Plant Physiol. (1986) 82, 423–427
0032-0889/86/82/0427/05/$01.00/0

Oxygen Isotope Exchange between Metabolites and Water during Biochemical Reactions Leading to Cellulose Synthesis[1]

Received for publication January 29, 1986 and in revised form May 28, 1986

LEONEL DA S. L. STERNBERG*[2], MICHAEL J. DENIRO[3], AND ROD A. SAVIDGE
Department of Earth and Space Sciences, University of California, Los Angeles, California 90024 (L.d.S.L.S., M.J.D.N.); *and Department of Forest Resources, University of New Brunswick, Bag Service Number 44555, Fredericton, N.B. E3B 6C2* (R.A.S.)

ABSTRACT

Cellulose was produced heterotrophically from different carbon substrates by carrot tissue cultures and *Acetobacter xylinum* (a cellulose-producing bacterium) and by castor bean seeds germinated in the dark, in each case in the presence of water having known concentrations of oxygen-18 (^{18}O). We used the relationship between the amount of ^{18}O in the water and in the cellulose that was synthesized to determine the number and ^{18}O content of the substrate oxygens that exchanged with water during the reactions leading to cellulose synthesis. Our observations support the hypothesis that oxygen isotope ratios of plant cellulose are determined by isotopic exchange occurring during hydration of carbonyl groups of the intermediates of cellulose synthesis.

The relationship between the stable oxygen isotope ratios of cellulose and the water used in its synthesis is relatively constant. Previous measurements showed that the $\delta^{18}O$ values (see "Materials and Methods" for definition of $\delta^{18}O$ values) of cellulose are 27 ± 3‰ higher than the water at the site of cellulose synthesis (1, 3, 9). The same relationship has also been observed in tunicates (1), which are heterotrophic cellulose-producing organisms. Oxygen isotope ratios of cellulose, in contrast to those of carbon and nonexchangeable hydrogen, are not affected by the photosynthetic mode utilized by a plant (10, 11).

Two models have been advanced to explain the relationship between the $\delta^{18}O$ values of cellulose and water in plants. In the first (3), it was assumed that two-thirds of the oxygen entering into the Calvin cycle is derived from CO_2 that is in isotopic equilibrium with leaf water, with the remaining one-third coming from the leaf water. This model also involves the assumption that after oxygen atoms from CO_2 and H_2O are incorporated into 3-phosphoglyceric acid, they do not undergo isotopic exchange with leaf water as they pass along the pathway leading to cellulose synthesis. The oxygen isotope ratio of cellulose can then be expressed by the equation

$$\delta^{18}O(CELL) = ([\tfrac{2}{3}]*\delta^{18}O[CO_2]) + ([\tfrac{1}{3}]*\delta^{18}O[H_2O]) \quad (1)$$

where $\delta^{18}O(CELL)$ is the $\delta^{18}O$ value of cellulose, $\delta^{18}O(CO_2)$ is the $\delta^{18}O$ value of CO_2 that is in isotopic equilibrium with leaf water, and $\delta^{18}O(H_2O)$ is the $\delta^{18}O$ value of the leaf water. Since CO_2 equilibrated with water at the temperatures at which plants grow has a $\delta^{18}O$ value about 41‰ higher than that of water (3), equation (1) can be rewritten as

$$\delta^{18}O(CELL) = ([\tfrac{2}{3}]*[\delta^{18}O(H_2O) + 41‰]) + ([\tfrac{1}{3}]*\delta^{18}O[H_2O]) \quad (2)$$

which simplifies to

$$\delta^{18}O(CELL) = \delta^{18}O(H_2O) + 28‰ \quad (3)$$

This model cannot explain why the relationship between the $\delta^{18}O$ values of cellulose and water is the same for tunicates, which do not fix CO_2, as it is for plants (1). Further, DeNiro and Epstein (1) observed that there is no temperature effect on the plant cellulose-water oxygen isotopic relationship, while the isotopic relationship between CO_2 and water with which it exchanges is temperature dependent.

The second model (1) is that oxygen isotope ratios of plant and tunicate cellulose are determined by isotopic equilibration during carbonyl hydration reactions of the intermediates of cellulose synthesis, reactions having the general form

$$H_2O + R_2CO \rightleftharpoons R_2C(OH)_2 \quad (4)$$

According to this model, the $\delta^{18}O$ values of carbonyl oxygens should be 27‰ higher then those of the water with which they equilibrate and should not be affected by reactions occurring after hydration during the pathway leading to cellulose synthesis.

