earlier work.[11] Combining the zero-field proton and deuterium data with the quadrupole coupling constants of the static molecule found by Chiba,[11] one can calculate $\langle \theta_y^2 \rangle$ for the H_2O molecule. In brief this is done by (1) calculating $\langle \theta_x^2 \rangle_{HDO}$ and $\langle \theta_z^2 \rangle_{HDO}$ using the formulas in reference 18 to correct for the reduced masses, (2) using these expressions to calculate $\langle \theta_y^2 \rangle$ from the zero-field HDO data, and (3) calculating $\langle \theta_y^2 \rangle_{H2O}$ by the reverse procedure in step 1. Using the explicit expressions for the field gradient tensor averaged by libration and the C_2 flipping, one obtains $\langle \theta_y^2 \rangle$ = 0.123(rad²). We note the librations have a relatively minor effect on the quadrupole spectrum, and the value of η near unity is primarily a consequence of the C_2 flips.[15] An advantage of the dipolar measurements is that the static dipole interaction is inherently axially symmetric and any asymmetry is the direct result of motion.

Discussion

The nonaxially symmetric dipolar tensor produced by libration is readily observable via the proton zero-field spectrum. The excellent agreement between the results of the two versions of the zero-field experiment as well as the results of computer simulations rules out the possibility of splittings due to residual fields. Our results for the mean square amplitudes of the librational modes are in fair agreement with earlier data,[11,18] especially when one considers that the exact librational modes might differ slightly from the inertial rotations assumed.[12] With the high resolution possible in the dipolar zero-field experiment one has a sensitive measure of relatively small changes in the dipolar tensor.

The zero-field NQR results for HDO demonstrate the high resolution of the experiment and the precision with which it can measure the asymmetry parameter. The parameters relating to the motion are underdetermined with a single NQR experiment since the quadrupolar frequencies are a function of the three librational modes, the rate of the 180° flips, as well as the values of $(e^2qQ/h)_0$ and η_0, the parameters of the static molecule. We note that room temperature deuterium NQR measurements of a hydrate are usually inaccessible to frequency domain techniques because of their relatively short T_1's and low quadrupolar frequencies. In contrast to typical frequency domain NQR methods there is no power broadening[13] and ideally no signal due to protons. This fact generally allows resolution of the ν_0 lines, a tremendous aid in the assignment of the spectra.

In summary the zero-field experiment has been demonstrated to provide information about the dipolar and electric field gradient tensors in a two spin system and thus provide information about its motional characteristics. In general these should provide complementary information since they possess unique principal axis systems and hence are affected differently by the different motions which occur in a system. The zero-field measurements have the significant advantage of being made with a powder sample whereas the earlier measurements required a single crystal.[11] This aspect should allow study of subtle motions in systems inaccessible to single crystal measurements including amorphous and polycrystalline materials as well as biological samples.

Deuterium Transfer in the Bioconversion of Glucose to Ethanol Studied by Specific Isotope Labeling at the Natural Abundance Level

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Abstract: Site-specific natural isotope fractionation measured by deuterium NMR (SNIF-NMR) was used for investigating the deuterium transfers occurring in the fermentation of sugars into ethanol. In contrast to carbon-13, which is usually assumed to be randomly distributed within the glucose skeleton, very large deviations with respect to a statistical repartition are determined for deuterium. By transforming glucose samples from different origins into acetates and nitrates, the absolute values of the D/H ratios in the nonexchangeable sites were obtained. The hydroxyl sites were considered to contribute to the isotope content of the starting water medium. The site-specific isotope parameters were determined for more than 50 ethanol samples obtained by fermenting glucose samples issued from different starches and sucroses in water media with different isotope ratios. Several concentrations of sugar, several strains of the same yeast, and different temperatures of fermentation were considered. The whole set of isotope parameters characterizing the nonexchangeable sites of glucose, the starting and end water media, and the methyl and methylene sites of ethanol was submitted to factor analysis. The results are satisfactorily reproduced on the basis of two main factors which may be associated with the independent contributions of the isotope ratios of glucose and water. The isotope parameters of the end products and of the starting materials were then considered to be related by a set of linear equations and the coefficients of the redistribution matrix were calculated by multidimensional analysis. Thus the isotope ratio of the methyl site of ethanol exhibits a strong sensitivity toward the nonexchangeable sites of glucose and depends to a lesser extent on the isotope content of the starting water medium. By contrast, no direct connection is found between glucose and the methylene site which is only sensitive, with a strong discriminating effect against deuterium, to the isotope content of water. A redistribution coefficient slightly less than unity (0.96 for a concentration of sugar of 100 g L⁻¹) is found between the isotope ratio of the end and starting water media. Although the bioconversion of glucose into ethanol occurs with high fractionation effects which are the source of high deuterium depletions in the methyl and methylene sites of ethanol as compared to glucose, constant redistribution parameters can be obtained in appropriate conditions and the isotope parameters measured in alcohols may be used as a fingerprint for characterizing the sugar and aqueous juice from which they have been produced. The site-specific natural isotope parameters of ethanol therefore constitute a faithful and powerful probe for investigating the physiological biochemical and climatological effects which have governed the photosynthesis of sugars in natural conditions.

