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Site-specific natural hydrogen isotope ratios in the characterization of plant cellulose

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ABSTRACT

Site-specific natural isotope fractionation of hydrogen studied by nuclear magnetic resonance has been applied to cellulose. A combination of n.m.r. and m.s. techniques has been used to measure the isotope ratios of cellulose nitrate, glucose pentanitrate, glucose penta-acetate, and ethanol obtained by fermentation. Fractionation effects during enzymic hydrolysis, chemical derivatization, separation, and purification have been investigated.

INTRODUCTION

Although the variation of the natural-abundance isotope ratios of plant metabolic hydrogen[1] is not as well understood as that of the $^{13}C/^{12}C$ ratios in the products of photosynthesis[2,3], it is recognized that hydrogen isotope discrimination depends on the mode of photosynthesis and environmental effects[4,5]. In order to eliminate fluctuations due to the variable composition of the organic matter, isotope ratios are usually determined on pure components. Moreover, possible perturbations of the isotopic content that result from the exchange of oxygen-bound hydrogens with water can be avoided by *O*-nitration[6]. Thus, cellulose nitrate has been selected frequently as a representative isotopic probe. The overall isotope composition of its *non-exchangeable carbon-bound hydrogens*, measured by mass spectrometry after combustion of the sample, is a valuable indicator of the environmental effects during the biosynthesis. In this respect, the total isotope contents of cellulose have been used frequently to estimate variations in the climatic temperature[7,8]. However complex isotopic variations may be produced during the evapotranspiration of leaf water and the incorporation of metabolic hydrogen, so that the results are not interpreted easily on the basis of a single isotopic variable.

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A new method, namely, site-specific natural isotope fractionation studied by nuclear magnetic resonance (SNIF-NMR)[9,10] now reinforces greatly the potential of isotope m.s. for the investigation of metabolic pathways. In particular, the method provides unique information on the botanical and even the geographical and temporal origin of plant reserves[11]. Deviations with respect to a random distribution of deuterium among the diastereotopic sites of a purified soluble molecule are observable by ^{2}H-n.m.r. spectroscopy and the molar fractions (f_i) of the individual isotopomers, or of groups of isotopomers, can be calculated from the integrated areas of the corresponding signals. Site-specific ratios, $(D/H)_i$, are then accessible by using an n.m.r. referencing technique[12] or by independent determination by m.s. of the overall deuterium content $(\overline{D/H})$. Thus, the site-specific parameters of the methyl (i = I) and methylene (i = II) groups of ethanol obtained by fermentation of carbohydrates characterize the biochemical and environmental effects during biosynthesis[11]. Methodologies have been developed to investigate simple sugars and starches. For starches, glucose, and ethanol, probes are derived[13] by hydrolysis and fermentation, respectively. We have now applied the SNIF-NMR method to cellulose because of the great interest in this biopolymer as a metabolic and palaeo-climatic indicator. Since cellulose cannot be investigated directly by ^{2}H-n.m.r. spectroscopy, isotope effects have been studied during the enzymic hydrolysis of cellulose to glucose, the fermentation of glucose to ethanol, and chemical transformations.

EXPERIMENTAL

Commercial cellulose (Sigma) was purified from the trunk pulp of hardwood from Canada.

Enzymic hydrolysis. — Cellulose (4 g) was hydrolysed for 24 h at 40° in 0.05M acetate buffer (200 mL, pH 5.8) in the presence of cellulase [4 g, Onozuka R10 (1→4)-β-D-glucan glucanohydrolase (EC 3.2.1.4 from *Trichoderma viride*)]. The enzyme contained cellobiase activity that effected the complete hydrolysis of cellulose to glucose. At the end of the hydrolysis, methanol (200 mL) and chloroform (200 mL) were added to the mixture. A large insoluble fraction (residual cellulose and cellulase) was precipitated and discarded, as was the chloroform layer. The yield of the hydrolysate was ~50%. The methanol layer that contained the glucose was concentrated to dryness and the residue was dissolved in water for further purification.