Sternberg and DeNiro (9) demonstrated that the $\delta^{18}O$ value of the carbonyl oxygen in a model compound, acetone, is 27‰ higher than the $\delta^{18}O$ value of the water with which it equilibrates. Further, no temperature effect was observed on the isotopic equilibrium between the carbonyl oxygen of acetone and water (9), consistent with the lack of a temperature effect observed in the relationship between the $\delta^{18}O$ values of cellulose and water. The available data thus are consistent with the proposal that carbonyl hydration reactions are responsible for establishing the oxygen isotopic relationship between cellulose and water.

In this study we tested two aspects of the carbonyl hydration model. The first was determining if isotopic exchange between carbonyl oxygens of metabolites and water occurs *in vivo*. The second involved determining the $\delta^{18}O$ values of metabolite oxygens that exchanged with water during reactions leading to cellulose formation. We grew carrot tissue cultures and *Acetobacter xylinum* (a cellulose-producing bacterium) heterotrophically and germinated castor bean seeds in the dark, all in the presence of water with known amounts of ^{18}O. We then determined (a) the percentage of oxygens in each substrate that exchanged with water during the metabolic steps leading to cellulose synthesis and (b) the magnitude of the isotopic fraction-

[1] Supported in part by National Science Foundation grant DMB 84-05003.

[2] Present address: Department of Biology, University of Miami, Coral Gables, FL 33124.

[3] Also in Archaeology Program.

ation that occurred during exchange. Our results continue to support the proposal that carbonyl hydration reactions determine the $\delta^{18}O$ values of plant cellulose.

MATERIALS AND METHODS

Carrot tissue cultures were grown as described by Murashige and Skoog (5). The growth media were prepared with waters having several $\delta^{18}O$ values and autoclaved. Cultures were grown for about 2 months, after which water from the callus tissues was extracted under vacuum as described previously (11) and reserved for isotopic analysis. Cellulose from the desiccated callus was then prepared for isotopic analysis as described elsewhere (1).

Acetobacter xylinum was grown as described by Schramm and Hestrin (8). Growth media with waters having several $\delta^{18}O$ values were prepared and autoclaved. After growth periods of about 2 weeks, liquid medium was collected by decantation, poisoned with mercuric chloride, and reserved for isotopic analysis. Cellulosic pellicles were desiccated and cellulose extracted as described previously (1).

Castor bean seeds were peeled, surface sterilized, and germinated in Erlenmeyer flasks with 75 ml of sterilized 1.3% agar solutions with waters having different $\delta^{18}O$ values. After 14 d, water from the agar of each culture was extracted by vacuum distillation and saved for isotopic analysis. Cellulose for oxygen isotope determination was extracted from the germinated seedlings (1).

Oxygen isotope ratios of water were determined by the CO_2 equilibration method (2), while those of cellulose and other organic substrates were determined following pyrolysis with mercuric chloride (3). Oxygen isotope ratios are expressed as δ values, where

$$\delta^{18}O = ([(^{18}O/^{16}O)_{sample}/(^{18}O/^{16}O)_{standard}] - 1]*1000‰ \quad (5)$$

The precision of $\delta^{18}O$ value determinations was ± 0.5‰ for organic substances and ±0.2‰ for water. $\delta^{18}O$ values are reported relative to Standard Mean Ocean Water (SMOW).

RESULTS

Figure 1 shows the relationship between the $\delta^{18}O$ values of cellulose of carrot tissue cultures and the $\delta^{18}O$ values of the water in which they were grown for experiments involving two carbon sources, glycerol and sucrose. The $\delta^{18}O$ values of heterotropically synthesized cellulose are affected by the $\delta^{18}O$ values of the water in which these cultures were grown. The least squares line is statistically significant at the $r = 0.99$ level for both glycerol and sucrose, with the line for the cultures grown on glycerol having a steeper slope than that for those grown on sucrose.

Figure 2 shows the relationship between the $\delta^{18}O$ values of cellulose from *Acetobacter xylinum* grown on glucose as a carbon source and the $\delta^{18}O$ values of the water in which the bacteria grew. At a given $\delta^{18}O$ value for water, less ^{18}O was incorporated into cellulose in *Acetobacter* than in carrot tissue cultures grown on either sucrose or glycerol.