The first issue of this journal, which appeared in 1879, began with a paper by D. P. Ricketts[1] which was devoted to the characterization of sugars from different origins. More than a hundred years later, the problem is still a challenge since the distinction

between glucose samples issued from starch or sucrose elaborated by various C3 plants cannot be established unambiguously. More generally, the control of the fermentation processes of sugars is the subject of renewed interest, since, due to the development of ethanol production from the biomass, alcoholic fermentation has shown itself to be of prime importance not only in beverage industries but as an energy source and chemical supply.[2] Therefore a need subsists, from both the analytical and biotechnical points of view, for a better understanding of the enzymatic steps and methodological parameters intervening in the bioconversion of sugars from various origins. For these purposes, *stable isotopes at the natural abundance level* are powerful tracers[3,4] since they offer a way of correlating the starting materials and the end products. Thus the carbon-13 isotope content, in particular, has been extensively investigated in natural compounds. Very precise values of the *isotope ratios* can be obtained by mass spectrometry, but since the method requires the burning of the organic matter into water and carbon dioxide on which the determination is run, only *overall* information is obtained directly. In some cases, carbon fractionation concerning specific molecular fragments was investigated,[5] but careful and tedious chemical transformations must then be performed and this approach has received very little attention in the field of deuterium analysis. Recently we have shown that a *site-specific natural isotope fractionation* is accessible on the basis of high-resolution deuterium NMR (SNIF-NMR)[6] and the method has been applied to origin recognition and to the characterization of the chemical or biochemical history of various natural or synthetic products.[7] The aim of this work is to investigate, on a quantitative basis, *the deuterium filiation throughout the fermentation process* from glucose and water to ethanol and water and to support the use of the ethanol molecule as a probe for studying in natural conditions the *biochemical and physiological factors* which govern the hydrogen cycle in photosynthetic conversions.

Theoretical Section

From a mechanistic point of view it is interesting to be able to describe the fate of the hydrogen or deuterium atoms issued from starting materials $S = S_A$, S_B, ... in the course of a bioconversion to products $Q = Q_A$, Q_B ... Conversely, knowing the relative probabilities that deuterium atoms in a given site, Q_j, of the products arise from the different sites S_i of the reactants may be of fundamental importance for the investigation of natural products since this would enable the isotope distribution in inaccessible starting compounds to be inferred from measurements performed on end products. For a *complete* bioconversion, $S_A + S_B + ... \rightarrow Q_A + Q_B + ...$, involving distinct starting and end products, it may be assumed that the deuterium content in every site of an end product, Q_j, can be described in terms of the deuterium contents in the different sites of the starting materials, S_i, by a set of linear equations.

In practice, the investigation of the deuterium contents performed by mass spectrometry usually provides the results under the form of *overall isotope ratios*, $\overline{(D/H)}^S$, $\overline{(D/H)}^Q$, of the starting

and end materials.[8] In addition the site-specific isotope distribution as measured by the SNIF-NMR method can be characterized by *selective isotope ratios* $(D/H)_i^S$, $(D/H)_j^Q$ associated with the diastereotopic sites.[9]

At the natural level, the deuterium fractional abundance reduces to the isotope ratio and this approximation, which is no longer valid in the case of ^{13}C,[5c] enables the deuterium distribution to be tentatively described on the basis of the site-specific isotope ratios by the set of linear equations ($j = 1$ to n and $i = 1$ to m)

$$(D/H)_j^Q \simeq a_{j1}(D/H)_1^S + ... + a_{ji}(D/H)_i^S + ... + a_{jm}(D/H)_m^S \quad (1)$$

or in matrix notation

$$[A]\vec{D}^S = \vec{D}^Q \quad (2)$$

where $\vec{D}^Q$ and $\vec{D}^S$ are the column vectors of the deuterium contents in the end products and starting molecules, respectively, and $[A]$ is the redistribution matrix. Conversely, the deuterium content of a given isotopomer of the starting products can then be expressed in terms of the contributions of the end materials.

In the set of equations 2 the matrix $[A]$ reflects the *technical and mechanistic aspects of the bioconversion* which can be assumed to be invariant in standardized experimental conditions. Thus providing the matrix $[A]$ has been previously determined by a series of *calibration experiments* it becomes possible to produce, from the vector $\vec{D}^Q$ deduced from SNIF-NMR measurements, a fingerprint of the deuterium contents of *disappeared* starting materials.

If we consider the case of alcoholic fermentation, this approach would enable isotope parameters of the glucose and water precursors to become accessible from a determination of the ethanol and water deuterium contents in the fermented juice. However, some complications occur in this system since the starting and end water products (S_B and Q_B) are chemically identical. Moreover the water sites are involved in *a chemical exchange*, with the hydroxyl sites of glucose and ethanol, that is fast on the fermentation time scale. Deuterium transfers therefore take place between these exchangeable sites all along the course of the fermentation and the corresponding deuterium contents are related through appropriate equilibrium constants. The liquid-phase equilibrium between water and ethanol has been investigated by various techniques[10] and we have also carried out a direct determination by the SNIF-NMR method

$$C_2H_5OH + HOD \rightleftharpoons C_2H_5OD + HOH \quad (K_E)$$
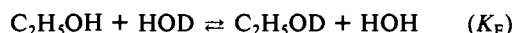
A value $K_E = 1.03$ will be adopted for estimating the (D/H) value of the hydroxyl site, III, of ethanol from the isotope ratio measured in the end water medium. No data are available for glucose but the isotope ratio of the exchangeable sites in the neat product can be derived on the basis of mass spectrometry determinations of the overall ratios in neat sugars and in their nitrate derivatives.

Although the seven nonexchanging sites of glucose are, in principle, diastereotopic, only three sets of sites (1), (5,6,6'), and (2,3,4) are clearly distinguished. Moreover, in most cases, because of the limited accuracy presently accessible in the quantification of the deuterium contents, these groups will be considered together in the statistical treatments. The fermentation reaction will therefore be described by two and three rank vectors $\vec{D}^S$ and $\vec{D}^Q$ written below in the transposed form:

$$(\vec{D}^S)^T = |D_G^{NE}, D_W^S| \quad (3)$$

$$(\vec{D}^Q)^T = |D_I, D_{II}, D_W^Q| \quad (4)$$

where D_W^S and D_W^Q refer to the starting and end exchanging

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water components, D_G^{NE} to the nonexchanging sites of glucose, and D_I and D_{II} to the methyl and methylene sites of ethanol.

Clearly the redistribution matrix cannot be determined in one experiment and a procedure founded on *regression analysis* is used to compute a whole row, $\bar{a}_j$, of matrix coefficients $\bar{a}_{ji}$ corresponding to a given isotopomer Q_j of the end product, Q. Let D_{jk} denotes a specific value of the deuterium content associated with the isotopomer j in experiment k. If q different values of D_j are available the results can be described by a set of q linear equations

$$\bar{D}_j^Q = [S]\bar{a}_j \qquad (5)$$

$[S]$ is a (q,m) matrix representing the measurements of the deuterium contents of the m groups of isotopomers individualized in the fermentation reactants and $\bar{D}_j^Q$ is the column vector of the q different values of the deuterium contents measured in the isotopomer Q_j.