A large part of the water-soluble pigments was retained by using 15 mg/mL activated carbon (Baker analyzed reagent). The suspension was filtered successively through 8- and 0.22-μm Millipore filters and loaded on to an octadecyl C18 column, preactivated with methanol–water (1:1) and eluted with water. The glucose in this eluate was purified by preparative h.p.l.c. (Waters 500) (Fig. 1). The extract was then loaded on a Si-NH$_2$ column (Prepack Bondapack NH$_2$, 10 μ, 57 × 300 mm) and eluted with acetonitrile–water (4:1) at 100 mL/min at room temperature for 38 min, and the products were detected with a differential refractometer. The eluate, which contained glucose, was then concentrated almost to dryness, the residue was redissolved in water, and the solution was concentrated. This process was repeated several times and the resulting syrup was maintained at 6° until crystallisation started. The glucose was then collected and dried on a vacuum line for 48 h.

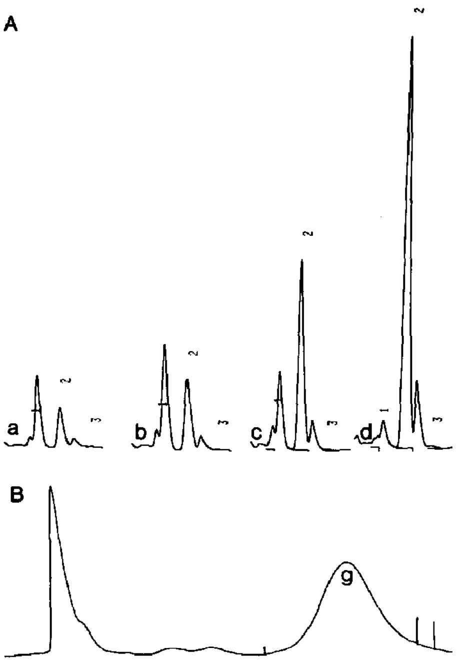

Fig. 1. A, Analytical h.p.l.c. of cellulose hydrolysate: 1(a), 2(b), 6(c), and 24 h (d) (1, cellobiose; 2, glucose; 3, unknown). B, Preparative h.p.l.c. (g, glucose).

Determination of the glucose contents. — The progress of the hydrolysis of cellulose was followed by analytical h.p.l.c. (Fig. 1). Filtered aliquots (0.45 μL) of the reaction mixture were injected into a Sugar Pack column (Waters) and eluted with water at 90° at 0.5 mL/min. The detector was a Waters 410 differential refractometer. The amount of glucose was determined by comparison with calibrated standard solutions. The presence of cellobiose as an early product of the reaction (Fig. 1) was checked by co-chromatography.

Nitration of compounds and δ^2H determination by mass spectrometry. — The procedure was adapted from refs. 14 and 15. Purified D-glucose (100 mg) was dissolved in a 2:1:1 mixture (2.4 mL) of pure HNO_3, AcOH, and Ac_2O. The mixture was stirred for 4 h, and glucose pentanitrate was then precipitated with cold water (12 mL), collected, rinsed with water to pH 5.5, and dried on a vacuum line. The same procedure was used for cellulose. Dried samples (~25 mg) of glucose and cellulose nitrate were burned in a vacuum combustion apparatus by classical procedures. Three combustions were performed for each sample. The resulting samples of water were introduced successively into the mass spectrometer (Aqua Sira equipped with a uranium furnace for the reduction of water). The last value was retained. The results are expressed either as absolute ratios in p.p.m. of ^{2}H with respect to ^{1}H, D/H, or as a relative isotope content, δD%, referred to the international standard (155.76 p.p.m.) according to the expression

$$\delta^2\mathrm{H} = \frac{(^2\mathrm{H}/^1\mathrm{H})\ \mathrm{sample} - (^2\mathrm{H}/^1\mathrm{H})\ \mathrm{standard}}{(^2\mathrm{H}/^1\mathrm{H})\ \mathrm{standard}} \times 1000.$$

The precision of the determination was ± 0.5 p.p.m., *i.e.*, ± 3‰.

