Non-Statistical $^{13}$C Distribution during Carbon Transfer from Glucose to Ethanol During Fermentation is Determined by the Catabolic Pathway Exploited

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Running title: $^{13}$C isotopic relationship in glucose fermentation

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**Background:** Different fermentation pathways should lead to distinctive isotope patterns in the products.

**Results:** Three pathways for glucose catabolism show discrete isotope patterns in ethanol.

**Conclusion:** Catabolism by different enzyme sequences leads to differential isotope redistribution patterns.

**Significance:** Learning how isotope fractionation occurs in nature is crucial for interpreting fractionation during biochemical and physical processes for traceability.

**ABSTRACT**

During the anaerobic fermentation of glucose to ethanol, the three micro-organisms, *Saccharomyces cerevisiae*, *Zymomonas mobilis* and *Leuconostoc mesenteroides* exploit, respectively, the Embden-Meyerhof-Parnas, the Entner-Doudoroff, and the Reductive Pentose-Phosphate pathways. Thus, the atoms incorporated into ethanol do not have the same affiliation to the atomic positions in glucose. The isotopic fractionation occurring in each pathway at both the methylene and methyl positions of ethanol has been investigated by isotopic quantitative $^{13}$C NMR spectrometry with the aim of observing whether an isotope redistribution characteristic of the enzymes active in each pathway can be measured. First, it is found that each pathway has a unique isotope redistribution signature. Second, for the methylene group a significant apparent kinetic isotope effect is only found in the reductive pentose-phosphate pathway. Third, the apparent kinetic isotope effects related to the methyl group are more pronounced than for the methylene group. These findings can (i) be related to known kinetic isotope effects of some of the enzymes concerned and (ii) can give indicators as to which steps in the pathways are likely to be influencing the final isotopic composition in the ethanol.

Efforts to understand the causes of isotopic fractionation$^1$ in the $^{13}$C/$^{12}$C ratio during metabolism are hampered by lack of data on the intramolecular $^{13}$C composition of the compounds involved. In particular, it is valuable to understand how different routes to the same product can influence isotope redistribution patterns. The anaerobic fermentation of glucose to ethanol can be carried out by a number of different pathways in which, although the substrate consumed and product accumulated are the same, the intermediate compounds and enzymes involved are not. It follows, therefore that, since isotopic fractionation is associated with reaction mechanism, different pathways should lead to different isotope patterns in the final product. The other principal factor that will determine the isotopic composition of a product is the position-specific isotopic composition of the substrate.

A combination of these two phenomena—position-specific isotope ratios in the substrate and position-specific fractionation during metabolism—determines therefore the final isotopic composition in the product(s) of fermentation. In previous studies, we have shown by isotopic quantitative-$^2$H NMR spectrometry that the isotopic fractionation in $^2$H is distinctive depending on whether the Embden-Meyerhof-Parnas (EMP)$^2$ pathway or the Reductive Pentose-Phosphate (RPP) pathway is used and that...
the hydrogen atoms in the product can be linked to the positions in the substrate (1,2).

Until recently, however, a similar approach to obtain $^{13}$C/$^{12}$C isotopic ratios was not possible. In order to exploit $^{13}$C NMR for the study of intramolecular $^{13}$C distributions several additional difficulties had to be overcome. The first is that the isotopic variation of $^{13}$C in natural compounds has a range about 10-fold less than $^2$H (about 50‰ and 500‰ respectively on the δ-scale). As a result, isotopic $^{13}$C NMR requires a 10-fold higher precision. Secondly, for the effective use of quantitative $^{13}$C NMR at natural abundance, uniform proton decoupling of $^{13}$C-$^1$H interactions is required. This was achieved by the use of adiabatic decoupling (3). Thirdly, slow relaxation times can lead to long acquisition times and associated potential instability, a difficulty largely solved by the use of relaxation agents (4). Now, this technique can be used for quantification of individual isotopomers by exploiting (i) the separation of the resonance signals from the different carbon positions due to their degree of shielding, and (ii) the direct relationship of the peak area to the amount of $^{13}$C resonating at a given frequency (5). Acquisition conditions need to be carefully defined and rigorously controlled in order to obtain repeatable spectra of sufficient quality for the required precision. Parameters such as concentration of sample, temperature, stability of the lock need to be the same, and spectra with a signal-to-noise ratio above 750 are recorded. The nOe (nuclear Overhauser effect) has to be eliminated and the efficiency of the $^1$H decoupling sequence must be uniform over the whole range of $^1$H chemical shifts (12 ppm). Studies (3,5-7) on the optimization of an NMR methodology for accurate and precise measurements of $^{13}$C/$^{12}$C isotope ratios have shed light on how the decoupling conditions of $^1$H in $^{13}$C single-pulse NMR experiments strongly affect the precision of measured peak surface areas. A major breakthrough was achieved with the development of an optimized adiabatic $^1$H decoupling sequence (3). Provided this is respected, individual $^{13}$C isotopomers can be observed and the absolute intramolecular distributions of $^{13}$C determined (8). A curve fitting based on a total-line-shape analyses is used to obtain the area under each peak, which provides the reduced molar fraction $f_i/f_i^0$ of $^{13}$C at each carbon position from which the δ$^{13}$C$_i$ (%) can be calculated (see Experimental Procedures and (9) for definitions). The extent to which individual positions are either enriched or depleted in $^{13}$C relative to the statistical mean indicates metabolically-induced isotope fractionation.

