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Site-Specific Hydrogen Isotope Fractionation in the Biosynthesis of Glycerol

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The nuclear magnetic resonance study of site-specific natural isotope fractionation (SNIF-NMR) produced in the glycolytic conversion of glucose into ethanol and glycerol provides isotopic transfer coefficients, a_{ij}, which relate sites i in the products to sites j in the reactants. The isotopic connection between the carbon-bound hydrogens of glycerol and those of glucose and water in fermentation reactions carried out with *Saccharomyces cerevisiae* has been investigated. The a_{ij} coefficients provide mechanistic information on the genealogy of the glycerol hydrogens, on the relative rates of triose phosphate isomerization and reduction of dihydroxyacetone phosphate into glycerol 3-phosphate, on the stereospecificity of the reduction, on the percentages of intra- and intermolecular hydrogen transfer occurring in the course of the reaction, and on the order of magnitude of the active overall kinetic and thermodynamic isotope effects. Thus a close connection is determined between sites 3 pro-R of glycerol (stereospecific numbering) and H_6 pro-R and to a lesser extent H_2 of glucose and between site 3 pro-S of glycerol and H_6 pro-S and H_1 of glucose. Moreover site 2 of glycerol, which exhibits a strong correlation with water is also partly connected with glucose. Possible changes in the values of the isotopic transfer coefficients, as a function of the composition of the medium or of the environmental conditions, enable mechanistic perturbations such as variations in the percentage of intermolecular transfers to be detected. Analyzed in terms of stereospecificity of the reduction step the isotopic results provide a direct chemical shift assignment of the enantiomeric pairs (1_S, 3_R) and (1_R, 3_S) of glycerol triacetate. The influence of added bisulfite, which strongly increases the yield in glycerol is estimated. The isotopic characterization of the bioconversion producing both ethanol and glycerol has been extended to the determination of the carbon isotopic parameters by isotope ratio mass spectrometry. Although it usually occurs as a by-product of the fermentation, glycerol can be considered as a useful complementary probe for characterizing the glycolytic pathway and for inferring various properties of the carbohydrate precursors.

INTRODUCTION

The investigation of site-specific natural isotope fractionation by nuclear magnetic resonance (SNIF-NMR) (*1*) is a powerful source of information on overall connections between individual atoms of reaction products and their precursor atoms in the starting materials (*2*). For a complex bioconversion, such as fermentation, this approach may provide the global isotopic balance involving specific molecular sites subjected to the whole set of successive and competitive reaction steps. Consequently the results

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can be interpreted in terms of individual genealogy of atoms, of relative contributions of intra- and intermolecular hydrogen transfers, and of the overall active kinetic or thermodynamic isotope fractionation effects affecting a specific molecular position. For a given standardized reaction, the matrix of isotopic coefficients connecting individual sites, or clusters of sites, in the products and in the reactants, may be used subsequently to infer isotopic properties of consumed reactants from the isotopic fingerprint of their products (*2*). It should be emphasized that investigating the isotopic behavior at natural abundance or close to natural abundance avoids the risk of perturbing the biochemical pathway through kinetic isotope effects in branched reaction steps. The determination of overall molecular carbon-13 contents by isotope ratio mass spectrometry (IRMS) has been frequently applied to the characterization of metabolic pathways in natural conditions (*3*). By isolating specific carbon sites, a more detailed interpretation of the biochemical mechanisms is accessible by IRMS (*4,5*) but only at the price of appropriate and often lengthy degradations of the investigated molecule. New information on the genealogy of the carbon skeleton of glycerol has recently been obtained in this way (*6*). In contrast to IRMS, the SNIF-NMR approach offers the advantage of simultaneously determining all the isotope contents of the diastereotopic molecular positions. This method is particularly well suited to the analysis of hydrogen isotope fractionation. For example, the glycolytic products, ethanol and water, can be used as isotopic probes to characterize sugar parents and in particular to obtain information on the metabolism of the photosynthesis and on the botanical origin of the precursors (*7*). Since the fermentation probe, ethanol–water, exhibits a strong reduction in the number of hydrogen isotope parameters as compared to the starting components, glucose and water for instance, complementary access to isotopic parameters of different molecules derived from sugars is desirable. In this perspective, the present study considers the isotopic aptitude of glycerol to characterize both the mechanistic pathway of its biosynthesis in a fermentation reaction and the isotopic properties of its carbohydrate precursors.

