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Some Considerations Regarding the Use of Amino Acid Racemization in Human Dentine as an Indicator of Age at Death

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ABSTRACT: An HPLC method is described for simultaneously obtaining the enantiomeric ratio of three amino acids (aspartic acid, serine, and glutamic acid) from dental collagen, with a view to using this information for estimating age at death. Results are reported from a sample of twenty three known age modern teeth, six known age 19th C. AD teeth, and two unknown age Romano-British teeth. It was found (as expected) that all three D/L ratios changed significantly with chronological age. Standard calibration techniques were used to estimate ages for the six 19th C. AD specimens from regression equations estimated from the modern specimens, and also to predict (for the first time) the error associated with such estimates. Errors using aspartic acid were found to be similar to those obtained by other methods of age estimation from dental evidence, serine, and glutamic acid providing much poorer age estimates. Additionally, a systematic difference in the age-enantiomeric ratio relationship was observed between modern and older dental samples. It is concluded that there is some fundamental difference in the observed enantiomeric ratios between modern teeth and older samples, possibly as a result of the chemical alteration of the dental proteins.

KEYWORDS: forensic science, forensic pathology, forensic odontology, forensic anthropology, amino acid racemization, aspartic acid, serine, glutamic acid, age at death, error estimation, collagen, dental, HPLC, chemical alteration, protein

It is well known that in certain proteins, such as those found in the eye lens (1), in dental tissue (2,3), in parts of the brain (4), and in vertebral discs (5), a slow amino acid racemization reaction takes place *in vivo*. This reaction proceeds throughout the lifetime of the host (and also after death, but probably at a reduced rate as a result of a presumed reduction in ambient temperature) and hence, in recent samples at least, the degree of racemization can be related to the chronological age at death of the host. Previous work has shown that the D/L ratio of aspartic acid from human dental collagen can give a good measure of age at death for modern individuals (and also in some forensic cases), but is less reliable for archaeological samples, even if relatively recent (18th Century AD) (6).

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Several groups of workers have used the racemization of aspartic acid in dental collagen and in other tissue for the estimation of age at death of recent individuals (5,7–14). In all cases, the method has relied on the use of a calibration curve derived from known-age modern material to determine the rate constant of the racemization procedure, followed by the use of this information to produce an age estimate on one or more unknown samples. Details of the calibration method vary—some workers plot $\ln\{(1 + (D/L))/(1 - (D/L))\}$ against age, others plot %D-asp against age. Some calculate the racemization constant explicitly, others use the plotted data simply to read off the value of predicted age from a measurement made on an unknown. What is, however, common to all published work is a lack of discussion of the statistical uncertainty associated with such age estimations. Estimates are invariably given as point estimates of age, without any consideration of the associated error. Although in some applications, it may be sufficient to give an approximate age estimate, for example, to pinpoint the identification of known missing persons, in general, it is necessary to know the exact distribution of the errors around the point estimate—especially if palaeodemographic information is required.

We have been interested for some time in the use of age estimation techniques from dental observations, and, in particular, in the correct calculation of the errors associated with such methods (15–17). We have also been working on the use of measurements of aspartic acid racemization for the estimation of age at death (6,18–21). We have now brought together these two strands of research to estimate for the first time the errors associated with the aspartic acid racemization method. While undertaking this research, we have observed a systematic variation in the relationship between age and extent of racemization of amino acids in dental collagen extract from modern and archaeological samples which cannot be explained simply by additional post mortem racemization. It must, we believe, reflect a fundamental difference in the racemization behavior of proteins in these two environments.

Materials and Methods

Sampling Procedures

From our previous experience and published data, we concluded that use of the same type of tooth throughout would reduce errors in estimation of the exact age of dentine, because it eliminates systematic variation due to different developmental rates for different teeth. Considerations of dental physiology and typical archaeological dentitions led to the conclusion that the use of upper (left and right) first pre molars was most appropriate. The effect of caries was also considered: Due to the possibility of contamination

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by amino acids from bacteria and the formation of secondary dentine at carious sites, it was decided to avoid carious samples where possible. Where it was impossible to avoid the use of carious or filled teeth, sampling was carried out with extreme care to avoid inclusion of any contaminated material.

