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Determination of the Natural Deuterium Distribution in Glucose from Plants having Different Photosynthetic Pathways

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The site-specific natural isotope fractionation of hydrogen in glucose was investigated by nuclear magnetic resonance spectroscopy (SNIF-NMR). Several glucose samples obtained from plants undergoing either C_3 (potato, barley, sunflower, wheat) or C_4 (maize, sorghum) photosynthetic metabolism were investigated. Since the glucose molecule is not suitable for direct SNIF-NMR analysis, several kinds of derivatives were synthesized. Three clusters of isotopomers were identified in glucose penta-acetate which is easily prepared. Up to 5–6 sites could be distinguished in 6 other derivatives. An integrated statistical analysis of the whole sets of cluster lines was performed in order to determine the individual isotope contents in the carbon-bound hydrogen positions of the glucose skeleton. Both the mole fractions of the monodeuterated isotopomers and the deuterium/hydrogen ratios of the individual sites thus become accessible. Using this approach, it is shown that similar trends in the isotopic distribution are exhibited within a given photosynthetic C_3 or C_4 family. Larger deviations with respect to a statistical distribution of deuterium are observed in glucose from C_3 plants as compared to C_4 plants. The methylenic sites 6,6′ in particular are significantly depleted in C_3 glucose as compared to C_4 glucose.

Keywords: C_3 Plants; C_4 plants; site-specific isotope contents; glucose; deuterium spectroscopy.

INTRODUCTION

Isotope labelling experiments have been extensively used in plant physiology for investigating photosynthetic pathways. Moreover mass spectrometric determination of overall natural ^{13}C, ^{2}H or ^{18}O contents of sugars have been shown to provide useful information on the biosynthesis of carbohydrates in natural environments (O'Leary, 1988; Smith and Ziegler, 1990; Yakir, 1992). In this context, site specific natural isotope fractionation studied by nuclear magnetic resonance (SNIF-NMR) (Martin and Martin, 1981) is a very attractive tool since it avoids the need for isotopic enrichment and enables information on metabolic and environmental effects to be deduced from the investigation of several molecular sites in large numbers of samples derived from plants grown under field conditions. Ethanol resulting from fermentation experiments performed in strictly controlled conditions provides a very faithful probe of the isotopic behaviour of the starting simple or complex sugars (Martin *et al.*, 1991). In particular, linear correlations have been found between the deuterium/hydrogen (D/H) isotope ratio of the methyl site of ethanol $(D/H)_I$ and the overall ratio $(\overline{D/H})$ of the non-exchangeable sites of glucose on one hand, and between the isotope ratio of the methylene site $(D/H)_{II}$ and the isotopic composition of the starting water medium $(D/H)^S_W$ on the other hand (Martin *et al.*, 1986).

However, most site-specific information contained in the glucose molecule is lost in the transformation to ethanol. Therefore it would be interesting from both a fundamental and practical point of view to have a direct and accurate access to the different isotopomers of glucose in order to connect them to both their precursors (ex. photosynthesis, neoglucogenesis) and metabolites (i.e. through glycolysis, biosynthesis etc).

A direct NMR determination of the $(D/H)_i$ ratios of glucose is not possible even at a very high field since the lines are broad and the chemical site discrimination is poor. It is necessary to prepare suitable derivatives of glucose which are fairly soluble, can be synthesized without significant isotopic fractionation and exhibit sufficient spreading of the ^{2}H chemical shifts of the non-exchangeable hydrogens. In principle up to 7 resonance signals of the carbon bound hydrogens may be expected in the ^{2}H spectrum of an appropriate glucose derivative since the prochiral methylenic sites 6,6′ are diastereotopic. However, in practice, these seven sites are rarely differentiated in a single molecular species. We have, therefore, combined results obtained for several kinds of chemical derivatives prepared from glucose extracted from plants with different carbon metabolisms, C_3 (Calvin) or C_4 (Hatch-Slack), but grown in similar environments. An integrated analysis of the whole population of isotope data has been undertaken in order to determine consistent sets of parameters and to appraise the extent by which photosynthetic metabolism exerts a detectable effect on the isotopic fingerprint.

