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VARIATIONS IN ASPARTIC ACID RACEMIZATION IN UNIFORMLY PRESERVED PLANTS ABOUT 11000 YEARS OLD

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Key Word Index—*Juniperus californica*; Cupressaceae; *Acacia greggi*; Leguminosae; *Nolina bigelovii*; Liliaceae; *Opuntia acanthocarpa*; Cactaceae; *Ephedra nevadensis*; Ephedraceae; aspartic acid racemization rates, variations in.

Abstract—Amino acid racemization forms a basis for determining the chronology and paleotemperature of old plant constituents. Disparity in the extent of aspartic acid racemization was found in different taxa of plants subjected to the same environmental history and found in close proximity within an ancient packrat midden. One taxon showed different rates of aspartic acid racemization in two different anatomical sites. Temperature, pH and time being virtually identical in this one micro-environment within the midden, the differences in racemization rates may have been ultimately derived from physiological variants among the plants. Thus, at least, aspartic acid racemization data should be used selectively.

INTRODUCTION

Application of the racemization rates of amino acids in wood for the determination of paleotemperatures or chronology necessitates the investigation of various factors. These include taxonomic and anatomical site dependency, the effect of the biochemical 'matrix' associated with amino acids in various plant tissues, their relative positions in peptides [1] and their kinetic determinants, all related to the physiology of the organisms. *Post mortem* environmental factors such as moisture content, pH, redox potentials, the nature and elemental abundances of the preserving substance and its environmental history, the extent of leaching during geological time, etc., must also be known to be able to accurately evaluate variations in amino acid racemization rates. It is often difficult or impossible to determine all of these factors. The purpose of this report is to emphasize the anatomical site and taxonomic dependency of aspartic acid racemization rates in a number of uniformly preserved, *ca* 11000-yr-old plant taxa. Obviously, such a study cannot be performed on the vast majority of plant taxa now living in which the D-amino acid enantiomers are essentially absent.

King and Hare [2] and King and Neville [3] reported that the rate of isoleucine epimerization in fossil planktonic foraminifera is strongly species dependent. Lee *et al.* [4] determined the activation energy for the epimerization of isoleucine to alloisoleucine in *ca* 3000-yr-old bristlecone pine heartwood. Engel *et al.* [5] determined the activation energies for the racemization of bound* aspartic acid, glutamic acid, proline and phenylalanine in *ca* 250-yr-old *Sequoia* heartwood. These two studies were conducted to help delineate the chronology and paleotemperature of the two plant taxa, respectively. However, the applicability of these activation energies to the racemization reactions in other plant taxa, or in other anatomical sites of these two respective taxa, still needs to be determined.

To establish whether or not differences exist in the rates of bound aspartic acid racemization in different plant taxa, components of various plants were isolated from the inerior of a packrat midden that was collected from a south facing rhyolite-rock shelter at 490 m elevation, 34° 16′ N latitude and 114° 25′ W longitude in the Whipple Mountains, San Bernardino County, California. The present mean annual temperature for this region is estimated to be 19.2° [6]. The radiocarbon date for this midden (Redtail Peaks No. 10A) is 10930 ± 170 B.P. (A-1616) on *Nolina bigelovii* (bear grass) from the midden. *Nolina bigelovii* no longer occurs near the collection site, thus ensuring that the date is well associated with this ecologically important species in the paleoflora.

Packrats are rodents in the genus *Neotoma* which build and live in relatively large structures containing local plant materials [7]. Waste areas termed middens are often located near the entrances of these structures. Middens often become dark, shiny and hard as the waste materials are cemented with urine. Such indurated middens become so resistant that they can be preserved until the shelter is weathered away if they remain relatively dry. Packrats are thorough collectors and ancient middens usually have well-preserved samples of the plants growing within *ca* 100 m range of the packrat's home [8–10]. Plant remains in ancient packrat middens in the southwestern United States provide a record of vegetation and inferred climatic regimes for the last 30000 yr [11, 12]. The juniper woodland ancient assemblage in Redtail Peaks No. 10A is at the beginning of the 11000–8000 yr period characterized by xeric woodlands in present desertscrub areas

* In this context, bound amino acids refer to those which cannot be extracted from plant tissues with 1.5 N HCl.

