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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

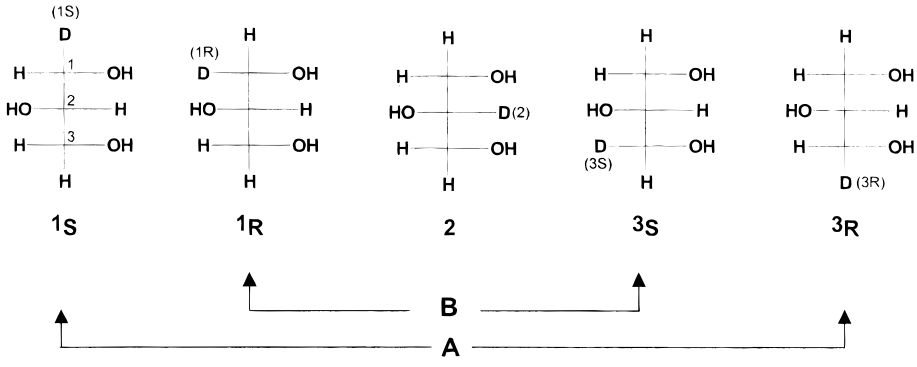

FIG. 1. Isotopomers of glycerol monodeuterated at the carbon-bound positions. The carbon atom numbered 3 (stereospecific numbering) was formerly carbon-3 of *sn*-glycerol 3-phosphate, which previously bore the phosphate group in the dihydroxyacetone phosphate (DHAP) precursor. Hydrogens at the pro-R 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