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EXPERIMENTAL

Materials. Pure (α)-D glucose samples were obtained by hydrolysis of barley, potato, sunflower and wheat starches (C_3 plants), and of maize and sorghum starches (C_4 plants). In some cases glucose samples were purchased from commercial sources and identified unambiguously as having a C_4 origin from their $^{13}C/^{12}C$ isotope ratio.

The hydrolysis was carried out in acidic solution as follows: 50 g of starch were added to 300 mL of 2% sulphuric acid solution. The mixture was then placed in an autoclave at 120 °C for 1 h and neutralized to pH 5–6 with $Ca(OH)_2$. After elimination of the $CaSO_4$ precipitate and treatment with activated charcoal, the filtrate was concentrated to a syrup which was allowed to stand for several days until crystallization was complete (yield > 80%).

In order to check the influence of the environmental conditions, expressed in terms of the deuterium content of the water used in the photosynthetic pathway, a laboratory plantation of potato and maize was irrigated with water having an isotope ratio of about 500 p.p.m. The plants were grown in normal conditions of temperature and humidity, but no recirculation of the atmosphere was provided. After about four months, the starch from both plants was extracted and the glucose samples prepared as described previously.

The glucose derivatives were prepared as described in the references quoted in the text.

NMR determination of the site-specific isotope ratios. The molar fractions, f_i, associated with the clusters of lines observed in the ^{2}H-NMR spectrum of a given glucose derivative were determined from the signal areas (Martin *et al.*, 1985) using the assumption that each of the individual peaks composing the cluster has a Lorentzian shape.

A dedicated curve-fitting procedure was implemented which involves a least square fitting of the experimental peaks. In order to avoid systematic errors due to improper convergence of the curve-fitting procedure, reasonable estimates of the three parameters of the Lorentzian curves (frequency, width at half-height and intensity) were obtained, before starting the iterative process, by scanning the spectra drawn on an expanded scale. A much higher degree of reproducibility is attained when this protocol is used instead of conventional curve-fitting procedures which are the source of drastic systematic errors when the signal-to-noise (S/N) ratio is relatively poor and the overlap of signals is important. Typical acquisition parameters (Martin and Martin, 1990) using a Bruker AM 400 or AM 500 spectrometer were: frequency of spectrometer, 61.4 MHz (9.4 T) and 76.7 MHz (11.1 T); spectral width, 4800 Hz and 6000 Hz to benefit from the oversampling efficiency (Delsuc and Lallemand, 1986; Wider, 1990); digital resolution, ca. 0.3 Hz/pt; broad band ^{1}H decoupling; ^{19}F locking device. The spectra were recorded using a solution of about 1.0 g of derivative in 1.5 mL of $CHCl_3$ and 0.5 mL of CCl_4 at 306 K. Up to 40,000 scans were often required in order to obtain a S/N ratio of the order of 40. The repeatability of NMR determination is about 2% (relative value), providing that systematic deviations are avoided by using the dedicated curve fitting procedure. The reproducibility is of the same order of magnitude.

Some spectra were recorded in more dilute solution in order to check the potential of natural abundance ^{2}H-NMR to study small quantities of glucose. Thus the spectrum of 150 mg of penta-acetate derivative (corresponding to less than 100 mg of glucose) was recorded at 76.7 MHz in 40 hours with an S/N ratio of about 15.

Table 1. Isotopic clusters involved in the integrated analysis of the hydrogen isotope distribution in glucoses from a C_4 or C_3 metabolic origin

Type of plants	Derivatives of glucose			Number of clusters
		Type	Number	S_i
C_4	**1**	Penta-*O*-acetyl	21	3
	2	Tetra-*O*-acetyl Cl	1	4
	3	Tetra-*O*-acetyl N_3	2	6
	4	Penta-*O*-acetyl CN	1	2
	5	Tetra-*O*-acetyl SCN	1	2
	6	Tri-*O*-acetyl Br, Br	1	5
C_3	**1′**	Penta-*O*-acetyl	13	3
	2′	Tetra-*O*-acetyl N_3	2	6
	3′	Penta-*O*-acetyl CN	1	2

Each type of glucose derivative is associated with a given number of S_i clusters. In the case of the penta-*O*-acetyl derivative (**1**) relatively large numbers of samples have been prepared by using glucose from 8 (C_4) and 9 (C_3) different botanical origins. In some cases several penta-acetates have been prepared from the same pool of glucose.

