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Aspartic acid racemization in tooth enamel from living humans

(racemization dating/living mammals/aging)

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ABSTRACT **The aspartic acid in human tooth enamel shows increasing racemization with age. This increase is not seen in the metabolically active protein hemoglobin. The rate constant for the racemization reaction of aspartic acid in human tooth enamel was found to be 8.29×10^{-4} yr^{-1}. This rate constant suggests that in any protein with a long *in vivo* lifetime, D-aspartic acid will accumulate with age (about 8% of total aspartic acid in enamel will be the D-enantiomer after 60 years). Thus, racemization may play some role in the aging process affecting metabolically stable tissues in long-lived homeotherms. Aspartic acid racemization in tooth enamel also provides a biochronological tool for assessing the age of living mammals.**

Racemization is a natural process which will eventually convert optically active compounds into a racemic mixture. The L-amino acids commonly found in living systems are the result of the stereochemical specificity of enzymes which utilize only the L-enantiomers. Racemization of amino acids in the proteins produced by living organisms would thus take place only after protein turnover has ceased. Recent studies (1–4) have shown that at 25° a period of about 100,000 years is required before all the L-amino acids present in living systems will have undergone complete racemization to an equilibrium mixture in which the ratio of D- to L-amino acids equals 1.0. (For amino acids with more than one asymmetric center, the ratio is different from 1.0, i.e., the ratio alloisoleucine/isoleucine equals approximately 1.3.)

The extent of racemization of amino acids may be used to estimate the ages of various fossil materials, including deep-sea sediments (5–7), shells (8–11), fossil bones (2–4, 12–15), and coprolites (16). Of the various amino-acid racemization reactions, the one involving aspartic acid has received the most attention. This amino acid has one of the fastest racemization rates of the stable amino acids (1). In bone, the half-life of aspartic acid racemization (the time required for the ratio of D- to L-enantiomers to reach 0.333) is about 15,000 years at 20° (3, 15). Thus, for temperate to near-equatorial environments, significant racemization of aspartic acid will have taken place in a bone during the last 40,000 years (the upper limit of radiocarbon dating).

The temperature dependence of the rate of the amino-acid racemization reaction can also be used to estimate the temperature history of a sample of known age. By determining the extent of aspartic acid racemization in a radiocarbon-dated bone, it is possible to calculate the *in situ* rate of racemization, and this rate constant can then be used to estimate the average temperature to which the bone has been exposed (17). Racemization analysis of radiocarbon-dated bones with ages less than about 12,000 years (i.e., post-glacial) have yielded rate constants which are proportional to the present-day average annual air temperatures of the sites where the bones were found (15).

Based on the correlations between aspartic acid racemization rates determined in fossil bones and environmental temperatures, we predicted that small but measurable amounts of D-aspartic acid should accumulate in structural proteins which are not turned over *during the life-time* of a warm-blooded animal. As a means of testing this prediction, we required proteins which are synthesized early in life and are not renewed. Also, the organism must have a long life span as well as a high body temperature to optimize our ability to detect racemization. Samples must be readily obtainable and of known age. Enamel from human teeth appeared to be one of the most suitable materials. In this paper, we report that with increasing age in humans, there is a corresponding increase in the extent of racemization of aspartic acid isolated from tooth enamel.

METHODS

Teeth extracted from patients of different ages were generously provided by several dentists. The teeth were first cleaned of all soft tissue and then placed in a desiccator for a minimum of 1 week. This step facilitated the separation of enamel from the underlying dentine, since enamel can be chipped away from a desiccated tooth with a mortar and pestle, while the dentine and cementum remain mostly intact. Carious or discolored fragments were discarded. The chips were then examined under ultraviolet light and fluorescing particles (the dentine) were removed (18). The non-fluorescing fragments (enamel) were then ground to a fine powder in the mortar. To insure the isolation of a pure enamel fraction, the powder was flotated (19) in a mixture of 87.8% tetrabromoethane and 12.2% acetone (ρ = 2.55 g/cm^3). At this density, enamel sinks while dentine floats to the surface. The isolated enamel was transferred to a test tube and washed three times with acetone and three times with double-distilled water. The samples were then hydrolyzed in 6 M double-distilled HCl in sealed tubes at 100° for 6 hr.‡ After hydrolysis, the HCl was evaporated and the residue was resuspended in a small volume of double-distilled water and desalted (4, 5).

