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Carbon, Hydrogen, and Oxygen Isotope Ratios of Cellulose from Plants Having Intermediary Photosynthetic Modes[1]

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ABSTRACT

Carbon and hydrogen isotope ratios of cellulose nitrate and oxygen isotope ratios of cellulose from species of greenhouse plants having different photosynthetic modes were determined. When hydrogen isotope ratios are plotted against carbon isotope ratios, four clusters of points are discernible, each representing different photosynthetic modes: C_3 plants, C_4 plants, CAM plants, and C_3 plants that can shift to CAM or show the phenomenon referred to as CAM-cycling. The combination of oxygen and carbon isotope ratios does not distinguish among the different photosynthetic modes. Analysis of the carbon and hydrogen isotope ratios of cellulose nitrate should prove useful for screening different photosynthetic modes in field specimens that grew near one another. This method will be particularly useful for detection of plants which show CAM-cycling.

There are three major photosynthetic pathways in plants: C_3,[2] C_4, and CAM. Carbohydrates in all three are synthesized by the Calvin cycle. In C_3 plants, CO_2 is fixed directly into the Calvin cycle, with the primary carboxylation product being a three-carbon compound (11). In C_4 and CAM plants, the initial carboxylation product is a four-carbon compound (10, 11) which is subsequently decarboxylated, with the resulting CO_2 being processed by the Calvin cycle as in C_3 plants. C_4 and CAM plants differ in that the first carboxylation reaction in C_4 plants is spatially separated from the Calvin cycle whereas in CAM plants it is temporally separated. In C_3 and C_4 plants, all CO_2 uptake occurs during the day, while in CAM plants CO_2 uptake occurs during the night (11). CAM plants also display a diurnal fluctuation in titratable acidity which is not observed for C_3 or C_4 plants (9).

In addition to these three major photosynthetic pathways, a number of variants have been discovered in recent years. The capacity of some C_3 plants to shift to CAM under specific environmental conditions is well documented for several species (11). A modification of CAM (referred to here as CAM-cycling[3]) has recently been reported (21). In species with CAM-cycling there is diurnal fluctuation of organic acids, but all gas exchange occurs during the day (9, 14, 21). More remarkable is the recent observation that in response to water stress some plants will shift directly from CAM-cycling to CAM-idling without going into CAM. This shift has been reported in *Peperomia obtusifolia*, *Peperomia peltifolia*, *Pereskia grandifolia*, and *Pereskia aculeata* (15).

One method of studying different photosynthetic pathways involves analysis of the stable carbon isotope ratios ($^{13}C/^{12}C$ ratios) of plant tissues. C_3 plants have characteristically lower $\delta^{13}C$ values (see "Materials and Methods" for definition of δ) than C_4 and CAM plants (1). $\delta^{13}C$ values, however, cannot be used to distinguish C_4 plants from CAM plants when the latter have grown in the CAM mode (1). Ziegler *et al.* (25) observed that the D/H ratios of CAM plants were much higher than those of C_3 and C_4 plants and that C_4 plants had higher δD values than C_3 plants for greenhouse-grown plants. These observations involved analysis of δD values for total plant matter. There are several problems associated with measurements of δD values of total plant matter which can be eliminated by measuring δD values of cellulose nitrate (6). Recently, Sternberg and DeNiro (17) demonstrated that hydrogen isotope ratios of cellulose nitrate in CAM plants were much higher than those of C_3 or C_4 plants growing in the field at the same site in Riverside County, California. CAM plants had δD values of $+56 \pm 13‰$ ($n = 11$), while C_3 and C_4 plants had δD values of $-69 \pm 35‰$ ($n = 9$) and $-27 \pm 4‰$ ($n = 2$), respectively. A second sample set containing all three photosynthetic modes collected in Val Verde County, Texas (L. Sternberg, M. J. DeNiro, and H. B. Johnson, submitted for publication) showed a similar distribution of cellulose nitrate hydrogen isotope ratios: CAM plants had δD values of $+51 \pm 10‰$ ($n = 12$) while C_3 and C_4 plants had δD values of $-42 \pm 17‰$ ($n = 16$) and $-33 \pm 12‰$ ($n = 18$), respectively. The results of these two studies indicate that for plants growing in the field at the same location, combined analysis of the $\delta^{13}C$ and δD values of cellulose nitrate allows for complete discrimination among C_3, C_4, and CAM plants.