The determination of KIEs, calculated from isotope fractionation, is recognized as an important way to obtain mechanistic information about enzymes, as they are determined by the rigidity of the bonds within the substrate and the transition state structure. They may be normal (KIE>1) or inverse (KIE<1), leading respectively to impoverishment or enrichment in the pertinent positions of the product. The magnitude of the isotope effect is, in general, greater for primary KIEs, in which a bond to the atom under consideration is broken. However, significant secondary isotope effects also occur: for example, a change in hybridization sp$^2$-sp$^3$ theoretically causes a normal KIE, while a change sp$^2$-sp$^3$ generates an inverse KIE (10). When a metabolic pathway consisting of a cascade of enzymes is studied, the observed fractionation will be the summed effect of the KIEs of the enzymes involved. Since the enzymes are probably not active under fully saturating conditions, as each product of an enzymatic reaction is the substrate for the next reaction, $k_{cat}$ is not accessible (11). Nevertheless, an effect on $V_{max}/K_m$ can be obtained and an apparent KIE ($^{iso}$KIE) potentially characteristic of the overall pathway under consideration.

That photosynthesis, wherein the assimilation of carbon dioxide by plants can involve three types of metabolism–C$_3$, C$_4$ and CAM–leads to different patterns of $^{13}$C distribution in the accumulated hexoses has been clearly demonstrated, initially by indirect analysis following fragmentation (12) and more recently with iq-$^{13}$C NMR (13,14). This shows that the photoassimilates are composed of mixtures of isotopomers that are determined by the physico-chemical processes (15) and the enzymes (16) involved in the particular metabolism exploited. These are determined by the presence of isotope effects in the Calvin cycle, and indicate the role of certain enzymes in the $^{13}$C isotope discrimination. For example, the aldol condensation of glyceraldehyde-3-phosphate (GAP) with dihydroxyacetone phosphate by fructose-1,6-bisphosphate aldolase (EC 4.1.2.13) to form fructose 1,6-bisphosphate could be identified as the origin of the relative $^{13}$C enrichment at the C-3 and C-4 positions of glucose (17).

However, very much less is known of the $^{13}$C fractionation implicit in the pathways utilizing glucose, the post-photosynthetic isotope...
fractionation. Three well-characterized pathways of microbial metabolism— the Embden-Meyerhof-Parnas, the Reductive Pentose-Phosphate, and the Entner-Doudoroff (ED) —convert glucose to ethanol and are exploited by a variety of micro-organisms to carry out the anaerobic fermentation of glucose. The first two of these are also active in plants.

Of these three pathways, only the EMP has been closely examined for position-specific $^{13}$C fractionation in ethanol biosynthesis (13,14,18), while some data from the RPP is also available (12). These studies clearly demonstrated that fermentation by the EMP route leads to $^{13}$C/$^{12}$C isotopic ratios for the methyl (CH$_3$) and methylene (CH$_2$) carbon positions that differ depending on the source glucose. However, in these studies the aim was complete degradation of glucose, so no information on KIEs during fermentation was obtained.

In order to understand better the origins of $^{13}$C isotope fractionation during fermentation, we have fermented glucose to ethanol using the three common pathways EMP, ED, and RPP. Ethanol is a convenient analyte for this type of study as it is readily produced in large quantities by easy-to-cultivate micro-organisms and can be obtained in pure form by distillation. Key features of the three target pathways are illustrated in Figure 1A and the origin of the carbons incorporated into ethanol are shown in Figure 1B.