EXPERIMENTAL

Materials

Commercial sugars from different origins have been used in the fermentation experiments. Their isotopic parameters are given in the tables. Variations in the isotope ratio at positions 1, 2 and 6, 6′ of glucose have been obtained by adding small quantities (34.8–90.5 mg) of α-D-glucose (from Aldrich) selectively labeled with deuterium (99.8%) at position 1, 2 or 6 to 100 or 150 g of corn glucose dissolved in water. Nantes tap water characterized by an isotopic ratio $(D/H)_W = 150$ ppm has been used in most fermentation experiments. In order to investigate the influence of the aqueous medium slightly enriched water (300–2000 ppm) was also prepared.

5.5 g of baker's yeast were used in all experiments. The composition of the fermentation medium denoted α was the following, for 1 L of solution: 150 g D-glucose, 1 g NH_4Cl, 1 g KH_2PO_4, two crystals of $MgCl_2$.

In some experiments the yield of glycerol was increased by adding to the solution, 15 g of Na_2SO_3 (β medium). In this medium, bisulfite interacts with acetaldehyde and partly inhibits its transformation into ethanol.

Extraction of Ethanol and Glycerol

In the conventional fermentation experiments, ethanol was extracted by fractional distillation (*2*). The residue was heated (about 100°C) to remove water until the volume was reduced to 1/3. The pH of the residue was adjusted to a value of 9 by adding wet $Ca(OH)_2$. After filtration on a buchner the solution was evaporated again, until a paste was obtained. Glycerol in the paste was extracted three times with ethanol/ether (2/1 v/v). The extraction solutions were combined and filtered. Glycerol was obtained, after vacuum evaporation (10 mm Hg) at 80°C, with a yield of 70%.

When Na_2SO_3 was added to the fermentation medium, the distillation and evaporation steps were perturbed by formation of a large quantity of foam. Evaporation had to be carried out by heating small portions added successively.

Synthesis of Glycerol Triacetate

Two grams (22 mmol) of glycerol, 10 ml (106 mmol) of acetic anhydride, and 1.5 g of sodium acetate were mixed in a round bottom flask and heated 30 min under reflux and stirring. The reaction mixture was poured into 80 ml of ice water and the solution was extracted three times with 30 ml of ether. The combined ether phases were washed successively with 30 ml of a sodium carbonate solution (150 $g.L^{-1}$) and 20 ml of a half-saturated sodium chloride solution. After drying over sodium sulfate, the ether and the impurities, acetic anhydride and acetic acid, were evaporated under vacuum. Four grams of triacetin was obtained (yield: 85%). The purity (>99%) was checked by ^{1}H-NMR.

Isotopic Determinations

The overall carbon isotope ratio of every molecular species, A, is expressed on the relative δ-scale:

$$\delta_A(‰) = \frac{(^{13}C/^{12}C)_A - (^{13}C/^{12}C)_{PDB}}{(^{13}C/^{12}C)_{PDB}} 1000, \quad [1]$$

where PDB denotes the international carbonate reference (Pee Dee Belemnite).

The site-specific hydrogen isotope ratios $(D/H)_i$ are defined (*8*) as:

$$(D/H)_i = \frac{D_i}{H_i} = \frac{N_{Di}}{P_i N_H}, \quad [2]$$

where D_i and H_i are the numbers of deuterium and protium atoms at site i, N_{Di} the number of isotopomers monodeuterated at position i, P_i the stoechiometric number of hydrogens at site i, and N_H the number of fully protonated molecules.