In this study, we extend measurements of carbon and hydrogen isotope ratios of cellulose nitrate to several species of greenhouse-grown plants that shift from C_3 to CAM or show CAM-cycling. Our results indicate the C_3 plants that can shift to CAM or show

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[2] Abbreviations: C_3 plants, plants in which the primary carboxylation occurs by ribulose bisphosphate carboxylase; C_4 plants, plants having Kranz anatomy in which the primary carboxylation occurs via P-enolpyruvate carboxylase; CAM plants, succulent plants having a diurnal acid flux in which the primary carboxylation occurs via P-enolpyruvate carboxylase; PDB, belemnite from the Peedee formation of South Carolina; SMOW, standard mean ocean water; PEP, P-enolpyruvate; RuBP, ribulose bisphosphate.

[3] In this paper, we use the term CAM-idling to refer to diurnal fluctuation of organic acid by plants whose stomates are closed both day and night, and the term CAM-cycling to refer to diurnal fluctuation of organic acid by plants whose stomates are open during the day and closed at night. In both cases, respiratory CO_2 is evidently fixed into malic acid at night.

CAM-cycling have higher δD values than obligate C_3 plants even when grown under conditions in which they show C_3-type gas exchange.

MATERIALS AND METHODS

Plants were grown from cuttings in a greenhouse in Riverside, California with an annual mean high temperature of 28°C and a mean low of 22°C. Plants were watered frequently to avoid water stress. Titratable acidity and CO_2 uptake were measured as described in Ting *et al.* (21). Samples of plant material for isotopic analysis were collected in one afternoon, air-dried at 50°C for several days, further desiccated in a freeze dryer, and then ground to a fine powder in a Wiley mill. Cellulose was extracted by the method of Wise (24). Cellulose oxygen isotope ratios were determined by the method of Rittenberg and Ponticorvo (16) as modified by Burk (2). Carbon and hydrogen isotope ratios of cellulose nitrate, prepared from cellulose as described elsewhere (3) were determined by a modified version of the Stump and Frazer method (13, 18). Isotope ratios are expressed as δ values, where

$$\delta = \left[\frac{R_{SAMPLE}}{R_{STANDARD}} - 1\right] \times 1000‰$$

and R represents $^{18}O/^{16}O$ for $\delta^{18}O$ values, D/H for δD values, and $^{13}C/^{12}C$ for $\delta^{13}C$ values. The standards are SMOW for $\delta^{18}O$ and δD values and the PDB carbonate for $\delta^{13}C$ values. The precision of the isotope analyses of cellulose and cellulose nitrate were ±2‰ for δD values, ±0.5‰ for $\delta^{18}O$ values, and ±0.2‰ for $\delta^{13}C$ values.

RESULTS

Based on biochemical and physiological characteristics, the plants which we analyzed were classified into five photosynthetic modes: (a) CAM plants, which have CO_2 uptake and stomatal conductance during the night and considerable diurnal acid flux (11); (b) obligate C_3 plants, which do not show diurnal acid fluctuation or a measurable amount of CO_2 uptake during the night (11); (c) C_4 plants, which, like C_3 plants, have no CO_2 uptake during the night and no diurnal acid fluctuation, but have Kranz anatomy and utilize the Hatch and Slack pathway (10); (d) $C_3 \rightarrow$ CAM plants, which behave as C_3 plants when well watered, but shift to CAM when water stressed (11); and (e) CAM-cycling plants, which show C_3-type gas exchange, but have diurnal fluctuation of organic acids. The criteria for classifying sampled plants into one of the five categories were based on previous publications or our own observations, as indicated in Table I.