A solution for $\bar{a}_j$ can be found in the least-square sense[12] by solving the equation

$$\bar{a}_j = [D]^{-1}[S]^T\bar{D}_j \quad \text{with} \quad [D] = [S]^T[S] \qquad (6)$$

Experimental Section

(a) Glucides and Fermentation Media. Glucides of different origin were selected on the grounds of their natural deuterium abundance. Maize glucose, cane sugar sucrose, grape glucose–fructose, barley glucose, beet-root sucrose, or potato glucose were used. The glucose species were obtained from appropriate hydrolysis of starch or sucrose when necessary.[19] In order to extend the range of deuterium contents available in natural glucose, we prepared α-D-glucose slightly enriched in site 1 by base-catalyzed exchange in HDO ($\simeq 200$, 250, 400 ppm) according to ref 11. The overall deuterium abundances were determined by mass spectrometry on the nitrate derivatives as described by Dunbar.[13] The purified glucose samples were transformed nearly quantitatively into their pentaacetate derivatives which are more suitable for the NMR investigation. The concentration of pentaacetate in $CHCl_3$ is maintained below 1.2 mol L^{-1} to avoid excessive line broadening. Moreover, the glucose samples extracted from natural products can be treated beforehand by a solution of EDTA to eliminate paramagnetic impurities.

Sterilized tap water was used to prepare slightly enriched HDO/H_2O solutions by the dilution technique. Deuterium-depleted waters were obtained either from distillation or from ice sheets cut off in Dumont d'Urville, Antarctic. The water deuterium content was determined according to the conventional procedure by mass spectrometry (VG Instruments, SIRA 9).

The synthetic fermentation medium contained carboxylic acids (tartaric, 3 g/L; malic, 6 g/L; citric, 0.3 g/L); salts (KH_2PO_4, 2 g/L, $(NH_4)_2SO_4$, 2 g/L; $MgSO_4\cdot7H_2O$, 0.2 g/L; $MnSO_4\cdot H_2O$, 0.01 g/L) asparagine, 2 g/L; and vitamines (biotine, 4 mg/L; mesoinositol, 0.5 g/L; thiamine, 0.1 g/L; pyridoxine, 0.1 g/L; nicotinic acid, 0.1 g/L; panthotenic acid, 0.1 g/L; p-aminobenzoic acid, 0.1 g/L). The medium was sterilized 15 min under pressure at 120 °C and the vitamin solution was submitted to ultrafiltration. The overall deuterium content of the water medium was determined before the fermentation.

The different strains of yeast considered for the fermentation belong to the species *Saccharomyces cerevisiae* and were used either in dried form as sold by their producers or as bakers' yeasts. The yeasts were reactivated in sterile water at 40 °C (dry yeasts) or directly in situ (bakers' yeast). The conditions of the fermentation were $T = 301$ K, pH 7, and c 100 g/L of water (unless otherwise stated).

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The fermentation was conducted in order to achieve complete transformation of the sugar into ethanol. The remaining glucose being always less than 1 g/L, thermodynamic isotope effects can be neglected.

The ethanol samples obtained from distillation in normalized conditions[9] were hydro-alcoholic mixtures titrating about 95% in volume. These mixtures were accurately titrated by the Karl–Fischer Technique and appropriate corrections[9] were applied in the calculation of the isotopic ratios $(D/H)_j$. The yield of ethanol extraction being higher than 99%, the fractionation effects produced on the ethanol parameters by distillation can be safely neglected.

(b) Measurement of Specific Isotope Ratios by ^2H NMR. We have used a Brüker AM 400 spectrometer fitted with a specific probe tuned on the deuterium frequency (61.2 MHz) and accepting 15-mm diameter tubes (Wilmad 7PP). A fluorine field-frequency lock and an automatic sample changer have been implemented. The spectrometer is controlled by an Aspect 3000 computer fitted with a CDC hard disk (^{96}Mo).

Careful preliminary adjustments of the spectrometer must be carried out.[14] Sealed tubes containing reference products are used for testing the response of the spectrometer. Thus, two samples of alcohol, from maize and beet root, containing a given amount of the reference $(CH_3)_2NCON(CH_3)_2(TMU)$ are used to check the long-term reproducibility of the spectrometer. The $\overline{(D/H)}$ values of the ethanols and of the TMU were determined by mass spectrometry in order to calibrate the NMR results on the V.SMOW scale.[8b] The reproducibility of the NMR measurements performed on the same ethanol sample is better than 0.2% in relative values. The $(D/H)_j$ ratios are usually obtained with a precision of ±0.2 ppm. A signal-to-noise ratio of at least 175 is necessary to achieve a good reproducibility. This is reached with 128 accumulations obtained with an acquisition time of 6.8 s ($\pi/2$ pulse width = 50×10^{-6} s) over a range of 1200 Hz (active memory 16K words). Ten spectra of each ethanol sample are successively recorded for statistical treatment, and eight samples can be automatically studied in 24 h with the aid of the changer robot. A program, written in PASCAL, enables in-line statistical calculations to be performed. This program can be called using the DISNMR-BRÜKER software system. This allows automatic treatment of the NMR measurements and calculation of the values of $(D/H)_I$, $(D/H)_{II}$, and $\overline{(D/H)}$ which are transferred into a data base administered by a DGITAL P350 computer.

The factor analysis, understood in terms of principal component determination,[12b] was performed with a program (Factorda) written in BASIC (Plus) for the DIGITAL P350 microcomputer.

Results

Natural sugars from different origins and several sugars slightly enriched on carbon position 1, as described in the Experimental Section, have been directly investigated. In order to study the deuterium filiation, these sugars have then been fermented into ethanol in controlled media.

