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Hydrogen and Oxygen Isotopic Fractionation During Heterotrophic Cellulose Synthesis

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ABSTRACT

Hydrogen and oxygen isotopic fractionation relative to medium water for two different carbohydrate metabolic pathways leading to cellulose synthesis were measured. This was accomplished by analysing stable hydrogen and oxygen isotope ratios of water and cellulose for seedlings. The seedlings had been germinated and heterotrophically grown in closed vessels from species having starch (*Triticum aestivum* L. and *Hordeum vulgare* L.) and lipids (*Ricinus communis* L. and *Arachis hypogaea* L.) as the primary substrate. Isotopic fractionation factors occurring during enzyme-mediated exchange of carbon-bound hydrogen with water or the addition of carbon-bound hydrogens from water during the synthesis of cellulose from either starch or lipids were similar (ranging from +144 to +166‰). About 34% and 67% of carbon-bound hydrogens were derived from water during the synthesis of cellulose from starch and lipid, respectively. Thus, the greater deuterium enrichment in cellulose from oil seed species associated with gluconeogenesis was caused by a greater proportion of water-derived carbon-bound hydrogens and not because of differences in fractionation factors. The proportion of carbon-bound hydrogens derived from water during these metabolic pathways was similar to that of oxygen derived from water. These results may explain the variability in D/H ratios of cellulose nitrate from terrestrial and aquatic plants.

Key words: δD, $\delta^{18}O$, cellulose, lipid, starch.

INTRODUCTION

D/H ratios of extracted and nitrated cellulose from terrestrial plants are quite variable, ranging from values showing deuterium enrichment to values showing depletion relative to the water available for photosynthesis (Ziegler, Osmond, Stichler, and Trimborn, 1976; Sternberg and DeNiro, 1983; Sternberg, DeNiro, and Ajie, 1984*a*; Luo and Sternberg, 1991). For terrestrial plants, this pattern is most significant when plants having Crassulacean Acid Metabolism (CAM) are compared to those having the C_3 or C_4 pathway. δD values of cellulose nitrate from CAM plants can be 100‰ higher than those of C_3 and C_4 plants. The variability in hydrogen isotopic composition of cellulose from terrestrial plants extends to submerged aquatic plants as well (Sternberg, DeNiro, and Keeley, 1984*b*). Thus, the observed variability in D/H should be attributed to biochemical effects rather than to evapotranspirative effects. This biochemical effect was further demonstrated by hydrogen isotopic analysis of the saponifiable lipid fraction of aquatic and terrestrial plants which showed little variability when compared to the variability of δD values of cellulose nitrate (Sternberg *et al.*, 1984*a*; Sternberg, DeNiro, and Ajie, 1986*a*). Thus, Sternberg *et al.* (1984*a*) hypothesized that variability in δD values of cellulose nitrate of plants (including CAM) is primarily due to isotopic effects occurring during carbohydrate metabolism. This hypothesis has been in part substantiated by the observations of Luo and Sternberg (1991), who observed higher δD values of leaf cellulose relative to that of leaf starch, indicating significant isotopic enrichment from the pathway of starch to cellulose.

We know of only one paper reporting on the hydrogen isotopic fractionation during carbohydrate metabolism. Yakir and DeNiro (1990) observed that heterotrophic plant cultures utilizing sucrose as the carbon substrate derived about 40% of their hydrogen from the medium water during cellulose synthesis. They also observed that the δD values of the hydrogen derived from water was about 158‰ higher than the δD value of the available water. In order to understand further the possible fractionations occurring during carbohydrate metabolism, we

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studied isotopic ratios of cellulose synthesized from either starch or lipids in the presence of water with a known isotopic composition. This was accomplished by heterotrophically growing seedlings having either starch (*Triticum aestivum* L. and *Hordeum vulgare* L.) or lipids (*Ricinus communis* L. and *Arachis hypogaea* L.) as their main substrate. The pathway leading from starch to cellulose only involves the hydrolysis of glucose α-1–4linkages and re-polymerization into β-1–4 linkages. Thus, one would expect that water would have a minimal isotopic effect during this metabolic pathway. With respect to cellulose synthesis from lipid, one would also expect a minimal isotopic effect of medium water, since lipids are saturated with hydrogen. On the other hand we would expect, for lipids, maximal oxygen exchange or derivation from water since lipids are relatively low in oxygen content. Previous studies, however, indicate that oxygen exchange during cellulose synthesis from lipids is similar to carrot tissue cultures grown on a glycerol substrate (Sternberg, DeNiro and Savidge, 1986*b*). The authors thus hypothesized that for lipid-storing seeds, glycerol from lipid hydrolysis must be the substrate for cellulose synthesis.

