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Natural Abundance Hydrogen Isotope Affiliation between the Reactants and the Products in Glucose Fermentation with Yeast

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In glucose fermentation, the hydrogen source of products such as ethanol and glycerol is the medium and the sugar. The site-specific natural isotope ratios of the products, $(D/H)_i$, and that of the medium and sugar, $(D/H)_k$, may be related by a matrix, **A**, of redistribution coefficients, a_{ik}, that characterizes the specific genealogies of the hydrogen atoms. $(\mathbf{D/H})_i = [\mathbf{A}](\mathbf{D/H})_k$, where $(\mathbf{D/H})_i$ and $(\mathbf{D/H})_k$ are the column vectors of the isotope ratios of sites *i* and *k* that can be measured by ^{2}H NMR. The complete redistribution matrix was determined in a set of isotope labeling experiments. Thus, we obtained a mathematical model representing the hydrogen isotope affiliation during alcoholic fermentation. It not only provides information about the biochemical reaction mechanism but also can be used to estimate the isotopic data of the products, based on those of the substrate and the medium. The results prove, in a quantitative way, that the metabolites contain isotopic information about the precursor in a biotransformation and can be used to identify its origin. The method established for the study of the hydrogen-transfer mechanism can be applied to other chemical and biochemical reactions.

KEYWORDS: Alcoholic fermentation, deuterium, ^{2}H NMR, isotopic labeling, origin authentification

INTRODUCTION

In 1981, G. J. Martin and M. L. Martin described the difference in the natural abundance "intramolecular" deuterium distribution in ethanols of different origin by ^{2}H NMR (*1*). Since then, a powerful tool of isotopic analysis has been developed. In contrast to isotope ratio mass spectrometry (IRMS), with which only the overall isotope ratio of a molecule can be obtained, quantitative ^{2}H NMR (SNIF NMR) can be used for the precise determination of the site-specific isotopic ratios of a molecule (*2*). At natural abundance, the stable hydrogen isotope, deuterium, occurs in the molecules of a compound essentially as monodeuterated isotopomers. The parameter measured by ^{2}H NMR is the ratio of the number of deuterium (*D*) atoms to that of protium (*H*) atoms at site *i* of the sample molecule, $(D/H)_i$ in ppm,

$$(D/H)_i = N_{D_i}/(P_i N_H) \quad (1)$$

where N_{D_i} is the number of site *i* deuterated isotopomers, P_i is the stoichiometric hydrogen number at site *i* (for example, for $-CH_3$, $P_i = 3$), and N_H is the number of unlabeled (totally protiated) molecules. $(D/H)_i$ at all sites of the sample compound can be determined when their ^{2}H NMR signals are sufficiently resolved.

^{2}H NMR was first used to identify the origin of ethanol and applied to the detection of added sugar in wine (*3*). In the alcoholic fermentation of sugars by yeast, the main product is ethanol, of which the carbon source is the sugar, while the hydrogen sources are the carbon-bound unexchangeable hydrogens and the medium, which contains the hydrogens of water and those of the hydroxyls of sugars. Since the natural abundance isotopic ratios of the unexchangeable hydrogens of sugars depend on their physiological and environmental conditions of photosynthesis (*4*), the isotopic distribution in the alcoholic fermentation products may reflect the origin of the carbohydrate precursors. It was found that the site-specific isotopic ratio of the methyl of ethanol $(D/H)_{CH_3}$ mainly depends on those of the unexchangeable hydrogens of sugars while that of the methylene group is strongly related to that of the fermentation medium (*5*, *6*). Thus, ethanol is a good isotopic probe for the recognition of the origin of its precursor, based on the $(D/H)_{CH_3}$ value. The ethanol probe is used in official analytical methods for the control of commodity authenticity (*7*). With the use of these methods, a question arises: Why can the natural abundance of isotope distribution in ethanol be used to characterize the origin of the sugar precursor? Or, how are the site-specific isotopic ratios of ethanol connected to those of the precursor and the fermentation medium? Only after resolving this problem can such methods of analysis be established on a firm scientific basis.