Isotope Ratio Mass Spectrometry (IRMS)

The overall $\delta^{13}C$ parameter of glycerol, sugars, and ethanol was measured using a Finnigan Delta E mass spectrometer coupled with a Carlo Erba NA 1500 elemental analyzer. The precision of the determination is better than 0.3 ‰ (*9*).

Nuclear Magnetic Resonance (SNIF-NMR)

The $(D/H)_i$ ratios of glycerol (investigated in the form of its triacetate) were measured by ^{2}H-NMR using a calibrated reference, tetramethylurea (TMU) (*8*), distributed by the Institute for Reference Materials and Measurements (IRMM) in Brussels [$(D/H)_{TMU}$ = 123.4 ppm]. The $(D/H)_i$ values were calculated from Eq. [3]:

$$(D/H)_i = \frac{P_{TMU}}{P_{iA}} \frac{m_{TMU}}{m_A} \frac{M_A}{M_{TMU}} \frac{S_{iA}}{S_{TMU}} (D/H)_{TMU}, \quad [3]$$

where *P*, *m*, and *M* are, respectively, the stoechiometric number of hydrogens, the mass and the molecular weight of the investigated compound, A, or of the reference, TMU. S_{iA} and S_{TMU} are the areas of signal i of A and of the methyl signal of tetramethylurea in the deuterium NMR spectrum.

The ^{2}H-NMR spectra were recorded, under broad band proton decoupling (Waltz-16), on a Bruker DRX 500 spectrometer equipped with a fluorine lock device. The experimental conditions were the following: recording frequency, 76.77 MHz; frequency window, 1200 Hz; memory size, 32 K; exponential multiplication associated with a line broadening of 0.5 Hz; acquisition time, 6.8 s; delay time, 1.2 s; scan number, 3360; temperature, 55°C. The $(D/H)_i$ values were calculated from the average over three spectra. Usually 2.5 g of sample, 0.17 g of TMU mixed with 150 mg of C_6F_6 (locking material), and 1 ml of CH_3CN were introduced, after filtration, into a 10-mm NMR tube.

In the case of ethanol the $(D/H)_i$ ratios were recorded on a Bruker DPX 400 spectrometer under the following conditions: frequency, 61.4 MHz; temperature, 303 K; frequency window, 1200 Hz; memory size, 32K; line broadening, 0.1 Hz; acquisition time, 6.8 s; delay, 0.1 s; scan number, 200. Three spectra were run successively for every sample. The NMR tube was prepared in the same way as for glycerol triacetate with the following quantities: 2.6 g ethanol, 1.3 g TMU, and 0.1 g C_6F_6.

The quantitative determinations were performed by using a dedicated algorithm based on a complex least square analysis of the signal (*10*) (SNIF-NMR Concept core system and Interliss program from EUROFINS SCIENTIFIC, Nantes, France). This theoretical treatment involves an automatic integrated management of all the experimental parameters, including the phases of the individual resonances and the baseline parameters.

RESULTS

Since the deuterium content of the hydroxyl groups is averaged by chemical exchange with the aqueous medium, only the carbon-bound hydrogens of glycerol will be considered. At natural abundance the five monodeuterated isotopomers, denoted $\mathbf{1_S}$, $\mathbf{1_R}$, **2**, $\mathbf{3_S}$, $\mathbf{3_R}$ (Fig. 1) are present, in proportions which depend on the overall mechanistic pathway and on the resulting isotope effects.