As dentine is formed from crown to root in a series of concentric cones over a number of years, it is expected that an age gradient should be observed from crown to root. Preliminary investigations, taking 1-mm horizontal sections from crown to root of a single tooth, after mechanically removing the secondary dentine, gave no evidence for such a gradient (Table 1). This is consistent with observations by other workers, although the more refined sampling procedure used by Saleh et al. (22) revealed a difference between primary and secondary (plus tertiary) dentine. For our purposes, it was decided, however, that rather than using a whole tooth (as often used by other workers), a horizontal slice ~1-mm thick should be taken at approximately the crown/root junction, just above the pulp chamber. Enamel is removed by a reproducible demineralization method (see below), and any pulp or secondary dentine present is mechanically removed with a scalpel and forceps, prior to hydrolysis of the primary dentine.

Demineralization Protocol

Remove any adhering organic matter from tooth surface by washing in water, followed by immersion in sodium hypochlorite (12% free Cl) (Fisons, Loughborough, UK) for at least 1 h. Rinse tooth thoroughly in water to remove all sodium hypochlorite and keep frozen at −18°C until required. Dry tooth to constant weight in a vacuum desiccator over phosphorus pentoxide. Place tooth in a universal sample tube with 20 mL 1M HCl (Fisons, Loughborough, UK). Seal tube and place on a roller. Allow demineralization to proceed at room temperature, with constant rolling for 36 h. Remove tooth from solution and rinse thoroughly with HPLC grade water (Alpha Q water system, Millipore, UK). Section tooth with microtome blade and forceps, to obtain a horizontal section approximately 1 mm in thickness from the crown/root junction. Ensure any secondary dentine or pulp material is removed. Place section in universal sample tube with 20 mL fresh 1M HCl, seal tube and place on roller for a further 12 h. Remove section from HCl, rinse thoroughly with HPLC grade water. Divide section into the required number of samples and weigh. Dry samples to constant weight in a vacuum desiccator. Weigh samples and proceed with hydrolysis.

TABLE 1—*%D-aspartic acid found in dental hydrolysates produced from 1 mm sections of a canine tooth, sample 1 being the root tip and 11 the crown. See text for preparation protocols.*

Position	Sample	%D-aspartic Acid	Standard Deviation (%)
Root tip	1	5.6	2.9
	2	7.8	2.7
	3	7.1	2.4
	5	5.0	2.1
	6	6.2	1.5
	8	8.9	2.0
	9	5.9	2.5
	10	10.2	2.6
Crown	11	6.3	2.1

Hydrolysis Protocol

Place demineralized, dried, weighed sample in Pyrex test tube. Add 500 μL 6M HCl (Fisons, Loughborough, UK). Freeze tube containing sample and HCl in solid CO_2 and alcohol mixture, and allow to thaw to remove dissolved oxygen. Repeat freezing, then allow to thaw while evacuating to about 50 μm Hg pressure. Seal tube after thawing is complete and while still under vacuum. Place in heating block at 110°C for 9 h. Open tube and dry sample down in a vacuum desiccator containing NaOH pellets and phosphorus pentoxide. Reconstitute samples in 500 μL HPLC grade water, mix thoroughly and allow to stand for at least 2 h prior to centrifuging and filtering samples (0.22 μm syringe filter). Store samples at 4°C until analysis.

Simultaneous Measurement of the Enantiomeric Ratio of Several Dental Amino Acids

Previous methods of measuring amino acid racemization have usually involved separation of the amino acid of interest (usually aspartic acid) by ion exchange chromatography, followed by derivatization and analysis by either GC or occasionally HPLC. Although these techniques appear to have been successful, it was considered that the ability to measure several amino acid ratios in the same sample should enhance the accuracy of the estimate of age at death. A method which involved derivatization of the amino acids with a chiral reagent, prior to separation by RP-HPLC, has now been investigated. This involved the use of *O*-phthaldialdehyde and *N*-isobutyryl-L- or D-cysteine (OPA/IBLC or OPA/IBDC) as the derivatizing reagent and a gradient mobile phase system of methanol/acetonitrile and sodium acetate buffer. The method had been shown previously to be applicable to complicated amino acid mixtures such as those from food protein samples (23). This previous work used a fluorescence detector but, because of the relatively large samples available from our dental collagenous proteins, we found that UV detection provided adequate sensitivity in this case.