Figure 3 shows the relationship between the $\delta^{18}O$ values of cellulose from castor bean seedlings germinated in darkness and the $\delta^{18}O$ values of the water available to them. The slope of the least squares line is similar to that observed for carrot tissue cultures grown on glycerol.

To check whether the isotopic exchange observed in tissue cultures was occurring *in vivo* and not during autoclaving, we autoclaved sucrose, glycerol, and glucose with ^{18}O-enriched water and with nonenriched water, removed the water by lyophilization, then determined the $\delta^{18}O$ values of the organic substrates. No significant differences were observed between the $\delta^{18}O$ values

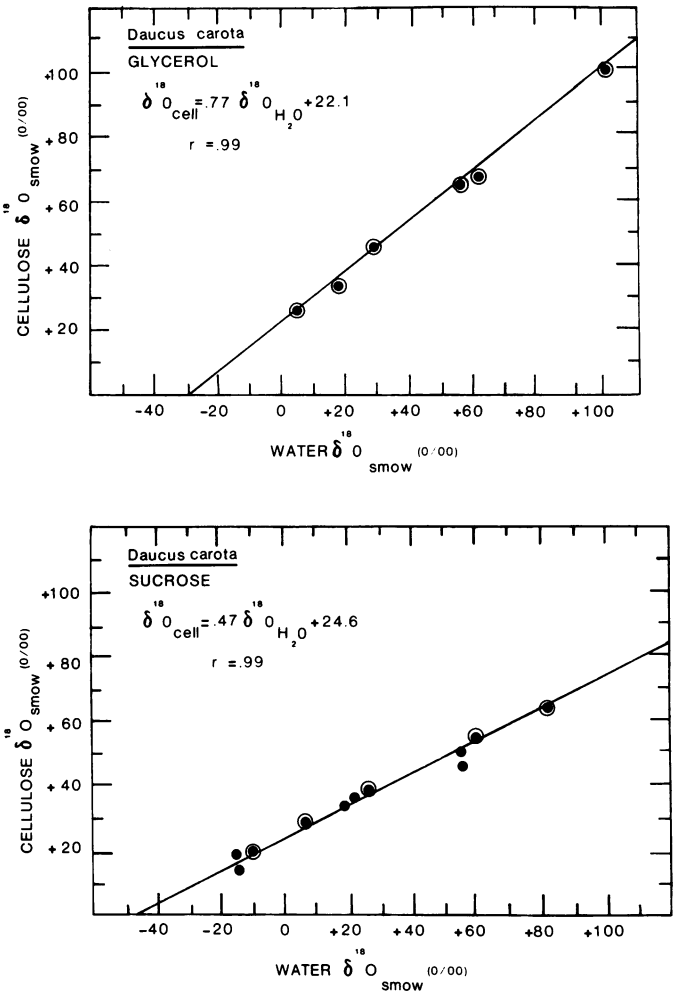

FIG. 1. Oxygen isotope ratios of cellulose from carrot tissue cultures grown on glycerol and sucrose *versus* oxygen isotope ratios of the water collected at the end of the growth period. Cultures were grown on sucrose both as suspension cultures (●) and as callus cultures (◉). For glycerol only callus cultures were grown.

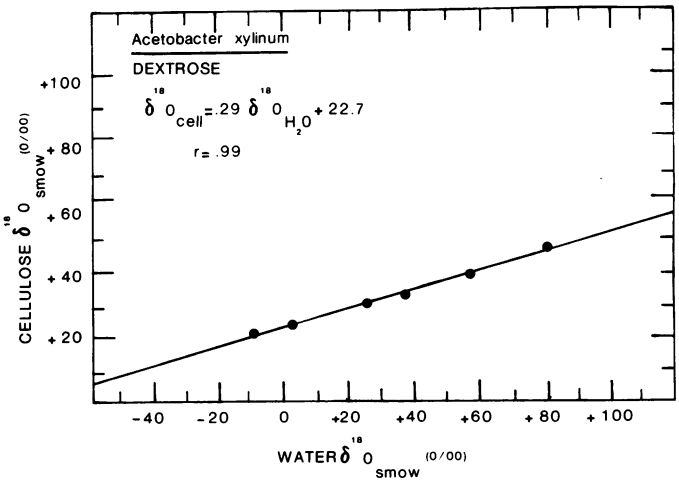

FIG. 2. Oyxgen isotope ratios of cellulose from *A. xylinum* grown in suspension on glucose *versus* oxygen isotope ratios of the water collected at the end of the growth period.