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The isotopic connection between the substrate, the medium, and the products in an alcoholic fermentation can be described by a mathematical model. The site-specific isotopic ratios of a product such as ethanol, $(D/H)_{i,p}$, is a linear function of the specific isotopic ratios of the carbon-bound hydrogen positions of the substrate and of the medium:

$$(D/H)_{i,p} = a_{im}(D/H)_m + \sum_j a_{ij}(D/H)_{j,s} = \sum_k a_{ik}(D/H)_k \quad (2)$$

in which $(D/H)_m$ is the isotopic ratio of the medium and $(D/H)_{j,s}$ is that of site j of the substrate. The terms a_{im} and a_{ij} are isotope redistribution coefficients; $m, j \subset k$. Since there are also several hydrogen sites in the product, the model can be expressed in matrix notation:

$$(\mathbf{D/H})_i = [\mathbf{A}](\mathbf{D/H})_k \quad (3)$$

where $(\mathbf{D/H})_i$ and $(\mathbf{D/H})_k$ are the column vectors of the site-specific natural isotopic ratios of the product and the substrate and where **A** is the redistribution matrix (*6*, *8*). From now on, $(D/H)_{i,p}$ and $(D/H)_{j,s}$ will be simplified as $(D/H)_i$ and $(D/H)_j$ in the text. The key to understanding the isotopic connection between the substrate and product molecules is the determination of the matrix **A** that characterizes the specific genealogies of the deuterium atoms. It also depends on the complex isotope effects during the biochemical reactions. Establishing the matrix **A** involves a detailed study of the deuterium-transfer mechanism in glycolysis and fermentation.

A can be evaluated by isotope labeling, and, thanks to ^{2}H NMR, the study can be carried out easily. Several sugars, such as glucose, fructose, and sucrose, can be used as a substrate in the fermentation. Since fructose can be considered as an intermediate in glycolysis, of which the first step is the conversion of glucose 6-phosphate (G6P) to fructose 6-phosphate (F6P), and since sucrose contains both glucose and fructose, it is most useful to study the isotope behavior of glucose, which has seven unexchangeable hydrogens. Essentially, the label is introduced either in the medium or at one position j of glucose by adding a small quantity of the deuterated isotopomer in one experiment while keeping the other isotopic ratios constant as in a reference experiment, in which only given starting materials at natural abundance are used. Hence, by measurement of the variation of $(D/H)_i$ of ethanol due to the labeling, the value of a_{ik} (a_{im} or a_{ij}) can be evaluated from a linear equation:

$$(D/H)_i = a_{ik}(D/H)_k + b \quad (4)$$

Then, when the labeling positions of the starting materials in a set of experiments are changed, **A** can be determined (*8*, *9*). During recent years, much work has been done and it has been established that the hydrogens of the methyl site of ethanol are strongly connected to the carbon-bound hydrogens 1, 2, 6, and 6′ of glucose, while those of the methylene site come mainly from fermentation water (*8*). However, the complete **A** has not yet been established and the validity of eq 3 has not been checked because of the lack of some labeled glucose molecules and because of the difficulty of measuring reliable natural $(D/H)_j$ values of glucose. Now these problems have been resolved, and the complete results are presented here.

MATERIALS AND METHODS

Materials. The reference glucose (produced from corn) was obtained from Prolabo. The (1-^{2}H$_1$)glucose, (2-^{2}H$_1$)glucose, and (6,6′-^{2}H$_2$)glucose were purchased from Aldrich. The (3-^{2}H$_1$)glucose and (4-^{2}H$_1$)glucose were purchased from Omicron. The isotopic purity of these isotopically substituted glucose molecules is at least 97%. D_2O (99.95%) was purchased from Eurisotop.

The dried yeast purchased from Val-Oeno was a mixture (50:50) of *Saccharomyces cerevisiae* and *Saccharomyces bayanus* used in wine production.

Alcoholic Fermentation and Ethanol Extraction. The composition of the fermentation medium was 150 g/L glucose and 3.75 g/L yeast. No other compounds were added to the medium (*10*). Glucose samples slightly enriched at specific positions were prepared by adding small quantities of the specifically deuterium-substituted glucose (~40 mg) to 120 g of the reference corn glucose dissolved in 800 mL of Nantes tap water (NTW). The variation of the $(D/H)_j$ was evaluated on the basis of the mass of the added enriched glucose obtained by weighing with a correction for its purity. Medium water of different (D/H) values was prepared by adding D_2O to Nantes tap water. The $(D/H)_w$ of both NTW and the labeled waters were determined by isotopic ratio mass spectrometry (IRMS) (*9*). The fermentation was performed under anaerobic conditions. The fermentation temperature was 30 ± 1 °C and the duration was 7 days. Ethanol was extracted by distillation under controlled conditions (*11*).

Ethanol Methylene Exchange with Water Mediated by Baker's Yeast. To a solution containing 20 mL of absolute ethanol (reference), 180 mL of water, NH_4Cl (2 g/L), KH_2PO_4(3 g/L), NaCl(3 g/L), $MgCl_2$ (60 mg/L), Na_2HPO_4 (6 g/L), and Na_2SO_4 (120 mg/L), 4 g of wet baker's yeast was added, corresponding to 1.6×10^9 cells/mL. After 45 days of incubation, the ethanol was extracted by distillation and analyzed by ^{2}H NMR.