Protocol for HPLC Analysis of Amino Acid Enantiomers, Derivatized with O-phthaldialdehyde/N-isobutyryl-L- or D-cysteine (OPA/IBLC, OPA/IBDC)

Derivatization Method—Dissolve 260 mM *N*-isobutyryl-L-or-D-cysteine (IBLC or IBDC) (Fluka Chemicals, Switzerland) with 170 mM *O*-phthaldialdehyde (OPA) (Sigma Chemicals, Poole, Dorset) in 1M potassium borate buffer, pH 10.4 ("Fluoraldehyde" reagent, Pierce, Rockford, Il, USA) to give IBLC/OPA or IBDC/OPA reagent respectively. Place 25 μL 0.4M sodium borate buffer, pH 10.4 (Hewlett-Packard, Waldbronn, Germany) in a microvial. Add 5 μL IBLC/OPA or IBDC/OPA, followed by 10 μL amino acid sample. Mix reagents twice using the autoderivatization program of ISS 200 Perkin Elmer autosampler, and allow the reaction mixture to stand for two minutes before analysis.

Preparation of Mobile Phases—Eluent A: Dissolve 6.26 g sodium acetate trihydrate (HPLC grade, Fisons, Loughborough, UK) in 1980 mL HPLC grade water (Alpha Q water system, Millipore, UK). Adjust pH to 6.2 by addition of 10% (v/v) acetic acid (Fisons, Loughborough, UK) and make up to 2 L with water. Filter through 0.45 μm filter (Millipore, UK). Eluent B: Add 600 mL methanol (HPLC grade, Fisons, Loughborough, UK) to 50 mL acetonitrile (HPLC grade, Fisons, Loughborough, UK). Helium is passed constantly through the eluents during preparation to

facilitate degasing, and eluents are kept under a positive pressure of helium.

Chromatographic Conditions

Instrument—Perkin Elmer LC Iss200 with Diode Array Detector 135. Column: 250 by 4.0-mm Hypersil ODS (5 μm) Shandon column, preceded by a 10 by 4-mm guard column packed with the same material (Shandon Scientific, Runcorn; UK). Mobile Phase: Linear gradient 0–57% B over 80 min. Eluent A: 23 mM sodium acetate solution; Eluent B: 600:50 methanol:acetonitrile. Injection volume: 20-μL derivatized amino acid. Flow rate: 1.0 mL/min. Column Temperature: 25°C. Detector wavelength: 340 nm.

Calibration Procedure

Standards of individual amino acids (Sigma Chemicals, Poole, Dorset) were run under the specified chromatographic conditions to obtain exact retention times for each of the expected dental amino acids. 0.02M solutions of the amino acids were prepared in 0.1 M HCl (Fisons, Loughborough, UK). Peak areas were calculated for each eluted peak using the automatic integration facility of the Perkin Elmer Nelson integrator. %D-amino acid was calculated thus: %D-amino acid = 100 × (D-amino acid peak area / (D-amino acid peak area + L-amino acid peak area)). To allow calculation of absolute quantities of amino acids separated, an internal standard was added to each amino acid sample (of known concentration) prior to derivatization. Equal volumes of sample and 0.02 M homoarginine (Sigma Chemicals, Poole, Dorset) were mixed thoroughly and then subjected to the derivatization and analysis procedures detailed above. When it was necessary to calculate absolute quantities of amino acids each set of peak areas was normalized to the peak area of the internal standard. This allowed comparison between samples as well as calculation of absolute values.

Results

A large collection of freshly extracted modern human teeth of known age at extraction has been built up through the co-operation of a number of dentists. This has allowed the analysis in triplicate of 23 upper first premolars, with a donor age range of 11 to 72 years. Triplicate samples were obtained by taking three separate aliquots from the dental hydrolysate which were then derivatized and analyzed independently. Measurement of peak areas for the D- and L-enantiomers of aspartic acid, glutamic acid, and serine allowed the calculation of the percentages of D- and L-amino acids for each of the tooth samples.

Initially it was decided to treat each of the three measurements of D-aspartic acid, D-serine, and D-glutamic acid as replicate independent observations, giving 69 data points for each amino acid (Table 2). Age was regressed as x against $\ln\{(1 + (D/L))/(1 - (D/L))\}$ for each amino acid as y. For each regression both pure error and lack of fit were calculated. A straight line model was used, because theory suggests that this relationship should be linear (24), but there was a significant lack of fit for all three amino acids (the smallest lack of fit being for aspartic acid, $F = 4.35$, $P < 0.001$, see Table 3). No other model (e.g., quadratic and higher, logarithmic, and exponential) could be found which did not display significant lack of fit. The initial assumption, i.e., that the three measurements for each specimen were independent replicates, was therefore rejected. The failure to find a regression model which did not display significant lack of fit was in this case because the component of variance ascribed to pure error was very small compared to that component of the variance attributed to lack of fit. This is because in this case the three separate aliquots from each dental hydrolysate were in effect just three measurements for the same tissue, all variation in D and L ratios being entirely due to derivatization and HPLC runtime variation. This is consistent with the observation that repeat runs can, according to Draper and Smith (25) 'wrongly detect non-existent lack of fit' if the repeat runs do not in fact meet rather stringent criteria as to what constitutes an independent replicate measurement. It is suggested that to guarantee independence of measurement for an individual separate hydrolysates from separate teeth be used, and that separate aliquots from the same hydrolysate only be used as a measure of preparation and instrumental error.