Table 1. D/L Aspartic acid ratios in various plant species isolated from a rat midden 10930 yr old

Juniperus sp. (seeds)*	*Juniperus* sp. (twigs)	*Acacia greggi* (pods)	*Acacia greggi* (twigs)	*Nolina bigelovii* (leaves)	*Opuntia acantho-carpa* (spines)	*Ephedra nevadensis* (seeds)
0.24	0.12	0.12	0.11	0.09	0.19	0.22
0.19	0.12	0.10	0.11	0.10	0.18	0.20
0.17	0.12	0.11		0.10	0.18	0.15
0.25	0.15	0.11				
0.20	0.13					
0.18						
0.19						
0.20						
0.18						
0.19						
$\bar{x} = 0.20$	0.13	0.11	0.11	0.10	0.18	0.19

Controls: D/L of soluble midden substance = 0.18;
D/L of modern *Juniperus osteosperma* seeds = 0.03

The instrumental error range is D/L = 0.001–0.01.
The above D/L ratios have been corrected by 0.02; this value was experimentally determined to be caused by (−)-2-butanol impurities and racemization during hydrolysis.

* It is possible that the fossil juniper seeds consist of 2 species as noted in the text; these 2 species of old seeds are morphologically indistinguishable and could account for the variations of the observed D/L ratios, i.e. 0.24 and 0.25.

[13]. The lower edge of the pinyon–juniper woodland was in these areas earlier.

Time, temperature, and other major variables controlling the rate of racemization must be identical to permit proper evaluation of the anatomical and taxonomical dependency of the racemization rates of amino acids in ancient plants. These conditions are met, to a large extent, in a 1 dm^3 volume microenvironment in the interior of the packrat midden, into which various plants of comparable age were incorporated at the time when the midden was constructed.

RESULTS

The D/L ratios of bound aspartic acid in plants from the Redtail Peaks No. 10A interior midden sample, of the soluble midden matrix and of modern *Juniperus osteosperma* seeds are shown in Table 1. A total of 10 separate juniper seeds and 5 juniper twigs were analyzed; the twigs had lower D/L aspartic acid ratios than the seeds. Three samples each of *Nolina bigelovii* and *Opuntia acanthocarpa*, and 6 samples of *Acacia greggi* (4 pods and 2 twigs) showed good agreement among their respective D/L aspartic acid ratios.

The current water content of the juniper seeds in the midden ranged from 1.5 to 5.9%. The juniper twig (*Juniperus californica*) contained 3.9% moisture and *Ephedra nevadensis* and *Opuntia acanthocarpa* contained 4.8% and 2.1% water, respectively. MS analysis of the gases released from a juniper seed at 75° under vacuum revealed that more than 99% of the vapor consisted of water and the rest was nitrogen. When the same sample was subsequently heated at 150° in vacuum, the MS showed additional but small fragment ions consistent with the fragmentation patterns of furfural and cresol, two known, common pyrolytic breakdown products of cellulose and lignin, respectively. All plant specimens which were analyzed and the whole midden were relatively but not absolutely dry and no correlation has yet been found between current moisture contents and the extent of aspartic acid racemization in the old plant samples. This does not, however, preclude the possibility that potential variations in the original moisture contents of individual plant specimens during the relatively short periods of incorporation and induration did not affect to some extent aspartic acid racemization. After induration, leaching by water would have destroyed the midden structure, therefore, this effect could be excluded.

The water soluble fractions of 4 randomly selected Redtail Peaks No. 10A midden samples had identical pH values (pH = 8.9). Thus, the pH of the soluble matrix fraction appears to be uniform throughout the midden. No evidence has yet been found which shows that the composition, redox potentials and the environmental history varied within the 1 dm^3 preserving substance from which the various taxa of plants were isolated.