Isotopic Probes of Glycerol

Unfortunately, due to insufficient chemical shift separation and relatively short transverse relaxation times the glycerol molecule itself is not a convenient probe for

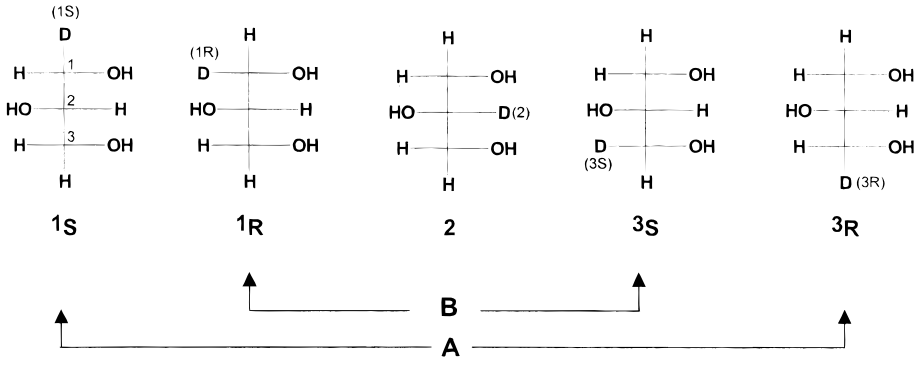

FIG. 1. Isotopomers of glycerol monodeuterated at the carbon-bound positions. The carbon atom numbered 3 (stereospecific numbering) was formerly carbon-3 of *sn*-glycerol 3-phosphate, which previously bore the phosphate group in the dihydroxyacetone phosphate (DHAP) precursor. Hydrogens at the pro-R and pro-S positions on carbon 1 and 3 (denoted 1S, 1R and 3R, 3S) have different origins. From a stereochemical point of view the four isotopomers $\mathbf{1_S}$, $\mathbf{1_R}$, $\mathbf{3_S}$, $\mathbf{3_R}$ correspond, respectively, to the four species (1S,2S), (1R,2S), (3S,2R), (3R,2R) resulting from a stereospecific reduction of the carbonyl site of DHAP. Two pairs of enantiomers ($\mathbf{1_S}$,$\mathbf{3_R}$) and ($\mathbf{1_R}$,$\mathbf{3_S}$) are therefore produced.

^{2}H-NMR. Derivatives, such as glycidal (*11*), obtained by a series of stereospecific enzymatic reactions could exhibit good discriminating potential. However, it should be emphasized that carrying out a large number of reaction steps, some of which are characterized by relatively low yields, is likely to introduce cumulated isotope fractionation effects, which should be carefully controlled at every step of the chemical pathway. As illustrated in Fig. 2, the simple triacetate derivative of glycerol exhibits reasonable chemical shift separation. For symmetry reasons only three carbon-bound sites, A, B, and 2, are distinguished. The assignment: A $\equiv \mathbf{1_S} + \mathbf{3_R}$ and B $\equiv \mathbf{1_R} + \mathbf{3_S}$ is confirmed later in this paper. Alternatively five carbon-bound hydrogen positions can be distinguished in the ^{2}H-spectrum of the derivative 2,2-dimethyl-1,3-dioxolane-4-methanol (glycerol acetonide), which is easily prepared. However, due to the lack of chiral specificity of the chemical conversion the corresponding prochiral positions on carbon 1 and 3 of glycerol have been scrambled and the ^{2}H spectrum exhibits two pairs of equal signals, one pair originating from ex-($\mathbf{1_S} + \mathbf{3_R}$) and the other from ex-($\mathbf{1_R} + \mathbf{3_S}$). Consequently, in spite of an increase in the number of diastereotopic sites with respect to the triacetate, the isotopic discrimination is not improved. Nevertheless this compound can be proposed as a useful complementary probe since it is characterized by relatively small line-widths.

Isotopic Ratios

The NMR determinations have been combined with the measurement, by IRMS, of the overall carbon-13 contents of both the starting materials and the glycerol and ethanol products (Table 1).