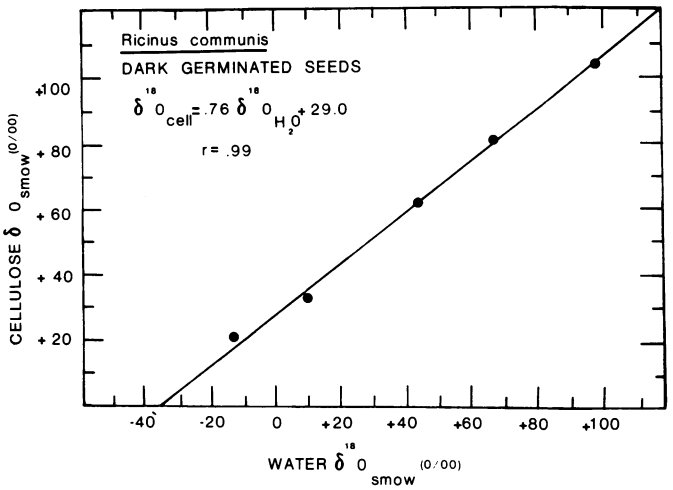

FIG. 3. Oxygen isotope ratios of cellulose from dark germinated castor bean seeds *versus* oxygen isotope ratio of the water collected at the end of the growth period.

of glycerol and sucrose autoclaved in water with different isotope ratios, but some exchange did occur with glucose (Table I). However, the exchange that occurred during autoclaving of glucose represents exchange between the carbonyl oxygen of the second carbon and water and thus would occur *in vivo* as well (Fig. 4). In the treatment discussed below, we include results from cultures grown on glucose and assume that *in vivo* processes account for our observations.

DISCUSSION

The observed linear correlation between the $\delta^{18}O$ values of cellulose and water in which the various cellulose-producing cells were grown can be interpreted as follows. The $\delta^{18}O$ value of cellulose is given by

$$\delta^{18}O(CELL) = n(\delta^{18}O[EO]) + ([1 - n]*[\delta^{18}O(NEO)]) \quad (6)$$

where n is the fraction of oxygens in a substrate that exchange during cellulose synthesis, $\delta^{18}O(EO)$ is the $\delta^{18}O$ value of the exchangeable oxygens after exchange, and $\delta^{18}O(NEO)$ is the $\delta^{18}O$ value of the nonexchangeable oxygens that do not exchange. Equation 6 can be understood by considering the extreme cases. If there is no exchange between the oxygens of the substrate and water in the steps leading to cellulose synthesis (*i.e.* n equals 0), the $\delta^{18}O$ value of the cellulose would be the same as that of the substrate and would not be affected by the $\delta^{18}O$ value of the water. Thus, the line describing the relationship between the $\delta^{18}O$ values of cellulose and water would have a slope of zero. The other extreme is when complete exchange between the oxygens of the substrate and water occurs (*i.e.* n equals 1), in which case the line describing the relationship between the $\delta^{18}O$ values of cellulose and water would have a slope of 1.

Equation (6) can be rewritten as follows

$$\delta^{18}O(CELL) = n*(\delta^{18}O[H_2O] + F) + ([1 - n]*\delta^{18}O[NEO]) \quad (7)$$

where the fractionation factor F is the difference between the $\delta^{18}O$ values of water and the exchangeable oxygen after exchange and $\delta^{18}O(H_2O)$ is the $\delta^{18}O$ value of the water available for growth. Equation 7 can be further simplified to the following:

$$\delta^{18}O(CELL) = n*\delta^{18}O(H_2O) + ([n*F] + [(1 - n)*\delta^{18}O(NEO)]) \quad (8)$$

Table I. *$\delta^{18}O$ Values of Substrates after Being Autoclaved with Waters Having $\delta^{18}O$ Values of (A) −15.2‰ and (B) +100.0‰, Respectively*