Determination of δ^{13}C by m.s. — The carbon isotope composition was obtained by automatic combustion of samples (a few mg) enclosed in stain capsules and introduced into a combustion apparatus (Carlo Erba) fitted with a mass spectrometer for natural abundance isotopic determinations (Finnigan Delta E). The CO_2 that resulted from the combustion was introduced with a helium flux into the spectrometer. The apparatus was calibrated using organic compounds with known δ^{13}C values. As defined for ^{2}H, the results are expressed as δ^{13}C (± 0.3‰) with respect to the international standard.

Acetylation of D-*glucose and isotopomeric characterisation by ^{2}H-n.m.r. spectrometry.* — Spectra obtained at 61.4 MHz were insufficient for reliable quantification of different groups of isotopomers. There was a strong resonance due to the rapidly exchanging hydroxyl hydrogens. Glucose penta-acetate is a better probe for the SNIF-

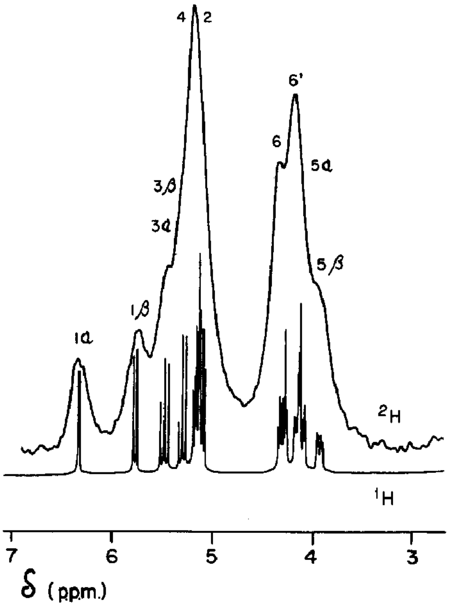

Fig. 2. The ^{1}H- (250 MHz) and ^{2}H- (61.4 MHz) n.m.r. spectra of α,β-D-glucose penta-acetate (1.3M in $CDCl_3$ and 2.6M in $CHCl_3$, respectively) prepared from purified D-glucose derived from cellulose (See Experimental).

NMR method, although the samples frequently contained different α,β-ratios. Acetylation of purified D-glucose (~1 g), performed with acetic anhydride–pyridine, gave the penta-acetate that contained 66% of the α form (Fig. 2). The proportion of the α form measured in aqueous solution tends to increase during the crystallisation.

The ^{1}H-n.m.r. spectra (250 MHz) were obtained with a Bruker WM 250 spectrometer on solutions in chloroform, and the assignments (Fig. 2) were checked by means of 2D COSY experiments. The ^{2}H-n.m.r. spectra (Fig. 2) were obtained at 61.4 MHz on a solution of D-glucose penta-acetate (0.8–1 g) in $CHCl_3$, using a Bruker AM400 spectrometer equipped with a $^2H\{^1H\}$ specific probe and an internal ^{19}F locking device. The acquisition conditions were as follows: sweep width, 2400 Hz, acquisition time, 1.70 s; broad-band decoupling; 45 000 scans were accumulated for each sample[12]. Exponential multiplication of the free induction decay associated with a line-broadening of 1 Hz was applied to improve the signal-to-noise ratio. The quantitative analysis of the different peaks was performed by Lorentzian curve fitting, using the program NMR1 (New Methods Research Inc., Syracuse, N.Y.).

Fermentation of glucose and isotopomeric characterisation by ^{2}H-n.m.r. spectroscopy. — The fermentations were conducted in Erlenmeyer flasks for 168 h on a solution of D-glucose (16.1 g) in tap water with a deuterium content of 149.9 p.p.m.[11]. Each mixture (100 mL, pH 6, 28°) contained 2 g/L of a wild strain of *Saccharomyces cerevisiae*. The deuterium content of the water medium at the end of the fermentation was 157.4 p.p.m. The ethanol was distilled under standardized conditions with a spinning-band column, and the yield recovered with a grade of 0.925 w/w was >95%. The ^{2}H-n.m.r. spectrum was obtained as described above.