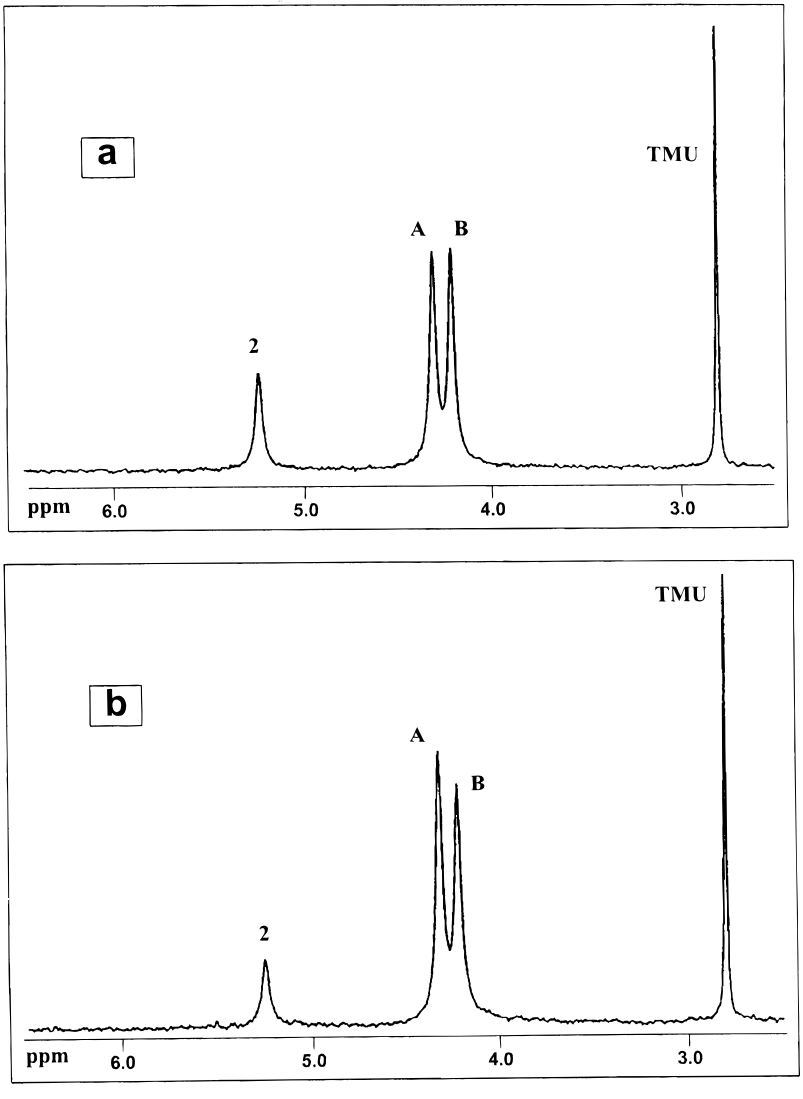

FIG. 2. ^{2}H-NMR spectra (at 76.8 MHz) of triacetates, prepared from glycerol obtained by fermentation of maize glucose (150g. L^{-1}) by *Saccharomyces cerevisiae*. The fermentation medium used in experiment a is described as α in the experimental section. Experiment b has been carried out, in the 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	152.1	100.2	—	110.4	125.3
				(0.8)	(0.7)	(1.0)		(0.3)	(0.6)
+ Na_2SO_3 (d) (e)	—	—	−16.9	142.1	151.8	92	—	108.3	120.7
				(0.7)	(0.4)	(1.9)		(0.3)	(0.9)
(M) H_1 enriched	585	—	−17.6	160.2	280.4	101.9	—	187.6	126
				(1.8)	(1.2)	(2.8)		(0.5)	(0.5)
+ Na_2SO_3 (d)	327	—	—	150.9	229.9	97.2	—	151	121.5
				(1.3)	(2.1)	(1.9)		(0.4)	(0.3)
(M) H_2 enriched	341	—	—	197	158.2	103.5	—	140.2	126.7
				(0.3)	(0.6)	(2.8)		(0.5)	(0.5)
+ Na_2SO_3 (d) (e)	337	—	−16.6	186.7	149.8	87.8	—	—	—
				(0.8)	(0.8)	(1.8)		—	—
(M) H_{66} enriched	376	—	−17.0	244.9	244.8	103.0	—	219.0 (f)	129.0
				(2.2)	(3.6)	(2.2)		(0.8)	(0.8)
+ Na_2SO_3 (d)	376	—	—	250.3	247.1	100.4	—	214.0 (f)	122.0
				(2.4)	(2.2)	(2.4)		(0.6)	(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.

out in a conventional medium and in a medium containing a relatively large amount of Na_2SO_3 with a view to increase the production of glycerol.

In order to avoid isotopic perturbations introduced by exchanges of the hydroxylic hydrogens, the overall deuterium content of the glucose skeleton was determined by IRMS on the trinitrate derivative (*7*). The starting sample of maize glucose used in most experiments is characterized by an overall isotope ratio of the carbon bound hydrogens $(D/H)_{GNE} = 155$ ppm. Both glycerol and ethanol are significantly depleted (Table 1) with respect to glucose, but, due to the high level of dilution, the isotope ratio of the aqueous medium is only slightly increased.

In order to evaluate the hydrogen connectivity with water the same maize glucose was also fermented in aqueous media characterized by different values of the isotope ratio $(D/H)^S_W$. The site-specific isotope parameters of both glycerol and ethanol are given in Table 2. The influence of a higher yield in glycerol produced by added Na_2SO_3 has also been considered.

Isotopic Balance

As expected, both the carbon and hydrogen isotope parameters of glycerol vary as those of ethanol as a function of the botanical origin of the sugar parent (Table 1).

