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Racemization Reaction of Aspartic Acid and Its Use in Dating Fossil Bones

(Olduvai Gorge/5,000–70,000-years-old range/hominids)

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ABSTRACT **In the time interval datable by radiocarbon, and at the temperatures of most archeological sites, a substantial amount of racemization of aspartic acid takes place. By determination of the amount of racemization of aspartic acid in bones from a particular location which have been dated by the radiocarbon technique, it is possible to calculate the *in situ* first-order rate constant for interconversion of the L- and D enantiomers of aspartic acid. Once this "calibration" has been calculated, the reaction can be used to date other bones from the deposit that are either too old to be dated by radiocarbon or that are too small for radiocarbon dating. The only assumption required with this approach is that the average temperature experienced by the "calibration" sample is representative of the average temperature experienced by older samples. This "calibration" technique is used herein to date bones from the Olduvai Gorge area in Tanzania, Africa.**

Only L-amino acids are commonly found in living organisms. However, recent studies have shown the occurrence of D isomers in fossil materials. The amino acids in fossil shells (1–3), sediments (4–6), and bones (7, 8) are partially racemized, with the amount of racemization increasing with the age of the fossil. This racemization reaction has obvious applications in geochronology, and it appears that the reaction can be used to date deep-sea sediments (4–6) and fossil bones (7, 8) found in certain environments.

Most studies have concentrated on isoleucine. The epimerization of L-isoleucine produces the nonprotein amino-acid D-alloisoleucine. (For the reaction involving isoleucine, the process is more properly termed epimerization rather than racemization.) L-Isoleucine and D-alloisoleucine are directly separable on an automatic amino-acid analyzer. In contrast, in order to determine the amount of racemization of other amino acids, a suitable diastereomeric derivative must first be synthesized. The isoleucine epimerization reaction in bone has a half-life (i.e., the time required for the ratio of alloisoleucine to isoleucine to reach 0.345) at 20° in excess of 100,000 years (8), and evidence suggests that the reaction can be used to date fossil bones too old to be datable by radiocarbon. The rate of the amino-acid racemization reaction is dependent upon temperature. Therefore, in order to use the reaction to calculate ages which are reasonably accurate, some estimate of the temperature history of a fossil bone must be available. Due to the problem of temperature uncertainties, the isoleucine epimerization reaction can apparently only be used to date fossil bones found in certain environments (7, 8) such as caves where paleotemperatures might be estimated from $^{18}O/^{16}O$ ratios in stalagmites and stalactites.

Preliminary results obtained from modern bone fragments heated at elevated temperatures show that amino acids such as aspartic acid and alanine are racemized substantially faster than is isoleucine (Bada, J. L., Kvenvolden, K. A. and Peterson, E., in preparation). These results indicate the following sequence of racemization rates: aspartic acid > alanine $\cong$ glutamic acid > isoleucine $\cong$ leucine. This pattern was also found in several fossil bones.

Of particular interest is the racemization reaction of aspartic acid; this reaction can be 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 interconversion of the L and D enantiomers of aspartic acid. In bone, this reaction has a half-life (i.e., the time required for the ratio of D- to L-aspartic acid to reach 0.333) at 20° of about 15,000–20,000 years (Bada, J. L., Kvenvolden, K. A. and Peterson, E., in preparation). Thus, in the time interval datable by radiocarbon, a substantial amount of racemization of aspartic acid will take place. This finding suggests that by determining the extent of racemization of aspartic acid in bones from a particular site which have been dated by the radiocarbon technique, it should be possible for one to calculate the *in situ* k_{asp} value for the deposit. Once this "calibration" has been done, the aspartic-acid racemization reaction can be used to date other samples from the area that are either too old to be dated by radiocarbon (i.e., older than 45,000 years), or for which insufficient amounts are available for radiocarbon dating (only a few grams are needed for amino-acid dating, compared to hundreds of grams needed for radiocarbon dating). This "calibration" procedure eliminates the need for evaluating the temperature history of a bone before it can be dated by the amino-acid racemization reaction. In this paper, we report how this approach was used to date fossil bones from the Olduvai Gorge area in Tanzania, Africa.