**FIGURE 1 here**

The anaerobic oxidative catabolism of glucose to pyruvate via the EMP pathway is exploited by a large range of organisms, including the yeast *Saccharomyces cerevisiae*, producing two moles of each of ethanol and CO$_2$ per mole of glucose (Fig. 1B). Within the present context, the critical feature is the homolytic splitting of the six-carbon unit by fructose 1,6-bisphosphate aldolase (EC 4.1.2.13) to form two 3-carbon units which isomerize then follow the same path from GAP to pyruvate, thence, by decarboxylation, to ethanol. The ED pathway, which is unique to prokaryotes, is less frequently exploited for anaerobic oxidative catabolism, the bacterium *Zymomonas mobilis* being an example of an organism that uses it in a strictly fermentative sense. The ED pathway also produces two moles of each of ethanol and CO$_2$ per mole of glucose and also involves the homolytic splitting of the six-carbon unit into two three-carbon units. However, the key enzyme here is 2-dehydro-3-deoxy-phosphogluconate aldolase (EC 4.1.2.14) which produces one mole of pyruvate and one mole of GAP. As a result the pattern of allocation of carbon to ethanol is modified (Fig. 1B). The RPP, in contrast to these two pathways, involves the heterolytic cleavage of the six-carbon unit into CO$_2$, a two-carbon and a three-carbon unit (Fig. 1B). While the three-carbon unit is again GAP and is metabolized to pyruvate as in the EMP and ED pathways, it is not converted to ethanol but to lactic acid. The two-carbon unit, acetyl-phosphate, is converted directly to ethanol without the intervention of pyruvate. This pathway is used primarily by heterolactic bacteria, including species of *Lactobacillus* and *Leuconostoc*.

Early studies of $^{13}$C fractionation concluded “…that glucose does not have large differences in the isotope ratios in the individual carbon atoms and that the Embden-Meyerhof reactions do not have a large carbon isotope effect.” (19). However, at that time (1961) isotope measurement by mass spectrometry (irm-MS) gave only a value for $\delta^{13}$C$_g$ (‰), the global or mean isotope content, and the technique is poorly adapted to the determination of the intramolecular distribution of isotopes. Subsequently, measurement of $\delta^{13}$C$_i$ (‰), the position specific isotope distribution, has proved the first part of the above conclusion to be incorrect (12,18): as yet, evidence is still to be presented that glycolysis proceeds without position-specific isotopic discrimination. In order to probe this, we have carried out an analysis of $\delta^{13}$C$_i$ (‰) in ethanol from the EMP pathway. In addition, we have examined whether fermentation by alternative pathways, by which the glucose is converted to ethanol by reactions involving alternative mechanisms will indicate distinct $^{13}$C appKIEs.

**EXPERIMENTAL PROCEDURES**

*Materials*—D-Glucose (batch # 071M014552V), L-ascorbic acid, KH$_2$PO$_4$ and (NH$_4$)$_2$SO$_4$, were obtained from Sigma-Aldrich (www.sigmaaldrich.com); K$_2$HPO$_4$, MgSO$_4$, sodium glycerophosphate, glycerol, Tris(2,4-pentadionato)chromium-(III) (Cr(Acac)$_3$) and casein-meat Peptone from Merck (www.merck.com); Tryptone and yeast extract (autolytic) from Biokar (www.biokardiagnostics.com); Soya-flour Peptone (prepared using papain) from Fluka (www.sigmaaldrich.com). The same supply of each component was used for all fermentations. DMSO-
was provided by Professor Michel Rohmer (Laboratory of the Chemistry and Biochemistry of Micro-organisms, Chemistry Institute, UMR7177, CNRS–University of Strasbourg, Strasbourg, France) and stored at -80°C in M17 medium (20) with 15 % (v/v) glycerol. *Zymomonas mobilis* ZM6 (DSMZ 3580) was provided by Professor Hervé Prevost (Unité de recherche Sécurité des Aliments et Microbiologie, ONIRIS, Nantes, France) and stored at -80°C before initiating the fermentation, D-glucose (300 g), (NH₄)₂SO₄ (12 g), KH₂PO₄ (4.5 g), MgSO₄ (1.5 g), yeast extract (15 g), were dissolved in 2 L of distilled water, the pH adjusted to 6.8, sterilized by autoclaving (121°C, 20 min) and transferred aseptically to the culture vessel. An overnight pre-culture of *Z. mobilis* was grown in medium (ZGM) composed of yeast extract (5 g/L), (NH₄)₂SO₄ (1 g/L), KH₂PO₄ (1 g/L), MgSO₄ (0.5 g/L), glucose (20 g/L), pH 5.0 were prepared. This and D-glucose (20 to 150 g/L) were introduced to the fermenter as described for *L. mesenteroides*.

**Quantification of ethanol—**Fermentation medium was diluted to give an ethanol concentration in the range 1-10 mg/mL. To 1 mL of this, 0.5 mL ethyl acetate was added. After vigorous agitation (3 min), the phases were separated by centrifugation (13500 g, 1 min) and the ethyl acetate phase recovered. Ethanol was quantified by GC on an Agilent 7820A gas chromatograph fitted with a Teflon turning band. Care was taken to ensure that at least 90 % of the ethanol present was recovered so as to avoid isotopic fractionation (21).