DISCUSSION

Overall carbon and hydrogen isotope content. — The carbon isotope ratio is a reliable criterion for the characterization of the photosynthetic pathway, since the uptake of CO_2 is accompanied by isotope fractionation which depends on the carboxylation mechanism. In C_3 plants, CO_2 directly enters the reductive pentose phosphate cycle in a carboxylation step mediated by ribulose bisphosphate carboxylase, which is the source of significant discrimination against ^{13}C. In contrast, the C_4 pathway first involves a low fractionating carboxylation step mediated by phosphoenolpyruvate carboxylase to give C_4 molecules, malate or aspartate, that are decarboxylated to supply CO_2 to the reductive pentose phosphate cycle. Whatever the climatic environment, C_3 plants usually exhibit a systematic ^{13}C depletion of ~13‰ as compared to C_4 plants. This characteristic property is partly the result of the constancy in the δ^{13}C value of the CO_2 in the atmosphere ($\delta^{13}C = -7.4‰$)[2]. In contrast, the ^{2}H content of rains and groundwaters, which are the source of hydrogen, undergoes relatively large variations under the influence of environmental effects. The overall ^{2}H content of cellulose nitrate obtained from plant cellulose covers a large range[4–8], from ~80 to 180‰. There is no systematic distinction between C_3 and C_4 plants. However, the isotope ratio of the metabolic hydrogen of succulent plants, which exhibit a crassulacean acid metabolism (CAM), is higher than that of C_3 and, to a lesser extent, C_4 plants. This ratio is sensitive to water stress and other environmental conditions[4,17,18]. The results given in Table I for cellulose nitrate are consistent with a C_3 origin of the commercial sample. However, the $\delta^{13}C$ value (−24.4‰) is at the upper limit of the ranges for C_3 plants (−23 to −39‰). Such relatively high values have been measured also for the carbohydrate of *Acer grandidentatum*, for example[16]. Similarly, the ^2H content of the cellulose nitrate is at the upper limit of the range observed for C_3 and C_4 plants[7,8,17,18]. Such relative enrichments in the heavy isotope may be related to the isotopic composition of the absorbed water, to special biosynthetic pathways for cellulose of the hardwood trees utilized, and to environmental effects that occur during photosynthesis. In addition, fractionation effects intervening in the course of the industrial purification procedure cannot be discounted.

TABLE I

Overall isotopic parameters of cellulose, glucose, and ethanol determined by mass spectrometry

Compound		$\delta^{13}C$ (‰)	*D/H (p.p.m.)*[b]	δD (‰)[b]
Cellulose		−24.4	154.0	−11.3
Glucose	1	−22.9	159.2	+22.1
	2	−23.3	158.9	+20.2
	3	–	157.7	+12.5
	mean	−23.1	158.6	+18.3
Ethanol	1	−24.9	108.4[c]	−304.1[c]
	2	−24.4	109.3[c]	−298.3[c]

[a] Three experiments (1–3) were performed on three glucose samples, and two fermentations were performed to give the corresponding ethanols 1 and 2. [b] Measured on the nitrate derivative. [c] Measured by n.m.r. intermolecular referencing[12] for the ethyl fragment.

During the hydrolysis of cellulose, cellobiose, which appeared early in the reaction, was hydrolysed eventually (Fig. 1). The isotope ratios of glucose, obtained in three independent experiments, were satisfactorily reproducible (Table I) and were consistent with those of the cellulose, although a slight enrichment in both ^{13}C and ^2H was observed. Considering the incomplete yield of the procedure, this enrichment may result from fractionation effects that intervened during the hydrolysis or the extraction and purification steps. It may also have been influenced by a heterogeneous isotopic composition of cellulose. The occurrence of moderate fractionation effects due to incomplete recovery is not an impediment to the development of an analytical method aimed at the characterization of the origin of the starting material. However, it requires that standardized procedures are used in the extraction and derivatization steps and that calibration charts are established. Such approaches have been elaborated in the use of the ethanol probe derived from starch hydrolysis.