TABLE 2

Isotopic Parameters of Glycerol and Ethanol Resulting from Fermentation of Maize Glucose (150g. L^{-1}) by *Saccharomyces cerevisiae* Carried Out in Water Characterized by Different Values of the Hydrogen Isotope Ratio $(D/H)^S_W$

Fermentation medium (a)		Glycerol (b)				Ethanol (c)	
$(D/H)^S_W$ (ppm)	medium	$(D/H)_A$ (ppm)	$(D/H)_B$ (ppm)	$(D/H)_2$ (ppm)	$(D/H)_t$ (ppm)	$(D/H)_I$ (ppm)	$(D/H)_{II}$ (ppm)
150	(α)	136.1	152.1	100.2	135.3	110.4	125.3
		(0.8)	(0.7)	(1.0)	(1.0)	(0.3)	(0.6)
(0.1)	+ Na_2SO_3 (β)	142.1	151.8	92.0	136.0	108.3	120.7
		(0.7)	(0.4)	(1.9)	(0.8)	(0.3)	(0.9)
389.9	(α)	284.1	270.0	230.0	267.6	151.7	304.8
		(3.1)	(2.7)	(2.8)	(2.9)	(0.3)	(0.4)
(4.5)	+ Na_2SO_3 (β)	277.2	255.0	215.4	256.0	157.3	298.3
		(2.3)	(0.7)	(1.9)	(0.8)	(0.4)	(1.0)
976.4	(α)	586.0	526.6	524.3	549.9	258.9	747.9
		(5.0)	(1.2)	(4.1)	(3.2)	(0.3)	(1.8)
(9.1)	+ Na_2SO_3 (β)	565.5	514.3	514.7	534.9	268.8	712.7
		(2.0)	(2.6)	(1.8)	(1.9)	(0.9)	(1.0)
1978.5	(α)	1119.8	977.9	1051.9	1049.5	434.0	1486.9
		(1.9)	(3.5)	(0.9)	(2.3)	(1.4)	(4.9)
(19.6)	+ Na_2SO_3 (β)	1079.0	944.1	1035.6	1016.4	460.4	1416.9
		(2.7)	(5.4)	(3.0)	(3.9)	(1.7)	(5.1)

Note. (a) The fermentation media are described in the experimental section. (b) The isotope ratio of the carbon bound hydrogens of glycerol have been determined on its triacetate derivative. Signals A, B, and 2 are identified in Fig. 1. $(D/H)_t$ is the overall isotope ratio of glycerol. The standard deviations (within parentheses) are calculated over three repetitions. (c) $(D/H)_I$ and $(D/H)_{II}$ correspond, respectively, to the methyl and methylene sites of ethanol.

In spite of a lower accuracy of the $(D/H)_i$ ratios measured on glycerol as compared to ethanol, similar trends in the values of $(D/H)_A$, $(D/H)_B$ on one hand and $(D/H)_i$ (methyl site of ethanol) on the other hand, are observed. Thus site B of glycerol is enriched in deuterium to the same extent (about 17 ppm) as the methyl site of ethanol when going from the cane C_4 precursor to the beet C_3 precursor. This behavior can be explained by the role of partly identical hydrogen parents in the carbohydrate material.

Since glycerol is only a minor product of the fermentation its isotopic parameters may be very sensitive to kinetic isotope effects intervening in the first steps of the glycolytic reaction. Thus the strong ^{13}C depletion already observed (*6*) in glycerol as compared to its glucose precursor must be compensated for by a corresponding enrichment of the other products. Since ethanol is also slightly depleted, the isotopic balance requires that carbon dioxide issued from sites 3 and 4 of glucose be enriched in ^{13}C. This behavior has been checked in independent experiments.

The isotopic balance is more difficult to establish in the case of hydrogen as a consequence of the participation of the large aqueous pool. Indeed since hydrogens are partly transferred from water with kinetic isotope effects significantly higher than unity (*12–14*) the deuterium depletions observed, at complete carbohydrate consumption, in ethanol and to a lesser extent in glycerol, must be accompanied by a small, hardly detectable, enrichment of the aqueous medium.

Significant differences in the isotope contents of the products are observed when Na_2SO_3 is added to the fermentation medium. The hydrogen isotope ratio of site 2 of glycerol in particular is modified with respect to fermentation in the absence of Na_2SO_3. This behavior probably depends partly on variable fractionation effects associated with higher yields of glycerol production (about 15 against 5%).