It was therefore decided to calculate the regression of the means of $\ln\{(1 + (D/L))/(1 - (D/L))\}$ as the best single measure as y (giving 23 data points per amino acid), against age as x, and use the three complimentary calibration curves relating $\ln\{(1 + (D/L))/(1 - (D/L))\}$ for each amino acid to estimate age for unknown specimens. In all cases, for the modern teeth, there was no evidence for a significant deviation from a linear model, and, in view of the theoretical relationship discussed above, a linear model was therefore accepted.

The analysis of variance tables for the regression of $\ln\{(1 + (D/L))/(1 - (D/L))\}$ against age for aspartic acid, serine, and glutamic acid from modern dental tissue are presented in Table 4 (see Figs. 1–3). All three amino acids displayed gradients which were significantly different from zero, but the increase in $\ln\{(1 + (D/L))/(1 - (D/L))\}$ glutamic acid is minimal over the age range investigated. This is to be expected due to the slower rate of racemization of glutamic acid (26). Both aspartic acid and serine show an accumulation of D-enantiomer of several percent over the age range studied.

Age estimates were made from six teeth, from six different individuals of known age and date of burial from the crypt of St. Barnabas church, West Kensington, London, and two teeth from two unspecified individuals of Romano-British origin. These estimates, and their associated errors are presented in Table 5. Estimates of age were calculated from the inverse regression equation as advocated by Lucy and Pollard (15) using the slopes and intercepts listed in Table 4 and the following equation:

$$\hat{x}_i = \frac{\left\{(1 + D/L)/(1 - D/L)\right\} - \hat{a}}{\hat{b}}$$

Estimates of associated error were calculated from the equation as given in Miller and Miller (27):

$$\hat{S}_{\hat{x}i} = \frac{\hat{S}_{y/x}}{\hat{b}} \sqrt{1 + \frac{1}{n} + \frac{(y_i - \bar{y})^2}{\hat{b}^2 \Sigma(x_i - \bar{x})^2}}$$

where:

$\hat{b}$ = estimate of the slope x on y
$\hat{a}$ = estimate of the intercept x on y
$\hat{x}_i$ = estimate of age for the unknown tooth
x_i = age for the i^{th} tooth
y_i = $\ln\{(1 + D/L)/(1 - D/L)\}$ for the unknown tooth for which age is being estimated
$\bar{x}$ = mean of ages in the reference sample

TABLE 2—*Triplicate measurements of ln{(1 + (D/L))/(1 − (D/L))} for all specimens used in the study with the exception of those featured in Fig. 5.*