Results of our isotopic analysis are shown in Table I and Figures 1, 2, and 3. Table I contains the $\delta^{13}C$ and δD values of cellulose nitrate, the $\delta^{18}O$ values of cellulose, and the photosynthetic mode for each species specified. Figure 1 shows the relationship between hydrogen and carbon isotope ratios of cellulose nitrate for the plants we analyzed. Figure 2 shows the relationship between oxygen and carbon isotope ratios of cellulose and cellulose nitrate, respectively. Figure 3 shows the relationship between the δD values of cellulose nitrate and the $\delta^{18}O$ values of cellulose.

DISCUSSION

Plants sharing the same photosynthetic mode are grouped in the plot of δD *versus* $\delta^{13}C$ values of cellulose nitrate shown in Figure 1. C_3 plants that shift either to CAM or have CAM-cycling are plotted as a single group, since no significant differences were observed between their δD or $\delta^{13}C$ values. Each of the four groups

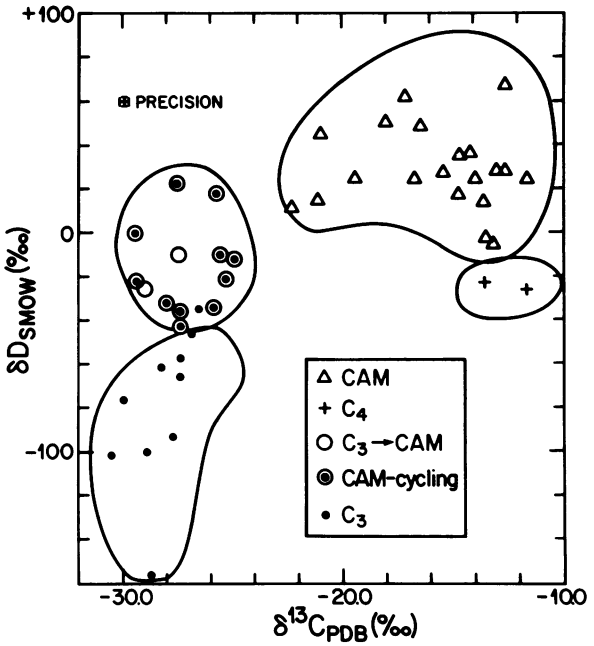

FIG. 1. δD values of cellulose nitrate plotted against $\delta^{13}C$ values of cellulose nitrate.

of plants segregate into different areas of the δD *versus* $\delta^{13}C$ plot. CAM plants are clustered in the upper right corner of the graph, C_4 plants are just below the CAM group, and C_3 plants are in the lower left corner of the graph. This distribution is unlike that for field samples, in which C_4 plants had δD values comparable to those of C_3 plants and much lower than the δD values observed for CAM plants (17; Sternberg *et al.*, submitted; I. P. Ting, L. Sternberg, and M. J. DeNiro, unpublished data). The C_3 plants that shift to CAM or show CAM-cycling cluster in the upper left corner of the δD *versus* $\delta^{13}C$ plot.

When oxygen isotope ratios of cellulose are plotted against carbon isotope ratios of cellulose nitrate, no distinct clustering of photosynthetic modes is apparent (Fig. 2). Within the group of plants with $\delta^{13}C$ values ranging from −30‰ to −25‰, there is a broad overlap in the oxygen isotope ratio among C_3 plants having $\delta^{18}O$ values ranging from +18.2‰ to +30.7‰, CAM-cycling plants having $\delta^{18}O$ values ranging from +25.4‰ to +32.9‰ and $C_3 \rightarrow$ CAM plants having $\delta^{18}O$ values ranging from +27.1‰ to +28.4‰. There is also considerable overlap in the oxygen isotope ratios of C_3 and CAM plants.