Mass spectrometric determination of the isotope contents. The overall ratio ($\overline{D/H}$) of the carbon-bound hydrogens was determined by isotope ratio mass spectrometry (IRMS) after transformation of the glucose sample into its hexanitrate derivative in order to eliminate the contribution of the exchanging hydrogen atoms. The nitrate was burnt in a Carlo Erba micro analyser to CO_2 and H_2O and the nitrogen oxides, which could subsequently poison the reduction metal, were reduced on a copper catalyst.

The combustion water was separated from the CO_2 by differential cooling and was reduced by Zn at 550 °C to hydrogen which was analysed using a VG Sira 9 mass spectrometer. The internal precision of the spectrometer was much higher than the external reproducibility of the whole analytical chain (same nitrate measured several times at different periods) which was, however, quite satisfactory (0.2 p.p.m. in absolute value or better than 0.2% in relative value).

The overall $^{13}C/^{12}C$ isotope ratios were also determined by IRMS. The measurements were carried out using a Finnigan Delta E spectrometer fitted with a Carlo Erba micro-analyser.

The isotopic parameters of a given compound, P, can be expressed as the ratios of the numbers of heavy and light isotopes of a given atomic species: $R_P^H = (D/H)_P$ and $R_P^C = (^{13}C/^{12}C)_P$. These absolute values are obtained by referring the experimental parameters to the corresponding isotope ratios of international standards. The water reference, V.SMOW, adopted for hydrogen (Gonfiantini, 1978) contains 155.76 p.p.m. of deuterium with respect to protium. A carbonate reference, PDB, ($^{13}C/^{12}C = 0.112372\%$) is used for referencing the carbon isotope ratios (Craig, 1957).

The isotopic parameters may also be converted into the relative δ-scale defined by equation 1

$$\delta_P = [(R_P/R_{ref.}) - 1]1000 \qquad \text{(eqn 1)}$$

where the reference is either V.SMOW or PDB.

Statistical computations. Equation 3 (see below) can be constructed from the two sets of data provided in Table 1. Up to 53 and 88 equations may therefore be written for the C_3

and C_4 pool, respectively. However, it is also possible to consider only the average values of S_i corresponding to a given type of derivative. In this case the results are described by only 11 and 27 equations for the C_3 and C_4 plants respectively, some of them being weighted by the corresponding number of individuals (as can be seen in Table 4). From a statistical point of view, the two approaches are not strictly equivalent, especially if the standard deviations of measurement change significantly from one derivative to the other. However, the precision is shown to be nearly the same whatever the derivative, providing that sites which are likely to undergo isotopic fractionation during the chemical derivatization are distinguished.

RESULTS AND DISCUSSION

In order to gain access to the hydrogen isotope ratios, $(D/H)_i$, of specific positions, i, in the glucose molecule, several kinds of functionalized tetra- or penta-*O*-acetyl pyranoses were prepared. The acetylation leading to α- or β-D-glucopyranose penta-acetate (**1**) for instance is conveniently performed by reacting the carbohydrate with an excess of acetic anhydride in the presence of imidazole (Wolfrom and Thomson, 1964). Isotope fractionation in the carbon-bound positions can be safely neglected in this transformation. In contrast the substituted position and even adjacent sites in 2,3,4,6-tetra-*O*-acetyl-(α or β)-D-glucopyranosyl chloride (**2**) (Williams *et al.*, 1993) or azide (**3**) (Paulsen *et al.*, 1974; Takeda *et al.*, 1980; Praly *et al.*, 1990), 2,3,4,5,6-penta-*O*-acetyl-D-glucononitrile (**4**) (Furneaux, 1983), 1,2,3,4-tetra-*O*-acetyl-6-deoxy-6-thiocyanato-α-D-gluco-pyranose (**5**) and 2,3,4-tri-*O*-acetyl-6-bromo-6-deoxy-α-D-glucopyranosyl bromide (**6**) (Williams *et al.*, 1993) may be subject to fractionation contributions resulting from kinetic isotope effects occurring in several reaction steps. The perturbed isotope ratios then appear as outliers in the statistical analysis and are excluded.