The aspartic acid fraction from each enamel sample was isolated from the other amino acids by separation on the automatic amino-acid analyzer. Since the eluate from the analyzer is a sodium citrate buffer, a second desalting step was required after the aspartic acid separation. Finally, the aspartic acid was converted to diastereomeric dipeptides by reaction with L-leucine-*N*-carboxyanhydride (20). The L-leucyl-L-aspartic acid and L-leucyl-D-aspartic acid dipeptides were separated on the amino-acid analyzer, as described elsewhere (4).

Hemoglobin was isolated from blood samples of humans

‡ A 6-hr hydrolysis was used instead of the normal 24 hr in order to reduce the acid-catalyzed racemization.

Table 1. Results of aspartic acid racemization analysis of human tooth enamel proteins

Age of enamel* (years)	Type of tooth	Age of individual (years)	D/L aspartic acid ratio: Amino acid analyzer	D/L aspartic acid ratio: Gas chromatography
5	Third molar	17	0.045	0.042
6	Premolar	11	†	0.045
8	Deciduous molar	8	0.030	
8	Premolar	13	0.043	0.038
15‡	Third molar	27-a	0.046	
15‡	Third molar	27-b	0.045	
15‡	Third molar	27-c	0.045	
23	Third molar	35	0.053	
35	Third molar	47	0.055	
42	Incisor	45	0.068	0.068
48	Canine	53	0.077	0.078
54	Third (?) molar	66	0.094	
56	First molar	58	0.082	
58	First (?) molar	60	0.088	
61	Second molar	67	0.112	
62	Incisor	65	†	0.085
66(?)	Second molar	72(?)	0.089	
69	Second molar	75	0.085	
72	Second molar	78	0.080	

* Because teeth, and their associated enamel, are laid down over approximately a 12-year period, we have calculated the age of the enamel by subtracting the following values from the age of the individual: incisor, 3 years; canine, 5 years; premolar, 5 years; first molar, 2 years; second molar, 6 years; third molar, 12 years (22).

† Insufficient amount of aspartic acid for dipeptide analysis was isolated.

‡ Analyses carried out on teeth from three different 27-year-old individuals.

of various ages by the method of Drabkin (21). The cells were washed several times with cold saline, then were lysed in a mixture of water and toluene (2:1). After centrifugation, the hemolysate was pipetted off. Small aliquots were hydrolyzed for 6 hr in 6 M HCl. The aspartic acid was separated first by ion exchange chromatography on Dowex 50W-X8 (4) to eliminate peptides which had not been completely broken down during the 6-hr hydrolysis. The aspartic acid fraction was further purified by separation on the amino-acid analyzer and then derivatized as described above.

The bovine serum albumin (Pentex) and bovine tendon collagen (Calbiochem) were hydrolyzed 6 hr in 6 M HCl as controls. The D/L aspartic acid ratio was determined using the procedure outlined above for the hemoglobin.

RESULTS

Table 1 summarizes the results of aspartic acid racemization analyses of tooth enamel from 19 individuals. These results clearly demonstrate that the D/L aspartic acid ratio increases with age, whether the age is taken to be the individual's chronological age or the true age of the enamel.

In order to show that the results in Table 1 are due to *in situ* racemization of amino acids in protein and are not in some way an artifact of the experimental procedure, we carried out aspartic acid racemization analysis of hemoglobin isolated from individuals of various ages. The results, which are summarized in Table 2, show that the proportion of D-aspartic acid in human hemoglobin does not increase with increasing age of the individual. Since the *in vivo* lifetime of human hemoglobin is 120 days (23), the turn-over rate is much faster than the aspartic acid racemization rate (see *Discussion* below). There should thus be no net accumulation of D-aspartic acid with age in hemoglobin or any other rapidly turned-over protein.

Table 2. Extent of racemization of aspartic acid in hemoglobin isolated from people of various ages

Age of individual	D/L aspartic acid ratio
23	0.011
32	0.010
52	0.009
76	0.011

Hydrolysates of six enamel samples were also analyzed by Etta Peterson and Keith Kvenvolden at NASA-Ames Research Center using gas chromatographic techniques, and these results are presented in Table 1. As can be seen, the D/L ratios we have obtained using the amino-acid analyzer to separate diastereomeric dipeptides are in excellent agreement with those obtained using this second, independent method. Furthermore, the gas chromatographic results indicate that there is no detectable racemization (i.e., less than 1% increase in the amount of D-enantiomer between an 11- and a 65-year old individual) of valine, alanine, leucine, proline, phenylalanine, or glutamic acid. This is what would be expected as a consequence of *in situ* racemization, since aspartic acid has the fastest racemization rate of these amino acids in bone (3) and in aqueous solution (24).