**Extraction of ethanol from fermentation medium—**Ethanol was recovered from the fermentation medium by distillation using a Cadiot column equipped with a Teflon turning band. Care was taken to ensure that at least 90 % of the ethanol present was recovered so as to avoid isotopic fractionation (21).

For *Z. mobilis*, fermentation culture medium (ZFM) (NH₄)₂SO₄ (12 g), KH₂PO₄ (3 g), MgSO₄ (1.5 g), yeast extract (15 g), were dissolved in 2 L of distilled water, the pH adjusted to 6.8, sterilized by autoclaving (121°C, 20 min) and transferred aseptically to the culture vessel. An overnight pre-culture of *Z. mobilis* was grown in medium (ZGM) composed of yeast extract (5 g/L), (NH₄)₂SO₄ (1 g/L), KH₂PO₄ (1 g/L), MgSO₄ (0.5 g/L), glucose (20 g/L), pH 5.0 were prepared. This and D-glucose (20 to 150 g/L) were introduced to the fermenter as described for *L. mesenteroides*.

**Quantification of D-glucose—**D-Glucose concentration was determined by HPLC on a Lichrosphere 100-NH₂ (250 x 46 mm, 5 μm) column eluted isocratically with acetonitrile/water (80:20) at 1 mL/min. Detection was by refractive index. Calibration was with pure D-glucose in the range 10 to 250 mg/mL.

**Isotope ratio measurement by mass spectrometry—**The global value for the whole molecule, δ¹³Cₐ (%), is the deviation of the carbon isotopic ratio R, relative to that of the international standard Vienna Pee Dee Belemnite, (V-PDB), Rᵥ-PDB. It is determined by isotope ratio measurement by mass spectrometry and calculated from:

\[
δ^{13}C_a (\%) = \left( \frac{R_S}{R_{V-PDB}} - 1 \right) \times 1000
\]
13C NMR acquisition conditions—Relaxation agent Cr(Acac)_3 solution (0.1 M) was prepared by dissolving 34.9 mg Cr(Acac)_3 in 1 mL DMSO-d_6 in a 4 mL vial. To this was added 600 µL ethanol and 100 µL DMSO-d_6 to act as lock. Following mixing, the solution was left 2-4 h, filtered to remove undissolved relaxation agent and transferred to a 5 mm NMR tube. Quantitative 13C NMR spectra were recorded at 100.6 MHz using a Bruker 400 NMR spectrometer fitted with a 5 mm 1H/13C dual+ probe. The temperature of the probe was set at 303 K. The offsets for both 13C and 1H were set at the middle of the frequency range. Inverse-gated decoupling was applied and the repetition delay between each 90° pulse was set at 10×T_1max of ethanol to avoid the nOe and to achieve full relaxation of the magnetization. The decoupling sequence used adiabatic full-passage pulses with cosine square amplitude modulation (ν_2max = 17.6 kHz) and offset independent adiabaticity with optimized frequency sweep (3). Each measurement consisted of the average of five independently recorded NMR spectra.

Spectral data processing—The positional isotopic distribution in ethanol was obtained from the 13C NMR spectrum essentially as described previously (9,22). To obtain S_i, the area under the 13C-signal for C-atom in position i (in this case the methyl and methylene positions), curve fitting based on a total-line-shape analyses (deconvolution) is carried out with a Lorentzian mathematical model using Perch Software (Perch™ NMR Software, http://www.perchsolutions.com). In this procedure, line-shape parameters are optimized in terms of intensities, frequencies, line-width, line-shape (Gaussian/Lorentzian, phase, asymmetry) by iterative fitted to a minimal residue. All line-fitting was performed by the same operator. Each S_i has to be corrected to compensate for the slight loss of intensity caused by satellites (13C–13C scalar coupling interactions) by multiplying by (1 + n×0.011), where n is the number of carbon atoms directly attached to the C-atom position i and 1.1% (=0.011) is the average natural 13C-abundance. (For ethanol, n=1 for both the methyl and methylene positions.)

The 13C mole fraction f_i, the area of the peak corresponding to the carbon position i divided by the sum of all the carbon sites of the molecule, is obtained by:

\[ f_i = \frac{S_i}{\sum_j S_j} \]

F_i is the statistical mole fraction for a carbon site i, that is the molar fraction for the carbon position i in the theoretical situation where there is a homogeneous 13C-distribution within the molecule:

\[ F_i = \frac{c}{C} \]

where c is the number of carbon equivalents in the molecule resonating at a given frequency and C is the total number of carbon atoms in the molecule (for ethanol F_i =1/2 for both the methyl and the methylene positions).