The assignment of the deuterium NMR signals to the specific isotopomers of the various glucose derivatives is directly derived from the complete analysis of the corresponding high field proton spectra since the ^2H chemical shifts are nearly identical to those of ^1H. Although the prochiral methylenic sites 6,6′ are diastereotopic they are rarely well separated on the ^2H-spectrum and we characterize them by a single average ratio, $(D/H)_6$. For a given functionalized-*O*-acetyl pyranose, we define a number $k(1 \leqslant k \leqslant 6)$ of more or less resolved ^2H signal clusters, $S_i(1 \leqslant i \leqslant k)$. Every cluster may correspond to one or several molecular sites, n_j. Denoting R_j, the site-specific isotopic parameter associated with a given isotopomer j, we may write an equation of type 2 for each signal cluster, S_i, pertaining to any of the p derivatives prepared from glucoses of either the C_3 or C_4 photosynthetic family

$$S_i = a_{i1}R_1 + a_{i2}R_2 + a_{i3}R_3 + a_{i4}R_4 + a_{i5}R_5 + a_{i6}R_6 \quad \text{(eqn 2)}$$

a_{ij} is a dummy variable which takes the value 1 or 0 according to whether isotopomer j resonates or not in the cluster S_i.

In matrix formulation the whole sets of equation 2 are represented by

$$[A]\mathbf{R} = \mathbf{S} \quad \text{(eqn 3)}$$

where $[A]$ is the $(m, 6)$ matrix containing the a_{ij} coefficients associated with the m observable clusters in the whole population, p, of the C_3 or C_4 glucose derivatives. **S** is a $(m, 1)$ vector which contains the observed ^2H intensities of the m signal clusters expressed either in molar fractions of the monodeuterated isotopomers, f_j, where

$$S_i = \sum_j a_{ij} f_j \quad \text{(eqn 4)}$$

or in units of isotope ratios (p.p.m.)

$$S_i = \sum_j \frac{a_{ij}}{n_j} (D/H)_j \quad \text{(eqn 5)}$$

R is the $(6, 1)$ vector composed either of the 6 site-specific molar fractions f_j or of the 6 site-specific isotope ratios $(D/H)_j$.

Although nearly all molecular sites are clearly identified in the proton spectra of the selected glucose derivatives, significant overlapping of the ^2H-NMR signals occurs for a number of isotopomers due to reduced chemical shift separation and quadrupolar broadening (Williams *et al.*, 1993). Thus, only the three clusters (1), (2,3,4) and (5,6,6′) are clearly distinguished in the ^2H-spectrum of α-glucose penta-acetate, **1**. More generally the overall isotope ratios $(\overline{D/H})$ of the different glucose derivatives can be expressed as a function of the quantified clusters, i, by

$$(\overline{D/H}) = \sum_i F_i (D/H)_i \quad \text{(eqn 6)}$$

where F_i is the statistical molar fraction of cluster i and $(D/H)_i$ the average isotope ratio of this cluster.

For the penta-acetate **1**

$$(\overline{D/H}) = \tfrac{1}{7}(D/H)_{(1)} + \tfrac{3}{7}(D/H)_{(2,3,4)} + \tfrac{3}{7}(D/H)_{(5,6,6')} \quad \text{(eqn 7)}$$

Only two clusters are observed in the glucononitrile **4** and

$$(\overline{D/H}) = \tfrac{4}{6}(D/H)_{(2,3,4,5)} + \tfrac{2}{6}(D/H)_{(6,6')} \quad \text{(eqn 8)}$$