The overall deuterium contents $\overline{(D/H)}_G$ of glucose samples issued from various starch and sucrose species were measured by mass spectrometry. However, these overall values may not correctly represent the original sugar since the deuterium content of the exchangeable hydroxyl sites, $(D/H)_G^E$, reflects that of the last aqueous medium. Moreover, unless strictly controlled experimental conditions are defined, discriminating fractionation effects are likely to be introduced by the physical treatments involved in the preparation step. Transforming the glucose into nitrate[13] enables this source of discrepancy to be eliminated, and the isotope ratios of the nonexchangeable sites, $(D/H)_G^{NE}$, can then be directly measured by mass spectrometry.

By the SNIF-NMR method, several site-specific isotope ratios associated with the nonexchangeable sites of glucose have been identified. This distinction and the accuracy of the determinations were improved by transforming the glucose samples into pentaacetate derivatives. In Figure 1 are shown two typical spectra of maize and potato glucose acetates. Such spectra enable the mole fractions of the monodeuterated species associated with the three sets of sites (5,6,6'), (2,3,4), and 1 to be determined (Table I). It should be noted that about 18 h are necessary to reach a signal-to-noise ratio of about 20–40 for the lines resulting from the nonexchangeable sites of the glucose skeleton. The repeatability of the NMR measurements is on the order of 2%, but due to the partial overlapping of the signals the accuracy accessible on the mole fractions does not exceed ±0.004. By resorting to intramolecular referencing[9] with respect to the acetate moiety, M, *absolute values* of the site-specific parameters, $(D/H)_i$, are

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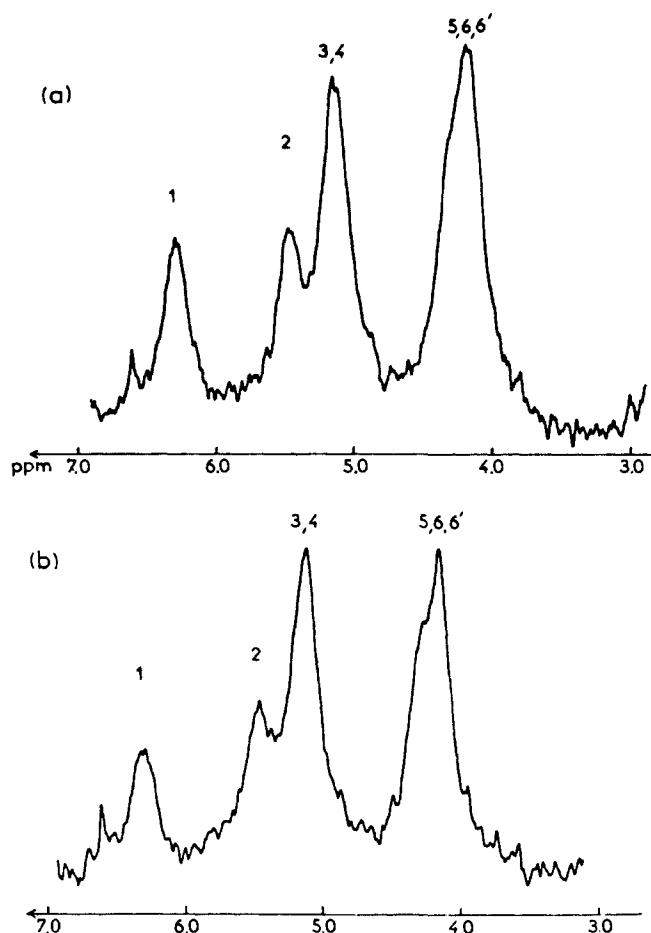*J. Am. Chem. Soc., Vol. 108, No. 17, 1986* 5119

Figure 1. Natural-abundance (61.4 MHz) ^{2}H NMR spectra of α-D-pyranose pentaacetate samples derived from (a) maize starch and (b) potato starch.

Table I. Effective Molar Fractions (f_i) of Monodeuterated Isotopomers and SNIF Ratios, (D/H)$_i$, Associated with Nonexchangeable Chemical Sites, i, of Two Samples of Glucose[a]

α-D-glucose pentaacetate	natural deuterium contents in site i			G_{NE}	
	1	2,3,4	5,6,6'	NMR	MS
maize					
F_i	0.143	0.428	0.428		
f_i	0.131	0.445	0.424		
(D/H)$_i$	139[b]	158[b]	150[b]	152[b]	151[b]
	138[c]	157[c]	149.5[c]		
potato					
f_i	0.109	0.492	0.398		
(D/H)$_i$	104[b]	157[b]	127[b]	136.5[b]	137.5[b]
	105[c]	158[c]	128[c]		

[a] Obtained from maize and potato starches. F_i denotes the statistical mole fractions of the nonexchangeable sites. The average values $\overline{(D/H)}_{NE}{}^G$ for maize or potato glucose were either computed from the NMR results by using the equation $\overline{(D/H)} = \sum F_i(D/H)_i$ or determined by mass spectrometry (MS). [b] These values were computed from eq 7 by using the intramolecular reference method. [c] These values were computed from $\overline{(D/H)}_{NE}{}^G$ (MS) and f_i according to the equation $(D/H)_i = (f_i/F_i)\overline{(D/H)}_{NE}{}^G$.