Tooth Number Condition		Known Age (Years)	D/L Aspartic Acid			D/L Serine			D/L Glutamic Acid		
			1	2	3	1	2	3	1	2	3
E180A	non carious	11	0.029	0.021	0.021	0.011	0.009	0.010	0.011	0.008	0.009
224(1)A	non carious	11	0.032	0.027	0.027	0.018	0.018	0.018	0.009	0.009	0.009
224(2)A	non carious	11	0.031	0.031	0.031	0.017	0.018	0.019	0.009	0.009	0.009
57A	non carious	13	0.032	0.029	0.029	0.012	0.010	0.010	0.009	0.009	0.010
88(i)A	non carious	15	0.035	0.029	0.029	0.011	0.011	0.012	0.008	0.007	0.007
88(2)A	non carious	15	0.029	0.029	0.029	0.012	0.013	0.012	0.008	0.008	0.008
65A	non carious	18	0.031	0.029	0.029	0.012	0.011	0.012	0.008	0.008	0.008
67A	non carious	18	0.031	0.031	0.031	0.012	0.013	0.012	0.009	0.009	0.008
E078A	restoration	23	0.030	0.029	0.029	0.026	0.025	0.026	0.007	0.008	0.008
E083A	caries	25	0.029	0.031	0.031	0.022	0.023	0.022	0.008	0.008	0.008
E172A	caries	29	0.035	0.032	0.032	0.020	0.022	0.017	0.009	0.008	0.009
E212A	caries	30	0.039	0.038	0.038	0.019	0.018	0.018	0.010	0.010	0.010
E194A	caries-restoration	35	0.040	0.039	0.039	0.024	0.022	0.023	0.008	0.007	0.007
15A	caries	38	0.042	0.040	0.040	0.023	0.025	0.026	0.008	0.008	0.008
E056A	caries-restoration	43	0.038	0.035	0.035	0.027	0.025	0.022	0.009	0.009	0.008
64A	non carious	46	0.045	0.046	0.046	0.022	0.021	0.022	0.009	0.009	0.009
E003A	caries	47	0.043	0.043	0.043	0.030	0.036	0.034	0.010	0.011	0.009
E191A	restoration	47	0.044	0.046	0.046	0.026	0.024	0.024	0.012	0.010	0.009
68A	non carious	49	0.046	0.049	0.049	0.017	0.024	0.022	0.008	0.009	0.008
121A	restoration	60	0.049	0.047	0.047	0.024	0.024	0.023	0.009	0.009	0.008
150A	caries	62	0.053	0.055	0.055	0.029	0.028	0.024	0.011	0.011	0.010
115A	restoration	69	0.046	0.047	0.047	0.022	0.026	0.027	0.009	0.009	0.009
14A	caries-restoration	72	0.058	0.055	0.055	0.028	0.027	0.028	0.012	0.012	0.011

TABLE 3—*Analysis of variance table age* (x) *regressed against ln{(1 + (D/L))/(1 − (D/L))} aspartic acid* (y) *from modern specimens, treating each measurement as a repeat measurement.*

Source	DF	Sum of Squares	Mean Square	F Ratio
Regression	1	0.1959	0.1959	
Residual	67	0.0030	0.0000	
Lack of fit	46	0.0024	0.0001	4.35
Pure error	46	0.0006	0.0000	$P = 0.00000$

$\bar{y}$ = mean of ln{(1 + D/L)/(1 − D/L)} for teeth in the reference sample

$\hat{S}_{xi}$ = estimated error for the i^{th} x (age) estimate

$\hat{S}_{y/x}$ = estimate of the standard deviation y on x

Because six of the unknown individuals from St. Barnabas church were of known age it was possible to calculate the true error between the age estimate and that known age.

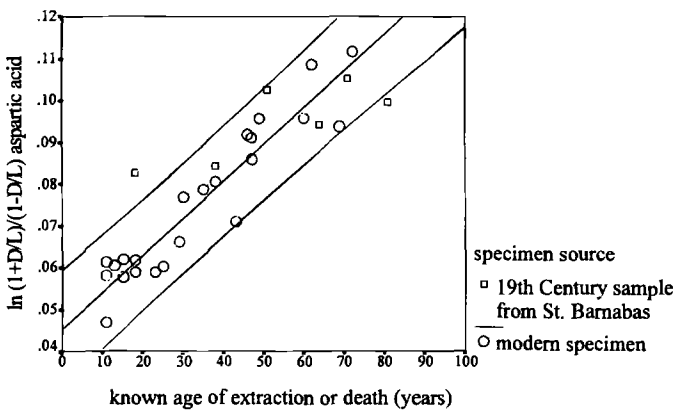

FIG. 1—*Plot of ln{(1 + (D/L))/(1 − (D/L))} aspartic acid against age with 95% confidence bands for St. Barnabas (19th C.) and modern dental samples.*

TABLE 4—*Analysis of variance tables and regression data for age* (x) *against ln{(1 + (D/L))/(1 − (D/L))} aspartic acid, serine, and glutamic acid, considering the means of triplicate measurements as the best single measure.*

Source	DF	Sum of Squares	Mean Square	F ratio	Slope	Intercept	R^2
Aspartic acid							
Regression	1	0.006530	0.006530	171.41	0.000880	0.045393	0.890860
Residual	21	0.000800	0.000038	$P < 0.001$			
Serine							
Regression	1	0.002040	0.002040	27.82	0.000492	0.023660	0.571210
Residual	21	0.001540	0.000073	$P < 0.001$			
Glumatic Acid							
Regression	1	0.000020	0.000020	6.00	0.000053	0.015958	0.026131
Residual	21	0.000070	0.000003	$P = 0.02$			

