Both curves may be considered to be linear in the D/L range of 0.1–0.5. It is obvious that the calibration curves can be used for age determination most satisfactorily in the linear range, (D/L between 0.1 and 0.5 where D-amino acids are present in well detectable amounts). The optimum D/L ratio can be found for each sample by analysing the amino acids best suited for age determination. E.g., for fossil bone samples of 11200 years the D/L ratio for His, Phe, Asp and Ala is 0.682, 0.473, 0.271 and 0.112, respectively. In this case the D/L ratios of Phe and Asp are recommended for determining the age

of samples, however the D/L ratios of His and Ala can be used to confirm the estimate based on the ratios of Phe and Asp.

Known age (Y) was regressed on D/L ratio (X_1) and $\ln[(1 + D/L)/(1 - D/L)]$ (X_2) for each of four amino acids (Phe, Asp, Ala and Ile) to produce prediction equations of the form $\hat{Y} = a + bX$. All eight regression equations produced r^2 values greater than 0.99. In each amino acid, $r^2_{X_1 X_2}$ was greater than 0.99 which means that X_2 was simply a coded value of X_1. The standard deviation of deviations from regression (standard error of estimate = $s_{Y.X}$) can be used to calculate the standard error of an individual estimate as

$$s^2_{\hat{Y}} = s^2_{Y.X}[1/n + (X - \overline{X})^2/\text{Sum}(X - \overline{X})^2]$$

with n = number samples used in estimating regression and $\text{sum}(X - \overline{X})^2$ being the sum of squares of deviations from the mean X. The value, $s_{\hat{Y}}$ was calculated for each regression for two situations ($X = \overline{X}$ and X = an extreme value). For Phe, Asp and Ala, mean values for D/L were 0.35 to 0.41 and extremes were approximately ± 0.30. Corresponding means for $\ln(X_2)$ were 0.75 to 0.90 and extremes were ± 0.75. For Ile, means were 0.16 and 0.32 with corresponding extremes at ± 0.06 and ± 0.12. The two $s_{\hat{Y}}$ values for each amino acid mean and extreme were averaged to produce the following values:

Amino acid	Mean	Extreme
Phe	189	329
Asp	226	458
Ala	382	988
Ile	311	514

A mean of estimates based on two amino acids would have a standard error of

S.E. = $\sqrt{(s^2_{\hat{Y}_1} + s^2_{\hat{Y}_2})/4}$ and 95% confidence limits can be established as

C.I. = Mean of two $\hat{Y}$ values $\pm$S.E. ($t_{0.05}$).

Since the average based on the smallest number of samples would have 15 degrees of freedom, the value of $t_{0.05}$ used in the following estimates was 2.13. The $\pm$ deviations were calculated for each pair of amino acids and are shown below. The confidence intervals at mean values are shown above the diagonal and confidence intervals at extreme values are below the diagonal:

	Phe	Asp	Ala	Ile
Phe	—	313	454	388
Asp	601	—	473	409
Ala	1109	1160	—	524
Ile	650	733	1186	—

If both D/L values were near the mean, we would be 95% confident that our estimate was in the range of mean $\hat{Y} \pm 313$ to 524 years. If both estimates were

based on extreme values of D/L, we would be 95% confident that our estimate was in the range of mean $\hat{Y} \pm 601$ to 1186 years. The confidence interval for each estimate of age of an unknown sample would be calculated individually.

Finally, the applicability of calibration curves is presented. As an example, one bone sample of unknown age was analysed for L- and D-amino acids and the following results were obtained:

L-His: 0.0697 mg, D-His: 0.0289 mg, $D/L_{His} = 0.428$.
Age calculated from calibration curve: 7100 year; S.E. = 337.
L-Phe: 0.0543 mg, D-Phe: 0.0138 mg, $D/L_{Phe} = 0.254$.
Age calculated from calibration curve: 6950 year; S.E. = 191.
L-Asp: 0.1346 mg, D-Asp: 0.0245 mg, $D/L_{Asp} = 0.182$.
Age calculated from calibration curve: 6900 year; S.E. = 465.

The estimated age of the sample is the mean value of the above estimates or 6980 years. This mean value has a standard error of 202 years and the 95% confidence interval would be 6554 to 7406 years.

Conclusions

The D- and L-amino acid composition was determined in fossil bone samples of known age. Ages were determined by the radiocarbon method. The D/L ratio was plotted as a function of time which resulted in a calibration curve which can be used for age estimation after the D- and L-amino acid contents in samples of unknown age have been determined. However, this method includes the analytical error of age estimation by the ^{14}C method, but the effects of temperature, pH and the composition of soil on AAR can be eliminated. The D/L ratio for 2 to 4 amino acids should be determined for each sample, and the mean value of estimated ages based on calibration curves is considered the best estimate of age of the fossil sample.

We have utilised this method very successfully for dating fossil bone samples from Hungary. The difference between the data from the calibration curve and those from ^{14}C dating was generally negligible. We were very cautious with both sample selection and preparation; the unknown samples were mainly of origin similar to those from which the calibration curves were formulated and sample preparation was carried out exactly the same for samples of known and unknown ages.

We are aware of the weak points of this method and the possible errors associated with ^{14}C dating. However, the results support the reliability of this method. Our calibration curves should not be used in other environments because of different conditions (e.g. temperature, pH, soil composition). However, based on these results, other calibration curves can be formulated for each environment based on methods described here.

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