Synthesis of Ethyl Mandelate. *S*-(+)-Mandelic acid was used. The synthesis of ethyl mandelate was performed according to reference 12.

^{2}H NMR Measurement. The deuterium NMR spectra were recorded at 61.4 MHz under broad-band proton decoupling using a Bruker DPX 400 spectrometer equipped with a ^{19}F lock device. Other conditions were the following: frequency window 1200 Hz, memory size 16K, scan number 500 for ethanol and 5000 for ethyl mandelate, and an exponential multiplication corresponding to a line broadening of 0.5 Hz. Six and three spectra were recorded for each sample of ethanol and ethyl mandelate, respectively. An average $(D/H)_i$ value was calculated from these measurements.

The $(D/H)_i$ values were determined using an external reference, TMU (tetramethylurea), of which the isotopic ratio $(D/H)_R$ was precisely calibrated by IRMS. $(D/H)_i$ was calculated from the following equation:

$$(D/H)_i = (D/H)_R P_R m_R M_S S_i/(P_i f m_S M_R S_R) \quad (5)$$

where P_i and P_R are the stoichiometric numbers of hydrogens at site i and in the reference. M_S, m_S, and M_R, m_R are the molecular weight and mass of the sample and the reference, respectively. f is the purity of the sample in mole fraction, and S_i and S_R are, respectively, the surface areas of the signals of the site i monodeuterated molecule and the reference in the ^{2}H NMR spectrum. Surface areas were determined using a curve-fitting program (Interlis from Eurofins, Nantes, France) (*13*).

RESULTS AND DISCUSSION

Ethanol has three unexchangeable hydrogen positions: methyl, pro-*R* and pro-*S* H of the methylene ($i = CH_3$, pro-*R* CH, and pro-*S* CH). When the last two are substituted by a deuterium, there are two isotopomers that are a pair of enantiomers. These can be observed separately by ^{2}H NMR when they are converted into two diastereoisomers (*14*) in reaction with *R*-(−)- or *S*-(+)-mandelic acid (*12*). Only when water and H-4 of glucose were labeled were $(D/H)_{pro-RCH}$ and $(D/H)_{pro-SCH}$ measured separately because in the other cases the $(D/H)_{CH_2}$ value remained constant. The $(D/H)_{CH_2}$ value is the average of $(D/H)_{pro-RCH}$ and $(D/H)_{pro-SCH}$ values. Glucose either monodeuterated at sites 1, 2, 3, and 4 or bideuterated at 6,6′ was used. C-5 deuterated glucose was not used because H-5 is lost to the medium and not transferred to the glycolytic products. In water labeling

Table 1. $(D/H)_i$ of Ethanol Produced in Alcoholic Fermentation with Labeled Waters and Glucose

medium		glucose		ethanol			
$(D/H)_m$, ppm	labeled site *j*	$(D/H)_j$ of the reference,[d] ppm	$(D/H)_j$ of the labeled site, ppm	$(D/H)_{CH_3}$, ppm	$(D/H)_{CH_2}$, ppm	$(D/H)_{pro-RCH}$, ppm	$(D/H)_{pro-SCH}$, ppm
149.4[a]	reference[d]			111.1(0.3)	123.2(0.3)	134.2(1.5)	112.2(1.2)
215.2[b]	reference[d]			122.9(0.4)	175.2(0.5)	188.0(2.2)	162.4(1.9)
284.7[c]	reference[d]			134.8(0.5)	228.3(0.5)	239.6(2.0)	217.0(1.8)
149.4[a]	1	173.8[e]	488.6	153.7(0.2)	123.1(0.5)		
149.4[a]	2	156.7[e]	478.0	141.2(0.2)	123.4(0.1)		
149.4[a]	3	146.4[e]	556.3	111.4(0.3)	123.2(0.4)		
149.4[a]	4	140.3[e]	457.5	111.8(0.3)	138.8(0.4)	162.6(0.6)	115.0(0.4)
	5	145.4[e]					
149.4[a]	6pro-*S*	151.7[e]	402.1	151.5(0.8)[f]	123.6(0.3)		
	6pro-*R*	142.7[e]	393.1	151.5(0.8)[f]			

[a] Calculated with $(D/H)_w = 149.1$ ppm (NTW) and $(D/H)_{OH}$ of glucose $= 159.4$ ppm. [b] $(D/H)_w = 217.3$ ppm. [c] $(D/H)_w = 289.4$ ppm. [d] The reference is a commercial corn glucose (*15*). [e] The $(D/H)_i$ values of the corresponding ethanol obtained in the same medium are given in the first line of data. [f] Because the labeled glucose is a bideuterated molecule, there are methyl mono- and bideuterated isotopomers (*8*). The value was calculated on the basis of the area of monodeuterated isotopomer + ($^1/_2$)(area) of the bideuterated isotopomer.

experiments, a corn glucose (reference for glucose) was used, whereas in glucose labeling experiments, Nantes tap water (NTW, reference for water) was used. The experimental results are summarized in **Table 1**. The data in the table show that the transfer of deuterium from the substrate (glucose) to the product (ethanol) is site-specific.