MATERIALS AND METHODS

Starch-storing fruits (*Triticum aestivum* L. and *Hordeum vulgare* L.), and lipid-storing seeds (*Ricinus communis* L. and *Arachis hypogaea* L.) were sterilized by soaking in 70% ethanol for 4 min and transferred to 20% commercial bleach for about 10 min. The materials were then washed three times with pre-sterilized water and spread on a pre-sterilized screen to remove excess moisture from the surface of the seeds. These treated seeds and fruits were sown in pre-sterilized plastic vessels with a medium containing different concentrations of deuterium and oxygen-18 in water. The vessels were capped, sealed with parafilm, to prevent evaporation, and transferred to a dark growth chamber for 10 d at 28 °C. Seedlings were collected, with careful removal of fruit or seed skin and seed leaves. These materials were then oven-dried at 70 °C.

Procedures for purification and nitration of cellulose and seed starch, isolation of hydrogen and carbon from nitrated cellulose and starch, and for isolation of oxygen from purified cellulose and starch all followed previous methods (Sternberg, 1989). Before nitration, starch samples (about 150 mg) were treated with 0·5 cm^3 85% phosphoric acid to dissolve granules and expose hydroxyl groups to the nitration solution. Lipids from the lipid-storing seeds were extracted and saponified by the method described by Northfelt, DeNiro, and Epstein (1981). The procedure for $\delta^{18}O$ determination of water samples was as in Epstein and Mayeda (1953).

All mass spectrometer analyses were performed on a VG-Isogas mass spectrometer (Dept. of Geology, University of Miami). Results are reported in δ values (‰), where

$$\delta(‰) = [(R_{sample}/R_{standard}) - 1] \times 10^3 \quad (1)$$

and the R is the D/H or $^{18}O/^{16}O$ ratios of sample or standard, respectively. All δD and $\delta^{18}O$ values are reported relative to SMOW.

RESULTS

The δD values of nitrated starch and $\delta^{18}O$ values of starch in fruits of *Triticum aestivum* L. and *Hordeum vulgare* L. and the δD values of saponified lipids in seeds of *Ricinus communis* L. and *Arachis hypogaea* L. are shown in Table 1.

The δD values of cellulose nitrate and $\delta^{18}O$ values of cellulose from the seedlings versus the respective δ values of water where the seedlings were grown are shown in Fig. 1. The δD and $\delta^{18}O$ values of cellulose for all species increased linearly with increasing concentration of deuterium and oxygen-18 in available water. All regression coefficients were above 0·97 with $P < 0·01$. Linear regression slopes of the two starch species were, on average, 0·34 and 0·35 for δD and $\delta^{18}O$ values, respectively, while for the oil storing seeds, the mean values were 0·67 and 0·71 for δD and $\delta^{18}O$ values, respectively. The relationship between the proportion of water-derived hydrogen and water-derived oxygen for each substrate is shown in Fig. 2. Fractionation factors for enzyme-mediated exchange or addition of carbon-bound hydrogens from medium water are shown in Table 2.

DISCUSSION

The relationship between δD and $\delta^{18}O$ values of heterotrophically produced cellulose versus the respective δD and $\delta^{18}O$ values of water available can be interpreted by the following equation:

$$\delta_{cellulose} = n(\delta_{water} + E) + (1 - n)\delta_{NE} \quad (2)$$

where $\delta_{cellulose}$, δ_{water} and δ_{NE} are the δD or $\delta^{18}O$ values of synthesized cellulose, water, and the non-exchangeable stable isotopes of the substrate, respectively (Sternberg *et al.*, 1986*b*). The slope (n) for the relationship indicates the proportion of carbon-bound hydrogen or oxygen that was derived from the medium water, during the pathway of cellulose synthesis, either through enzyme-mediated exchange or addition. The fractionation factor E for the enzyme-mediated exchange or addition reaction can be solved by using the value of the intercept for the above relationship and the following equation with certain assumptions (Sternberg *et al.*, 1986*b*):

$$\text{Intercept} = nE + (1 - n)\delta_{NE} \quad (3)$$

TABLE 1. *The δD and/or $\delta^{18}O$ values of main storage materials, starch in fruits of* Triticum aestivum *L. and* Hordeum vulgare *L., and lipid in seeds of* Ricinus communis *L. and* Arachis hypogaea *L.*

