PERGAMON

Organic Geochemistry 30 (1999) 1193–1200

Organic Geochemistry

Note

Fractionation of hydrogen isotopes in lipid biosynthesis

Alex L. Sessions[a], Thomas W. Burgoyne[b], Arndt Schimmelmann[b], John M. Hayes[a,*]

[a]*Department of Geology and Geophysics, Woods Hole Oceanographic Institution, Woods Hole, MA 02543, USA*
[b]*Biogeochemical Laboratories, Departments of Chemistry and of Geological Sciences, Indiana University, Bloomington, IN 47405, USA*

Received 17 April 1999; accepted 16 June 1999
(Returned to author for revision 12 May 1999)

Abstract

Isotopic compositions of carbon-bound hydrogen in individual compounds from eight different organisms were measured using isotope-ratio-monitoring gas chromatography–mass spectrometry. This technique is capable of measuring D/H ratios at natural abundance in individual lipids yielding as little as 20 nmol of H_2, and is applicable to a wide range of compounds including hydrocarbons, sterols, and fatty acids. The hydrogen isotopic compositions of lipids are controlled by three factors: isotopic compositions of biosynthetic precursors, fractionation and exchange accompanying biosynthesis, and hydrogenation during biosynthesis. δD values of lipids from the eight organisms examined here suggest that all three processes are important for controlling natural variations in isotopic abundance. *n*-Alkyl lipids are depleted in D relative to growth water by 113–262‰, while polyisoprenoid lipids are depleted in D relative to growth water by 142–376‰. Isotopic variations within compound classes (e.g., *n*-alkanes) are usually less than ~50‰, but variations as large as 150‰ are observed among isoprenoid lipids from a single organism. Phytol is consistently depleted in D by up to 50‰ relative to other isoprenoid lipids. Inferred isotopic fractionations between cellular water and lipids are greater than those indicated by previous studies. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Hydrogen isotopes; irmGCMS; Isotope fractionation; Lipids; Acetogenic; Isoprenoid; Biosynthesis

1. Introduction

We have developed an analytical system capable of measuring the D/H ratio of nanogram quantities of individual organic compounds with a precision of 5‰ or better. Variations in the natural abundance of D in C-bound hydrogen clearly record both environmental (Yapp and Epstein, 1982; Sternberg, 1988) and biochemical effects (Estep and Hoering, 1980; Yakir and DeNiro, 1990). Furthermore, isotope effects for hydrogen are commonly large (Bigeleisen, 1965). Therefore, the ability to examine hydrogen isotopic variation at the natural abundance level in individual organic compounds should prove to be quite useful. At present, with the exception of specific analyses of cellulose, almost nothing is known about hydrogen isotopic variability in individual compounds. As an initial examination, therefore, we extracted lipids from eight separate organisms and measured the D/H ratio of individual components in those extracts.

The purpose of this study was to examine the natural range of fractionations between cellular water and

* Corresponding author. Fax: +1-508-457-2183.
E-mail address: jhayes@whoi.edu (J.M. Hayes)

0146-6380/99/$ - see front matter © 1999 Elsevier Science Ltd. All rights reserved.
PII: S0146-6380(99)00094-7

lipids, among lipids of similar and disparate biosynthetic origin, and among lipids from different organisms. To this end, we examined two cultures of unicellular marine algae (*Alexandrium fundyense*, a dinoflagellate, and *Isochrysis galbana*, a haptophyte), two species of multicellular marine algae (*Fucus vesiculosis* and *Ascophyllum* sp., both brown algae), a submergent higher plant (*Zostera marina*, a sea grass) and an emergent higher plant (*Spartina alterniflora*, a marsh grass), a bacterial culture (*Methylococcus capsulatus*, a methanotroph), and the leaves of a carrot plant (*Daucus carota*). Aquatic organisms were selected preferentially to minimize isotopic differences between cellular and growth water due to evapotranspiration (Edwards, 1993). *Daucus* was selected to examine isotopic characteristics related to a recently discovered biosynthetic pathway (Schwender et al., 1996).