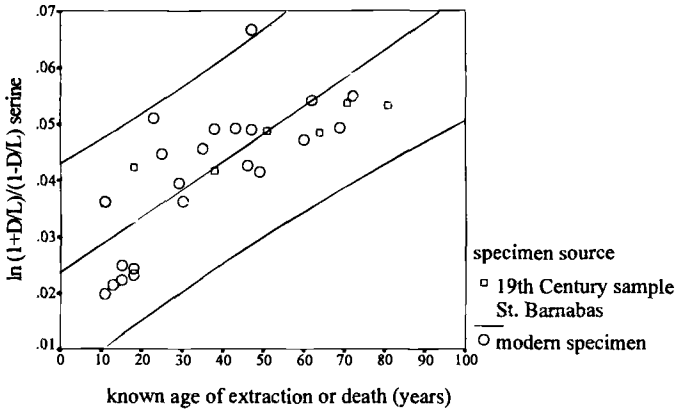

FIG. 2—*Plot of ln{(1 + (D/L))/(1 − (D/L))} serine against age with 95% confidence bands for St. Barnabas (19th C.) and modern dental samples.*

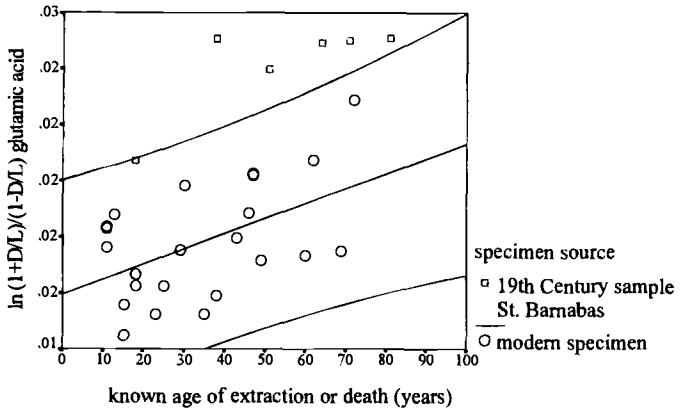

FIG. 3—*Plot of ln{(1 + (D/L))/(1 − (D/L))} glutamic acid against age with 95% confidence bands for St. Barnabas (19th C.) and modern dental samples.*

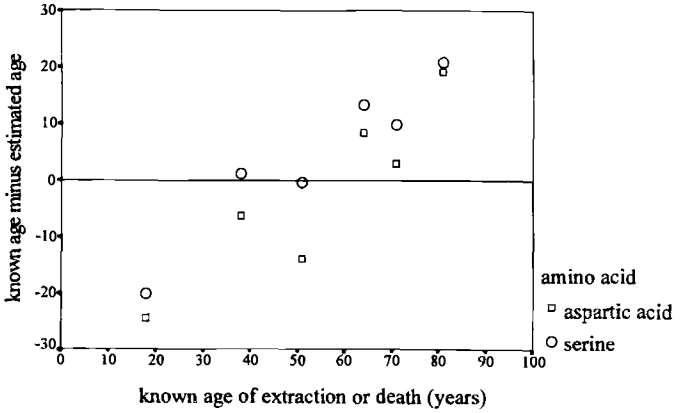

FIG. 4—*Plot of known age against true error in estimated age for aspartic acid and serine for six 19th Century specimens. (Estimated age obtained by using the modern data as a calibration curve for the 19th C. samples. True error is difference between estimated age and known age).*

Discussion

Using the modern calibration curves to estimate age at death of the ancient (in this case, early 19th C. AD) samples shows some relationship between estimated and actual age (in this case known), but significant systematic discrepancies are apparent. There is a marked tendency for young individuals to have estimated ages which are too old, and older individuals to have estimated ages which are too young, as seen in Fig. 4. Although similar tendencies have been attributed to the regression procedure itself, this bias is removed by using an inverse regression procedure (as discussed in Aykroyd et al. unpublished observations), and the discrepancies observed here are not therefore believed to arise from problems of calibration. Moreover, this observation is consistent with previous measurements on other archaeological samples. If the lines of regression for values of ln{(1 + (D/L))/(1 − (D/L))} for aspartic acid and serine from 19th C. samples are calculated in exactly the same way as was done for modern specimens, a different regression line is estimated, and it is clear that highly significant differences exist between the gradients calculated for modern and 19th C. specimens (see Table 6 and Figs. 1–3). This suggests that the estimated slopes of the regression equations for aspartic acid and serine are significantly smaller for the 19th C. specimens, and the intercepts significantly larger, giving regression lines which are not parallel (28).