Hence, the site-specific reduced molar fraction can be defined as:

\[ f_i/F_i \]

From the reduced molar fraction δ^{13}Ci, the specific isotope composition of the carbon position i, is obtained from the isotope composition of the whole molecule (δ^{13}Cg) measured by irm-MS as follows. A_i (%), the isotopic abundance for carbon position i, is obtained from:

\[ A_i = A_g \times f_i/F_i \]

where A_g is the isotopic abundance of a whole molecule and is obtained from:

\[ A_g = \frac{R_g}{(R_g + 1)} \]

with

\[ R_g = \left( \frac{\delta^{13}C_g}{1000} + 1 \right) \times R_{V-PDB} \]

where \( R_{V-PDB} = 0.0112372 \). Then:

\[ R_i = A_i/(1 - A_i) \]

hence

\[ \delta^{13}C_i(\%) = \left( \frac{R_i}{R_{V-PDB}} - 1 \right) \times 1000 \]

Calculation of Isotope Effects—Kinetic isotope effects were calculated using a modified form of the Biegeleisen equation:

\[ KIE = \frac{k_L}{k_H} = \frac{\ln(1 - f_{eth})}{\ln\left[ 1 - \left( \frac{f_{eth} \times R_{eth.t}}{R_{G,0}} \right) \right]} \]
where \( k_1 \) and \( k_2 \) are the rates of overall reaction with light \((^{12}\text{C})\) and heavy \((^{13}\text{C})\) isotopomers respectively, \( f_{\text{eth}} \) is the fraction of product formed \(=([\text{ethanol}]/[\text{ethanol theoretical}]) \) determined by GC for ethanol and HPLC for the initial \([\text{glucose}]\), \( R_{\text{eth}, t} \) is the isotopic ratio for ethanol at time \( t \), determined by iq-\(^{13}\text{C} \) NMR, \( R_{G,0} \) is the mean isotopic ratio for the relevant positions in glucose at \( t=0 \), determined by iq-\(^{13}\text{C} \) NMR as described previously (23).

**RESULTS**

In order to calculate the \( ^{\text{app}} \)KIE values, the position-specific \(^{13}\text{C} \) content of the accumulated product, ethanol, was determined. To obtain the greatest degree of isotopic fractionation between substrate and product, fermentation to a low \( f_{\text{eth}} \) is advantageous. Fermentations in anaerobic conditions adapted to each micro-organism were sampled in the range 10-70\% of advancement of the reaction, ethanol recovered by distillation and the iq-\(^{13}\text{C} \) NMR spectra recorded (Fig. 2).

**FIGURE 2**

From this, the \( \delta^{13}\text{C}_i \) values were calculated for the methyl and methylene positions (see Experimental). The mean for the \( f_i/F_i \) for each fermentation (5 spectra) and the mean±SEM values of \( \delta^{13}\text{C}_i \) are given in Table 1.

**TABLE 1**

From the calculated \( \delta^{13}\text{C}_i \) data, the position-specific KIEs were calculated using the modified Biegeleisen equation. These are given in Table 2.

**TABLE 2**

**DISCUSSION**

*Unique features of the EMP pathway*—The position-specific \( ^{\text{app}} \)KIE observed for the fermentation of \( ^{\text{D}} \)glucose by \( S. \text{cerevisiae} / S. \text{bayanus} \) culture is not significant for the \( \text{CH}_2 \) position and only shows a small normal effect for the \( \text{CH}_3 \). Thus the previous proposal that the Embden-Meyerhof reactions do not have a large carbon isotope effect (19) is substantiated by direct measurement. However, as the \( ^{\text{app}} \)KIE determined on the final product (ethanol) reflects the sum of counteracting normal and inverse KIEs, this does not exclude the possibility of fractionation having occurred at several steps in the pathway, which consists of a series of thirteen enzymatic reactions.

In addition, each value is the mean of the isotope effects on the \( \text{C}-2/\text{C}-5 \) and \( \text{C}-1/\text{C}-6 \) positions of \( ^{\text{D}} \)glucose for the \( \text{CH}_2 \) and \( \text{CH}_3 \), respectively.