With the data of **Table 1**, linear equations were obtained.

$$(D/H)_{CH_3} = 0.18(D/H)_m + 85 \quad (6)$$

$$(D/H)_{pro-RCH} = 0.78(D/H)_m + 19 \quad (7)$$

$$(D/H)_{pro-SCH} = 0.78(D/H)_m - 4 \quad (8)$$

$$(D/H)_{CH_3} = 0.14(D/H)_1 + 88 \quad (9)$$

$$(D/H)_{CH_3} = 0.09(D/H)_2 + 96 \quad (10)$$

$$(D/H)_{pro-RCH} = 0.09(D/H)_4 + 122 \quad (11)$$

$$(D/H)_{CH_3} = 0.16(D/H)_{6pro-S} + 87 \quad (12)$$

$$(D/H)_{CH_3} = 0.16(D/H)_{6pro-R} + 88 \quad (13)$$

In principle, because $(D/H)_i$ is a linear function of $(D/H)_{j\text{ or }m}$, each of these equations can be established using only two $(D/H)_i$ values and their corresponding $(D/H)_{j\text{ or }m}$ values. For instance, when position 1 of glucose was labeled, $(D/H)_1$ was 488.6 ppm and $(D/H)_{CH_3}$ for the corresponding ethanol was 153.7 ppm. These values were compared with $(D/H)_1 = 173.8$ ppm of the reference glucose and its ethanol produced in the same water (NTW) with $(D/H)_{CH_3} = 111.1$ ppm (**Table 1**). On the basis of the two sets of data, eq 9 was obtained. Equations 6−8 were established with $(D/H)_i$ values of ethanol obtained in three water labeling experiments by linear regression. $(D/H)_m$ is a weighted mean value of all hydroxyls calculated on the basis of the $(D/H)_{OH}$ of glucose and $(D/H)_w$ and the quantities of water and glucose in the medium (*9*). The a_{im} values are slightly different from those determined in the previous work using $(D/H)_w$. Since the hydrogens of glucose hydroxyls are considered as part of the medium, the intercept value in eqs 6−8 corresponds only to the contribution of unexchangeable deuteriums of glucose. Equations 9−13 were obtained in glucose labeling experiments performed in the NTW. Each of them was calculated with only two sets of data: $(D/H)_j$ values of the reference and a labeled glucose and $(D/H)_i$ values of the corresponding ethanol (*8*, *9*). In previous work, the values of a_{CH_31}, a_{CH_32}, a_{CH_36}, $a_{CH_36'}$, a_{CH_21}, a_{CH_22}, a_{CH_26}, and $a_{CH_26'}$ have been evaluated on the basis of only the variation of the specific isotopic ratios, $\Delta(D/H)_j$, ignoring the absolute $(D/H)_j$ values of the reference glucose (*8*). In this work, since we used a glucose with carefully determined $(D/H)_j$ values (*15*), the labeling experiments were repeated. The a_{ij} values obtained in the repeated experiments are in good agreement with the values from previous work, given that the uncertainty in the slope (a_{ik}) is $\pm(0.01-0.02)$ and $\pm(3-5)$ at the intercept (b).

Complex scrambling of hydrogens may occur in glycolysis. According to the hydrogen-transfer mechanism in glycolysis and ethanol formation (**Scheme 1**), there are hydrogens from the medium in both the methyl and methylene groups of ethanol, while hydrogens 1, 2, 6pro-*R* and 6pro-*S* of glucose can be found in the methyl position. There may also be a relationship between the hydrogen at position 4 of glucose and the pro-*R* methylenic site of ethanol because of NADH recycling.