The site-specific hydrogen isotope parameters determined in fermentation experiments involving glucose samples from different botanical origins and isotopically modified maize glucose are collected in Table 1. Several series of fermentation

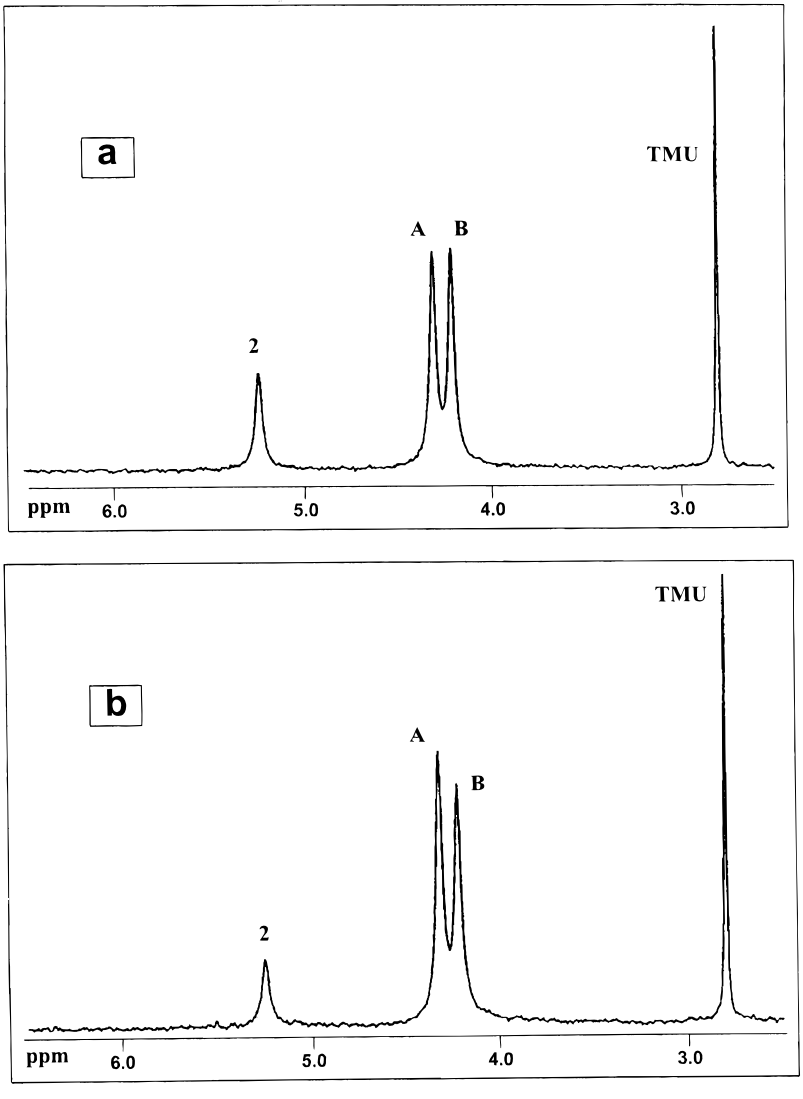

FIG. 2. ^{2}H-NMR spectra (at 76.8 MHz) of triacetates, prepared from glycerol obtained by fermentation of maize glucose (150g. L^{-1}) by *Saccharomyces cerevisiae*. The fermentation medium used in experiment a is described as α in the experimental section. Experiment b has been carried out, in the fermentation medium denoted β, with glucose slightly enriched (327 ppm) at position 2. TMU denotes the natural abundance deuterium signal of the isotopic reference, tetramethylurea (delivered by IRMM in Brussels). Spectra a and b have been registered at 55°C in CH_3CN. An exponential multiplication corresponding to a line broadening of 0.5 Hz has been applied. The signals are assigned in Fig. 1.

experiments have been carried out, in otherwise identical experimental conditions, with maize glucose samples either normal or enriched on sites 1, 2, 6, 6′, at levels ranging from 327 to 585 ppm. The results are compared for fermentations carried