We suspect that field samples encompassing these photosynthetic modes would show a different distribution in a plot of cellulose nitrate δD values *versus* $\delta^{13}C$ values. The upper left cluster with CAM-cycling and $C_3 \rightarrow$ CAM plants (Fig. 1) would probably be composed mostly of CAM-cycling plants. Under field conditions, a certain amount of water stress would probably occur, so that $C_3 \rightarrow$ CAM plants would be operating in the CAM mode part of the time. This would cause the $\delta^{13}C$ values of $C_3 \rightarrow$ CAM plants to move towards values typical of CAM plants. Hence, $C_3 \rightarrow$ CAM plants would shift over towards the CAM cluster on a plot of δD *versus* $\delta^{13}C$ values. The $\delta^{13}C$ values of CAM-cycling plants will not be affected by the availability of water, because there is no net nocturnal fixation of CO_2 in these plants regardless of the watering regime. Thus, CAM-cycling plants should occupy the same part of a plot of δD *versus* $\delta^{13}C$ values under any conditions. It should thus be possible to distinguish CAM-cycling plants from C_3 plants based on differences in the δD values of their cellulose nitrate.

Considerable variation was observed in the hydrogen isotope ratios of cellulose nitrate from the plants sampled here (Table I).

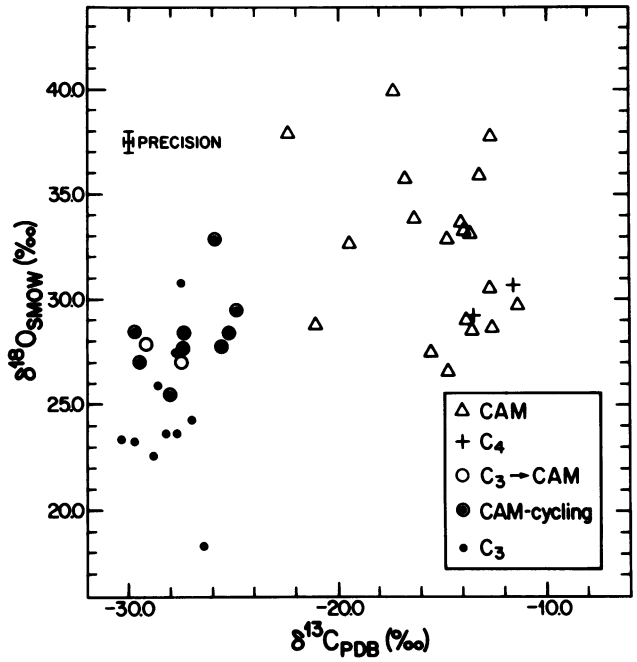

FIG. 2. $\delta^{18}O$ values of cellulose plotted against $\delta^{13}C$ values of cellulose nitrate.

A pattern emerges from this sample set when the δD values are considered in terms of the photosynthetic modes the plants utilize: CAM plants had the most positive δD values, with mean values of +30 ± 19‰ (n = 20), while C_3 and C_4 plants had more negative δD values, averaging −80 ± 35‰ (n = 10) and −25 ± 2‰ (n = 2), respectively. CAM-cycling and $C_3 \rightarrow$ CAM plants had δD values for cellulose nitrate of −16 ± 22‰ (n = 11) and −19 ± 12‰ (n = 2), respectively. This is the first report of δD values for plants which show CAM-cycling. Our observations for $C_3 \rightarrow$ CAM plants are consistent with those of Ziegler *et al.* (25), who reported that the total organic matter of plants capable of undergoing the C_3 to CAM shift have higher δD values than those of C_3 plants, even when the former are performing C_3 photosynthesis.