Substrate	A	B
Sucrose	+33.2‰	+33.2‰
Glucose	+24.4‰	+35.9‰
Glycerol	+4.5‰	+5.1‰

With this equation and the least squares lines shown in Figures 1 to 3, we can calculate two parameters. The first is the fraction of oxygens in the substrate that are exchangeable with water during the steps leading to cellulose synthesis. This fraction is given by n, which is the slope of the line obtained when $\delta^{18}O(CELL)$ is plotted against $\delta^{18}O(H_2O)$. The second is F, the difference in the $\delta^{18}O$ values between exchangeable oxygen (after exchange) and water, which is obtained from the intercept of the same line, which is

$$n*F + ([1 - n]*\delta^{18}O[NEO]) \quad (9)$$

To calculate F, we measured the $\delta^{18}O$ values of the substrates added to the growth media, and assumed that the $\delta^{18}O(NEO)$ values were similar to that of the total oxygen in each substrate (*i.e.* that there were not substantial intramolecular oxygen isotopic differences in the substrates). We were forced to make this assumption because methods for determining the $\delta^{18}O$ values of oxygen at different positions within molecules are not available. If this assumption is not valid, the fractionation factors, but not the percentages of exchangeable oxygen, we calculated and reported in Table II are in error.

The results of the calculations described above, given in Table II, can be used to test two aspects of the carbonyl hydration model. The first is whether or not the observed exchange of carbonyl oxygens with water is similar to that expected for a given substrate and its subsequent intermediates in the pathway leading to cellulose synthesis. The second is whether or not the carbonyl oxygens that exchange with water have $\delta^{18}O$ values 27‰ higher than the water with which they exchanged, as predicted by the carbonyl hydration model.

The amount of exchange expected for a particular substrate can be determined by counting how many of its oxygen atoms pass through carbonyl positions, the only oxygen-containing moieties that exchange under physiological conditions (7), during the pathway leading to cellulose. We did this by following the fate of each oxygen during the reactions shown in Figure 4. For glycerol, the oxygen attached to the middle carbon will become a carbonyl oxygen when glycerol is converted to dihydroxyacetone phosphate. Dihydroxyacetone phosphate will then be converted to glyceraldehyde 3-phosphate, and the oxygen attached to the first carbon will become a carbonyl oxygen. Thus, two out of three oxygens in glycerol can exchange with water. We assumed for the calculation of potentially exchangeable oxygens in glucose that glucose and fructose are in rapid equilibration with each other. For the synthesis of cellulose from glucose there are two exchangeable carbonyl oxygens (out of six total); one is on the first carbon of glucose and the other on the second carbon when glucose is converted to fructose. For sucrose, in addition to the four carbonyl oxygens (two for glucose and two for fructose), there is one oxygen that comes from water when sucrose enters the pathway at the monosaccharide level, making 5 of 11 oxygens potentially exchangeable. The results of our analysis, presented in Table II, indicate a good agreement between the predicted exchange based on carbonyl oxygen counts and that we calculated from our experimental observations.

Our calculation of the fractionation factor for the exchangeable oxygens for glycerol is in good agreement with that which would be necessary to make the $\delta^{18}O$ values of cellulose 27‰ higher than that of water at the site of synthesis (Table II). For sucrose

Table II. *Predicted and Observed Percentage of Oxygens of Different Substrates that Exchanged with Water during Cellulose Synthesis, as well as Fractionation Factors (Differences between $\delta^{18}O$ Values of Exchangeable Oxygens after Exchange and $\delta^{18}O$ Values of Water) Calculated for Different Substrates*

Species	Substrate	Exchangeable Oxygen		Fractionation Factor
		Observed	Predicted	
		%		
Daucus carota	Sucrose	47	45[a]	16.3‰
	Glycerol	77	67[b]	27.3‰
Acetobacter xylinum	Glucose	29	33[c]	15.4‰[d]
Ricinus communis	Fatty acids and/or glycerol	76	ND[e]	ND[f]

[a] 5 of 11 total oxygens exchangeable. [b] 2 of 3 total oxygens exchangeable. [c] 2 of 6 total oxygens exchangeable. [d] Calculated by assuming that the change in the $\delta^{18}O$ value of glucose that occurs during autoclaving does not affect the $\delta^{18}O$ value of the nonexchangeable oxygens. [e] Not determinable because stoichiometry of substrate not known. [f] Not determinable because of $\delta^{18}O$ (NEO) not known.