Coefficients of Isotopic Transfer Between Reactants and Products

A set of linear equations of type 4 is expected to relate the isotopic ratios of the fermentation products, i, to those of the starting materials, j (*2*).

$$(D/H)_i = a_{ij}(D/H)_j + C_i \qquad [4]$$

The a_{ij} coefficients connecting sites i = A = $(\mathbf{1_S} + \mathbf{3_R})$, B = $(\mathbf{1_R} + \mathbf{3_S})$ and 2 of glycerol to sites j = 1 to 6 of glucose and j = w of water are accessible from the determination of the isotopic ratios, $(D/H)_i$, of glycerol obtained in fermentation experiments carried out with glucose samples and water characterized by different deuterium distributions, $(D/H)_j$. In principle such experiments could be performed by using only natural reactants with different isotopic distributions. However, in the present state of accuracy of the isotopic determinations performed on glucose (*23*) it was preferred to resort, in addition, to artificial modifications of the isotopic contents through addition of very small quantities of selectively ^2H-enriched glucoses (Table 1). The coefficients a_{ij} of the investigated correlations are gathered in Table 3.

DISCUSSION

Although detailed investigations of the individual reaction step of glycolysis have been carried out and various kinetic isotope effects have been determined (*12 13,16–*

TABLE 3

Isotopic Coefficients a_{ij} Connecting Sites *i* of Products to Sites *j* of Reactants in Fermentation Experiments Carried Out with *Saccharomyces cerevisiae*

a_{ij} (a) *j*	*i*	Glycerol				Ethanol	
		A	*B*	*t*	2	I	II
1	α	0.04	0.22	0.10	0.0	0.13	0.0
	β	0.03	0.23	0.11	0.01	0.13	0.0
2	α	0.18	0.02	0.08	0.0	0.09	0.02
	β	0.13	0.0	0.05	0.0	—	—
6, 6′ (b)	α	0.29	0.25	0.21	0.0	0.29	0.03
	β	0.29	0.25	0.22	0.02	0.28	0.03
W	α	0.53	0.45	0.50	0.52	0.18	0.74
	β	0.51	0.43	0.48	0.52	0.19	0.71

Note. (a) The sites considered in the starting medium are $j = 1,2,6,6'$ of glucose and $j = W$ of water. Sites $i =$ I(methyl) and II(methylene) of ethanol and sites $i = A$, B, and 2 (Fig. 1) of glycerol are observed in the products. Two series of experiments have been conducted in parallel with maize glucose fermented either in a conventional medium (α) or in a medium containing a high concentration of Na_2SO_3 (β) (see Experimental Procedures). (b) Since the starting glucose contains species bideuterated at postion 6, isotope effects are observed on the chemical shifts corresponding to isotopomers bideuterated on carbon 3 of glycerol and on carbon I of ethanol. This phenomenon introduces distorsions of the line shape, which are somewhat detrimental to the accuracy of the determinations.

21) the proportions of hydrogens at the different sites of glycerol, which have been transferred from water, in a fermentation reaction conducted at natural isotopic abundance, are not known. These proportions may depend to some extent on the experimental conditions of the reaction. In this respect the SNIF-NMR method is expected to provide an efficient source of information on the overall participations of intra- and intermolecular transfers in biosyntheses carried out with different microorganisms and in different media (*14*,*22*,*23*).

From the point of view of the hydrogen isotopes the transformation of glucose into glycerol occurring in the course of the glycolytic pathway can be characterized by transfer coefficients a_{ij}, which enable the carbon-bound hydrogen atoms of glycerol, i, to be connected to those of glucose, j (Eq. [4]).