Five enzymes have the potential to generate an isotopic fractionation on positions \( \text{C}-2 \text{c} \); and/or \( \text{C}-5 \text{c} \) on their route to become the \( \text{CH}_2 \) of ethanol (Fig. 3). Two of these, glucose-6-phosphate (G6P) isomerase (EC 5.3.1.9) and triose phosphate isomerase (EC 1.2.1.12) catalyze steps that are unique to the EMP pathway, while three others, enolase (EC 4.2.1.11), pyruvate kinase (EC 2.7.1.40), and the pyruvate dehydrogenase complex (EC 1.2.4.1+EC 2.3.1.12+EC 1.8.1.4) are common to at least two of the three pathways studied (Fig. 1). It should be noted that any primary KIE associated with fructose-1,6-bisphosphate aldolase will not manifest itself, as the \( \text{C}-3 \) and \( \text{C}-4 \) are both lost as \( \text{CO}_2 \). G6P isomerase causes a \( \text{sp}^3/\text{sp}^2 \) change in hybridization at the \( \text{C}-2 \), a modification commonly associate with a normal secondary kinetic isotope effect (24). That this is manifest is highly probable, as glucose isomerase (EC 5.3.1.5) from \( S. \text{muralis} \) has an inverse isotope effect in the direction fructose to glucose (18), which would act to impoverish the \( \text{C}-2 \) of F6P derived from G6P, by about 15\% at equilibrium. This depletion is much higher than that measured in ethanol, implying that further steps compensate. While a potential candidate is triose phosphate isomerase, since during the reaction an \( \text{sp}^2/\text{sp}^3 \) change in hybridization occurs at the \( \text{C}-2 \) position (Fig. 3A), it can be reasonably argued that this is unlikely, as the limiting step of the reaction (proton transfer from His95 of the active site) does not involve a primary \(^{13}\text{C} \) isotope effect (25). The \( \text{C}-2 \) position is involved in further reactions, notably enolase, pyruvate kinase, and pyruvate dehydrogenase, which are all susceptible to introducing isotopic fractionation. These enzymes are all located in the part of the EMP which is common to other pathways and are discussed below.

*Unique features of the ED pathway*—The fermentation of \( ^{\text{D}} \)glucose by \( Z. \text{mobilis} \) gives values of \( ^{\text{app}} \)KIE similar to those obtained for the fermentation of \( ^{\text{D}} \)glucose by \( S. \text{cerevisiae} / S. \text{bayanus} \) culture (Table 2), with no significant isotope effect at the \( \text{CH}_2 \) but a larger normal effect for the \( \text{CH}_3 \) of \( ^{\text{app}} \)KIECH3=1.0035±0.0002. As with the G6P to F6P transformation, the \( \text{C}-2 \) undergoes an \( \text{sp}^3/\text{sp}^2 \) transition in the conversion of 6-phosphogluconate to 2-keto-3-deoxy-6-phosphogluconate (KDPG) by phosphogluconate
dehydratase (EC 4.2.1.12). Although the mechanism has been defined (26) and the C-2G undergoes cleavage of the C–H bond and ketonization (Fig. 4), the lack of any KIE indicates that, as with triose phosphate isomerase, this is not the kinetically limiting step. However, the lack of any KIE supports the suggestion that the G6P to F6P isomerization in the EMP pathway is indeed responsible for a small fractionation, since this step is absent in the ED pathway and the C-2G, unlike the C-5G, does not undergo the steps common to the three pathways (Fig. 1).

The normal \( \text{appKIE}_{C^1H_3} \) is considerably greater than for the EMP pathway. This may reflect the origin of the \( \text{CH}_3 \) being the C-3G+C-6G in this pathway. The C-3G position is potentially subjected to a primary KIE during the fission of the C-3G–C-4G bond by the action of KDPG aldolase which cleaves KDGP to yield directly pyruvate and GAP. Thus, the C-3G enters the \( \text{CH}_3 \) of ethanol, in marked contrast to the EMP pathway. Although no \( ^{13}\text{C} \) KIE studies have been done on the KDPG aldolase, its mechanism (27) is closely similar to that of fructose 1,6-bisphosphate aldolase (28), for which a normal KIE of 1.0254 has been determined (17). This value is in consensus with the \( \text{appKIE}_{C^1H_3} \) found here.