Ziegler *et al.* (25) proposed that CAM plants are enriched in deuterium relative to C_3 and C_4 plants because of their ability to maintain metabolic activity under water stress. They argued that, under water stress, plant water becomes enriched in deuterium during evaotranspiration (23), and this enrichment is passed on to the organically bound hydrogen. If this proposal is correct, plants with higher cellulose nitrate δD values should also have higher cellulose $\delta^{18}O$ values, since evapotranspiration also causes enrichment of ^{18}O in plant water (8) and consequently in cellulose (4). Thus, there should be a correlation between δD values of cellulose nitrate and $\delta^{18}O$ values of cellulose. We did not observe such a correlation in two previous sample sets (17; Sternberg *et al.*, submitted) and concluded that isotopic fractionations occurring during biochemical reactions, rather than those occurring during evapotranspiration, are responsible for the hydrogen isotope differences between CAM plants and C_3 and C_4 plants. In this sample of greenhouse-grown plants, there is a weak but significant correlation (r = 0.63; m = 6.88; b = −212) between cellulose nitrate δD values and cellulose $\delta^{18}O$ values (Fig. 3). We suggest that this correlation is somehow related to the fact that the plants were grown in an artificial environment, because field-collected samples growing in a single location and encompassing all the photosynthetic modes analyzed here show no such correlation (Ting *et al.* unpublished data).

We did not observe any significant correlation (r = 0.07; m = 0.409; b = 35.52) between δD and $\delta^{13}C$ values of cellulose nitrate in CAM plants (Fig. 1). In contrast, Ziegler *et al.* (25) observed a correlation between δD and $\delta^{13}C$ values of total organic matter of CAM plants. The basis for the difference between these observations can be understood by considering the factors which

Table I. *Hydrogen and Carbon Isotope Ratios of Cellulose Nitrate and Oxygen Isotope Ratios of Cellulose for Plants of the Indicated Photosynthetic Mode*

The information used to determine photosynthetic mode is indicated under the Reference column.

Plant Species	δD	$\delta^{13}C$	$\delta^{18}O$	Reference
		‰		
C_3 plants				
Lycopersicum esculentum	−157	−28.7	+25.9	[a]
Nicotiana tabacum	−100	−28.9	+22.5	[a]
Persea americana	−35	−26.4	+23.0	[a]
Parthenium argentatum	−77	−29.9	+23.0	[a]
Rhizophora mangle	−47	−27.0	+24.2	[a]
Frankenia grandifolia	−62	−28.2	+23.7	[a]
Samanea saman	−67	−27.6	+23.7	[a]
Glycine max	−94	−27.7	+27.3	[a]
Senecio vulgaris	−107	−30.4	+23.4	[a]
Senecio tropaeolifolius	−60	−27.4	+30.7	[b]
CAM-cycling plants				
Cissus tuberosa	−36	−25.8		[b]
Pelargonium crassicaule	−33	−28.0	+25.4	[b]
Peperomia orba	−1	−29.5	+27.0	[b]
Senecio mikaniodes	−23	−25.2	+28.3	[b]
Cissus quadrangularis (leaf)	+19	−25.9	+32.9	22
Adenia keramanthus	−22	−29.9	+28.3	[b]
Peperomia peltifolia	−12	−25.5	+27.7	9
Peperomia obtusifolia	−14	−24.9	+29.6	9
Pereskia grandifolia	−44	−27.4	+27.6	14
Pereskiopsis velutina	−38	−27.5		14
Peperomia campotrichia	+23	−27.7	+28.4	[b]
$C_3 \rightarrow$ CAM plants				
Carpobrotus edulis	−28	−29.2	+27.8	[c]
Portulacaria afra	−10	−27.5	+27.1	20
CAM plants				
Alluaudia humbertii	+30	−12.9	+28.1	[b]
Cissus quadrangularis (stem)	+48	−21.2	+28.9	22
Opuntia basilaris	+63	−17.2	+40.0	[c]
Bryophyllum tubiflorum	+27	−16.0	+25.8	[c]
Cereus peruvianus	+66	−12.3	+30.6	[c]
Euphorbia resinifera	+29	−12.7	+37.7	[b]
Hoya kerri	+26	−14.1	+33.2	[b]
Zygocactus truncatus	+50	−16.4	+33.8	[c]
Peperomia scandens	+14	−22.3	+38.0	[b]
Senecio articulata	−7	−13.3	+35.6	[b]
Ananas comosus	+26	−11.7	+29.8	[b]
Hoya carnosa	+29	−15.4	+27.3	[c]
Bryophyllum daigremontiana	+37	−14.2	+33.4	[c]
Xerosicyos danguyi	+38	−14.6	+26.4	[c]
Cissus quinguangularis	+17	−21.2		[b]
Aloe vera	−1	−13.7	+28.4	[c]
Senecio medley-woodii	+50	−13.9	+33.3	[b]
Yucca schidigera	+17	−13.9	+29.0	12
Anacampseros baeseckei	+26	−19.5	+32.8	[b]
Senecio radicans	+18	−14.9	+33.0	[b]
C_4 plants				
Cynodon dactylon	−24	−13.5	+29.1	5
Distichlis stricta	−27	−11.7	+30.6	5