For the four clusters of the chloride **2**

$$(\overline{D/H}) = \tfrac{1}{7}(D/H)_{(1)} + \tfrac{1}{7}(D/H)_{(3)} + \tfrac{2}{7}(D/H)_{(2,4)} + \tfrac{3}{7}(D/H)_{(5,6,6')} \quad \text{(eqn 9)}$$

Different arrangements are identified in the ^2H spectra of the α- and β-azides **3**

$$(\alpha) \quad (\overline{D/H}) = \tfrac{1}{7}(D/H)_{(1)} + \tfrac{1}{7}(D/H)_{(3)} + \tfrac{2}{7}(D/H)_{(2,4)} + \tfrac{3}{7}(D/H)_{(5,6,6')} \quad \text{(eqn 10)}$$

$$(\beta) \quad (\overline{D/H}) = \tfrac{1}{7}(D/H)_{(1)} + \tfrac{3}{7}(D/H)_{(2,3,4)} + \tfrac{1}{7}(D/H)_{(5)} + \tfrac{2}{7}(D/H)_{(6,6')} \quad \text{(eqn 11)}$$

and nearly all sites are separated in the dibromide **6**

$$(\overline{D/H}) = \tfrac{1}{7}(D/H)_{(1)} + \tfrac{1}{7}(D/H)_{(2)} + \tfrac{1}{7}(D/H)_{(3)} + \tfrac{1}{7}(D/H)_{(4)} + \tfrac{1}{7}(D/H)_{(5)} + \tfrac{2}{7}(D/H)_{(6,6')} \quad \text{(eqn 12)}$$

Finally five clusters are observed in the thiocyanato derivative **5**

$$\overline{(D/H)} = \tfrac{1}{7}(D/H)_{(1)} + \tfrac{1}{7}(D/H)_{(3)} + \tfrac{2}{7}(D/H)_{(2,4)} + \tfrac{1}{7}(D/H)_{(5)} + \tfrac{2}{7}(D/H)_{(6,6')} \quad \text{(eqn 13)}$$

Similar equations hold for the molar fractions f_i but in this case the summation over the different clusters is equal to unity.

Taking into account the whole set of derivatives prepared from glucose samples pertaining to the C_3 or C_4 photosynthetic family, and eliminating the sites subjected to strong fractionation effects in the course of the derivatization, the retained numbers of clusters are given in Table 1.

Although most investigated glucose samples were extracted from plants grown in the western part of France, the climatic conditions which prevailed during the photosynthesis were not strictly identical. Variations in the environmental conditions, combined with physiological differences within every C_3 or C_4 population, may be the source of significant differences in the values of the isotopic parameters. In this respect, the site-specific isotope ratios of ethanol obtained by fermentation of carbohydrates extracted from a given plant species grown in different geographical area have been shown to exhibit a climatic significance (Martin and Martin, 1990). This behaviour partly explains the noticeable dispersion of the overall hydrogen and carbon isotope ratios measured by isotope ratio mass spectrometry, as reflected by the relatively large values of their standard deviations (Table 2). However, although the deuterium content of water entering the photosynthetic cycle has a crucial effect on the overall deuterium content of glucose it influences to a lesser extent the distribution of ^2H among the molecular sites. Thus when potato and maize plants are grown under similar conditions, with the exception for the isotopic ratio of the irrigation water used, the total deuterium content of glucose increases with the enrichment of water but the mean molar fractions of the isotopomers resonating in the three clusters identified in glucose penta-acetate remain nearly identical, within experimental accuracy (Table 3). At a first level of approximation, no sub-classes will therefore be considered within the two metabolic C_3 and C_4 pools of glucose samples.