DISCUSSION

Racemization in enamel can be considered an *in vivo* kinetic experiment conducted at about 37°. The racemization reaction of aspartic acid is written as

$$\text{L-aspartic acid} \underset{k_{asp}}{\overset{k_{asp}}{\rightleftharpoons}} \text{D-aspartic acid} \qquad [1]$$

where k_{asp} is the first-order rate constant for the interconversion of the D- and L-enantiomers. The rate expression for aspartic acid racemization is

$$\frac{-d[\text{L-Asp}]}{dt} = k_{asp}[\text{L-Asp}] - k_{asp}[\text{D-Asp}] \qquad [2]$$

When the extent of racemization is small (i.e., D/L ratio is low), the k_{asp}[D-Asp] term in Eq. 2 can be considered negligible. Integration of the rate expression thus yields the irreversible first-order rate equation

$$\ln\{1 + \text{D/L}\} = k_{asp} \cdot t + b \qquad [3]$$

where the b term is the constant of integration.

Fig. 1 shows a plot in the form of Eq. 3 of the data given in Tables 1 and 2. A least squares fit of the data yields the equation

$$\ln\{1 + \text{D/L}\} = 8.29 \times 10^{-4}\ \text{yr}^{-1} t_e + 0.033 \qquad [4]$$

where t_e is the age of the enamel (years). The correlation coefficient (r), which is a measure of the relationship between age and ln {1 + D/L}, equals 0.921. Therefore, the proportion of the total variation of the ln {1 + D/L} terms that is accounted for by the relationship with age is $100\ r^2$ = 85% (25). The standard deviation between the experimental and calculated points is ±0.0084.

Reproducibility of the D/L aspartic acid measurements is

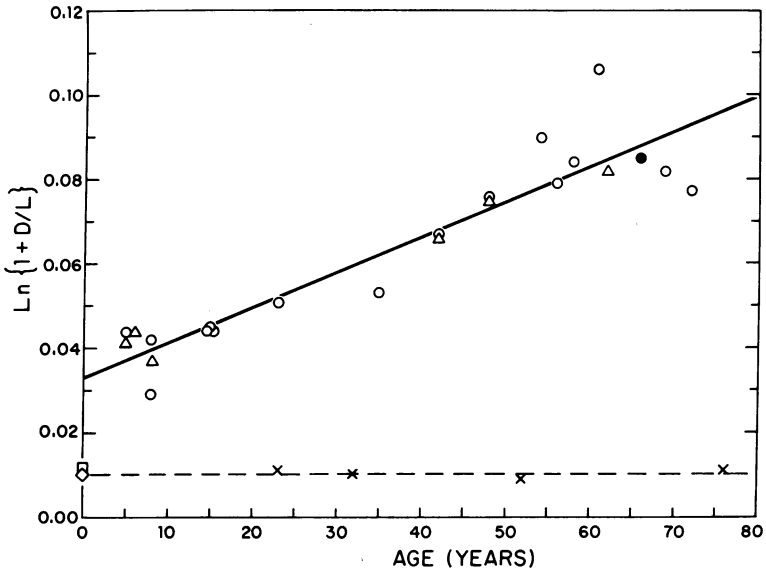

FIG. 1. Plot of ln {1 + D/L} for aspartic acid against age of the enamel and against age of the individual for the hemoglobin values. The slope (8.29×10^{-4}yr^{-1}) of the enamel line is equal to k_{asp} for tooth enamel at about 37°. ○, measurement using amino-acid analyzer method; △, measurement using gas chromatographic method (NASA); ●, determination (amino-acid analyzer) on tooth enamel from an individual whose exact age is not known but is estimated at 72 years (this point was not used to calculate the least squares fit); determinations (amino-acid analyzer) on hemoglobin, ×; albumin, ◇; and bovine tendon collagen, □.

demonstrated by analyses of teeth from three different 27-year-old individuals. The average of the D/L ratios is 0.0453 ± 0.0004. Therefore, the error which arises from the measurement of the D/L ratio is small. The scatter in D/L ratios for enamel samples older than 50 years (these have a standard deviation 2.4 times that for the younger samples) may possibly be due to (*a*) variable and, in general, poor preservation of the enamel or (*b*) small differences in the temperature of the mouth between various individuals.

