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Hydrogen, Oxygen, and Carbon Isotope Ratios of Cellulose from Submerged Aquatic Crassulacean Acid Metabolism and Non-Crassulacean Acid Metabolism Plants

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ABSTRACT

Isotope ratios of cellulose and cellulose nitrate from aquatic Crassulacean acid metabolism (CAM) and non-CAM plants were determined. Cellulose oxygen istope ratios for all plants that grew together were virtually identical, whereas large differences were observed for hydrogen isotope ratios of cellulose nitrate between CAM and non-CAM plants. Carbon isotope ratios of cellulose nitrate did not differentiate CAM from non-CAM plants.

Most terrestrial plants can be divided into three major photosynthetic types. Among C_3 and C_4 plants, the initial carboxylation step occurs during the day and is catalyzed by ribulose 1,5-bisphosphate carboxylase or by PEP[1] carboxylase, respectively, while in plants having CAM, the initial carboxylation step occurs at night and is catalyzed by PEP carboxylase (1). Aquatic plants have more variable and unusual photosynthetic modes than terrestrial plants. While some aquatic species have been shown to have CAM (11–14), no aquatic plant is known to have the C_4 photosynthetic mode. Some aquatic plants such as *Elodea canadensis* (7) and *Eleocharis acicularis* (Keeley, unpublished observations) assimilate carbon in the light with both PEP carboxylase and ribulose 1,5-bisphosphate carboxylase. These species differ from C_4 plants in that they lack Kranz anatomy and both carboxylases seem to operate simultaneously on the same CO_2 pool. Thus, our present understanding of aquatic plant photosynthesis indicates that some plants have CAM, some are C_3 plants and some are not comparable to the terrestrial modes.

It is now well established that the carbon isotope ratios of terrestrial plants are, to a large extent, dependent on the photosynthetic pathway they employ (1, 2, 16). Terrestrial C_4 and CAM plants (operating in the CAM mode) have $\delta^{13}C$ values (see "Materials and Methods" for definition of δ) that average about −13‰, while C_3 plants have $\delta^{13}C$ values that average about −25‰ (1, 2, 16). However, the carbon isotope ratios of aquatic plants (both freshwater and marine) are influenced by several factors other than the primary carboxylation step (3, 15, 23), so that it may not be feasible to use $\delta^{13}C$ values as an indicator of photosynthetic type for these plants.

Recently it has been shown that hydrogen isotope ratios of organic matter from terrestrial plants are also related to photosynthetic mode (17, 19, 20, 22, 23). For example, δD values of cellulose nitrate from field grown CAM plants are from 45‰ to 223‰ higher than those observed for C_3 and C_4 plants growing in the same vicinity (17, 20). In this study, we present stable isotopic analysis of aquatic plants which have known photosynthetic modes, and demonstrate that hydrogen isotope ratios of aquatic plant cellulose nitrate can be used as an indicator of photosynthetic mode. Further, we show that the differences in δD values between aquatic CAM and non-CAM plants are similar to the differences observed between terrestrial CAM plants and C_3 and C_4 plants. These observations are consistent with the proposal that isotopic fractionations occurring during biochemical steps (rather than during evapotranspiration) are responsible for the hydrogen isotopic differences between terrestrial CAM and non-CAM plants (17, 29, 20).

MATERIALS AND METHODS

Plants were cultured in the laboratory at Occidental College by layering the bottom of two aquaria with soil having sporelings and seeds of *Isoetes howellii*, an aquatic CAM plant (13), and *Chara contraria*, *Eleocharis acicularis*, and *Ranunculus aquatilis*, which are aquatic non-CAM plants (12). The aquaria were filled with water and covered with a glass sheet to minimize evaporation, which can cause changes in hydrogen and oxygen isotope ratios of the water. Cultures were started in January 1983 and maintained at room temperature (22.5 ± 2.5°C). Previous studies indicated that *I. howellii* operated in the CAM mode when grown under these conditions (11, 13). Plant material was harvested in April 1983. Water samples were collected at the beginning and end of the experiment.