Equations 6−8 confirm the presence of deuterium from water at both methyl and methylene positions. From a_{ij} values of eqs 9−13, the conversion rates of deuterated glucose molecules to ethanol can be evaluated (*9*). In the biotransformation, n_{RH} totally protiated glucose molecules are completely converted to $2n_{RH}$ ethanol molecules.

$$\underset{n_{RH}}{\text{glucose(H)}} \rightarrow \underset{2n_{RH}}{2\ \text{ethanol(H)}}$$

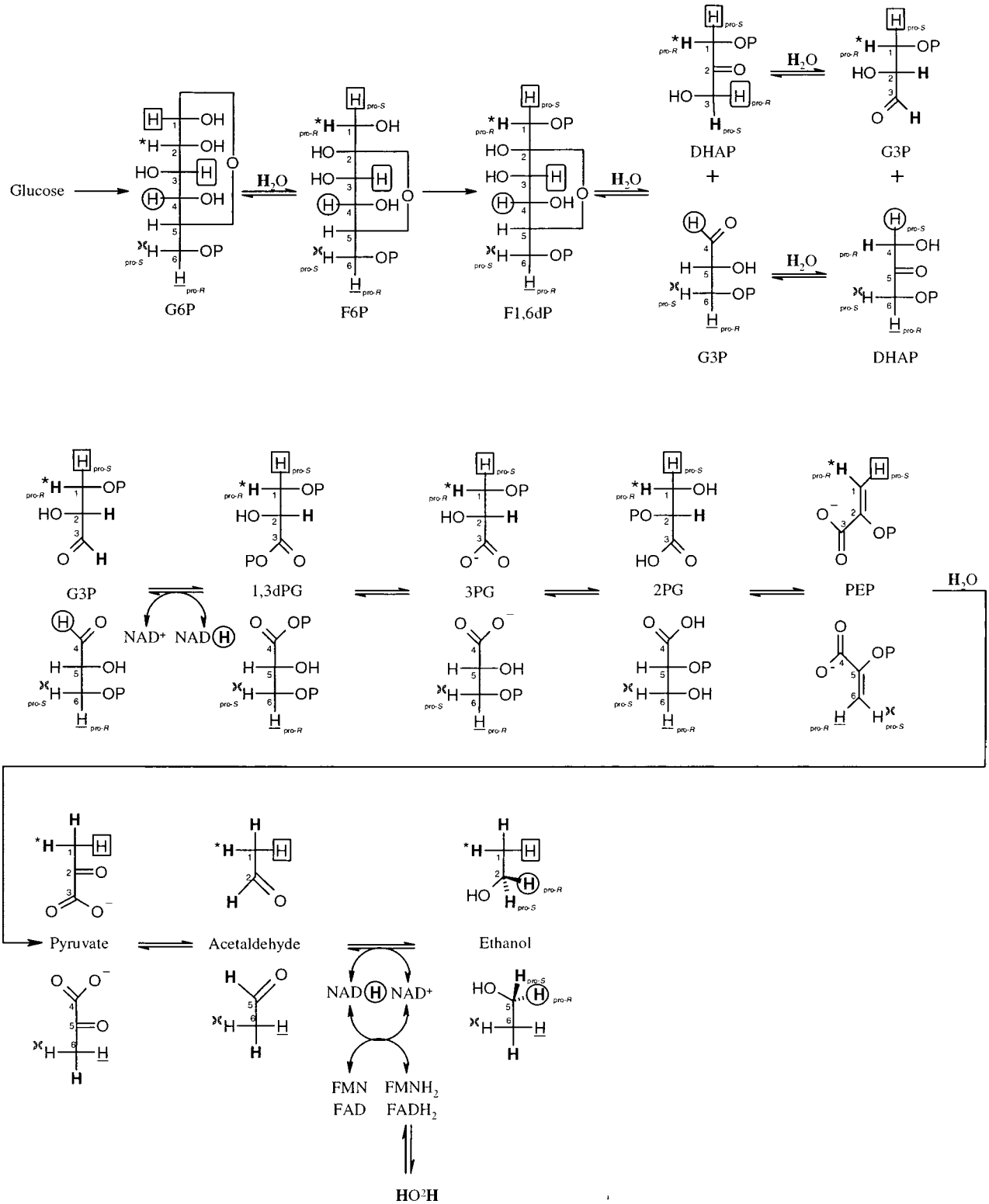
n_{RD} site j monodeuterated glucose molecules were converted to xn_{RD} site i monodeuterated ethanols, where x is the conversion rate of site j deuterated glucose ($0 \le x \le 1$).

$$\underset{n_{RD}}{(j\text{-}^2H_1)\text{glucose}} \rightarrow \underset{xn_{RD}}{x(i\text{-}^2H_1)\text{ethanol}\ (D_j)}$$