2. Experimental

2.1. Isotopic measurements

Hydrogen isotopic compositions of individual compounds were measured using an isotope-ratio-monitoring gas chromatography–mass spectrometry (irmGCMS) system developed in our laboratory. The system is similar to that described by Scrimgeour *et al.* (1999) and Tobias and Brenna (1997), but with several important improvements. The effluent from a conventional GC using He carrier gas is fed into a graphite-lined alumina tube held at 1400°C, at which temperature organic compounds are quantitatively pyrolyzed to graphite, H_2, and CO (Burgoyne and Hayes, 1998). An open split transmits 200 μl/min of the resulting gas stream to a Finnigan MAT 252 mass spectrometer. An electrostatic filter in front of the mass-3 Faraday cup reduces scattered $^4He^+$ reaching that collector to < 75 femtoamps at 200 μl/min He.

Mass-2 and -3 ion currents are recorded at 4 Hz and all data are processed using Visual Basic computer codes that we have developed. Raw mass-3 ion currents are corrected for contributions from H_3^+ on a point-by-point basis: each mass-3 data point is corrected based on the corresponding mass-2 data point using the equation, $i_{HD} = i_3 - K(i_2)^2$, where i_2 and i_3 are the raw mass-2 and -3 ion currents, i_{HD} is the corrected ion current, and K is the H_3^+ factor. We have tested this correction scheme extensively, and find that it reliably corrects for H_3^+ currents for a wide variety of peak shapes and sizes, retention times, and sample compositions. Following correction for H_3^+, ion currents are integrated, ion–current ratios are calculated, and isotope ratios are computed and standardized using techniques similar to those used in carbon irmGCMS (Ricci *et al.*, 1994). The working concentration range for this system (to obtain ~5‰ precision) is 20–100 nmoles H_2 injected on-column per sample component.

All values for δD reported here are relative to VSMOW. A laboratory standard containing 15 homologous *n*-alkanes, varying in concentration over a six-fold range and varying in δD over a 210‰ range, was analyzed daily. Values of δD for these alkanes were determined by offline combustion to a precision of ±1.6‰. The alkane standard was used to (1) determine the H_3^+ factor for the day, (2) normalize δD values to the VSMOW scale, and (3) monitor the stability of the system. The H_3^+ factor is determined as the value of K that minimizes the mean absolute error for the 15 peaks of varying size. Values of K determined using this method are generally within 5% of those determined conventionally (i.e., by observation of i_3/i_2 at varying values of i_2; Friedman, 1953). Regression of δD (irmGCMS) on δD (offline) for these standards provides a normalization line analogous to that used in batchwise analyses (i.e., so that δD values for VSMOW and SLAP are 0 and −428‰, respectively; Coplen, 1988). Values of R^2 for this regression always exceeded 0.99, and normalization results in corrections of < 10‰ (commonly < 5‰) to any δD value. The mean precision of measurement of δD for these alkanes over the 3-month period of this study was ±4.2‰ ($n = 33$ injections) and the root-mean-square error ($\equiv \sqrt{\Sigma d^2/n}$, where $d = \delta D_{measured} - \delta D_{known}$ and n = number of values for d) was 5.3‰ ($n = 491$ measurements) with no systematic bias due to peak size or retention time.

Values of δD for sample compounds were determined by reference to coinjected *n*-alkane standards. Where possible, four to six *n*-alkanes were coinjected with each sample; two of these were used as isotopic reference peaks, with the remaining alkanes providing tests of analytical accuracy. To avoid analyzing isotopically exchangeable H from carboxyl and hydroxyl positions, and to improve chromatography, alcohols and fatty acids were derivatized as trimethylsilyl ethers and esters using bis-(trimethylsilyl)-trifluoroacetamide (BSTFA). BSTFA is an attractive derivatizing reagent because (1) it can be used for both alcohols and acids; (2) hot, acidic conditions which might promote hydrogen isotopic exchange are avoided; and (3) all hydrogens in the derivatizing reagent are in the trimethylsilyl (TMS) groups, so that the δD of the derivatizing H can be measured directly by irmGCMS analysis of the BSTFA peak. To test this procedure, we measured the δD of non-exchangeable H in a cholestanol standard by first measuring the acetate, ketone, and trifluoroacetate derivatives using conventional, offline techniques, then measuring the TMS derivative using irmGCMS. The three offline measurements produced derivative-