MATERIALS AND METHODS

The bones from the Naisiusiu Beds were sent to us by M. D. Leakey, Centre for Prehistory and Paleontology of the National Museum, Nairobi, Kenya. The actual location in Olduvai Gorge where the bones were collected is described elsewhere (9). About 10 g of bone were used for the amino-

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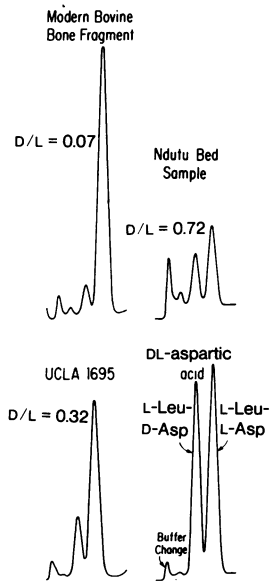

FIG. 1. Part of the amino-acid analyzer printouts for the various bone samples showing the diastereomeric dipeptides L-leucyl-D-aspartic acid and L-leucyl-L-aspartic acid. A 56 × 0.9-cm column filled with Beckman–Spinco AA-15 resin was used for the separation. The column was eluted with pH 3.24 buffer for 40 min, then the buffer was switched to pH 4.25 for the remainder of the run. The buffer change, L-Leu-D-Asp, and L-Leu-L-Asp peaks come at 72, 79, and 83 min, respectively, with this elution sequence. The small peak that elutes between the buffer peak and L-Leu-D-Asp is glycine that was not completely separated from aspartic acid on the Dowex 50 (H^+) column.

acid analysis; 412 g were used for the radiocarbon age determination.

The Ndutu Bed bone was provided by R. L. Hay, University of California, Berkeley. The sample was from a site located at the rim of the Gorge, between the Main and Side Gorges and 150 m south of archeological site FLK-NNI and 70 m west of the recently excavated site in the Masek Beds. At the sampled locality, the Ndutu Beds comprise 1.5 m of horizontal unstratified zeolitic eolian tuff, the lithology and mineral content of which indicate assignment to the upper unit of the Ndutu Beds. A piece of bone weighing about 10 g was used for the amino-acid analysis.

Several pieces of the Eyasi I Hominid bone amounting to 6.3 g were generously provided by H. Müller-Beck, University of Tübingen. Microanalysis of the pieces (determined at the Univ. of California, Los Angeles by the micro-Dumas method) gave 2.0% fluorine and 0.20% nitrogen, the same as those reported by Oakley and Campbell (10) for the skull Eyasi I. These measurements demonstrate the contemporaneity of the separate pieces and the skull. Before the amino-acid analysis, the Eyasi bones were refluxed in diethyl ether for 30 days in order to remove the preservative (shellac) that had been applied to the bones. After this treatment, the bones were processed in the manner described below.

Before amino-acid analyses, the fossil bones were carefully cleaned by repeated washings in dilute hydrochloric acid (0.2 M), using ultrasonication to loosen dirt and adhering soil. After cleansing, the bones were hydrolyzed in excess double-distilled 6 M HCl for 24 hr. Upon completion of the hydrolysis, the HCl solution was evaporated to dryness and the residue was dissolved in double-distilled water and desalted on unused Dowex 50W-X8 (100–200 mesh) resin, which had been cleaned with NaOH and regenerated with doubly-distilled HCl. The amino acids were eluted from the resin with 1.5 M NN_4OH prepared by dissolving NH_3 gas into double-distilled water. The effluent was concentrated in a rotary evaporator at 53° under reduced pressure.

The ratio of the D- to L- enantiomers of aspartic acid was determined by preparing diastereomeric dipeptides using the procedure described by Manning and Moore (11). Before the dipeptide synthesis, the aspartic acid was separated from the total amino-acid mixture by ion-exchange chromatography (12) on Dowex 50W-X8, using 1.5 M HCl to elute the aspartic acid. This separation step was necessary because the dipeptide synthesis of the total amino-acid mixture isolated from the bone resulted in a large number of peaks, many of which overlapped one another. After the ion-exchange separation, the isolated aspartic acid was taken up in borate buffer (pH = 10.4), the solution was cooled to 2–4°, and a small amount of L-leucine-*N*-carboxyanhydride (obtained from Cyclo Chemical Co.) was added to the solution in order to synthesize L-leucyl-D-aspartic acid and L-leucyl-L-aspartic acid. A Beckman–Spinco model 118 automatic amino-acid analyzer was used to separate the diastereomeric dipeptides.