DISCUSSION

Table 1 shows that taxonomically different old plant specimens of identical age, which have the same environmental histories, can vary in their extent of bound aspartic acid racemization. These interspecies variations may, at least to a noticeable extent, be a function of the anatomical sites that were available for analysis. For example, *Juniperus californica* seeds and *Ephedra nevadensis* seeds have similar D/L aspartic acid ratios, as do *Juniperus californica* twigs and *Acacia greggi* twigs. Different anatomical sites in the same taxon show different rates of racemization, as is evident from the differences in the D/L ratios between juniper seeds and juniper twigs. Anomalous D/L ratios (0.24, 0.25) for two of the juniper seeds in Table 1 may reflect a different juniper species, i.e. *Juniperus osteosperma*. Ancient seeds of *Juniperus californica* and *Juniperus osteosperma* are morphologically indistinguishable. While *Juniperus californica* was the predominant species of juniper present at the time of incorporation of the midden, the presence of *Juniperus osteosperma* cannot be excluded. However, the rate of bound aspartic acid racemization shows not only anatomical site but also taxonomical dependency, within the limits of available plant contituents, as is shown in Table 1.

The variations in the rates of aspartic acid racemization among some of the ancient plant constituents in the interior of this particular midden are apparently not the result of differences in age, temperature, moisture content pH, or experimental error. It is equally unlikely that substantially different moisture contents and leaching could have persisted in the water permeable and soluble 1 dm^3 size midden sample. Therefore, it is probable that some types of complex physicochemical relationships existing between bound aspartic acid and its host lignin–cellulose, etc., matrix, and/or the relative position of aspartic acid in the amino acid sequences of peptides/proteins in different taxa and anatomical sites control bound aspartic acid racemization rates.

Although the chemical structure of lignin has not yet been fully elucidated, differences in abundances of monomeric and large structural units of lignin exist between major groups of plants. Lignin extracted from

gymnosperms, e.g. *Juniperus* sp., *Ephedra nevadensis* consists primarily of 4-hydroxy-3-methoxyphenylpropane monomeric units derived from coniferyl alcohol [14]. Lignin extracted from dicotyledonous angiosperms, e.g. *Acacia greggi*, *Opuntia acanthocarpa*, contains monomers of 3,5-dimethoxy-4-hydroxyphenylpropane in addition to monomers of 4-hydroxy-3-methoxyphenylpropane [15]. Lignin extracted from monocotyledonous angiosperms, e.g. *Nolina bigelovii* contains monomers of 4-hydroxyphenylpropane in addition to the above mentioned monomers found in dicotyledons [14]. Some 4-hydroxyphenylpropane moieties are also found in gymnosperms. The minimum mw of cellulose in plants can vary from 50000 to 2500000 [16]. Variations in less abundant components of plant tissues, e.g. resins, alcohols, proteinaceous matter, mineral constituents, etc., could be even more dependent on species and, of course, anatomical site, than variations in lignin and cellulose [17]. Variations in hydrocarbons in various taxa of higher plants have been studied in detail, e.g. Eglinton *et al.* [18], Nagy *et al.* [19].

Since bound aspartic acid racemizes at different rates in different anatomical sites and taxa of plants isolated from the Redtail Peaks No. 10A midden, the activation energies and Arrhenius frequency factors obtained for aspartic acid racemization from one anatomical site of a plant taxon is most probably not applicable to all other anatomical sites of the same taxon, or to other plant taxa, in evaluating paleotemperatures or chronology.