[a] Based on general knowledge, lack of succulency, or absence of Kranz anatomy.

[b] Unpublished observations on gas exchange and titratable acidity.

[c] Sternberg *et al.*, submitted.

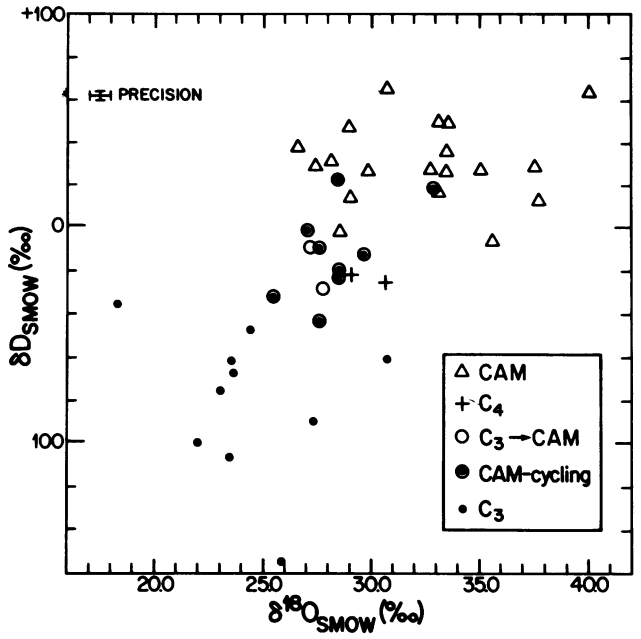

FIG. 3. δD values of cellulose nitrate plotted against $\delta^{18}O$ values of cellulose.

affect the $\delta^{13}C$ and δD values of CAM plants. The $\delta^{13}C$ values of CAM plants are determined primarily by the relative proportions of carbon fixed by PEP carboxylase and by RuBP carboxylase. Plants utilizing CAM (and thus fixing CO_2 primarily via PEP carboxylase) have higher $\delta^{13}C$ values than when they grow in the C_3 mode (in which CO_2 is fixed by RuBP carboxylase). The δD values of CAM plants (as well as those of other plants) are influenced by the D/H ratios of the meteoric waters available to the plants and by the isotopic fractionations that occur during water uptake and subsequent metabolism. Hydrogen isotope ratios of the meteoric water available to plants varies with geograhical location, such that meteoric waters from warmer and drier regions have higher D/H ratios than waters from cooler and more humid regions (7). In the study of Ziegler *et al.* (25), plants which were utilizing CAM were growing in warmer and drier environments than plants which were utilizing the C_3 pathway. Thus, the correlation between δD and $\delta^{13}C$ values observed by Ziegler *et al.* (25) is not necessarily related to physiological factors. In our greenhouse sample set, this complication is eliminated, since all plants were watered with water of the same isotopic composition. The range in $\delta^{13}C$ values (−22‰ to −12‰) indicates that the plants we analyzed ranged from those fixing most of their CO_2 via RuBP carboxylase to those in which CO_2 is fixed almost exclusively by PEP carboxylase (9). The absence of a significant correlation between δD and $\delta^{13}C$ values indicates that there are processes occurring in CAM plants which affect hydrogen isotope ratios independent of those that determine the carbon isotope ratios.

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