FIG. 3. Reaction mechanism for the formation of glycerol from glucose. The fate of hydrogens from sites 1(□) 2(○) and 6-pro-S of glucose (△) is tentatively illustrated ignoring possible exchanges with the aqueous medium. The steps which may be responsible for intermolecular exchanges are mentioned (H_2O). In order to illustrate the genealogy of the glycerol atoms the numbering adopted for the carbon atoms follows that of glucose. The stereospecific numbering corresponding to *sn*-3-glycerol phosphate is given within circles in the last glycerol formula. G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; $F_{1,6}P_2$, fructose 1,6-diphosphate; DHAP, dihydroxyacetone phosphate; G3P, glyceraldehyde 3-phosphate; *sn*-Gly 3 P, *sn*-3-glycerol phosphate; Gly, glycerol.

(G6P)
(F6P)
(F1,6P2)
(DHAP)
(G3P)
(DHAP)
(G3P)
(sn-Gly 3-P)
(Gly)
Glycerol
NADH
NADH
(H2O)
(H2O)
(H2O)

Isotopic Connection with Glucose and Hydrogen Genealogy

It has been assumed that glycerol produced in a glycolytic reaction involving glucose originates from the two fragments C_1 - C_2 - C_3 and C_4 - C_5 - C_6 of the glucose skeleton (Fig. 3). These fragments result from the splitting of fructose 1,6-biphosphate in the aldolisation step leading to the trioses, dihydroxyacetone phosphate, DHAP, and glyceraldehyde 3-phosphate, G3P.

If the equilibrium between DHAP and G3P mediated by triose phosphate isomerase is relatively fast, the glycerol molecules obtained through a reversible NADH-dependent reduction of DHAP catalyzed by glycerol dehydrogenase may be issued from both parts of the starting glucose. In these conditions carbons 1, 2, and 3 of glycerol (Fig. 1) are derived from, respectively, carbons 3 + 4, 2 + 5, and 1 + 6 of glucose. Similarly, on the basis of the present knowledge of the glycolysis mechanism, each of the four hydrogens bound to carbons 1 and 3 (Fig. 1) are expected to have the following double origins (Fig. 3): $\mathbf{1_S}$, introduced from water at the aldolization step and from H_4 of glucose with possible exchange with water; $\mathbf{1_R}$, from H_3 and H_5 of glucose, but exchange with water intervenes at the triosephosphate isomerization step; $\mathbf{3_R}$, from H_2 of glucose but with partial intermolecular transfer at the glucose phosphate isomerization step and from the pro-R H_6 position of glucose; $\mathbf{3_S}$, from H_1 and $H_{6\ pro\text{-}S}$ of glucose. Hydrogen at position 2 is introduced from NADH by glycerol phosphate dehydrogenase. Full scrambling of the two glucose moieties should be characterized in particular by significant values of the coefficients a_{i1}, a_{i2}, $a_{i\ 6pro\text{-}R}$, and $a_{i\ 6pro\text{-}S}$ involving sites $1,2,6_{pro\text{-}R}$, $6_{pro\text{-}S}$ of glucose. A maximum theoretical value for full conversion of 0.25 is then assigned to these coefficients as a result of the fourfold degeneracy of the structure.

In a second hypothesis where the reduction of DHAP into glycerol is faster than the reversible isomerization of the triose phosphates, G3P $\rightleftarrows$ DHAP, the glycerol molecule would result solely from the C_1 - C_2 - C_3 fragment of glucose and the origin of each of the four hydrogens at positions 1 and 3 would be restricted to the first of the two sources described above. Such an hypothesis has been favoured in the case of yeast fermentation carried out in the absence of Na_2SO_3 (*6*). Significantly different values of the coefficients a_{ij} of the transfer matrix, [A], connecting reactants and products are expected in this mechanistic possibility. In particular the coefficient a_{i6} involving both sites 6 of glucose should be now close to zero.