The mean internal isotopic distribution of deuterium in sites 1 to 6 of glucose can be computed by resorting either to the molar fractions of the monodeuterated isotopomers (eqn 4) or to the isotope ratios (eqn 5). The vectors, **S**, of the intensity parameters S_i expressed in terms of both units are given in Table 4. In the case of the glucose penta-acetates, the retained values of the cluster parameters S_i result themselves from an averaging over the data obtained for several glucose samples from a C_3 or from a C_4 origin. The mean values of f_j and $(D/H)_j$ calculated for the two photosynthetic populations are collected in Table 5. The site-specific isotope ratios have been computed directly by solving the matrix eqn 3 built on the S_i parameters expressed in p.p.m. units. The $(D/H)_j$ ratios have also been calculated by means of eqn 14 from the computed molar fractions f_j and the average overall deuterium content of glucose $(\overline{D/H})$ measured by isotope ratio mass spectrometry on the nitrate derivatives of glucose

$$(D/H)_j = \frac{f_j}{F_j}(\overline{D/H}) \quad \text{(eqn 14)}$$

Significant deviations with respect to a statistical distribution of deuterium along the glucose skeleton are observed. Such variations in the natural deuterium contents at the different sites of a given molecular species have now been directly determined by the SNIF-NMR method in a great number of plant products (Martin and Martin, 1990). From a similar point of view, by resorting to the previous degradation of compounds such as lipids (Monson and Hayes, 1982) or glucose (Rossmann *et al.*, 1991) into appropriate derivatives it has also been observed by mass spectrometry that the distribution of ^{13}C may be non-statistical. In addition the results summarized in Table 5 demonstrate that the photosynthetic metabolism exerts a noticeable

Table 2. Mean values of the overall isotopic parameters of the two sets of C_3 and C_4 glucose samples

C_3	$n=9$	$(\overline{D/H}) = 139$ (2.5) p.p.m.	$\delta^{13}C‰ = -26.0$ (0.5)
C_4	$n=8$	$(\overline{D/H}) = 151.5$ (1.5) p.p.m.	$\delta^{13}C‰ = -11.0$ (0.5)

The numbers in brackets represent the standard deviation of the whole population investigated. The carbon isotope content measured by isotope ratio mass spectrometry is expressed in the δ-scale

$$\delta(‰) = 1000[(\overline{^{13}C/^{12}C}) - (\overline{^{13}C/^{12}C})_{PDB}]/(\overline{^{13}C/^{12}C})_{PDB}$$

where PDB is the international reference for the carbon isotope ratios.

Table 3. Comparison of the internal distribution of deuterium in potato and maize glucose samples extracted from plants grown in natural and slightly ^2H-enriched irrigation water

Specific molar ratios	Site (1)	Sites (2,3,4)	Sites (5,6,6')	$(\overline{D/H})$
Statistical distribution	0.143	0.428	0.428	
Potato (C_3)				
Natural water 150 p.p.m.	0.095	0.475	0.43	140.9
Enriched water 500 p.p.m.	0.105	0.485	0.41	175.0
Maize (C_4)				
Natural water 150 p.p.m.	0.145	0.455	0.400	151.9
Enriched water 500 p.p.m.	0.145	0.45	0.405	181.0

The overall $(\overline{D/H})$ isotope ratio is in p.p.m. and the isotopic distribution is expressed in terms of molar fractions of the three clusters of isotopomers identified in the deuterium spectrum of glucose penta-acetate.

Table 4. Values of the isotopic clusters S_i(vector S of eqn 3) calculated in terms of isotope ratios, $(D/H)_i$, (p.p.m.), or of molar fractions, f_i, for the derivatives of glucose described in Table 1

Origin	i	j	N_o	N_c	$(\overline{D/H})_{tot}$	S_i f_i	S_i $(D/H)_i$
C_4 plants	1	1	9	15	152.2	0.134	144.3
	2	2	2	2	153.0	0.144	154.1
	3	3	4	4	153.0	0.161	171.3
	4	4	2	2	153.0	0.152	162.2
	5	5	2	2	153.0	0.112	119.8
	6	6,6′	3	3	152.0	0.293	156.7
	7	2,3,4,5	1	1	150.0	0.562	149.5
	8	2,3,4	6	12	152.2	0.439	155.9
	9	2,4	2	2	153.0	0.311	167.3
	10	5,6,6′	7	13	152.2	0.421	149.5
C_3 plants	1	1	10	14	140.5	0.109	106.6
	2	2	1	1	136.0	0.151	143.3
	3	3	1	1	136.0	0.143	136.3
	4	4	1	1	136.0	0.191	182.0
	5	5	1	1	136.0	0.115	108.2
	6	6,6′	2	2	136.5	0.261	124.6
	7	2,3,4,5	1	1	137.0	0.637	152.7
	8	2,3,4	8	12	141.2	0.478	157.5
	9	5,6,6′	8	12	141.2	0.415	136.8