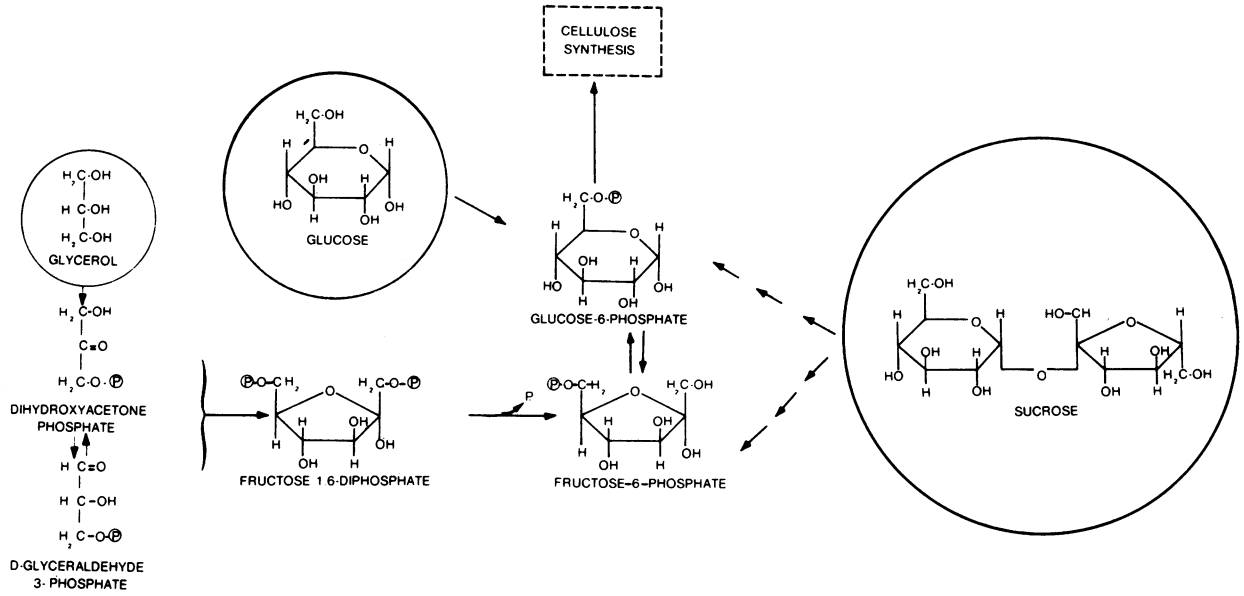

FIG. 4. Pathways to cellulose formation from different substrates used in this study.

and glucose, however, the fractionation factor is substantially less than the required 27‰. Based on consideration of the pathways by which oxygen from glycerol, glucose, and sucrose reach cellulose (Fig. 4), we propose that the oxygens of glycerol that pass through carbonyl positions reach isotopic equilibrium with water, while those of the sugars, which enter the pathway to cellulose after those from glycerol, do not. The time required for the carbonyl oxygens of the various intermediates in the cellulose pathway to reach isotopic equilibrium with water are not known. However, 50% of the oxygen in dihydroxyacetone phosphate exchanges with water and reaches isotopic equilibrium in less than 10 s, while half-equilibration of the carbonyl oxygens of fructose 6-P and fructose 1,6-diP takes 166 min and 29.5 min, respectively (4, 6). If the times required to reach isotopic equilibrium are of the same order of magnitude as the *in vitro* half-equilibration times, it seems likely that oxygens entering from glucose or sucrose will not reach equilibrium with water before they are transformed into chemical moieties (*e.g.* hydroxyl groups) that do not exchange with water under physiological conditions (7). Further study of the *in vivo* residence time of oxygen in carbonyl positions of the various intermediates shown in Figure 4 will be necessary to verify this explanation of the discrepancy between the fractionation factors observed for glucose and sucrose (Table II) and that predicted by the carbonyl hydration model.