These deuterated ethanol molecules are denoted by D_j. The other totally protiated moiety of the deuterated glucose may become ethanol. Because their quantity is very small, it can be neglected. According to the definition (eq 1), for site j of glucose, since $P_j = 1$ for $j = 1$, 2, 3, 4, 6pro-*R*, and 6pro-*S*, we have

$$(D/H)_j = n_{RD}/n_{RH} \quad (14)$$

Scheme 1. Mechanism of Glycolysis and Ethanol Formation



For site i of ethanol, we have

$$(D_j/H)_i = xn_{RD}/[(P_i)(2n_{RH})] \quad (15)$$

It should be noted that $(D_j/H)_i \neq (D/H)_i$ because D_j is produced only by a site j deuterated glucose molecule, while the deuterium of site i of ethanol may also be of other origins. The ratio of eq 15 to eq 14 gives

$$(D_j/H)_i/(D/H)_j = x/(2P_i) \quad (16)$$

with $(D_j/H)_i = (D/H)_i -$ [the intercept of $(D/H)_i$ vs $(D/H)_j$] $= b$ in eq 4] because the intercept is the specific isotopic ratio of site i of ethanol concerning only the deuterium of other sites $k \neq j$ of glucose and of the medium. Thus, $(D_j/H)_i/(D/H)_j = a_{ij}$, and the conversion rate x of a site j deuterated glucose molecule to a site i deuterated ethanol molecule can be evaluated with

$$x\,(\%) = 2P_i a_{ij} \times 100 \quad (17)$$

Table 2. Conversion Rate of the Deuterated Glucose Isotopomers in Alcoholic Fermentation

deuterated site *j* of glucose	deuterated site *i* of ethanol	*x* (%)
1	CH_3	84
2	CH_3	54
3		0
4	pro-*R*CH	18
6pro-R	CH_3	96
6pro-*S*	CH_3	96

Table 3. Exchange between the pro-*R* Site of Ethanol and Water Mediated by Baker's Yeast

$(D/H)_w$, ppm	$(D/H)_{CH_3}$, ppm	$(D/H)_{CH_2}$, ppm	$(D/H)_{pro-RCH}$, ppm	$(D/H)_{pro-SCH}$, ppm
	100.1(0.3)[a]	128.6(0.5)[a]	139.0(1.8)[a]	118.2(1.6)[a]
150	99.9(0.2)	129.1(0.6)	140.0[b]	118.2
186	99.8(0.3)	130.0(0.4)	141.8[b]	118.2
285	99.8(0.5)	134.0(0.4)	149.8[b]	118.2

[a] Starting ethanol. [b] Calculated values in assuming $(D/H)_{pro-SCH} = 118.2$ ppm, $(D/H)_{pro-RCH} = 2[(D/H)_{CH_2} - 118.2]$.

The conversion rates of different deuterated glucose isotopomers are listed in **Table 2**.

The results show clearly that hydrogens at sites 1, 2, 6pro-*R*, and 6pro-*S* of glucose are strongly connected to the methyl site of ethanol. Whereas deuteriums at site 1 or the two 6 positions are transferred to a great extent, the degree of transfer is lower for deuterium 2 because in the first step of glycolysis (the conversion of G6P to F6P) the transfer of hydrogen is partially intramolecular (*16*). These results are in accord with the glycolytic mechanism as discussed previously (*8*). Theoretically, deuterium at site 1 of glucose should be intact and totally transferred to the methyl site of ethanol as is that at site 6, but its conversion rate is only 84%. This is similar to the case of glycerol formation (*9*). The cause of the loss of the deuterium at position 1 of glucose during the alcoholic fermentation cannot be determined from the available data. The labeling of position 3 of glucose has no effect on the $(D/H)_i$ of the ethanol (that means $a_{ij} = 0$). According to the glycolytic mechanism, this hydrogen is lost to the medium as is hydrogen 5.

The results also confirm that there is a relationship between hydrogen at position 4 of glucose and the pro-*R* methylenic site of ethanol. A detailed study of the intramolecular deuterium transfer was performed in this work. Since the reduction of acetaldehyde to ethanol catalyzed by alcohol dehydrogenase is stereospecific (the attack of H from NADH is by the *re* face), the deuterium in the *S*-enantiomer is that of the acetaldehyde formed during the decarboxylation of pyruvate and transferred from the medium water, whereas that of the *R*-enantiomer is transferred from NAD^2H during the reduction of the acetaldehyde by alcohol dehydrogenase (*17*).

NADH is regenerated in the oxidation step of glyceraldehyde 3-phosphate to 1,3-diphosphoglycerate (**Scheme 1**). The intercept value of eq 7 is considerably superior to zero and implies there is deuterium of sugar origin at the pro-*R* site. Furthermore, eqs 9−13 show that the deuterium comes only from C-4 of glucose. Theoretically, the intercept value of these equations cannot be negative, but in eq 8, it has a value of −4, which is due to experimental error in both the mandelate and water isotopic analyses. However, the fact that it is very close to zero implies there is no deuterium from sugar at the pro-*S* CH site.