Table 1
δD measurements for individual lipids

Species/compound	δD (‰)[a]	σ (‰)[b]
Cultured specimens		
Alexandrium fundyense (dinoflagellate)		
Hydrocarbons		
C_{22} alkadiene[d]	−204	10
Fatty acids		
14:0 + 14:1	−232	NA[c]
16:0	−227	NA[c]
22:4	−218	NA[c]
Sterols[e]		
$27\Delta^{5}$	−323	8
$30(4\alpha,23,24)\Delta^{22}$	−295	9
$29(4\alpha,24)\Delta^{22}$	−311	1
Other isoprenoids		
phytol	−357	13
Isochrysis galbana (haptophyte)		
Hydrocarbons		
C_{31} alkadiene[d]	−113	6
Fatty acids		
14:0 + 14:1	−216	18
16:0	−209	10
18:1 (2 isomers)	−181	7
Sterols[e]		
$28\Delta^{5,22}$	−258	11
Methylococcus capsulatus (methanotroph)		
Fatty acids		
14:0	−144	8
16:0 + 16:1	−161	5
Sterols[e]		
$28(4\alpha)\Delta^{8(14)}$	−234	6
$29(4,4)\Delta^{8(14)}$	−234	6
Native specimens		
Ascophyllum sp. (brown alga)		
Fatty acids		
14:0	−208	7
16:0	−210	6
16:1	−213	8
18:1	−189	4
Sterols[e]		
$29\Delta^{5}$	−219	11
Fucus vesiculosis (brown alga)		
Fatty acids		
14:0	−187	8
16:0	−167	4
18:1	−157	10
Sterols		
$29\Delta^{5,24(28)}$	−208	4
Zostera marina (sea grass)		
Hydrocarbons		
n-C_{17} alkane	−167	6
n-C_{19} alkane	−159	3
n-C_{21} alkane	−147	6
squalene	−241	8
Sterols[e]		
$27\Delta^{5}$	−253	1
$27\Delta^{5,24}$	−205	24
$29\Delta^{5}$	−194	6
Spartina alterniflora (marsh grass)		
Hydrocarbons		
n-C_{25} alkane	−150	10

Table 1 (*continued*)

Species/compound	δD (‰)[a]	σ (‰)[b]
n-C_{26} alkane	−170	11
n-C_{27} alkane	−166	1
n-C_{28} alkane	−164	4
n-C_{29} alkane	−169	3
n-C_{30} alkane	−161	19
n-C_{31} alkane	−160	7
n-C_{32} alkane	−145	NA[c]
n-C_{33} alkane	−155	15
Ketones		
C_{33} alkanone	−178	16
Triterpenoids		
lupenone	−142	6
pentacyclic triterpenone[d]	−144	5
lupenol	−171	1
pentacyclic triterpenol[d]	−165	3
pentacyclic triterpenol[d]	−151	8
Other isoprenoids		
phytol	−278	4
Daucus carota (carrot)		
Hydrocarbons		
n-C_{23} alkane	−141	4
n-C_{24} alkane	−135	18
n-C_{25} alkane	−158	6
n-C_{26} alkane	−139	7
n-C_{27} alkane	−166	3
n-C_{29} alkane	−162	2
n-C_{30} alkane	−134	15
n-C_{31} alkane	−113	21
n-C_{32} alkane	−117	11
Alcohols		
n-C_{22} alkanol	−186	11
n-C_{24} alkanol	−192	12
n-C_{26} alkanol	−192	6
n-C_{28} alkanol	−197	7
n-C_{30} alkanol	−180	15
Fatty acids		
12:0	−262	8
14:0	−217	7
16:0	−172	6
16:1	−204	17
18:0, 18:1, 18:2, 18:3	−190	7
C_{24} hydroxy-fatty acid	−193	12
Sterols[e]		
$29\Delta^{5,22}$	−283	7
$29\Delta^{5}$	−292	7
Triterpenoids		
Δ-amyrin + β-amyrin	−252	10
pentacyclic triterpenols[f]	−239	6
pentacyclic triterpenol[d]	−226	16
pentacyclic triterpenol[d]	−231	16
Other isoprenoids		
caryophyllene ($C_{15}H_{24}$)	−308	6
sesquiterpene[d] ($C_{15}H_{24}$)	−320	7
phytadiene	−345	2
phytol	−376	4

[a] Corrected for TMS derivatization.
[b] Standard deviation of 3 to 5 replicate analyses.
[c] Only one analysis available.
[d] Specific identity could not be determined.
[e] Leading number indicates total C and parenthesized numbers indicate positions of methyl substituents (where known).
[f] Three coeluting triterpenol peaks.

corrected δD values of −247, −250, and −253‰ (all ±1.5‰), and the irmGCMS measurements produced a corrected δD of −256 ± 3‰.