accessible, providing the deuterium content (D/H)$_M$ of the pool of acetic anhydride reactant has been determined beforehand. Thus the isotope ratio associated with P_i isochronous nuclei of the investigated glucose pentaacetate which contains P_M (=15) hydrogen positions is calculated from

$$(D/H)_i = \frac{P_M}{P_i} \frac{f_i}{f_M}(D/H)_M \qquad (7)$$

where f_i and f_M are the mole fractions of the monodeuterated

Table II. Influence of the Deuterium Content of the Medium on the Deuterium Distribution in Ethanols[a]

	starting water[b]		reaction products		
	(D/H)$_W^{I c}$	(D/H)$_W^{S c}$	(D/H)$_I^c$	(D/H)$_{II}^c$	(D/H)$_W^{Q c}$
	Experiment A				
(b)	120.0	120.9$_5$	107.7	102.9	124.4
(c)	149.7	149.8$_5$	112.0	124.2	151.9
(c)	150.2	150.2	113.3	125.0	152.3
(d)	194.4	193.5	124.0	162.6	193.8$_5$
(d)	205.4	204.3	125.3	165.3	204.0$_5$
(d)	259.8	257.3	134.1	199.1	254.8$_5$
(d)	262.3	259.8	135.0	201.7	257.1$_5$
(d)	327.7	323.6	145.0	237.7	318.2
(d)	395.3	389.5	161.1	281.6	381.3
(d)	400.6	394.7	161.5	291.5	386.3
	Experiment B				
(a)	99.6$_5$	102.2	97.1	82.2$_5$	104.9
(c)	149.3$_5$	149.7$_5$	110.6	123.4	151.6
(d)	199.4	197.1	125.1	159.3	198.7
(d)	249.3	244.7	137.6$_5$	202.6	245.2
(d)	298.8	291.8$_5$	153.4$_5$	237.0$_5$	292.3
(d)	349.6	340.2	165.1	279.8	339.7
(d)	365.8	355.6$_5$	167.8$_5$	292.1$_5$	353.4
	Experiment C				
(b)	130.0	130.4	88.1	106.1	133.3
(c)	148.9	149.0	93.0	121.5	152.0$_5$
(d)	155.4$_5$	155.7	94.0	128.0	158.2$_5$
(d)	198.6$_5$	200.0	103.9	164.5	199.6$_5$
(d)	249.4	252.0	111.1	188.3	246.5$_5$
(d)	301.1	305.0	122.6	239.2	295.9
(d)	348.5	353.6	135.3	283.9	343.9

[a] Obtained from three series of fermentation of glucose or sucrose samples in controlled conditions (100 g L^{-1} glucose, *S. cerevisiae*, 94 h, T = 298 K). Experiments: A, maize glucose (D/H)$_G^{NE}$ = 151.5 ppm; B, cane sucrose (D/H)$_G^{NE}$ = 150.8 ppm; C, beet glucose (D/H)$_G^{NE}$ = 137.0 ppm. [b] (a) Antarctic water, (b) distillated water, (c) Nantes tap water (NTW), (d) Slightly enriched water from NTW and D$_2$O 95%. [c] The (D/H) indices mean respectively, WI, water initially used, WS, starting water medium taking into account the exchange phenomena, I, CH$_2$DCH$_2$OH, II, CH$_3$CHDOH, W^Q, reacted water and, GNE, nonexchangeable atoms of glucose.

isotopomers i and M. Alternatively the mass spectrometry determination of the isotope ratio of the nonexchangeable sites, (D/H)$_G^{NE}$, also offers the possibility of calibrating, in an absolute way, the mole fractions obtained in SNIF-NMR. As shown in Table I the results obtained by both procedures are in satisfactory agreement. In the present experimental conditions the accuracy on the isotope ratios of the specific sites, i, is not better than ±2.5 ppm for sites 2 to 6. For the unique site 1, the two-sided confidence interval is limited to ±4 ppm. This explains that the determination of redistribution coefficients associated with the individual sites of glucose will not be presently attempted.

The investigated sugars have been fermented in synthetic media with a controlled isotope content and the site specific isotope ratios of the methyl (I) and methylene (II) sites of ethanol have been determined by NMR. Precise measurements have also been performed on the final water by mass spectrometry. In Table II are given the results associated with the fermentation of maize glucose and beet root sucrose samples in aqueous media with different deuterium contents. Results obtained for several concentrations of maize glucose in the same water are collected in Table III, and the data corresponding to the fermentation of sugars with different deuterium contents in water issued from the same pool are given in Table IV. It should be emphasized that a signal-to-noise ratio of 250 is reached within about 12 min for the methyl and methylene sites of ethanol. This enables an accuracy of about ±0.2 ppm on the (D/H)$_j$ ratios to be easily attained.

Discussion

Following the approaches defined in the theoretical section, we have checked the possibility of determining redistribution coefficients on the basis of the isotope parameters of the final products,

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Table III. Influence of the Concentration of Cane Sucrose (m in g L^{-1}) on the Deuterium Distribution in the Reacted Products Ethanol and Water[a]

starting compounds		reaction products		
m	(D/H)$_w$^{S}	(D/H)$_I$	(D/H)$_{II}$	(D/H)$_w$^{Q}
		Experiment E		
50	149.3	108.5$_5$	120.9	149.7$_5$
100	149.4$_5$	110.9	122.3$_5$	150.7
150	149.6	109.8	121.3	151.6
200	149.8$_5$	110.6	121.9	152.0
250	150.0	110.8	122.6	152.5
300	150.1$_5$	110.4$_5$	123.5	153.7
		Experiment F		
100	149.4$_5$	110.3	122.1	151.6$_5$
125	149.5	110.4	122.5	152.2
150	149.6	110.1	122.3	152.7
175	149.7	110.4	123.3	153.4
200	149.8$_5$	110.6	122.5	153.9

[a] Fermentation conditions: *S. cerivisiae*, 94 h, T = 298 K. The isotope ratio of the nonexchangeable sites of the corresponding glucose is 150.8 ppm and that of the starting water medium is 149.2 ppm. Linear dependencies of the isotope content of the end water medium on the concentration of sucrose are observed (slopes $\simeq$ 0.02 and constant term 149.2). Experiments E and F were performed under the same experimental conditions but with two different strains of *S. cerivisiae*.