The radiocarbon age of the Naisiusiu Bed bone was determined at the Univ. of California, Los Angeles Radiocarbon Laboratory by use of the method described by Protsch (13).

RESULTS

The Olduvai Gorge sequence consists of several stratigraphic sections, the oldest of which has an age of several million years. The uppermost part of the Gorge is upper Pleistocene (14, 15); this stratigraphic section is subdivided into a younger formation, the Naisiusiu Beds, and an older formation, the Ndutu Beds (9). Bones from the middle section of the Naisiusiu Beds were used to "calibrate" the aspartic-acid racemization reaction for Olduvai Gorge. Radiocarbon analysis of the collagen fraction isolated from the bones yielded an age of of 17,550 ± 1,000 yr (UCLA-1695). This age agrees closely with the age (9) of 17,000 yr (Lamont-no.?) determined on ostrich eggshell found in the same stratigraphic horizon and in close proximity to the bones.

Part of the amino-acid analyzer printout of the Naisiusiu Bed bone is shown in Fig. 1. Also included in Fig. 1 is the printout for a modern bovine-bone fragment and a DL-aspartic-acid mixture. The ninhydrin color yield is lower for the L-D dipeptide than for the L-L dipeptide (11). Thus, in order to obtain the actual ratio of D- to L- aspartic acid in a sample, the measured ratio must be multiplied by a conversion factor. The factor determined for our analyzer was 1.30.‡

The first-order rate constant (k_{asp}) for interconversion of the D and L enantiomers of aspartic acid can be calculated from the equation [see refs. (6) and (8) for derivation]

$$\ln\left[\frac{1 + \text{D/L}}{1 - \text{D/L}}\right] - \ln\left[\frac{1 + \text{D/L}}{1 - \text{D/L}}\right]_{t=0} = 2 \cdot k_{asp} \cdot t, \quad [2]$$

‡ We carefully tested the dipeptide method by preparing samples with D to L-aspartic-acid ratios ranging from 0.1 to 0.75, then converting these to the diastereomeric dipeptides. In all cases the D- to L-aspartic acid ratios estimated with the amino-acid analyzer were within 1–2% of the known values.

where t = time (in years) and D/L is the aspartic acid enantiomeric ratio in the bone. Kinetic studies at elevated temperatures have shown that the epimerization reaction of isoleucine in bones follows this equation essentially to equilibrium (8). The $t = 0$ term in Eq. [2] can be evaluated by setting t equal to zero and using D/L = 0.07, the aspartic acid enantiomeric ratio determined for a modern bovine bone carried through the same analytical steps as the fossil bones. The calculated value for the $t=0$ term is 0.14.

The D to L-aspartic-acid ratio determined for the Naisiusiu Bed bone was 0.32. Substitution of this ratio and the radiocarbon age of 17,550 ± 1000 yr into Eq. [2] yields $k_{asp} = 1.48 \pm 0.09 \times 10^{-5}\ yr^{-1}$; this value thus represents the average value of k_{asp} for Olduvai Gorge over about 18,000 yr.

It should be possible to use this k_{asp} value to estimate the age of other fossil bones from Olduvai Gorge. To test this proposal, amino-acid analyses were performed on a bone from the Ndutu Beds. The Ndutu Beds are older than the Naisiusiu Beds and appear to be beyond the range of radiocarbon dating (9). The Ndutu Bed bone was found to have a D to L aspartic-acid ratio of 0.72; part of the amino-acid analyzer printout for this bone is also shown in Fig. 1. Substitution of D/L = 0.72 and $k_{asp} = 1.48 \pm 0.09 \times 10^{-5}\ yr^{-1}$ into Eq. [2] gives an age of 56,000 ± 3,500 yr.

Analysis of fossil shells from the upper unit of the Ndutu Beds indicated a radiocarbon age of older than 29,000 yr (9). Based on mineral alterations, the upper Ndutu Beds have been estimated (9) to range in age from 30,000 to 70,000 years old. The artifacts associated with these beds appear to represent a Middle Stone Age culture (9). Thus, the age of 56,000 yr deduced for the Ndutu Bed bone using the aspartic-acid racemization reaction is in agreement with the general age range predicted from other information.