2.2. Sample collection and preparation

Alexandrium clone #GTCA28 was grown in a 1-l culture on f/2 medium at 15°C with a 14:10 h light: dark cycle. *Isochrysis* clone T-ISO was grown in a 20-l culture on f/2 medium at 20°C under continuous illumination. The f/2 medium for both cultures was prepared from sterile-filtered Vineyard Sound seawater, which has a salinity of 31.5–32.0‰. Both cultures were filtered onto precombusted 0.7-μm glass fiber filters (Whatman GF/F) prior to extraction. *Ascophyllum* and *Fucus* were collected from Quisset Harbor, Woods Hole, MA. *Spartina* was collected from a salt marsh on Penzance Point, Woods Hole, and *Zostera* was collected from Woods Hole Harbor. *Daucus* with attached leaves was obtained from a local grocery, and only the leaves were analyzed. The *Methylococcus* culture analyzed here was the same as that described by Summons *et al.* (1994) and was obtained in dried form. All native specimens were collected in February 1999.

Samples were extracted with an Accelerated Solvent Extractor (Dionex) using dichloromethane (DCM)/methanol (90:10 v/v) with three 5-min extraction cycles at 100°C and 1000 psi. Total lipid extracts from *Ascophyllum, Spartina*, and *Daucus* were saponified by reacting with methanolic NaOH at 75°C for 6 h, then neutral and acid fractions were extracted into hexane and combined. Lipids were separated into classes by elution from disposable, solid-phase extraction cartridges containing 500 mg of an aminopropyl stationary phase (Supelclean LC-NH2). Fractions collected were: hydrocarbons (4 ml hexane), ketones (6 ml hexane/DCM 3:1), alcohols (5 ml DCM/acetone 9:1), and fatty acids (8 ml DCM/formic acid 4:1). Each fraction was analyzed by GCMS to identify compounds of interest.

3. Results and discussion

Results are summarized in Table 1. Standard deviations of replicate analyses typically ranged from 3 to 12‰. Worse precision (up to 24‰) was obtained for some compounds where coeluting or especially small peaks were measured. When these were included in order to provide a conservative overview of the results, the pooled estimate of the standard deviation of a single analysis was 9.2‰ (375 degrees of freedom in 155 sets of replicates). The arithmetic mean error for the coinjected alkanes that served as test compounds in these analyses was 0.3‰, and the root-mean-square error was 7.6‰. Considering all possible sources of error in the measurement of unknown compounds, we estimate that the δD value measured for each compound is accurate to within ± 15‰. However, relative differences between compounds in the same chromatogram are probably accurate to within ± 5‰.

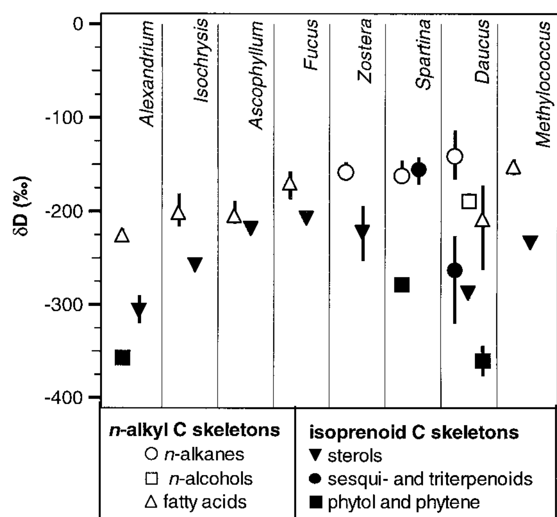

Fig. 1. Summary of δD measurements for compound classes. Each symbol represents the mean for that class, and the bars represent the range of measured values.

Results are summarized graphically in Fig. 1. Several points emerge. First, for any of the eight organisms examined, the differences between compound classes (e.g., *n*-alkanes, sterols, fatty acids, etc.) are greater than those within any one class. Second, where isotopic compositions of growth water are known, all lipids are depleted in D relative to growth water by 150‰ or more. Third, compounds from a given class (e.g., sterols) can have substantially different δD values in different organisms, despite growing in water with the same hydrogen isotopic composition.

3.1. Variations of δD within organisms

Compound classes exhibit restricted ranges in δD, with individual lipids in each class generally falling within a range of < 50‰. Variations between classes are much larger, with differences of 50–150‰ being common. Polyisoprenoid lipids are generally depleted relative to acetogenic (*n*-alkyl) lipids (compare filled and open symbols, Fig. 1), but there are also differences between compound classes with biosynthetically equivalent carbon skeletons (e.g., *n*-alcohols versus *n*-alkanes in *Daucus*). Based on compounds examined in *Daucus*, the range of variability appears to be somewhat greater within the polyisoprenoid lipids than within the acetogenic lipids.