Table IV. Influence of the Deuterium Content of the Starting Glucose on the Deuterium Content in Ethanols Obtained by Fermentation under Standard Conditions[a]

origin of the glucose	glucose (D/H)$_G$^{NE}	reaction products			
		(D/H)$_w$^{S}	(D/H)$_I$	(D/H)$_{II}$	(D/H)$_w$^{Q}
maizes					
enriched[b]	187.8	151.0	140.7	124.1	155.0
enriched[c]	168.4	150.2	126.7	123.8	154.0
enriched[d]	159.1	149.8	120.2	121.4	153.5
natural	151.5	150.2	113.3	125.0	152.3
cane sugars	151.3	149.3	112.6	122.9	152.0
	150.8	149.4	110.9	122.3	150.7
grapes	145.0	155.8	101.7	129.6	160.3
	144.2	155.3	100.5	129.0	159.9
	143.4	154.9	99.4	127.5	159.7
	142.6	154.4	97.4	124.8	159.1
	141.8	154.0	96.2	124.2	158.8
C$_3$ cereals[e]	142.0	149.8	98.0	122.0	151.8
tubers[f]	137.0	149.6	93.1	121.1	151.3

[a] The results are in reasonable agreement with previous determinations of the overall deuterium contents of some glucose and ethanol samples from similar origins performed by mass spectrometry.[13,4,15,20] [b,c,d] The site 1 of maize glucose was enriched by exchange in water with deuterium contents 395.3, 259.8, and 194.4 ppm respectively. [e] Wheat, barley. [f] Beet root, potato.

ethanol and water, and of those of the starting components, glucose and water. For a given site j of the products, vectors $\vec{D}_j$ are built from sets of experiments selected from the tables and, eventually, from complementary results published previously.[9] The matrix [S] of the deuterium contents may include the isotope ratios associated with the starting aqueous medium and either three sets (5,6,6'), (2,3,4), (1) or two sets (1 + 5,6,6'), (2,3,4), or, due to the limited accuracy, one set, of isotopomers involving the nonexchangeable sites of glucose. As far as the starting water is concerned, it should be noted that the deuterium content of the effective aqueous medium is not strictly equal to that of the water pool used in the experiment, (D/H)$_w$^{1}, since a fast exchange takes place with the five hydroxyl groups of glucose. Corrected values, (D/H)$_w$^{S}, have therefore been calculated by assuming an equilibrium constant, K_G, close to unity and using isotope values of the glucose exchangeable sites derived from the mass spectrometry measurements of the glucose samples and of their nitrates.

Analysis of the Main Factors Contributing to the Isotope Parameters. In a first step we have submitted the overall data of the deuterium contents to a factor analysis in order to obtain the true dimension of the space. In this approach the isotope ratios
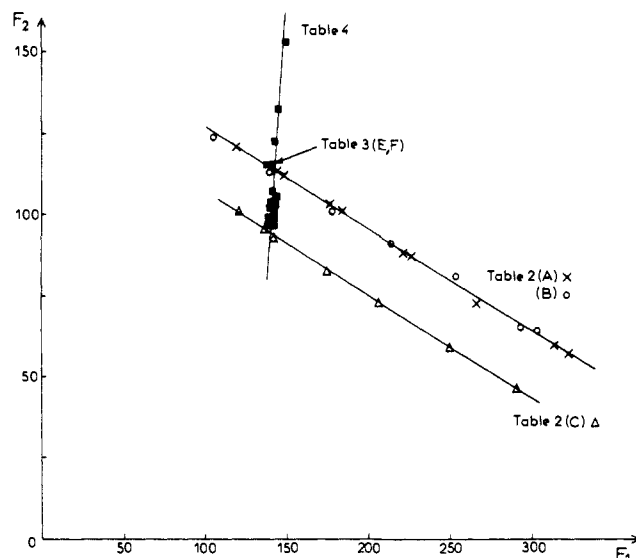
Figure 2. Representation in the plane of the two main factors of the whole population of experiments concerning the deuterium transfer from glucose and water into ethanol and water isotopomers. The relative contribution of factor F_2 decreases when the deuterium content of the fermentation medium increases (F_1) for experiments A–F.

(D/H)$_{i,j}$ of the starting and reaction products (eq 3 and 4) are the variables for which a number of observations can be performed depending on the experimental conditions. The diagonalization of the covariance matrix computed from the (48,5) data matrix yields two major eigenvalues which represent almost completely the overall variance: λ_1 = 3.78 (75.5%) and λ_2 = 1.19 (23.8%). The factor space is therefore bidimensional and the original data can be reproduced within errors which are of the same order of magnitude as the experimental ones ($\pm$1 ppm for the isotope ratios of glucose and $\pm$0.25 ppm for the isotope ratios of water). The correlation matrix computed from the centered data shows that the isotope contents of the initial and final water, (D/H)$_w$^{S}, (D/H)$_w$^{Q}, and those of the ethyl group of ethanol, (D/H)$_I$, (D/H)$_{II}$, are highly correlated whereas (D/H)$_G$^{NE} appears to be significantly related only to (D/H)$_I$. The physical meaning of the two factors can be appreciated by considering the corresponding (48,2) row matrix. Thus in Figure 2 are shown the transformed data points in the space of the two main factors. Three straight lines are clearly identified. Two of them are characterized by the same slope. They are associated with the sets of experiments A + B on one hand and C on the other hand (Table II) in which a given sugar was fermented in aqueous media of variable deuterium contents

$$A + B: \quad F_2 = 156.9 - 0.302F_1 \qquad (8)$$

$$C: \quad F_2 = 139.2 - 0.316F_1$$

The third line which results from the experiments described in Table IV exhibits a steep positive slope (3.94) and a negative intercept. In these experiments the same pool of water was utilized for fermenting different sugars. From these behaviors it is concluded that whatever the experimental conditions used (nature of the yeast, temperature, ...) the results are simply governed by two independent contributions which may be associated with the nonexchangeable sites of the sugar and with the water medium.

Multidimensional Determination of Redistribution Parameters. As considered in the theoretical section, multidimensional statistical treatments were then performed in order to determine isotope redistribution parameters. Thus an experimental matrix was built on the basis of q = 34 experiments carried out with isotopically different waters and various sugars at a given concentration (100 g L^{-1}). If the set of equations (3) is allowed to include a constant term to be defined, several types of correlations characterized by multiple correlation coefficients better than 0.95 are obtained.