Site-specific re-partition in glucose. — Three sets of molecular positions are distinguished in the ^{2}H spectrum of D-glucose penta-acetates (Fig. 2). The α,β ratio can be determined from the ^{1}H-n.m.r. spectrum. As the difference in chemical shifts between the resonances of H-1 reaches 1.2 p.p.m., the area of the D-1α signal can be measured individually. The area of the D-1β signal can be estimated by curve-fitting and the results expressed in terms of mole fractions associated with the three groups of hydrogen atoms, namely, at positions 1,2/4, and 5/6 (Table II). The confidence intervals, estimated from three independent measurements performed on the same sample of D-glucose penta-acetate, were ± 0.013, ± 0.006, and ± 0.007, respectively. The mole fractions associated with the three sets of molecular sites deviate significantly from the values corresponding to a random distribution of ^{2}H. Sites 2–4 are slightly enriched with respect to sites 5–6 and site 1 is depleted significantly. The isotope ratios, $(D/H)_i$, can be calculated (Table II) by resorting to the values of the overall ^{2}H contents obtained by m.s. The results agree reasonably with those obtained for glucose derived from starches[11], although the values measured are closer to those observed for the maize C_4 species than to those of such C_3 species as wheat or potato. The relative magnitudes of the effects of biochemical and technological fractionation are being studied further.

TABLE II

Site-specific isotope parameters[a] of D-glucose penta-acetates derived from cellulose (Table I)

	Glucose					
	F_i			$(D/H)_i$		
	C-1	*C-2,3,4*	*C-5,6,6'*	*C-1*	*C-2,3,4*	*C-5,6,6'*
1	0.125	0.445	0.430	139	165	160
2	0.120	0.440	0.440	134	163	163
3	0.130	0.440	0.430	144	163	160
mean	0.125	0.442	0.433	139	164	161
Stat.	0.143	0.428_5	0.428_5			
	Ethanol					
	F_i^c			$(D/H)_i^d$		
	I		*II*	*I*		*II*
1	0.566		0.434	102.6		117.2
2	0.567		0.433	103.4		118.1
Stat.[b]	0.6		0.4			

[a] F_i refers to the mole fractions of the monodeuterated isotopomers. [b] Statistical mole fractions. [c] Only the isotopomers of the ethyl fragment are considered. I and II refer to the methyl and methylene group respectively. [d] Obtained by n.m.r. intermolecular referencing[12].

Site-specific isotope contents in ethanol obtained by fermentation. — Since ethanol obtained by fermentation is an accurate probe for inferring the isotopic behaviour of starch precursors[11], glucose obtained from cellulose has been fermented under standardized conditions. The values of the site-specific isotope ratios of the ethanol so obtained are in reasonable agreement with those of ethanol derived from starches from C_3 plants[13]. However, the relatively high value of $(D/H)_I$ reflects an enrichment of sites 1,6, and 6′ of glucose extracted from the commercial cellulose sample as compared to glucoses from wheat or barley, for example, for which $(D/H)_I$ is frequently found to be 96–100 p.p.m.

The determination of site-specific natural isotope ratios by ^{2}H-n.m.r. spectroscopy has several stringent requirements. Due to limitations in sensitivity, relatively large amounts (typically 1 g) of pure samples are needed. Because of the low resolution of ^{2}H-n.m.r. spectra, high-field n.m.r. is required for several individual isotopomers, or at least groups of isotopomers, to be distinguished. In addition, due to signal broadening associated with fast quadrupolar relaxation of deuterium, the method is not suitable for large molecules that have a slow molecular reorientation. From these points of view, the suitable spectroscopic properties of ethanol derived by fermentation ensure good reproducibility of the n.m.r. results (the confidence interval was usually better than 0.3 p.p.m. for the methyl and methylene isotope ratios). Although the situation is less favourable for D-glucose penta-acetate (Fig. 2), three sets of isotope parameters could be quantified with reasonable accuracy (confidence interval better than 1.5 p.p.m.). By defining the experimental conditions which minimize the fractionation effects or by deriving appropriate correction factors in standardized conditions, the strategy described enables sets of isotope parameters that are representative of cellulose to be obtained. Metabolic and environmental effects can now be studied by SNIF-NMR, using the widespread cellulose probe already considered as a reliable indicator by mass spectroscopists.

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