Plants and water samples from Siesta Lake (2,440 m elevation, Tuolumne County, CA) were collected in August 1983. The following plant species were collected from the lake: *Isoetes bolanderi*, an aquatic CAM plant (14), and *C. contraria*, *Fontinalis antipyretica*, and *Calitriche longipedunculata*, which are aquatic non-CAM plants (12). Only specimens which did not exhibit evidence of emergence were collected.

Plant samples were cleaned, then dessicated at 50°C. Dried plant material was ground in a Wiley mill and cellulose was extracted and nitrated as described previously (19). Cellulose oxygen isotope ratios and hydrogen and carbon isotope ratios of cellulose nitrate were determined as in previous studies (19). Water hydrogen and oxygen isotope ratios were determined by standard methods (4, 9). Isotope ratios are expressed as δ values,

[1] Abbreviations: PEP, P-enolpyruvate; SMOW, standard mean ocean water; PDB, Peedee belemnite.

where

$$\delta\ (‰) = \left[\frac{R_{sample}}{R_{standard}} - 1\right] \times 1000$$

and R represents $^{18}O/^{16}O$ for oxygen, D/H for hydrogen and $^{13}C/^{12}C$ for carbon. The standard is SMOW for oxygen and hydrogen isotope ratios and PDB carbonate for carbon isotope ratios. The precision of isotopic analyses was ± 2‰ for δD values, ±0.5‰ for $\delta^{18}O$ values, and ±0.2‰ for $\delta^{13}C$ values.

RESULTS

Results of our hydrogen and oxygen isotope analysis for laboratory grown plants are shown in Figure 1A. The δD values for the CAM plant (*I. howellii*) were about 30‰ to 75‰ higher than those for the non-CAM plants. The δD values of cellulose nitrate from *I. howellii* were about 50‰ higher than the water in which it grew, while the non-CAM plants had δD values from +18‰ to −14‰ relative to the same water. The oxygen isotope ratio of cellulose for all laboratory grown plants were similar, averaging 28.6 ± 1.8‰ higher than the oxygen isotope ratio of the water in which the plants grew.

Analysis of plants from Siesta Lake (Figure 1B) produced similar patterns. *I. bolanderi*, the CAM species, had δD values from 37‰ to 109‰ higher than those of aquatic non-CAM plants. However, the δD values of the cellulose nitrate from the CAM plant was only 2‰ higher than the δD value of the lake water at the time of collection. The differences between the δD values of the cellulose nitrate of the non-CAM plants and the δD value of Siesta Lake water at the time of collection were also lower than the corresponding differences observed for non-CAM plants grown in the laboratory. For all field samples, the average difference between oxygen isotope ratios of cellulose and the oxygen isotope ratio of the water at time of collection was 26.2 ± 1.2‰, slightly lower than that observed in the laboratory.

The $\delta^{13}C$ values of cellulose nitrate for all samples are shown in Table I. CAM plants did not have significantly different $\delta^{13}C$ values than those of non-CAM plants, in either the laboratory grown or the field grown samples.