Thus for the *methyl site* of ethanol

$$(D/H)_I = 1.1(D/H)_G{}^{NE} + 0.23(D/H)_w{}^S - 90 \quad (R = 0.95)$$
$$(9)$$

The standard deviations (S) on the three parameters are respectively 0.09, 0.01, and 13. Looser correlations are obtained when experiments performed with different concentrations of sugars are included in the statistical treatment, a situation that reflects differences in the isotopic content of the water medium in the course of the fermentation. It is noted that in contrast to the theoretical model, a constant term with a relatively high value is required to fit the experimental results. Forcing the equations to intercept the origin leads to a decrease in the coefficient relative to the sugar but results in a drastic decrease in the quality of the correlation since the correlation coefficient is then lowered to $R = 0.88$. Several factors may contribute to the discrepancy between the experimental behavior and the theoretical model. Thus the constant term in eq 9 certainly incorporates a fractionation contribution due to the growth of the yeast in the course of the fermentation process but this effect is very limited in the conditions of the experiments (see Experimental Section). Similarly, although redistribution equations of type (2) are not strictly valid for isotope ratios, this source of deviation is expected to be small at the high level of isotopic dilution of deuterium. More important sources of discrepancy may be found in the following considerations: Due to the restricted range exhibited by the isotope ratios of the nonexchangeable sites of glucose, a relatively limited accuracy is accessible. Moreover, since the deuterium distribution among the nonexchangeable sites is not uniform (Table I) the use of average of parameters is not really justified on a theoretical basis. Some artefacts may also be introduced when selectively labeled sugars are considered. In addition some mechanistic effects are likely to occur since, as the reaction evolves, changes in the isotope ratio of the aqueous medium occur which are reflected in the deuterium content of the ethanol.

However, according to these results it can be concluded that the deuterium content of the methyl site of ethanol is about *5 times more sensitive toward the nonexchangeable sites of glucose than toward the starting water*. This result supports the exploitation of the site-specific parameters of alcohols as origin identificators of the sugar materials.[6]

By contrast, in the bidimensional correlation of the methylene parameter, $(D/H)_{II}$, with $(D/H)_G{}^{NE}$ and $(D/H)_w{}^S$ $(R = 0.98)$, only the water parameter, which is associated with a slope of about 0.74 (± 0.02), is significative. As shown by the Snedecor test, no real meaning can be attached to the slope, -0.04 ($S = 0.1$), calculated for the sugar variable, and a monodimensional treatment gives the following correlation

$$(D/H)_{II} = 0.74(D/H)_w{}^S + 18 \quad (R = 0.986) \quad (10)$$
$$S = 0.2 \qquad S = 20$$

It can therefore be concluded that in the diluted media considered, *the methylene site of ethanol has no important direct connection with glucose*. As in the case of the methyl site, the results do not fit the theoretical model very precisely; however, the correlation with the deuterium content of the starting water is only slightly degraded ($R = 0.985$) by forcing the intercept to be zero (slope $= 0.75 \pm 0.03$). This behavior may be indicative of a slight indirect relationship to glucose via the modification of the aqueous medium occurring in the course of the fermentation.

As regards the isotope content of the *final aqueous medium*, only a very slight dependency on the glucose parameters is noted and the variations in the deuterium content of the final water follows, with a slope slightly smaller than unity, the variations in the deuterium content of the starting water. Thus for the set of experiments performed with a concentration of 100 g L^{-1} (Tables II and IV)

$$(D/H)_w = 0.04(D/H)_G{}^{NE} + 0.96(D/H)_w{}^S + 1.3$$
$$(R = 0.999) \quad (11)$$

the standard deviations on the slopes and the constant term being respectively 0.02, 0.003, and 3.7. In a realistic range of isotope parameters for natural sugars and natural waters, a change in

the concentration from 50 to 400 g L^{-1}, for example, exerts only small effects on the isotope parameters of the alcohols. Nevertheless for a given sugar fermented in aqueous media issued from a common pool, a regular increase (0.012–0.019 ppm/(g/L^{-1})) in the isotope ratio of the final water accompanies the increase in the concentration (Table III).

As far as *the behavior of the water component* is concerned it is also noted that, for a given sugar species, the deuterium content of the aqueous medium increases in the course of the fermentation as long as the isotope ratio of the water is smaller than, or close to, that of the sugar, whereas the final water is depleted with respect to the initial water for heavier waters (Table II). This behavior can again be interpreted on the basis of a participation of nonexchangeable sites of glucose with the water medium. In addition it is observed that the depletion of the final water with respect to the starting one does not occur as soon as the deuterium content of the starting water exceeds that of the sugar. This behavior can be explained by the fact that the coefficients for redistribution from glucose to ethanol are significantly smaller than unity.

Site-Specific Dependency with Respect to Glucose. Due to the limited accuracy of the site-specific determinations concerning the diastereotopic sites of the glucose samples, no quantitative relationships involving *these individual sites* have been attempted. However, it can be noted that the variations exhibited by the isotope ratio of the methyl site of ethanol issued from the fermentation of different glucose species in the same water reflect the large changes in the isotope parameters associated with the *anomeric site 1 and the set of sites 6,6'*. The sensitivity of the ethanol probe to the nature of the sugar therefore corroborates a filiation from the anomeric and hydroxymethylene groups of glucose whereas at least part of the other cyclic sites, which seem to be less dependent on the origin of the sugar, are probably involved in an exchange with water in the course of the bioconversion, as considered previously.

Conclusion

To conclude, although a simple redistribution matrix built on four independent components, sugar, ethanol, and initial and final water (eq 1), does not fit the experimental results very precisely, *a set of coefficients characterizing the sensitivity of the isotope ratios of the products to the starting components*, and vice versa, can be retained. Thus in the range of concentrations investigated, it can be considered that the variations in the deuterium content of the methyl site respond, with a coefficient of about 1.05, to variations in the isotope ratio of the nonexchangeable sites of glucose, whereas the coefficient giving the response to variations in the isotope ratio of the starting water medium is only about 0.23. By contrast the methylene site of ethanol is related by a factor 0.74 to the isotope variations of the water medium. Finally, for fermentation in diluted media ($\simeq 100$ g L^{-1}), the deuterium content of the final water has a small but significant dependence on the isotope parameter of glucose (coefficient 0.04) and variations in the deuterium content of the initial water are transmitted with a factor 0.96 to the final water.