For the methylenic hydrogens, the situation is more complex because there is an exchange between the methylenic pro-*R* deuterium and water due to an indirect exchange of the pro-*S* hydrogen of NADH with water via flavin (*18*, *12*). The degree of exchange was studied by incubation of ethanol in water of different isotopic compositions in the presence of baker's yeast (*12*). The results showed that the percentage of ^2H involved in the exchange was limited, even in very enriched medium. We repeated the experiment in slightly enriched water (**Table 3**) and found

$$(D/H)_{pro-RCH} = 0.07(D/H)_w + 128 \qquad (18)$$

From eq 18, the percentage of deuterium involved in the exchange can be evaluated as 7.9% = 100 × (139 − 128)/139. According to the glycolysis mechanism, G3P issues from the two moieties (C1−C2−C3 and C4−C5−C6) of glucose (**Scheme 1**). In G3P formation, the hydrogen of C-4 of glucose in the (C4−C5−C6) moiety remains intact whereas that of C-3 of glucose in the (C1−C2−C3) moiety is lost in the medium because of the stereospecifity of G3P/DHAP interconversion (*19*). Hence, G3P(C1−C2−C3) transfers a hydrogen of water origin and G3P(C4−C5−C6) transfers a hydrogen of C-4 of the glucose to NAD^+. Since ethanol is the main product, nearly all DHAP molecules are isomerized to G3P and then transformed to ethanol. Taking into account the 7.6% exchange, there would be 53.8% of water D and 46.2% of C-4 D in the *R*-enantiomer if there were no isotope effects and no other exchange processes. The experimental data deviate considerably from this estimation because of complex inter- and intramolecular D/H exchange and isotope fractionation in G3P/DHAP interconversion and NADH regeneration (*20*−*22*).

Unfortunately, with the available data, it is difficult to evaluate the kinetic or equilibrium isotope effects in hydrogen transfer from the medium to the methyl or methylene of ethanol. Nevertheless, some isotopic fractionation information can be obtained from a_{im}. At position *i* of ethanol, there are deuterium atoms (D) and protium atoms (H) from the unexchangeable sites of glucose (s) and the medium (m).

$$(D/H)_i = \frac{D_i}{H_i} = \frac{D_{i,s} + D_{i,m}}{H_{i,s} + H_{i,m}} \qquad (19)$$

Table 4. Calculated and Experimental $(D/H)_i$ of the Products in the Fermentation of a Corn Glucose[a] in Nantes Tap Water[b]

	$(D/H)_i$, ppm					
	ethanol			glycerol		
	$(D/H)_{CH_3}$	$(D/H)_{pro-RCH}$	$(D/H)_{pro-SCH}$	$(D/H)_A$	$(D/H)_B$	$(D/H)_C$
calculated	108.0	128.9	116.5	144.9	151.8	94.7
experimental	111.1	134.2	112.2	143.3	160.7	99.6

[a] For the site-specific isotopic ratios of the glucose, see **Table 1**. [b] In Nantes tap water $(D/H)_m = 149.4$ ppm.

If we characterize the complex isotope effects by a global isotopic fractionation factor (IFF) during the hydrogen transfer from the medium to site i of the product,

$$\mathrm{IFF}_{i,m} = \frac{(D/H)_m}{(D/H)_{i,m}} = \frac{(D/H)_m}{D_{i,m}/H_{i,m}} \tag{20}$$

In the experiments with labeled water (medium), we have

$$(D/H)_i = \frac{D_{i,s} + D_{i,m}}{H_{i,s} + H_{i,m}} = a_{im}(D/H)_m + \frac{D_{i,s}}{H_i} \tag{21}$$

After rearrangement, eq 21 becomes

$$\frac{D_{i,m}}{H_{i,s} + H_{i,m}} = a_{im}(D/H)_m \tag{22}$$

from which

$$\frac{1}{a_{im}} = \frac{(D/H)_m}{D_{i,m}/(H_{i,m} + H_{i,s})} > \frac{(D/H)_m}{D_{i,m}/H_{i,m}} = \mathrm{IFF}_{i,m} \tag{23}$$

Although $H_{i,m}$ and $H_{i,s}$ are unknown, we have

$$\mathrm{IFF}_{i,m} < 1/a_{im} \tag{24}$$

as in the case of i = pro-RCH. But if $D_{i,s}$ and $H_{i,s} = 0$ (no hydrogen transferred from the substrate to i), for example, when i = pro-SCH, $\mathrm{IFF}_{i,m} = 1/a_{im}$. Thus, $\mathrm{IFF}_{\mathrm{pro}-R\mathrm{CH},m} < {}^1/_{0.779} = 1.28$ and $\mathrm{IFF}_{\mathrm{pro}-S,m} = {}^1/_{0.775} = 1.29$. It can be concluded that the global isotope fractionation during the transfer of hydrogen from the medium to the S-enantiomer is normal (protium is more easily transferred than deuterium) and is more significant than that for the R-enantiomer formation.