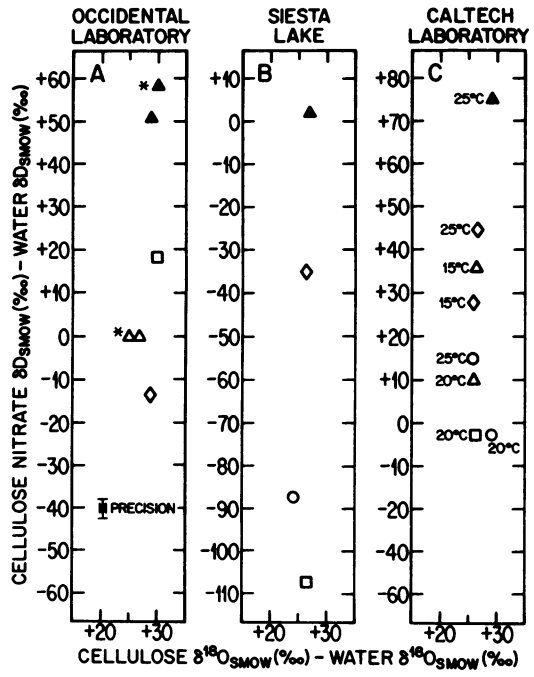

FIG. 1. δD values of cellulose nitrate and $\delta^{18}O$ values of cellulose from aquatic plants relative to the δD and $\delta^{18}O$ values of the waters in which they grew (A) in the lab at room temperature at Occidental College, (B) in Siesta Lake, or (C) in the lab at the indicated temperature at Caltech (8). Points marked with an asterisk indicate plants grown together in a second aquarium at Occidental College. Note sliding of δD scales for the different graphs. For the plants from Occidental College, the CAM plant is *I. howellii* (▲) and the non-CAM plants are *E. acicularis* (□), *R. aquatilis* (△), and *C. contraria* (◇). For the plants from Siesta Lake, the CAM plant is *I. bolanderi* (▲) and the non-CAM plants are *C. contraria* (◇), *C. longipedunculata* (○), and *F. antipyretica* (□). For the plants from Caltech, the CAM plant is *V. spiralis* (▲) and other plants, whose photosynthetic modes are not known, are *Ludwigia natans* (◇), *Ceratopteris* sp. (□), *Hygrophila polysperma* (○), and *Synnema triflorum* (△).

DISCUSSION

Results of isotopic analysis presented here demonstrate the feasibility of using δD values of cellulose nitrate as an indicator of photosynthetic mode in aquatic plants. Cellulose nitrate from aquatic CAM plants had δD values 33‰ to 109‰ higher than the δD values of cellulose nitrate of aquatic non-CAM plants growing in the same waters, either in the lab or in Siesta Lake. Consistent with these results is the observation that among laboratory grown submerged aquatic plants analyzed in a previous study (8) (Figure 1C), one species (*Vallisneria spiralis*) had an exceptionally high δD value relative to the other plants that were analyzed. Recent measurements of CO_2 uptake patterns in this species indicate that it has CAM (10). From the results presented here, we would predict that the other species shown in Fig. 1C are non-CAM plants. δD values of cellulose nitrate might also be a useful indicator of CAM-like metabolism observed in marine plants, since the δD values of cellulose nitrate from marine plants such as *Laminaria agardhii* and *Macrocystis* sp., which show dark fixation of inorganic carbon (6, 21), are exceptionally high relative to the δD values for other marine plants growing in their vicinity (8). The use of δD values of cellulose nitrate from aquatic plants as an indicator of photosynthetic mode offers a distinct advantage over the use of $\delta^{13}C$ values. Although carbon isotope ratios can differentiate CAM from non-CAM plants among terrestrial plants, they do not differentiate aquatic CAM from non-CAM plants. Our measurements and previous measurements (8) of carbon isotope ratios of cellulose nitrate from aquatic plants did not distinguish between CAM and non-CAM plants (Table I).

There is an apparent discrepancy in the relationship between the δD value of cellulose nitrate and the δD value of the water in which the plants grew (Δδ D values) for field and laboratory grown plants. Field grown plants displayed a greater deuterium depletion relative to the water in which they grew compared with laboratory grown plants. As an example, *C. contraria* had Δδ D values of −35‰ and −14‰ for field and laboratory grown plants, respectively. This discrepancy can be understood when one considers that when the *Chara* species grew, the water in Siesta Lake may have had a lower δD value relative to the water sample that was collected, while for the laboratory samples the isotope ratio of the water remained constant throughout the growth period. The $\delta^{18}O$ values also reflect this discrepancy. Previous work has established that the relationship between the $\delta^{18}O$ values of cellulose and the $\delta^{18}O$ values of the water in which plants grew is relatively constant for all aquatic plants that have been studied (data summarized in [18]). By using the $\Delta\delta^{18}O$ value for *Chara* grown in the laboratory and the $\delta^{18}O$ value of cellulose from field grown *Chara*, we calculate that *Chara* grew in Siesta Lake when the time-averaged $\delta^{18}O$ value of the water was 2.6‰ less than what we measured for the single water sample collected along with the plants. The δD value of the time-averaged water would have been 21‰ less than what we measured, since one