N_o is the number of different glucose samples involved in the determination of the considered isotopic cluster i, and N_c is the number of experiments. $(\overline{D/H})_{tot}$ is the average value of the hydrogen isotope ratios of the carbon-bound positions measured by IRMS on the pentanitrate derivatives of the glucose samples contributing to the defined cluster. The matrix [A] (eqn 3) is formed from the a_{ij} variables defined in eqn 2 on the basis of the cluster constitution given in the two first columns of the table and further described in eqns 6 to 13.

influence on the isotopic distribution. In particular, a significant impoverishment in deuterium is exhibited at sites 1 and 6,6′ of glucose from a C_3 origin as compared to C_4 glucose.

Although only plants grown in rather similar environments have been considered, the dispersion of the results, which largely exceeds experimental accuracy, reflects the influence of botanical and environmental factors. Since these contributions have a more prominent effect on the overall deuterium content than on the relative distribution, approximate values of the site-specific isotope ratios of a given glucose, P, can be obtained (eqn 14) by resorting to the mean values of f_j and to the value $(\overline{D/H})_P$ measured on the nitrate prepared from sample P. However, further investigations must be carried out in order to appraise in more detail the influence of the physiological and environmental effects within a given metabolic type.

We have shown that ethanol obtained by fermentation in strictly controlled conditions constitutes a faithful probe of the isotopic behaviour of glucose (Martin *et al.*, 1986). The CH_2DCH_2OH isotopomer of ethanol in particular is expected to be closely related to the isotopomers associated with positions 1,6,6′ of glucose. The sensitivity of the $(D/H)_I$ parameter of ethanol to the metabolic and botanical origin of the plant precursor is fairly consistent with the significant differences detected in the $(D/H)_1$ and $(D/H)_{6,6'}$ values of glucose from C_3 and C_4 origins. Thus a mean increase of about 30 p.p.m. is determined between the $(D/H)_{1,6,6'}$ values of C_3 and C_4 glucose. This behaviour is paralleled by an increase of about 18 p.p.m. (Martin *et al.*, 1991) in the deuterium content of the methyl site of ethanol resulting from the same C_3 and C_4 plant species.

From a mechanistic point of view, it is concluded that the sites 1,6,6′ of glucose which are the most sensitive to the photosynthetic metabolism are those which are directly related to sites 1 and 5 of the ribulose 1,5-bis phosphate (Irvine *et al.*, 1992). In contrast, the other sites, 2 to 5, of glucose which undergo a levelling effect are introduced in the course of reduction steps with NADPH or are engaged in exchange processes with water in specific isomerization reactions involving the three or six carbon intermediate molecules of the photosynthetic pathway.

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Table 5. Calculated mean values of the molar fractions, f_j, and of the site-specific isotope ratios $(D/H)_j$ of the carbon-bound hydrogens of glucoses extracted from C_3 and C_4 plants

	Site	1	2	3	4	5	66′	$(\overline{D/H})_{MS}$
	F_j	0.143	0.143	0.143	0.143	0.143	0.286	–
C_4	f_j	0.133	0.143	0.151	0.151	0.118	0.298	–
C_4	$(D/H)_j$	141.8	152.5	161.0	161.0	125.8	158.8	152.3
C_3	f_j	0.109	0.149	0.141	0.189	0.145	0.269	–
C_3	$(D/H)_j$	106.2	145.2	137.4	184.1	141.3	131.0	139.2

$(\overline{D/H})_{MS}$ is the average value of the overall deuterium content (p.p.m.) measured by isotope ratio mass spectrometry of the nitrate derivatives of the glucose samples. For the considered population, the 95% confidence intervals are: $\Delta(f_j)$: 0.01; $\Delta((D/H)_j)$: 10 p.p.m.; $\Delta((\overline{D/H})_{MS})$: 1.5 to 2.5 p.p.m.

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