Our results indicate that oxygen isotope exchange between metabolic intermediates and water occurs in a well integrated and relatively undisturbed system such as the germinating castor bean seeds. In the case of the castor bean seeds the substrate available for cellulose synthesis is mostly lipids, which consist primarily of glycerol and fatty acids. If the seeds were using fatty acids or total lipids as the substrate for cellulose synthesis the slope of the regression line between the $\delta^{18}O$ values of cellulose and water available for growth would be close to one because these substrates are oxygen-poor (*i.e.* most of the oxygen in cellulose would have to come from water). If the seedlings were using glycerol liberated from lipid metabolism as the substrate for cellulose synthesis a slope similar to that observed for carrot tissue cultures grown on glycerol would be expected. The observed slope of the linear regression between the $\delta^{18}O$ values of cellulose and the water used by the castor bean seedlings, 0.76, was virtually identical to that observed for carrot tissue cultures grown on glycerol, 0.77. Thus, germinating castor bean seeds may be using glycerol as the primary substrate for cellulose synthesis. $\delta^{18}O$ values were measured on glycerol prepared from lipids extracted from the castor bean seeds (method of extraction to be reported elsewhere). The fractionation factor F, calculating as above using the results shown in Figure 3 and the $\delta^{18}O$ values of this extracted glycerol for $\delta^{18}O$(NEO), was +25.6‰, a value similar to that observed for carrot tissue cultures grown on

glycerol and close to the 27‰ predicted by carbonyl hydration model.

The results presented in this study are consistent with the hypothesis that the oxygen isotope ratios of cellulose are determined by isotopic effects occurring during carbonyl hydration reactions of the intermediates of cellulose synthesis. We have observed that the percentages of oxygen that exchanged with water for different substrates agreed with the percentages expected based on counts of carbonyl oxygens in a substrate and in subsequent intermediates leading to cellulose synthesis. Thus, carbonyl oxygens readily exchange with water *in vivo*. The carbonyl oxygens that do exchange with water rapidly, such as those in the intermediates found in the pathway of cellulose synthesis from glycerol, have the fractionation factor necessary to make the $\delta^{18}O$ value of cellulose 27% higher than that of the water at the site of its synthesis. In closing, we note that every oxygen coming into the Calvin cycle passes through a carbonyl stage in phosphoglyceraldehyde, which would allow for rapid isotopic exchange between water and newly assimilated oxygen prior to cellulose formation in all plants, regardless of photosynthetic mode.

LITERATURE CITED

1. DeNiro MJ, S Epstein 1981 Isotopic composition of cellulose from aquatic organisms. Geochim Cosmochim Acta 42: 485–506
2. Epstein S, T Mayeda 1953 Variations of ^{18}O content of water from natural sources. Geochim Cosmochim Acta 42: 213–224
3. Epstein S, P Thompson, CJ Yapp 1977 Oxygen and hydrogen isotopic ratios in plant cellulose. Science 218: 1209–1215
4. Model P, L Ponticorvo, D Rittenberg 1968 Catalysis of an oxygen exchange reaction of fructose 1,6-diphosphate and fructose 1-phosphate with water by rabbit muscle aldolase. Biochemistry 7: 1339–1347
5. Murashige T, F Skoog 1962 A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473–497
6. Reynolds SJ, DW Yates, CI Pogson 1971 Dihydroxyacetone phosphate: Its structure and reactivity with α glycerophosphate dehydrogenase, aldolase and triose phosphate isomerase and some possible metabolic implications. Biochem J 122: 285–297
7. Samuel D, BL Silver 1965 Oxygen isotope exchange reactions of organic compounds. Adv Phys Org Chem 3: 123–186
8. Schramm M, S Hestrin 1954 Factors affecting production of cellulose at the air/liquid interface of a culture of *Acetobacter xylinum*. J Gen Microbiol 11: 123–129
9. Sternberg L, MJ DeNiro 1983 Biogeochemical implications of the isotopic equilibrium fractionation factor between oxygens atoms of acetone and water. Geochim Cosmochim Acta 47: 2271–2274
10. Sternberg L, MJ DeNiro, JE Keeley 1984 Hydrogen, oxygen and carbon isotope ratios of cellulose from submerged aquatic Crassulacean acid metabolism and non-Crassulacean acid metabolism plants. Plant Physiol 76: 68–70
11. Sternberg L, MJ DeNiro, ME Sloan, CC Black 1986 Compensation point and isotopic characteristics of C_3/C_4 intermediates and hybrids in *Panicum*. Plant Physiol 80: 242–245