These behaviors offer direct mechanistic information on the fate of the hydrogen atoms and on fractionation effects occurring in the course of the fermentation. Thus it is verified that no direct connection with the hydrogen atoms of glucose occurs in the mechanism of formation of the methylene group of ethanol. This result is in accordance with an introduction into the methylene site, of hydrogens originating from the water medium in the course of the decarboxylation step of pyruvate and enzymatic reduction of acetaldehyde.[15,17] As shown by the low value of the redistribution factor ($\simeq 0.7$), these mechanisms occur with a noticeable fractionation effect which explains the strong deuterium depletion observed in the methylene site. This behavior substantiates the occurrence of strong isotope effects in enzymatic incorporation of hydrogen atoms from water into organic molecules.

As regards the methyl behavior, only a mean redistribution coefficient with respect to the nonexchangeable sites of glucose has been calculated. This parameter indicates a direct filiation

between the methyl group and the glucose skeleton, and a schematic pathway of the Embden–Meyeroff cycle in which all the hydrogen atoms bound to carbon atoms in the sugar would be involved in proton transfers with the fermentation aqueous medium may be excluded. From the redistribution coefficients calculated above, the influence of glucose appears to strongly predominate over that of water. However, since the introduction of hydrogen isotopes issued from water may occur with strong discriminating effects against deuterium, the redistribution coefficients cannot be considered as directly representing the relative numbers of hydrogens derived from the sugar and water materials. More specific information can be advanced on the basis of the large deviations with respect to a statistical distribution, observed in the isotope contents of the different sites of glucose. Thus the strong depletion occurring in the methyl site of ethanol as compared to the overall isotope content of glucose should be put together with the relatively low isotope parameters of the anomeric site and to a lesser extent of the methylene sites 6,6′ of glucose. In the hypothesis of similar values of the fractionation factors accompanying the introduction of deuterium atoms from water into the methyl and methylene sites of ethanol, the number of hydrogens n_1^W issued from water and entering the methyl site can be estimated. Thus on the basis of the coefficients $a_{22} \simeq 0.7$ and $a_{12} \simeq 0.23$ deduced from the investigation of $(D/H)_{II}$ and $(D/H)_I$, respectively, a value $n_1^W \simeq 2$ is calculated. In this hypothesis, the glucose skeleton would therefore contribute four hydrogens to the methyl groups of the two ethanol molecules issued from

the fermentation of one molecule of glucose, the two remaining hydrogens coming from the water medium.

A priori it could be considered that the strong fractionation effects intervening in the biochemical synthesis of ethanol render its use as a probe for characterizing the starting materials questionable.[15] However, the present results demonstrate that constant redistribution parameters are involved in standardized conditions. From a practical point of view these results therefore legitimate the use of the isotope parameters of the fermentation products as a fingerprint of the parent compounds, i.e., glucose and water. More generally since we have proved that hydrolysis of starch and inversion of sucrose occur without modification of the natural deuterium distribution in the glucose unit of these glucides,[19] the isotope parameters measured in ethanol and water samples issued from the fermentation of the various forms of glucides offer a faithful image of the original natural species and therefore contain invaluable geographical, climatological, and biochemical information. This fingerprint concept is the basis of a powerful tool in the quality control of foods and beverages and in tracer studies of photosynthesis reactions and bioconversion processes.[6]

Registry No. EtOH, 64-17-5; D₂, 7782-39-0; D-glucose, 50-99-7; sucrose, 57-50-1.

Why the Accumulation of Electron Density Appears Weak or Absent in Certain Covalent Bonds[†]

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Abstract: The standard deformation density, molecular density minus spherical atom densities, may appear small or even negative in certain covalent bonds. For example, in difluorine subtraction of the electron densities of spherically averaged F atoms, which include all components of the ^2P $(2s^2 2p^5)$ ground state, results in the standard deformation density. Contour maps show an electron density deficit along the bond axis in both the internuclear bonding region and the lone-pair regions beyond the nuclei and accumulation in the π regions perpendicular to that axis. This result resembles experimental maps of the deformation density of the covalent bond between electronegative atoms such as that between the O atoms of peroxides. Our analysis shows that one can interpret this type of deformation density as a combination of constructive interference (covalent bond formation) and atomic reorientation, polarization, promotion, and hybridization (atom preparation for bonding). Subtraction of the electron densities of one component of the ^2P ground state of the F atoms, oriented with singly occupied $2p_z$ orbitals along the σ direction, results in maps with weak accumulation of charge in the bonding region between the nuclei, deep troughs of density deficit near the nuclei, no change along the π regions, and accumulation of charge in the lone-pair regions beyond the nuclear centers. Subtraction of the electron densities of optimally hybridized valence-state F atoms matches the lone-pair densities of atoms and molecule, so that the total difference is dominated by a localized bonding orbital density difference. This map reveals not only the accumulation of charge in the internuclear region but also the concomitant depletion of charge in the nonbonding regions beyond the nuclear centers which together are the signature of the covalent bond. Thus, if one views bond formation in two steps, atom preparation then bond formation, one easily sees the origin of the loss of electron density in the bond region caused when two spherical F atoms form a bond.

Recently, the number of studies of the electron density distribution, $\rho(\vec{r})$, in molecules by means of X-ray diffraction have increased.[1] Part of the interest arises from the promise, given by the theorem of Hohenberg and Kohn,[2] of the existence of a direct relationship between the electron density and the energy of the ground-state molecule. Most experimental studies measure

what is called the standard deformation density, $\Delta\rho(\vec{r})$, which is defined as the molecular electron density minus the electron density of the promolecule[3] made up of the superposition of isolated, neutral, spherically averaged, ground-state atoms.

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