TABLE 1

Isotopic Parameters of the Reactants: (Sugar + Water) and of the Products (Glycerol + Ethanol + Water) of Fermentation Reactions Conducted with *Saccharomyces cerevisiae* in Two Different Fermentation Media

	Starting materials (a) Isotopic parameters Glucose		Products (b) Isotopic parameters						
			Glycerol (c)				Ethanol		
Origin	$\Delta(D/H)$ (ppm)	$\delta^{13}C$ (‰)	$\delta^{13}C$ (‰)	$(D/H)_A$ (ppm)	$(D/H)_B$ (ppm)	$(D/H)_2$ (ppm)	$_D{}^{13}C$ (‰)	$(D/H)_I$ (ppm)	$(D/H)_{II}$ (ppm)
Beet sugar	—	−23.6	—	128.6	129.2	98.8	−25.6	92.1	125.6
+ Na_2SO_3 (d)	—	−23.6	—	124.1	122.8	103.0	—	—	—
Cane sugar	—	−10.7	—	132.5	146.5	86.6	−11.9	109.0	123.9
+ Na_2SO_3 (d)	—	−10.7	—	136.3	141.2	103.4	—	—	—
Wine	—	—	—	137.3	139.2	105.7	−27.3	97.9	125.0
Maize glucose (M)	—	−10.6	−16.8	136.1 (0.8)	152.1 (0.7)	100.2 (1.0)	—	110.4 (0.3)	125.3 (0.6)
+ Na_2SO_3 (d) (e)	—	—	−16.9	142.1 (0.7)	151.8 (0.4)	92 (1.9)	—	108.3 (0.3)	120.7 (0.9)
(M) H_1 enriched	585	—	−17.6	160.2 (1.8)	280.4 (1.2)	101.9 (2.8)	—	187.6 (0.5)	126 (0.5)
+ Na_2SO_3 (d)	327	—	—	150.9 (1.3)	229.9 (2.1)	97.2 (1.9)	—	151 (0.4)	121.5 (0.3)
(M) H_2 enriched	341	—	—	197 (0.3)	158.2 (0.6)	103.5 (2.8)	—	140.2 (0.5)	126.7 (0.5)
+ Na_2SO_3 (d) (e)	337	—	−16.6	186.7 (0.8)	149.8 (0.8)	87.8 (1.8)	—	— —	— —
(M) H_{66} enriched	376	—	−17.0	244.9 (2.2)	244.8 (3.6)	103.0 (2.2)	—	219.0 (f) (0.8)	129.0 (0.8)
+ Na_2SO_3 (d)	376	—	—	250.3 (2.4)	247.1 (2.2)	100.4 (2.4)	—	214.0 (f) (0.6)	122.0 (0.3)

Note. (a) The initial concentration of glucose was 150 $g.L^{-1}$. $\Delta(D/H)$ represents the specific enrichment. The starting fermentation water is Nantes tap water which is characterized by an isotope ratio $(D/H)_W^S = 150$ ppm. The overall isotope ratio of the carbon bound (nonexchangeable) hydrogens of the maize glucose pool used for producing the isotopically modified reactants is $(D/H)_{GNE} = 155$ ppm. (b) The isotope ratio of the end fermentation water, $(D/H)_W^Q$ was only slightly modified with respect to the starting values $(D/H)_W^S$. Values of 150.3 (1.1) have been measured in experiments performed with maize glucose. The standard deviations (within parentheses) are calculated over three repetitions. (c) The isotopic parameters of the carbon-bound hydrogens of glycerol are determined on the triacetate derivative. (d) The fermentation medium (β) contains a high concentration of Na_2SO_3 which significantly enhances the production of glycerol (≃15%). (e) The starting concentration of glucose was 100 $g.L^{-1}$. (f) These values integrate both monodeuterated and bideuterated species which are observed separately on the ^2H spectrum.