The isotopic coefficients a_{A6} and a_{B6} connecting sites 6, 6′ of glucose to sites A and B of glycerol, exhibit relatively high values (Table 2), which prove that, even in the absence of Na_2SO_3, glycerol is derived from both fragments, 1-2-3 and 4-5-6, of glucose scrambled at the triosephosphate isomerization step. The observed values of a_{A6} and a_{B6} may be compared to the theoretical values 0.25, which would correspond to complete transfer from positions 6 pro-R and 6 pro-S of glucose to sites 3 pro-R and 3 pro-S, respectively, of glycerol in conditions of full scrambling of the two moieties issued from fructose 1,6-bisphosphate. The experimental results are in agreement with a situation where both the aldolase and triosephosphate isomerase steps are close to equilibrium. This behavior is very similar to that of the coefficients $a_{I\ 6pro\text{-}R}$ and $a_{I\ 6pro\text{-}S}$, which connect sites 6, 6′ of glucose to the methyl site of ethanol, the major product of the fermentation reaction (*14*). In addition the absence of

noticeable isotope effects on a_{A6} and a_{B6} is consistent with purely intramolecular transfers associated with small secondary isotope effects. High levels of connection are also observed between sites 1 and 2 of glucose and, respectively, positions 3 pro-S and 3 pro-R of glycerol since the coefficients a_{B1} and a_{A2} reach values of about 0.22 and 0.18, whereas the theoretical values corresponding to complete intramolecular transfers are equal to 0.25. Partial exchange with the aqueous medium occurring when hydrogen at position 2 of glucose 6-phosphate is transferred to position 1 of fructose 6-phosphate at the isomerization step mediated by phosphoglucose isomerase (*19*,*30*,*31*) may be responsible for a lowering of the a_{A2} coefficient with respect to the theoretical value.

More generally, ignoring possible fractionation contributions resulting from secondary kinetic isotope effects in conditions of incomplete transformation, the ratio of the experimental to the theoretical values of the a_{ij} coefficients involving sites $j = 1$ to 6 of glucose and $i =$ A,B,2 of glycerol would represent the proportion of hydrogens directly transferred from glucose to glycerol at every position. The present results therefore characterize a high degree of intramolecular transfer from, on one hand hydrogens 6 pro-R and, to a lesser extent, 2 of glucose to position 3 pro-R of glycerol and, on the other hand from 6 pro-S and 1 of glucose to 3 pro-S of glycerol. It may be concluded that glycerol is potentially a good isotopic probe for characterizing disappeared sugar precursors (case of alcoholic beverages for instance).

Stereospecificity of the Enzymatic Reduction of DHAP

The values of the coefficients a_{ij} connecting sites 1, 2, 6, 6′ of glucose to signals A and B of glycerol are also representative of the stereospecificity of the reduction step by glycerol dehydrogenase.

Thus the evolution of the spectrum of triacetin prepared from glycerol, itself derived from a glucose sample slightly enriched at site 2, is illustrated in Fig. 2. A relative increase in the intensity of signal A accompanies the deuterium enrichment at position 2 of glucose. This behavior corroborates the stereospecific character of the reduction step of DHAP mediated by glycerol dehydrogenase (*24–27*) since a nonstereospecific reaction would lead to similar enhancements of the A and B signals. Moreover since signal A has been tentatively assigned, on the basis of a conformational analysis using ^{1}H-NMR parameters (*28*,*29*), to both the pro-R position at the pro-R carbon and the pro-S position at the pro-S carbon of glycerol its ^{2}H-enrichment provides direct proof of the *si*-face type of the reduction of DHAP. The hydrogen atom at position 2 of glucose initially transferred to the pro-R site on carbon 1 of fructose 1,6-diphosphate (*19, 30*) and subsequently occupying the pro-R position on carbon-3 of DHAP must be associated with the pro-R position on carbon-3 of a *sn*-glycerol 3-phosphate molecule characterized by a stereochemistry R of carbon-2 (Fig. 3). Conversely, admitting the *si*-face character of the reduction of DHAP by glycerol phosphate dehydrogenase, the present experiments corroborate the chemical shift assignment of the ^{1}H- and ^{2}H-NMR signals A and B to, respectively, the $\mathbf{1_S} + \mathbf{3_R}$ and $\mathbf{1_R} + \mathbf{3_S}$ positions (Fig. 1). Although the considered reaction had already been indirectly investigated, this example illustrates the advantages of the method for directly elucidating the stereochemistry of individual reaction steps occurring in complex bioconversions.