Three potential sources of isotopic variability in

lipid hydrogen can be identified: (1) the isotopic composition of biosynthetic precursors, (2) isotope effects (including exchange of organic H with H_2O) associated with biosynthetic reactions (Martin *et al.*, 1986), and (3) the isotopic composition of hydrogen added, commonly from NADPH, during biosynthesis (Smith and Epstein, 1970; Luo *et al.*, 1991). This third control, which is not related to the flow of substrates used in assembly of the carbon skeleton, is notably different from those encountered in carbon-isotopic studies (Hayes, 1993). Below we discuss evidence that isotopically different pools of NADPH may exist within cells; this suggests that compound-specific hydrogen isotopic analyses will provide information about compartmentalization of biosynthesis in cells, as well as about biosynthetic pathways.

The depletion of D in isoprenoid lipids relative to acetogenic lipids was first described by Estep and Hoering (1980). Our results confirm and extend, but do not explain, this finding. Acetogenic lipids, sterols, and pentacyclic triterpenols and all use acetyl-CoA — either directly or via the formation of mevalonic acid (MVA) — as the biosynthetic precursor (Abeles *et al.*, 1992). Slightly more hydrogen in acetogenic lipids derives from NADPH (~50%) as compared to isoprenoid lipids derived from the MVA pathway (~37%). If the acetogenic-versus-isoprenoid contrast (roughly −200‰ versus −250‰) were attributed to this difference alone, it would require that NADPH-derived hydrogen is substantially enriched ($\delta D \approx 0$) and acetate-derived hydrogen is highly depleted ($\delta D \approx -400$). More plausibly, as noted by Estep and Hoering (1980), the depletion of D in polyisoprenoids relative to *n*-alkyl lipids is due to isotope effects associated with the two pathways.

In *Daucus*, fatty acids and *n*-alcohols have similar δD values, but are depleted in D by ~50‰ relative to *n*-alkanes. Estep and Hoering (1980) also found substantial hydrogen isotopic differences (up to 54‰) between fatty acids and hydrocarbons separated from three higher plants and from *Trichodesmium*. In that case, however, the pattern was reversed from that observed here, with fatty acids in the higher plants D-enriched relative to the hydrocarbons. Given that the carbon skeletons of fatty acids, *n*-alcohols, and *n*-alkanes are all formed via the same biosynthetic pathway, these differences must be due to changes in the isotopic composition of acetate starting material, or of NADPH, or both. These changes could occur through time (e.g., as growth rate changes), or could exist spatially within the cell or plant at any given time. There appears to be a slight trend toward D-enrichment with longer chain length in the fatty acids. If real, this may also reflect synthesis in different parts of the plant.

Substantial hydrogen isotopic variations occur between classes of polyisoprenoid lipids. In particular, phytol is consistently depleted relative to sterols and pentacyclic triterpenoids by 50–140‰. Part of this large variability may be due to isotopically different pools of NADPH in the cytosol, where triterpenols are synthesized, and the chloroplasts, where phytol is synthesized. In plants, $NADP^+$ is reduced to NADPH by two processes: electron-transport chains associated with photosynthesis in the chloroplasts, and during oxidation of sugars in the pentose-phosphate pathway in the cytosol (Abeles *et al.*, 1992). The sources of hydrogen for NADPH are very different in these two cases (H_2O in photosynthesis, and C-bound H from sugars in the pentose-phosphate pathway), so it is plausible that isotopically distinct pools of NADPH might exist within these different cellular compartments.

Isotopic differences between phytol and triterpenols may also be due in part to different biosynthetic pathways. Schwender *et al.* (1996) have recently described a novel pathway for polyisoprenoid synthesis, in which glyceraldehyde phosphate and pyruvate — rather than three molecules of acetyl-CoA — condense to form I-deoxy-D-xylulose phosphate (DOXP), the immediate precursor of isopentenyl phosphate. The phylogenetic distribution of this novel pathway is still under investigation. Of the species included here, only *Daucus* has been examined. *Daucus* uses the well-known MVA pathway to synthesize cytosolic isoprenoids, including sterols, while using the novel DOXP pathway to synthesize plastidic isoprenoids, such as phytol (Lichtenthaler, 1999). In general, the DOXP pathway appears to be closely related to chloroplasts, and it is presumed to be a relict biochemical pathway preserved from the endosymbiotic origin of chloroplasts (Disch *et al.*, 1998). If differences between the DOXP and MVA pathways are responsible for the observed depletion of phytol relative to triterpenols, this would require either that DOXP and pyruvate are D-depleted relative to acetate, or that there is a larger isotope effect associated with the DOXP pathway.