It is recognized that the number of ancient teeth reported here is too small to draw definitive conclusions about differences in measured racemization between modern and ancient samples. In a previous study (6), we measured %D-aspartic acid in 16 modern dental samples and 46 (largely) 18th Century specimens from the vaults of Christ Church, Spitalfields (29), although the sample preparation and measurement protocols were different from those described here, and therefore the two sets of data may not be directly comparable. We have therefore plotted separately ln{(1 + (D/L))/(1 − (D/L))} aspartic acid against age for these samples (Fig. 5). Again, the regression equations for the modern and 18th Century specimens were highly significantly different (see Table 6), with the 18th Century samples displaying a similar lower slope and higher intercept as seen in the St. Barnabas material above. Comparison of Figs. 1 and 5 show that both these sets of data, obtained from different skeletal collections, and measured by different chromatographic techniques, show very similar patterns of behavior when compared with modern data.

The fact that the regression lines for modern and ancient measured enantiomeric ratios against age for aspartic acid (and serine) are not parallel, and in fact appear to intersect at an age within a normal human lifespan, suggests that the difference is not simply due to an additional component of the enantiomeric ratio accumulating at a different rate post mortem. If this were the case, then we would expect the ancient line to lie uniformly above that of the modern samples, and it could not therefore explain the observation that in older individuals the observed enantiomeric ratio is actually less in ancient samples than in modern teeth.

We can suggest several possibilities for the cause of this observation, but none are as yet tested. We know that the demineralization and hydrolysis procedures used to release the amino acids from the mineralized collagen for enantiomeric assay induce a small degree of additional racemization. It is assumed that, under strictly controlled conditions, this is constant for all samples, and simply has the effect of introducing a small positive constant offset into the regression curve. Our observations above could suggest that this may not be the case when modern and ancient collagens are treated identically. It is possible that the ancient collagen extract responds differently to the extraction procedure, resulting in a different degree of induced racemization to that experienced by modern collagen. This might be the case if the ancient collagen were already partially degraded before extraction began. A second possibility stems from the fact that we, like most other workers

TABLE 5—*Results from the calibration of 19th Century specimens from St. Barnabas against known modern specimens using aspartic acid, serine, and glutamic acid.*

Specimen	Year of Death	Known Age	ln (1+D/L)/(1−D/L) Aspartic	Calibrated Age	Error on Calibration	Real Error	ln(1+D/L)/(1−D/L) Serine	Calibrated Age	Error on Calibration	Real Error	ln (1+D/L)/(1−D/L) Glutamic	Calibrated Age	Error on Calibration	Real Error
CW1A	Romano-British	Unknown	0.09	47.30	7.19		0.06	66.38	18.82		0.03	202.26	70.62	
CW2A	Romano-British	Unknown	0.09	54.13	7.26		0.07	103.14	22.13		0.04	447.21	155.39	
WK6A	1853	18	0.08	42.38	7.17	−24.38	0.04	38.04	17.76	−20.04	0.02	89.40	39.94	49.46
WK11A	1832	38	0.08	44.19	7.18	−6.19	0.04	36.86	17.75	1.14	0.03	170.64	60.75	109.89
WK12A	1831	51	0.10	64.94	7.43	−13.94	0.05	51.23	18.07	−0.23	0.02	149.98	54.67	95.30
WK17A	1839	64	0.09	55.57	7.27	8.43	0.05	50.60	18.04	13.40	0.02	167.63	59.84	107.78
WK30A	1839	71	0.11	68.14	7.50	2.86	0.05	61.20	18.51	9.80	0.03	169.30	60.35	108.95
WK31A	1826	81	0.10	61.80	7.37	19.20	0.05	60.09	18.46	20.91	0.03	170.96	60.85	110.11

TABLE 6—*Significance tests for regression parameters between amino acids from ancient and modern dental specimens.*