**Unique features of the RPP pathway**—The fermentation of D-glucose by \( L. \text{mesenteroides} \) shows a distinctly different isotopic fractionation compared with the EMP or ED pathways. Three features are striking: (i) the CH\(_2\) position shows a normal \( \text{appKIE}_{C^1H_2}=1.0058\pm0.0007 \), (ii) the \( \text{appKIE}_{C^1H_2}=1.0057\pm0.0011 \) is of a similar magnitude, and (iii) both values are greater than for the other pathways (Table 2). As in the EMP and ED pathways, cleavage of the C-3G–C-4G bond occurs, but, crucially, it is only the (C-2–C-3)G unit that is converted to ethanol, the (C-4–C-5–C-6)G yielding D-lactic acid (Fig. 1). It is found that this difference has a major influence on the \( \text{appKIE} \) values. An analysis of the data is simplified, especially for the \( \text{CH}_3 \) of ethanol, as the C2G is only involved in one reaction: phosphogluconate dehydrogenase (decarboxylating) (EC 1.1.1.44), which simultaneously ketonizes the C-2G position and cleaves the C-1G–C-2G bond, and could therefore involve a primary KIE (Fig. 5). The CH\(_2\) position similarly is derived from a cleavage reaction, that catalyzed by phosphoketolase (EC 4.1.2.9) which cleaves the C-3G–C-4G bond and again, therefore could involve a primary KIE. The C-5G does undergo further reactions, but the only susceptible to any isotopic fractionation in the final alcohol dehydrogenase (EC 1.1.1.1/EC 1.1.1.12) in which a change in hybridization sp\(^2\)-sp\(^3\) occurs (see below).

The C-1 of D-glucose is lost during the decarboxylation of 6-phosphogluconate to ribulose-5-phosphate catalyzed by 6-phosphogluconate dehydrogenase (decarboxylating). Decarboxylation is characteristically associated with a normal primary KIE (29,30). The mechanism of 6-phosphogluconate dehydrogenase has been elucidated (31,32) and a \( ^{13}\text{C} \) KIE=1.0209±0.0005 determined for the C-1G during decarboxylation by the enzyme from the yeast \( \text{Candida utilis} \) (31). While no data are available for the C-2G position, a KIE of a similar magnitude is probable (33), so an \( \text{appKIE} \approx1.006 \) is compatible with the proposed mechanism. It should be noted that, as reactions are not occurring under \( V_{max}/K_m \) conditions, values of \( \text{appKIE} \) obtained in vivo can be expected to be lower than values of KIE obtained under in vitro conditions.

Considering the origin of the \( \text{CH}_3 \) of ethanol, a KIE associated with the cleavage of the C-3G–C-4G bond is probable (34). Although no isotopic data are available for this reaction, its similarity to the better-studied fructose-1,6-bisphosphate aldolase (35) for which a normal KIE of 1.0254 (17) has been determined, making it probable that the \( \text{appKIE}_{C^1H_2}=1.0057 \) is due to the activity of this enzyme. That this value is smaller than determined for the fructose-1,6-phosphate aldolase might reflect the sp\(^2\)-sp\(^3\) change during the alcohol dehydrogenase step, which is likely to manifest a small secondary inverse KIE (see below).

**Features common to the EMP and ED pathways**—Both the EMP and ED pathways produce ethanol from GAP via pyruvate (Fig. 1A). Only the reaction by which 2-phosphoglycerate is converted to phosphoenolpyruvate involves a change in hybridization state of sp\(^2\)-sp\(^3\) for both the C-5G and C-6G, and could therefore manifest a secondary \( ^{13}\text{C} \) KIE. This is catalyzed by enolase in an elimination reaction involving a carbanion intermediate (36,37). The slow step of the reaction, the abstraction of the H at the \( \beta \)C (i.e. the C-2G or C-5G) is associated with a strong \( ^2\text{H} \) isotope effect: subsequent enolization of the \( \gamma \)C (i.e. the C-1G or C-6G) is not. Therefore, it can be concluded that this step is unlikely to contribute to the \( \text{appKIE}_{C^1H_3} \) of ethanol. Furthermore, any normal isotope effect at the \( \gamma \)C is likely to be reversed by an inverse isotope effect in the subsequent reaction catalyzed by

\[ ^{13}\text{C} \text{ isotopic relationship in glucose fermentation} \]
pyruvate kinase in which an sp\(^2\)-sp\(^3\) change in hybridization occurs.

The only common step in which a primary KIE might occur is pyruvate dehydrogenase (decarboxylating) (EC 1.2.4.1), responsible for the bond cleavage within the pyruvate dehydrogenase complex; Primary \(^{13}\text{C}\) KIEs have been shown for both carbon positions involved in the decarboxylation, the \(\beta\)C (i.e. the C-2\(_G\) or C-5\(_G\) of glucose) showing a \(^{13}\text{C}\) KIE=1.0213±0.0017 and 1.0254±0.0016 for the enzyme from bacteria (Escherichia coli) and yeast (S. cerevisiae) respectively (29). Furthermore, a small secondary effect on the \(\gamma\)C of 1.0031±0.0009 was found. It can therefore be proposed that this enzyme makes a major contribution to the values of \(^{app}\text{KIECH}_{3}\) obtained of 1.0014±0.0002 and 1.0035±0.0002 for the ethanol from S. cerevisiae and Z. mobilis fermentations respectively.