Based on the data presented here, we cannot distinguish the source of isotopic variability in isoprenoid lipids. Differing biosynthetic precursors, isotope effects and exchange associated with biosynthesis, isotopically distinct pools of NADPH, or all three may be responsible for the observed pattern. If different isotope effects can be positively associated with the DOXP and MVA pathways, compound-specific D/H measurements would become a valuable tool for studying the distribution and importance of this novel biosynthetic pathway.

3.2. Isotopic fractionation

We calculate values for ε (isotopic fractionation) by

Table 2
Hydrogen isotopic fractionations[a]

Compound-specific measurements, this study[b]

Sample	$\varepsilon_{fa/w}$	$\varepsilon_{ak/w}$	$\varepsilon_{st/w}$	$\varepsilon_{ph/w}$
Alexandrium	−226		−310	−357
Isochrysis	−202		−258	
Ascophyllum	−205		−219	
Fucus	−170		−208	
Zostera		−158	−223	
Spartina		−162		−278

Bulk lipid measurements, previous studies

Sample	$\varepsilon_{l/w}$	$\varepsilon_{sl/w}$	$\varepsilon_{nsl/w}$	Reference[c]
Cyanobacteria	−175			1
Macrophytes		−152	−232	1
Red algae	−139			2
Brown algae	−178			2
Green algae	−132			2
Macrophytes	−125			3

[a] Abbreviations: water (w), alkanes (ak), fatty acids (fa), sterols (st), phytol (ph), bulk lipids (l), saponifiable lipids (sl), non-saponifiable lipids (nsl); $\varepsilon_{a/b} \equiv 1000[(\delta_a + 1000)/(\delta_b + 1000) - 1]$.

[b] Calculated using the mean δD for each compound class, and assuming $\delta D_w = 0‰$.

[c] (1) Estep and Hoering (1980); (2) Sternberg *et al.* (1986); (3) Sternberg (1988).

estimating that the δD value of the cellular water for all samples except *Daucus* and *Methylococcus* was close to 0‰. For those organisms that were submerged in ocean water (*Alexandrium, Isochrysis, Ascophyllum, Fucus*, and *Zostera*) this is straightforward. Cellular water in *Spartina* may have been enriched relative to the growth water by evaporation, but the nearly identical δD values for alkanes from *Zostera* and *Spartina* suggest that this was minimal. The isotopic compositions of water in the *Daucus* and *Methylococcus* cultures are unknown.

Fractionation factors are defined and summarized in Table 2. Values for $\varepsilon_{\text{fatty acid/water}}$ range from −170 to −226‰ (mean = −200‰), values for $\varepsilon_{\text{alkane/water}}$ range from −158 to −162‰ (mean = −160‰), and values for $\varepsilon_{\text{sterol/water}}$ range from −208 to −310 (mean = −244‰). By comparison, fractionations between bulk lipids and water measured by offline techniques range from −125 to −178‰ (mean = −150‰). The observed fractionation between water and alkanes is indistinguishable from that previously observed between water and bulk lipids, but fractionations between fatty acids and water are nearly 50‰ larger on average. *n*-Alkanes were the most D-enriched lipid compounds observed in this study and fatty acids were in most cases the most abundant lipid components; it therefore appears unlikely that the smaller fractionations measured by previous studies are due solely to differences in the relative abundance of these components. A possible explanation is that components of the bulk extracts which do not appear in GC analyses (e.g., lipoproteins) are D-enriched relative to the lipids we examined. In addition, bulk lipid measurements may incorporate hydrogen from H_2O or exchangeable oxygen-bound positions, which would be expected to produce anomalously large δD values (Schimmelmann, 1991).