Table I. $\delta^{13}C$ *Values of Cellulose Nitrate*

The samples were taken from aquatic plants of the indicated photosynthetic type grown in the lab at room temperature at Occidental College, in the lab at Caltech at the indicated temperatures by DeNiro and Epstein (8), or in Siesta Lake. Results marked with an asterisk indicate plants grown together in a second aquarium at Occidental College.

Plant Species	Location	Photosynthetic Mode	$\delta^{13}C$
Chara contraria Braun ex. Kutzing	Occidental	non-CAM	−15.7
Eleocharis acicularis (L.) R. & S.	Occidental	non-CAM	−25.6
Isoetes howellii Engel	Occidental	CAM	−29.2, −24.3*
Ranunculus aquatilis L.	Occidental	non-CAM	−13.4, −16.8*
Calitriche longipedunculata Morong.	Siesta Lake	non-CAM	−24.1
Chara contraria Braun ex. Kutzing	Siesta Lake	non-CAM	−25.3
Fontinalis antipyretica Hedw.	Siesta Lake	non-CAM	−27.1
Isoetes bolanderi Engel	Siesta Lake	CAM	−24.1
Ceratopteris sp.	Caltech	non-CAM	−39.0 (20°C)
Hygrophila polysperma T. Anders.	Caltech	non-CAM	−24.9 (20°C), −33.7 (25°C)
Ludwigia natans Ell.	Caltech	non-CAM	−32.5 (15°C) −32.3 (25°C)
Synnema triflorum (Roxburgh ex Nees) O. Kuntze	Caltech	non-CAM	−36.5 (15°C) −32.8 (20°C)
Vallisneria spiralis L.	Caltech	CAM	−31.5 (25°C)

unit of change in the $\delta^{18}O$ value of the lake water would be accompanied by 8 units of change in the δD value (5). The $\Delta\delta D$ value of field grown *Chara* relative to the δD value of the time-averaged water is −14‰, which is the same value as was observed in the laboratory grown *Chara*. Using the same logic, we calculate that *I. bolanderi* from Siesta Lake had a $\Delta\delta D$ value of +35‰ relative to the time-averaged δD value of the lake water, which is similar to what we observed in the laboratory for *I. howellii*.

The differences of up to 100‰ in the δD values of CAM and non-CAM in aquatic plants demonstrates that fractionations occurring during biochemical reactions can account for similar differences observed between terrestrial CAM plants and C_3 and C_4 plants, as has been previously suggested (17, 19, 20). The aquatic CAM plants studied here have only about 40% of their biomass produced via the CAM pathway (Keeley, unpublished results), so that greater hydrogen isotopic differences can be expected between non-CAM plants and CAM plants which have more or all of their biomass fixed via the CAM pathway. The similarity in the relationship between oxygen isotope ratios of cellulose and the water in which the plants grew for aquatic non-CAM and CAM plants suggests that there are no significant differences in the oxygen isotope fractionations occurring during biochemical reactions for the different photosynthetic modes.