CONCLUSION

From the available data, the numerical form of eq 3 can be established for ethanol biosynthesis in glucose fermentation (some a_{ij} values are the mean of that obtained in this work and in previous work (*8*)), as shown in **Chart 1**. In fermentation,

Chart 1.

$$\begin{pmatrix} (D/H)_{\mathrm{CH_3}} \\ (D/H)_{\mathrm{CHpro}-R} \\ (D/H)_{\mathrm{CHpro}-S} \end{pmatrix} = \begin{pmatrix} 0.13 & 0.09 & 0 & 0 & 0 & 0.16 & 0.16 & 0.18 \\ 0 & 0 & 0 & 0.09 & 0 & 0 & 0 & 0.78 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0.78 \end{pmatrix} \begin{pmatrix} (D/H)_1 \\ (D/H)_2 \\ (D/H)_3 \\ (D/H)_4 \\ (D/H)_5 \\ (D/H)_{6\mathrm{pro}-R} \\ (D/H)_{6\mathrm{pro}-S} \\ (D/H)_m \end{pmatrix} \tag{25}$$

besides the main product ethanol (95−97%), there is a minor product, glycerol (3−5%), whose biosynthesis is also related to the glycolysis. The mechanism of deuterium transfer of glycerol formation was studied recently (*9*). The corresponding matrix equation can be expressed as shown in **Chart 2**.

Chart 2.

$$\begin{pmatrix} (D/H)_{\mathrm{A}} \\ (D/H)_{\mathrm{B}} \\ (D/H)_{\mathrm{C}} \end{pmatrix} = \begin{pmatrix} 0 & 0.16 & 0 & 0.03 & 0 & 0.27 & 0 & 0.53 \\ 0.22 & 0 & 0.07 & 0 & 0 & 0 & 0.24 & 0.44 \\ 0 & 0 & 0 & 0.10 & 0 & 0 & 0 & 0.53 \end{pmatrix} \begin{pmatrix} (D/H)_1 \\ (D/H)_2 \\ (D/H)_3 \\ (D/H)_4 \\ (D/H)_5 \\ (D/H)_{6\mathrm{pro}-R} \\ (D/H)_{6\mathrm{pro}-S} \\ (D/H)_m \end{pmatrix} \tag{26}$$

For glycerol, three sites can be distinguished by ^{2}H NMR: site A, which is (1*S*,2*S*)- and (1*R*,2*R*)-(1-^{2}H$_1$)glycerol; site B, which is (1*R*,2*S*)- and (1*S*,2*R*)-(1-^{2}H$_1$)glycerol; site C, which is (2-^{2}H$_1$)glycerol. In the two biosynthesis routes, the transfer pathways of the 1,2 and the two 6 hydrogens are very similar. The 3 and 4 hydrogens are different. This is also in agreement with the reaction mechanism.

The complete mathematical model representing the hydrogen isotope affiliation during alcoholic fermentation not only can provide information on the reaction mechanism but also can be used to estimate the isotopic data, based on those of the substrate and the medium. For the commercial corn glucose and Nantes tap water used in this work as references, the calculation of the $(D/H)_i$ of ethanol and glycerol based on the $(D/H)_j$ of the glucose and $(D/H)_m$ are shown in **Table 4**. Taking into account the uncertainty in determining $(D/H)_i$, $(D/H)_j$, $(D/H)_m$, and a_{ik}, the agreement between the calculated and experimental results is satisfactory. The study proves, in a detailed and quantitative way, that the metabolites contain isotopic information about their precursor in a biotransformation and can be used for the identification of its origin. This method of quantitative isotope tracing close to natural abundance established in our study of isotope affiliation can be applied to other chemical and biochemical transformations.

ABBREVIATIONS USED

SNIF NMR, site-specific natural isotope fractionation studied by nuclear magnetic resonance; IRMS, isotope ratio mass spectrometry; NTW, Nantes tap water; IFF, global isotope fractionation factor; G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; F1,6dP, fructose 1,6-diphosphate; DHAP, dihydroxyacetone phosphate; G3P, glyceraldehyde 3-phosphate; 1,3dPG, 1,3-diphosphoglycerate; 2PG, 2-phosphoglycerate; PEP, phosphoenol pyruvate; NADH/NAD$^+$, nicotinamide adenine dinucleotide (reduced form/oxidized form); FMNH$_2$/FMN, flavin mononucleotide (reduced form/oxidized form); FADH$_2$/FAD, flavin adenine dinucleotide (reduced form/oxidized form).

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