3.3. Variations in δD between organisms

Differences in δD of up to 80‰ that cannot be reconciled as stemming from differences in cellular water exist between the same compounds in different organisms (e.g., compare $\varepsilon_{\text{phytol/water}}$ in *Spartina* and *Alexandrium* in Table 2). These differences appear to be most pronounced between the cultured organisms (*Alexandrium, Isochrysis*, and *Methylococcus*) and *Daucus* versus the native specimens (*Ascophyllum, Fucus, Spartina*, and *Zostera*). Values of $\varepsilon_{\text{sterol/fatty acid}}$ range from −70 to −118‰ in the former group, and from −18 to −46‰ in the latter. $\varepsilon_{\text{triterpenol/alkane}}$ is −112‰ in *Daucus* and +8‰ in *Spartina*, and $\varepsilon_{\text{phytol/alkane}}$ is −274‰ in *Daucus* and −138‰ in *Spartina*. Thus, the depletion of D in isoprenoid relative to acetogenic lipids is greater in the actively growing cultures and in *Daucus* than in the native specimens collected in winter. If the differing extents of isotopic fractionation observed here represent a more general pattern, they should be useful for understanding the underlying mechanisms, which could involve differences in photosynthetic activity, growth rate, water temperature, light intensity, nutrient supply, or other parameters.

A correlation between D-depletion in lipids and photosynthetic activity would not be surprising, as a similar situation is observed for carbohydrate metabolism. Photosynthesis produces C-bound H in carbohydrates which is D-depleted relative to tissue water by 100–170‰ (Estep and Hoering, 1981; Yakir and DeNiro, 1990). In contrast, "heterotrophic processing" of carbohydrates within plants (such as isomerization of triose phosphates and interconversion of fructose-6-phosphate and glucose-6-phosphate) results in the exchange of up to 50% of C-bound H with tissue water, with those exchanged positions becoming D-enriched by up to 158‰ relative to the tissue water (Yakir and DeNiro, 1990). Luo and Sternberg (1991) found that starch from chloroplasts was highly depleted relative to cytoplasmic cellulose in the same plant, due probably to this effect. Thus, the δD value

of carbohydrates in plants is thought to represent a balance between autotrophic and heterotrophic metabolism, and can potentially be used as an indicator of photosynthetic status in plants (Yakir, 1992). Our results suggest that a similar pattern may exist in lipids.

4. Conclusions

We have measured δD values of 80 individual lipids from eight separate organisms using a newly-developed analytical system. This system is flexible, robust, and accurate, and provides a means for measuring D/H ratios in many geochemically important organic compounds, including hydrocarbons, sterols, and fatty acids.

Although the data presented here are limited in scope, they suggest several generalizations: (1) There is little isotopic variability within specific compound classes in individual organisms, but there is significant variability between compound classes, even among those that share common biosynthetic origins. Part of this variability may be due to isotopically distinct pools of NADPH used for hydrogenation during biosynthesis. (2) Phytol is significantly and consistently depleted in D relative to sterols and other triterpenols. This difference suggests that the hydrogen-isotopic characteristics of the MVA and DOXP pathways for synthesis of isoprenoid lipids may differ significantly. If so, this contrast may facilitate further study of the occurrence of the DOXP pathway. (3) The estimated isotopic fractionation between cellular water and individual lipids is larger than previous estimates using bulk lipid measurements.

Acknowledgements

The authors wish to thank Peter Sauer for assistance collecting samples and preparing cholestanol derivatives, Sean Sylva for preparing and analyzing GCMS samples, and Chris Reddy and Kai-Uwe Hinrichs for helpful discussions. We also thank Scott Gallager, David Kulis, Don Anderson, and Linda Jahnke for supplying culture samples. Marilyn Fogel and one anonymous reviewer provided constructive comments. This project was supported by National Science Foundation grant OCE-9711284 to JMH, and a National Science Foundation postdoctoral fellowship to TWB.

Associate Editor—**J. Curiale**

References

Abeles, R.H., Frey, P.A., Jencks, W.P., 1992. Biochemistry. Jones and Bartlett, Boston.

Bigeleisen, J., 1965. Chemistry of isotopes. Science 147, 463–471.

Burgoyne, T.W., Hayes, J.M., 1998. Quantitative production of H_2 by pyrolysis of gas chromatographic effluents. Analytical Chemistry 70, 5136–5141.

Coplen, T.B., 1988. Normalization of oxygen and hydrogen isotope data. Chemical Geology 72, 293–297.

Disch, A., Schwender, J., Müller, C., Lichtenthaler, H.K., Rohmer, M., 1998. Distribution of the mevalonate and glyceraldehyde phosphate/pyruvate pathways for isoprenoid biosynthesis in unicellular algae and the cyanobacterium *Synechocystis* PCC 6714. Biochemical Journal 333, 381–388.