LITERATURE CITED

1. BENDER MM 1971 Variation in $^{13}C/^{12}C$ ratios of plants in relation to the pathway of photosynthetic carbon dioxide fixation. Phytochemistry 10: 1239–1344
2. BENDER MM, I ROUHANI, HM VINES, CC BLACK JR 1973 $^{13}C/^{12}C$ ratio changes in Crassulacean acid metabolism plants. Plant Physiol 52: 427–430
3. BENEDICT CR, WWL WONG, JHH WONG 1980 Fractionation of the stable isotopes of inorganic carbon by seagrasses. Plant Physiol 65: 512–517
4. BIGELEISEN J, ML PERLMAN, HC PROSSER 1952 Conversion of hydrogenic materials to hydrogen for isotopic analysis. Anal Chem 24: 1356–1357
5. CRAIG H 1961 Isotopic variations in meteoric water. Science 133: 1702–1703
6. CRAIGIE JS 1963 Dark fixation of C^{14}-bicarbonate by marine algae. Can J Bot 41: 317–325
7. DEGROOTE D, RA KENNEDY 1977 Photosynthesis in *Elodea canadensis* Michx. Plant Physiol 59: 1133–1135
8. DENIRO MJ, S EPSTEIN 1981 Isotopic composition of cellulose from aquatic organisms. Geochim Cosmochim Acta 42: 495–506
9. EPSTEIN S, T MAYEDA 1953 Variations of ^{18}O content of water from natural sources. Geochim Cosmochim Acta 42: 213–224
10. HELDER RJ, MV HARMELAN 1982 Carbon assimilation pattern in the submerged leaves of the aquatic angiosperm: *Vallisneria spiralis* L. Acta Bot Neerl 31: 281–295
11. KEELEY JE 1982 Distribution of diurnal acid metabolism in the genus *Isoetes*. Am J Bot 69: 254–257
12. KEELEY JE, BA MORTON 1982 Distribution of diurnal acid metabolism in submerged aquatic plants outside the genus *Isoetes*. Photosynthetica 16: 546–553
13. KEELEY JE, G BOWES 1982 Gas exchange characteristics of the submerged aquatic crassulacean acid metabolism plant, *Isoetes howellii*. Plant Physiol 70: 1455–1458.
14. KEELEY JE, CM WALKER, RP MATHEWS 1983 Crassulacean acid metabolism in *Isoetes bolanderi* in high elevation oligotrophic lakes. Oecologia 58:63–69
15. SACKETT WM, WS MOORE 1966 Isotopic variations of dissolved inorganic carbon. Chem Geol 1: 323–328
16. SMITH BN, S EPSTEIN 1970 Two categories of $^{13}C/^{12}C$ ratios for higher plants. Plant Physiol 47: 380–384
17. STERNBERG L, MJ DENIRO 1983 Isotopic composition of cellulose from C_3, C_4, and CAM plants growing in the vicinity of one another. Science 220: 947–948
18. STERNBERG L, MJ DENIRO 1983 Biogeochemical implications of the isotopic equilibrium fractionation factor between the oxygen atoms of acetone and water. Geochim Cosmochim Acta 47: 2271–2274
19. STERNBERG L, MJ DENIRO, IP TING 1984 Carbon, hydrogen and oxygen isotope ratios of plants having intermediary photosynthetic modes. Plant Physiol 74: 104–107
20. STERNBERG L, MJ DENIRO, HB JOHNSON 1984 Isotope ratios of cellulose from plants having different photosynthetic pathways. Plant Physiol 74: 557–561
21. WILLENBRINK J, BP KREMER, K SCHMITZ, LM SRIVASTARA 1979 Photosynthetic and light-independent carbon fixation in *Macrocystis*, *Nereocystis*, and some related Pacific Laminariales. Can J Bot 57: 890–897
22. ZIEGLER H, CB OSMOND, W STICKLER, D TRIMBORN 1976 Hydrogen isotope discrimination in higher plants: correlation with photosynthetic pathways and environment. Planta 128: 85–92
23. ZIEGLER H 1979 Diskriminierung von Kohlenstoff-und Wasserstoffisotopen: Zusammenhange mit dem Photosynthesemechanismus und den Standortbedingungen. Ber Dtsch Bot Ges Bd 92: 169–184