It should be emphasized that the only assumption concerning the temperature, and therefore the value of k_{asp}, made in this calculation is that the average temperature at Olduvai Gorge over the last 18,000 yr is representative of the average temperature over the last 56,000 yr. This assumption seems justified based on the estimated temperature fluctuations during the Pleistocene glacial cycles for the tropical Atlantic Ocean (16) and for a cave in New Zealand (17). The estimated average temperatures in these environments over the last 20,000 yr is only about 0.5° higher than the average temperature over much longer periods. If a similar situation existed in Olduvai Gorge, then the age of the Ndutu Bed bone would be increased from 56,000 yr to about 61,000 yr, assuming that the aspartic-acid racemization reaction has the same temperature dependence as the isoleucine reaction (8), which is the case in aqueous solution (18).

The value of k_{asp} determined above should be applicable not only to Olduvai Gorge, but also to bones from sites in the general vicinity. In fact, we have used the aspartic-acid racemization reaction "calibrated" by the Olduvai Gorge bones to estimate the age of some hominid bones found at Lake Eyasi, a site located about 65 k southwest of Olduvai Gorge. Fragmentary pieces of three hominid skulls were found on the northeast shore of Lake Eyasi in 1935 by a German expedition (19). A reconstruction of one of the skulls (Eyasi I) has been generally considered to display *Homo erectus* affinities (19, 20). However, the associated faunal and cultural material were upper Pleistocene (21, 22). More recent authors (23) have suggested that the Lake Eyasi hominids might belong to the African Neanderthals, and could thus be contemporaneous with other African hominids such as Broken Hill, Saldanha, and Florisbad. Fauna associated with the Saldanha and Florisbad hominids have recently been dated by radiocarbon (13); the ages are Saldanha, 40,680 ± 2000 yr (UCLA 1742) and Florisbad, 38,683 ± 2000 yr (UCLA 1745B). The Broken Hill skull has been roughly estimated (24) to have an age of about 40,000 yr, i.e., early Middle Stone Age. There have been no attempts to estimate the age of the Lake Eyasi hominids by radiocarbon because of the small amount of bone which was found, and because of the unavailability of any associated faunal matrial.

The ratio of D- to L-aspartic acid for the Eyasi I Hominid bone was found to be about 0.5. Based on the k_{asp} value determined for Olduvai Gorge, this ratio indicates an age of 34,000 yr. The only factor that could make this age greatly in error is if the temperatures at Lake Eyasi and Olduvai Gorge were substantially different. However, this is not the case: the present day climates in the two regions are essentially the same (R. L. Hay, personal communication). The 34,000-yr age does indeed make the Lake Eyasi hominids broadly contemporary with Saldanha and Florisbad, and possibly with Broken Hill also. A more detailed discussion of the archeological and anthropological implications of the Lake Eyasi results will be given elsewhere.

DISCUSSION

These results illustrate well the application of the amino-acid racemization reaction to the dating of fossil bones. By use of the approach described here, the aspartic-acid racemization reaction can be used to date fossil bones that have ages ranging from 5,000 yr up to 60,000 and 70,000 yr, the actual range dependent upon the temperature of the location. By use of a similar approach with other amino acids such as alanine, which racemize at rates slower than that of aspartic acid, it should be possible to date bones as old as 100,000–150,000 yr. With the isoleucine epimerization it might be possible to date bones as old as several hundred thousand years, but it will be difficult to "calibrate" this reaction since only small amounts of epimerization would take place during the time interval datable by radiocarbon. It may be possible to calibrate the isoleucine reaction by use of bones dated by the racemization of other amino acids.

The amino-acid dating method extends well beyond the time range applicable to radiocarbon. Furthermore, because of the small amounts of material needed, even bones thought to be datable by radiocarbon but which were available only in small quantities could be dated by use of the appropriate amino-acid racemization reaction. By using expendable faunal material to "calibrate" the racemization reaction, only a few grams of valuable hominid material would have to be used for amino-acid dating. Thus, the amino-acid racemization reaction should prove to be a valuable tool in paleoanthropology and provide a powerful chronological aid in unraveling the history of human evolution.

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