EXPERIMENTAL

Taxonomically distinct plant specimens were collected from, and identified in, a 1 dm^3 sample from the interior of the solidified Redtail Peaks No. 10A midden. These specimens included *Ephedra nevadensis* (Mormon tea) seeds, *Opuntia acanthocarpa* (staghorn cholla) spines, *Nolina bigelovii* (bear grass) leaves, *Acacia greggi* (Catclaw acacia) pods and twigs, and *Juniperus* cf. *californica* (California juniper) seeds and twigs. Individual seeds, spines, leaves, twigs, and pods were analyzed separately and repeatedly for bound D/L aspartic acid ratios. All glassware employed in these experiments was cleaned in hot conc H_2SO_4/HNO_3 (17:3). All solvents used in these analyses were freshly redist prior to analysis from spectral or pesticide-grade reagents. The ancient plant samples were first washed extensively with triple dist H_2O using ultrasonication. Next, the plant samples were crushed and washed thoroughly with 1.5 N HCl (redist) followed by triple redist H_2O to remove any possible soluble (i.e. non-bound) amino acid contaminants [20]. The samples were then hydrolyzed in 6 N HCl (redist) for 24 hr at 100° in pyrex test tubes sealed under N_2. The hydrolysates were filtered, and the filtrates were evaporated to dryness. The resulting residues were placed on precleaned Bio Rad AG 50-X8, 50–100 mesh cation exchange columns and the amino acids were subsequently eluted with 1 M NH_4OH after washing with H_2O. Amino acid carboxyl moieties were esterified with (+)-2-BuOH (Norse Lab., Los Angeles, Calif.) which had previously been acidified by passing dry HCl gas through the alcohol to obtain 2 to 4 N anhydrous solns [21, 22]. After the evaporation of excess alcohol, the samples were acylated with pentafluoropropionic anhydride in CH_2Cl_2 [23]. The relative abundances of the resultant aspartic acid diastereomeric derivatives were quantitatively determined by GLC with an FID detector, coupled to an automatic digital integrator. A Carbowax-20M 46 m × 0.5 mm id SCOT capillary column was used. He (8.5 ml/min) was the carrier gas. The injection temp was 140°. For each determination the column was kept isothermal at 90° for 20 min and then programmed from 90° to 150° at 1°/min. The H_2O soluble portion of the bulk midden matrix and modern juniper seeds (*Juniperus osteosperma*) were also analyzed by this procedure; their respective D/L aspartic acid ratios were used for controls. Also, as a further precaution against laboratory amino acid contamination a complete experimental procedural blank was performed following the steps described above for the plant analyses.

The moisture contents of 5 juniper seeds from the Redtail Peaks midden were determined in order to evaluate whether there is a correlation between different D/L aspartic acid ratios and the amount of H_2O currently present in these seeds. In addition, the moisture contents of one *Ephedra nevadensis* seed, one *Juniperus californica* twig and one *Opuntia acanthocarpa* spine from the same midden were also determined. The 5 juniper seeds were split in half; one half of each seed was analyzed for D/L aspartic acid ratios. The remaining juniper seed-halves and the *Ephedra nevadensis* seed, *Juniperus californica* twig and *Opuntia acanthocarpa* spine were, without any prior aq. treatments, weighed and placed in a vacuum oven together with strong desiccants and heated at 75° for 4.5 hr. The samples were subsequently weighed again and then heated for an additional 5 hr to ensure that there was no further wt loss. To ascertain that the wt loss was attributable to H_2O, an additional fossil juniper seed was split in half; one half of the seed was analyzed for its D/L aspartic acid ratio while the other half of the seed was heated at 75° and 150° under vacuum in a modified MS gas inlet system. The released gases were trapped under liquid N_2 and then introduced and directly analyzed by MS.

The pH of H_2O extracts of 4 randomly selected 1 cm^3 bulk samples from the Redtail Peaks midden were determined. The samples were weighed and then ultrasonicated in triple dist H_2O. The H_2O extracts were rotary evaporated and weighed. The dry residues were dissolved in proportion to their dry wt in small quantities of triple distl H_2O. pH Determinations were made using a calibrated pH meter equipped with an Ag–AgCl glass electrode and a standard frit junction reference electrode.

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