Species	δD (‰)		$\delta^{18}O$ (‰)
	Nitrated starch	Saponified lipids	Starch
T. aestivum L.	−83	—	+29·5
H. vulgare L.	−74	—	+30·1
R. communis L.	—	−136	—
A. hypogaea L.	—	−196	—

TABLE 2. *Fractionation factors for the addition of carbon-bound hydrogens or oxygens from medium water during carbohydrate metabolism calculated as in Sternberg* et al. *(1986b)*

Species	*T. aestivum* L.	*H. vulgare* L.	*R. communis* L.	*A. hypogaea* L.
Fractionation factors (‰)				
Hydrogen isotope	+152	+166	+144	+84
Oxygen isotope	+20	+22	—	—

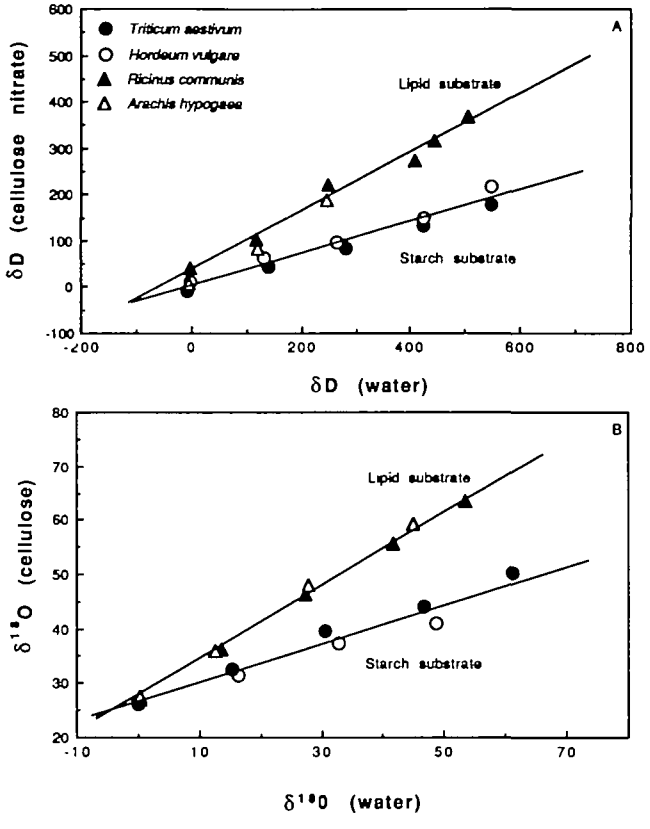

FIG. 1. The δD values of nitrated cellulose and $\delta^{18}O$ values of cellulose from seedlings versus the respective δD (A) and $\delta^{18}O$ (B) values of water where the seedlings were grown; for wheat (*Triticum aestivum* L., $\delta D_{cell} = -5{\cdot}2 + 0{\cdot}33\ \delta D_{water}$, $r^2 = 0{\cdot}998$ and $\delta^{18}O_{cell} = 26{\cdot}4 + 0{\cdot}31\ \delta^{18}O_{water}$, $r^2 = 0{\cdot}993$), barley (*Hordeum vulgare* L., $\delta D_{cell} = 11{\cdot}1 + 0{\cdot}35\ \delta D_{water}$, $r^2 = 0{\cdot}984$ and $\delta^{18}O_{cell} = 27{\cdot}1 + 0{\cdot}38\ \delta^{18}O_{water}$, $r^2 = 0{\cdot}996$), castor bean (*Ricinus communis* L. $\delta D_{cell} = 40{\cdot}6 + 0{\cdot}63\ \delta D_{water}$, $r^2 = 0{\cdot}983$ and $\delta^{18}O_{cell} = 27{\cdot}0 + 0{\cdot}69\ \delta^{18}O_{water}$, $r^2 = 0{\cdot}999$), and peanut (*Arachis hypogaea* L., $\delta D_{cell} = 5{\cdot}9 + 0{\cdot}72\ \delta D_{water}$, $r^2 = 0{\cdot}991$ and $\delta^{18}O_{cell} = 27{\cdot}3 + 0{\cdot}72\ \delta^{18}O_{water}$, $r^2 = 0{\cdot}999$). All the seeds or fruits were germinated and grown in closed vessels without light and with a temperature of 28 °C for 10 d.