		Slope					Intercept				
Source of Dentine	Amino Acid	Slope Modern	Slope Pre C20th	*t*	DF	Probability	Intercept Modern	Intercept Pre C20th	*t*	DF	Probability
St. Barnabas	Aspartic Acid	0.0009	0.0003	$t = 27838$	25.0000	$p < 0.0001$	0.0454	0.7693	$t = -551$	25.0000	$p < 0.001$
	Serine	0.0005	0.0002	$t = 226888$	25.0000	$p < 0.0001$	0.0237	0.0372	$t = -126$	25.0000	$p < 0.001$
Spitalfields	Aspartic Acid	0.0009	0.0004	$t = 52708$	85.0000	$p < 0.0001$	0.0004	0.0385	−1841.0000	85.0000	$p < 0.0001$

in this field, do not characterize the collagen extract before hydrolysis, and cannot therefore be sure that the material we are hydrolyzing is pure collagen. If, during the post mortem period, cross-linking reactions were to occur between the acid insoluble collagenous protein and some of the normally acid soluble molecules present in dentine which might contain faster racemizing amino acid residues, then the resulting unpurified collagen extract might have enhanced enantiomeric ratios compared with extracts from modern collagen of the same age. This model cannot, however, fully explain the observations, because it does not account for the lower enantiomeric ratio in older individuals. Other explanations are also possible, and it is likely that some combination is necessary. It is known that the measured enantiomeric ratio for amino acids in various molecular size fractions of proteins extracted from fossil shells shows a significant variation, suggesting that the rate of racemization depends on the molecular weight distribution of the extracted protein (30). All of these possibilities must be further investigated before the age of ancient individuals can be reliably estimated by calibration against curves derived from modern samples, and might suggest that the enantiomeric ratio derived from mineralized collagen samples might depend on the exact state of the collagen before processing begins.

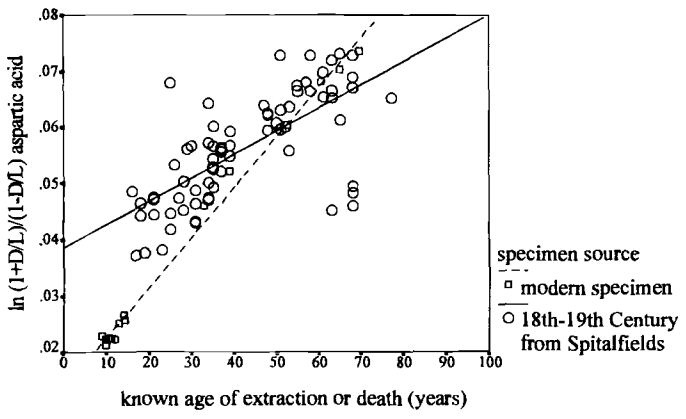

FIG. 5—*Plot of ln{(1 + (D/L))/(1 − (D/L))} aspartic acid with regression lines depicted for 18th C. specimens (Spitalfields) and modern specimens.*

Conclusions

We believe that this work supports two principal conclusions, one regarding the magnitude of the error associated with the age estimation using amino acid racemization techniques on dental collagen, and the other on the comparability of measurements made on modern and ancient material. Reliable though amino acid age estimation techniques may appear to be, when errors are properly taken into account through a statistically rigorous calibration, it can be seen that the method produces a substantial expected error, in this case averaging around 7.5 years about the mean estimated values, at the 68% level of confidence (1 standard deviation). Although apparently large, this average error is in fact slightly smaller than that calculated for the best of the morphological methods applied to dental samples (16). We must conclude that no method of age estimation from dental observations can truly be regarded as precise—it would probably be safe to apply a ±15 year error margin (95% confidence) to any age estimate produced from aspartic acid racemization measurements on dental collagen. We can also conclude that, of the three amino acids quantified in this work (aspartic acid, serine, and glutamic acid), only aspartic acid provides age estimations which are comparable with morphological methods.

Perhaps the most significant observation is the systematic difference between the regression equations obtained from modern and 18th/19th C. AD specimens. The observed accumulation of both D-aspartic acid and D-serine seems to be systematically different between modern and ancient samples of the same age, the differences for aspartic acid being seen in two different sets of data, from two different crypts, prepared and measured in two different ways. This must raise questions about the use of amino acid age estimation on older material (both forensic and archaeological), if the method is calibrated against modern measurements without any further understanding of the effect of the degradation of collagen on the observed amino acid enantiomeric ratios. It should be noted, however, that the systematic differences between estimated and true ages produced by the non concordance of the two regression lines does not influence the size of the estimated error discussed above, and the conclusion drawn about the error estimate applicable to amino acid racemization age determinations will also apply to regressions carried out using modern material as the unknown.

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