Edwards, T.W.D., 1993. Interpreting past climate from stable isotopes in continental organic matter. In: Swart, P.K., Lohmann, K.C., McKenzie, J., Savin, S. (Eds.), Climate Change in Continental Isotopic Records. American Geophysical Union, Washington, DC, pp. 333–341.

Estep, M.F., Hoering, T.C., 1980. Biogeochemistry of the stable hydrogen isotopes. Geochimica et Cosmochimica Acta 44, 1197–1206.

Estep, M.F., Hoering, T.C., 1981. Stable hydrogen isotope fractionations during autotrophic and mixotrophic growth of microalgae. Plant Physiology 67, 474–477.

Friedman, I., 1953. Deuterium content of natural water and other substances. Geochimica et Cosmochimica Acta 4, 89–103.

Hayes, J.M., 1993. Factors controlling ^{13}C contents of sedimentary organic compounds: principles and evidence. Marine Geology 113, 111–125.

Lichtenthaler, H.K., 1999. The I-deoxy-D-xylulose-5-phosphate pathway of isoprenoid biosynthesis in plants. Annual Reviews of Plant Physiology 50, 47–65.

Luo, Y-H., Sternberg, L.dS.L, Suda, S., Kumazawa, S., Mitsui, A., 1991. Extremely low D/H ratios of photoproduced hydrogen by cyanobacteria. Plant and Cell Physiology 32, 897–900.

Luo, Y-H., Sternberg, L.dS.L, 1991. Deuterium heterogeneity in starch and cellulose nitrate of CAM and C3 plants. Phytochemistry 30, 1095–1098.

Martin, G.J., Zhang, B.L., Naulet, N., Martin, M.L., 1986. Deuterium transfer in the bioconversion of glucose to ethanol studied by specific isotope labeling at the natural abundance level. Journal of the American Chemical Society 108, 5116–5122.

Ricci, M.P., Merritt, D.A., Freeman, K.H., Hayes, J.M., 1994. Acquisition and processing of data for isotope-ratio-monitoring mass spectrometry. Organic Geochemistry 21, 561–571.

Schimmelmann, A., 1991. Determination of the concentration and stable isotopic composition of non-exchangeable hydrogen in organic matter. Analytical Chemistry 63, 2456–2459.

Schwender, J., Seemann, M., Lichtenthaler, H.K., Rohmer, M., 1996. Biosynthesis of isoprenoids (carotenoids, sterols, prenyl side-chains of chlorophylls and plastoquinone) via a novel pyruvate/glyceraldehyde 3-phosphate non-mevalo-

nate pathway in the green alga *Scenedesmus obliquus*. Biochemical Journal 316, 73–80.

Scrimgeour, C.M., Begley, I.S., Thomason, M.L., 1999. Measurement of deuterium incorporation into fatty acids by gas chromatography/isotope ratio mass spectrometry. Rapid Communications in Mass Spectrometry 13, 271–274.

Smith, B.N., Epstein, S., 1970. Biogeochemistry of the stable isotopes of hydrogen and carbon in salt marsh biota. Plant Physiology 46, 738–742.

Sternberg, L.dS.L, 1988. D/H ratios of environmental water recorded by D/H ratios of plant lipids. Nature 333, 59–61.

Sternberg, L.dS.L, DeNiro, M.J., Ajie, H.O., 1986. Isotopic relationships between saponifiable lipids and cellulose nitrate prepared from red, brown, and green algae. Planta 169, 320–324.

Summons, R.E., Jahnke, L.L., Roksandic, Z., 1994. Carbon isotopic fractionation in lipids from methanotrophic bacteria: relevance for interpretation of the geochemical record of biomarkers. Geochimica et Cosmochimica Acta 58, 2853–2863.

Tobias, H.J., Brenna, J.T., 1997. On-line pyrolysis as a limitless reduction source for high-precision isotopic analysis of organic-derived hydrogen. Analytical Chemistry 69, 3148–3152.

Yakir, D., 1992. Variations in the natural abundance of oxygen-18 and deuterium in plant carbohydrates. Plant, Cell and Environment 15, 1005–1020.

Yakir, D., DeNiro, M.J., 1990. Oxygen and hydrogen isotope fractionation during cellulose metabolism in *Lemna gibba L*. Plant Physiology 93, 325–332.

Yapp, C.J., Epstein, S., 1982. Climatic significance of the hydrogen isotope ratios in tree cellulose. Nature 297, 636–639.