The value of the *b* term in Eq. 3, calculated from our data, is 0.033. The *b* term should not be zero since some racemization occurs during acid hydrolysis (4). The average D/L ratio for the hemoglobin, albumin, and collagen equals 0.011, and this value thus represents the amount of acid-catalyzed racemization during the 6-hr hydrolysis. The difference between this and the enamel value is 0.022. Possible explanations for this difference are: (*a*) this residual represents the amount of D-aspartic acid incorporated during synthesis of enamel proteins, although this seems unlikely; (*b*) there is a slightly larger amount of racemization during the acid hydrolysis of enamel due to the influence of neighboring amino-acid residues (20); and/or (*c*) there is a small amount of racemization resulting from catalysis by calcium released from enamel during the acid hydrolysis step.

The fact that the aspartic acid in tooth enamel shows an increasing extent of racemization with age may have implications concerning the mechanism of aging in humans and other long-lived mammals. Approximately 40 years ago, Kuhn (26, 27) suggested that the racemization of amino acids in synthetic enzymes could be responsible for the aging process. Our results indicate that this mechanism cannot be important for globular proteins (e.g., those involved in catalysis, transport, immune response, etc.) because their turnover rates are about 5 orders of magnitude faster than the racemization rate at mammalian body temperatures. Our enamel results do suggest, however, that for metabolically inert proteins [e.g., proteolipid protein in the myelin sheath (28), possibly certain collagen components in connective tissue (29), and a β-crystallin fraction in the lens (30)], there would be increasing amounts of D-aspartic acid with increasing age. Based on the tooth enamel results, D-aspartic acid could constitute 8% of the total aspartic acid present in totally stable proteins after about 60 years. If this amount of D-aspartic acid has a significant effect on the stability and function of structural proteins, then racemization, in addition to other age-related reactions such as deamidation of asparaginyl and glutaminyl residues (31), may contribute to that part of the aging process associated with these proteins.

There is another, less hypothetical implication concerning racemization in structural proteins and aging. Essentially, our technique estimates an age for the enamel protein, which has an *in vivo* lifetime approximately as long as the lifetime of the organism. With structural proteins taken from tissues having shorter *in vivo* lifetimes, the method estimates the age of the corresponding tissue. Using aspartic acid racemization, it may be possible to estimate the *in vivo* lifetimes of proteins which have turn-over rates too slow to be determined by conventional labeling techniques.

An obvious application of Eq. 4 is that it can be used to determine the age of any long-lived mammal, provided a tooth with suitable enamel can be obtained. Just as the racemization reaction of amino acids provides a useful tool in geochronology, it may develop a similar function in "biochronology." Age profiles of natural populations of long-lived mammals can now be constructed, and this should be of particular importance to ecologists and conservationists. Similar studies of primitive human groups may benefit biological anthropologists. Demographers and anthropologists investigating the history of human reproductive rates and population sizes (32) need age profiles of skeletal populations, but age at death cannot be accurately assessed beyond 40–45 years with current morphological techniques (33). Under suitable conditions, where there has been no racemization since death, i.e., relatively cold temperatures, or within a few hundred years of the burial, the age of an individual at death could be deduced using the extent of aspartic acid racemization in tooth enamel from skeletons in cemetery populations.

Probably the most interesting application of Eq. 4 would be to examine claims of unusual longevity among the peoples living in Ecuador, Hunza, and Georgian Russia. Some doubt has been raised about the authenticity of the Russian centenarians (34). Verification of extreme longevity would be of considerable importance to gerontological research.

CONCLUSIONS

Amino-acid racemization dating is a technique originally developed to date events on the geological time scale. In this paper, we have demonstrated its potential as a method for assessing the ages of living mammals and their tissues. Although the techniques of physical anthropology can assign ages to skeletal materials, no reliable means has yet been established for estimating the age of a living person. Aspartic acid racemization in tooth enamel represents the first chemical quantitative method for doing so. The fact that the aspartic acid in metabolically inert proteins may undergo slow racemization with increasing age may also have implications concerning the mechanism of the aging process associated with these proteins.

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