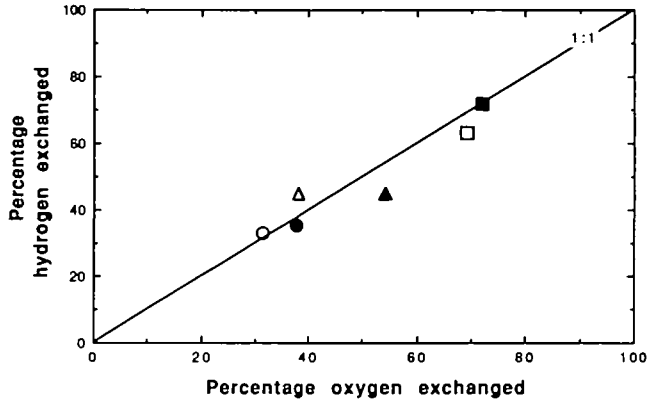

FIG. 2. Relation between the proportion of water-derived hydrogens versus water-derived oxygens during the synthesis of cellulose for various substrates. *Triticum aestivum* L., white circle; *Hordeum vulgare* L , black circle; *Ricinus communis* L., white square and *Arachis hypogaea* L., black square; *Lemna gibba* (cited from Yakir and DeNiro, 1990), white triangle; and *Zea mays* L. (Yakir unpublished data), black triangle. Line describes a 1 to 1 relationship between the two axes.

The proportion of carbon-bound hydrogen derived from water during cellulose synthesis is much greater for lipids (67%) than for starch (34%), although the reductive state of lipids is greater than carbohydrates. For any substrate, the proportion of water-derived hydrogen is the same as the proportion of water-derived oxygen (Fig. 2). It has been previously established that oxygen labelling of metabolic intermediates by water during the synthesis of cellulose occurs during carbonyl hydration in conjunction with isomerization reactions. The isomerization reactions may also explain the labelling of carbon-bound hydrogens by water during carbohydrate metabolism. Although exchange of hydrogen with water in the hexose isomerization reaction is relatively slow (50% in D-glucose-6-P→fructose-6-P), exchange can occur readily at the triose isomerase level (98% in D-glyceraldehyde-3-P→dihydroxyacetone-P, Rose 1975). Further investigations will be necessary to determine whether these reactions may in part be responsible for deuterium labelling by water during the synthesis of cellulose for different substrates.

Values for the fractionation factor for the enzyme-mediated exchange or addition reaction between carbon-bound hydrogens and water for both starch and oil seed species, except for peanut, ranged from +144 to +166‰ (Table 2), similar to +158‰ obtained by Yakir and DeNiro (1990) using *Lemna gibba* with a sucrose substrate. Thus, the greater deuterium enrichment in oil seed species can not be caused by specific chemical reactions of gluconeogenesis with a higher fractionation factor, but rather are caused by the greater amount of water-derived hydrogen during cellulose synthesis. We do not know why cellulose synthesis from peanut seeds had a lower fractionation factor of 84‰. It is possible that in peanut, with a higher protein content, carbohydrate synthesis from amino acids may have a different fractionation factor. Further, since the isomerization can be relatively slow it may be that this fractionation

factor value reflects exchanges which have not reached equilibrium.

Our results explain previously observed patterns of isotopic ratios in plants. Firstly, these data show that there is a net isotopic enrichment relative to water during the synthesis of cellulose from starch, thus explaining deuterium enrichment in leaf cellulose relative to starch (Luo and Sternberg, 1991). Secondly, this paper shows that there is a greater proportion of hydrogens added by water and hence a greater net isotopic enrichment during cellulose synthesis in the lipid-storing seeds than in the starch-storing fruits (Fig. 1). Thirdly, these findings support the conclusion by Yakir and DeNiro (1990) that water will donate a considerable proportion of hydrogens to carbohydrates during carbohydrate metabolism and that this reaction will cause an overall deuterium enrichment in carbon-bound hydrogens relative to water. In fact, the fractionation factors for these reactions are remarkably similar regardless of what substrate was used for cellulose synthesis. Finally these results may explain why cellulose nitrate from CAM plants is enriched in deuterium relative to that of C_3 and C_4 plants. We hypothesize that since CAM plants have a large amount of carbohydrates synthesized via gluconeogenesis, these carbohydrates have a greater proportion of carbon-bound hydrogens which have undergone the exchange observed here with its concomitant deuterium enrichment.

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