Isotopic Connections with the Aqueous Medium

Both the a_{iw} and a_{ij} coefficients connecting glycerol sites, i, to water, w, on one hand and to glucose sites, j, on the other hand, are expected to provide, respectively, direct or indirect information on the participation of water in the hydrogen genealogy of glycerol.

Since, whatever the yield of the bioconversion, strong isotope effects may accompany hydrogen transfer from the large aqueous pool, the coefficients a_{iw} involving water are not directly exploitable for estimating the percentage of hydrogen originating from water. However, in principle, for a given site such as $i = 2$ that is strongly connected with water, the proportion of hydrogen originating from water can be indirectly estimated from the ratios of the experimental and theoretical values of the a_{ij} coefficients involving glucose. In fact the deficit with respect to the maximum value for $j = 1$ to 6 may be attributed to the participation of water. An overall value of the kinetic isotope effect characterizing hydrogen transfer from water can then be calculated. This approach has enabled a limit value of 1.4 to be assigned to the global kinetic isotope effect accompanying intermolecular transfers from water to the methylene group of ethanol in the fermentation reaction (*14*). Moreover it was shown that hydrogen transfer from NADH to the pro-R methylenic position of ethanol in the reduction step of acetaldehyde conveys only relatively low indirect connection with site 4 of glucose (*14*). In the case of glycerol the absence of connection between site 2 and sites 1,2,6,6′ of glucose is confirmed (Table 3). This site is strongly connected with water and a primary kinetic isotope effect of about 2 would be estimated from the a_{2W} coefficient in the hypothesis of a unique origin from the aqueous medium. However, possible connection with site 4 must be considered. The value of a_{24} is not directly accessible from the present results but the significant variations observed in the D/H value of site 2 of glycerol as a function of the origin of the sugar precursor (*32*) suggest a tighter connection with the glucose skeleton than in the case of the methylene group of ethanol. This connection is corroborated by the behavior of the C_2 parameter in equation 4 : $(D/H)_2 = 0.52(D/H)_w + 23.4$. In spite of the restricted precision reached on this extrapolated parameter it may be concluded from its noticeable value that a significant proportion of hydrogens in site 2 of glycerol is not derived from the aqueous medium.

In this respect it should be noted that in the glycolytic pathway the aldehyde hydrogen of G3P transferred to NADH after the triose phosphate isomerization step is related to hydrogen-4 of glucose and to the pro-S hydrogen on DHAP, itself introduced from water at the aldolization step. Another source of exchange with water, which has been shown to intervene in the absence of G3P (*16,33–35*), involves the pro-S hydrogen of the CH_2OH group of DHAP. Since this exchange enzymatically catalyzed by aldolase also affects DHAP molecules resulting from isomerization of the G3P moiety, it may be responsible for a loss of connectivity between H-4 of glucose and both the 1-pro-S site in signal A and the H_2 position of glycerol.

It should also be emphasized that the strong connection of clusters A and B with carbon-bound hydrogens of glucose is further confirmed by the behavior of the C_2 parameter of Eq. [4], involving j = water. Thus C_2, which measures the average contribution of hydrogens not issued from water, is of the order of 70 ppm for A and

reaches about 85 ppm for B. This result is in agreement with the highest proportion of intermolecular hydrogen transfer affecting hydrogen formally originating from site 2 of glucose and ending at cluster A as compared to hydrogen originating from site 1 and ending at cluster B. Another contribution to the difference between the C_2 values could result from different deuterium contents at the 6 pro-R position of glucose connected to B and at the 6 pro-S position connected to A.

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