Following on from this proposal is the possibility to estimate the effect isotopic caused by the KDPG aldolase on C-3\(_G\) by comparing the \(^{app}\text{KIECH}_{3}\) calculated for the two types of fermentation. The \(^{app}\text{KIECH}_{3}\) for Z. mobilis is 1.0035 and for S. cerevisiae it is 1.0014. The \(^{app}\text{KIECH}_{3}\) observed for Z. mobilis can be estimated as the average of the \(^{app}\text{KIECH}_{3}\) for S. cerevisiae and the KIE caused by KDPG aldolase, since no other reaction is likely to cause fractionation at that position (C-3\(_G\)) in the upper portions of these two pathways (see Fig. 1 and above). By calculation, the deduced KIECH\(_3\) for KDPG aldolase on the C-3\(_G\) is 1.0021. While only 10% the magnitude of the primary KIE determined for fructose-1,6-bisphosphate aldolase (17), this once again indicates that reactions are occurring under conditions far from \(V_{\max}/K_m\). In addition, these two enzymes differ mechanistically (27). It is notable, however, that the value obtained is of the same magnitude as for the RPP pathway (Table 2), in which a similar cleavage catalyzed by phosphoketolase, is observed.

Features common to all three pathways—The only step common to all three pathways in the final step: the reduction of acetaldehyde to ethanol, during which a change of hybridization sp\(^2\)-sp\(^3\) occurs that could induce a small secondary inverse KIE. The yeast and liver enzymes gave intrinsic \(^{13}\text{C}\) equilibrium isotope effects of 1.0164 and 1.0149, respectively, with benzyl alcohol as substrate (38). However, the value was highly dependent on the C-H bond distance in the transition state, which will not be equivalent in acetaldehyde. Only in the case of L. meseneroides is the \(^{app}\text{KIECH}_{3}\) of the same order. However, as any isotope effect is predicted to be inverse, it appears that this step does not influence the final \(^{13}\text{C}\) value of the CH\(_3\) group.

**CONCLUSIONS**

The application of iq-\(^{13}\text{C}\) NMR to the study of fermentation pathways has made possible the detection of weak \(^{app}\text{KIEs}\) in the formation of ethanol from D-glucose. These are manifest because the transformation is to an end-product which accumulates. Thus, despite the potential reversibility of a number of the individual steps in each pathway, the overall vector is driven by the metabolic need to regenerate NAD\(^+\) in order that metabolism can continue. Hence, the pathway overall is a unidirectional closed system and any isotope fractionation associated with reverse reactions will be negated. While it is confirmed that glycolysis (EMP) essentially proceeds without isotopic discrimination, as previously supposed (19), the data support the previous observation that the methylene group becomes relatively enriched during fermentation and the methyl relatively impoverished (13). As indicated in Table 2, a normal KIE is found for the CH\(_3\), which will lead to impoverishment, and a negligible or slightly inverse KIE is found for the CH\(_2\). Furthermore, rather more significant KIEs are seen to be present in the ED and RPP routes of D-glucose catabolism.

The relatively small size of the \(^{app}\text{KIEs}\) observed and the absence of comprehensive mechanistic data on all the enzymes involved limits the extent to which the data can be interpreted. Nevertheless, a number of enzymes can be implicated, notably those involved in key steps characteristic to each pathway. Little can be concluded for the EMP pathway, other than that isotopic effects are relatively small and fully consistent with a series of secondary isotope effects in which normal and reverse effects counteract, giving an overall negligible effect for the \(^{app}\text{KIECH}_{3}\) and a very small normal \(^{app}\text{KIECH}_{2}\). For the ED pathway, a net estimate for the KIE at C-3\(_G\) due to KDPG aldolase can be postulated. This effect is relatively small for a primary KIE, which suggests that isotopic fractionation due to other enzymes is also playing a contributory role. The RPP pathway shows more intense isotopic fractionation at both the methyl and methylene positions. These are satisfactorily explained by a normal KIE for both positions associated with the action of phosphoketolase. The data obtained serve to focus
attention on these enzymes, for which more details of their specific properties need to be obtained.
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FOOTNOTES
1Isotopic fractionation is the selection of one isotopeomer versus another during a physical or (bio)chemical process which leads to a non-statistical distribution of isotopes in the population of isotopeomers within the final product and the residual substrate.
2The abbreviations used are: FP6, fructose-6-phosphate; G6P, glucose-6-phosphate; GAP, glyceraldehyde-3-phosphate; KDPG, 2-keto-3-deoxy-6-phosphogluconate; EMP, Embden-Meyerhof-Parnas pathway; ED, Entner-Doudoroff pathway; RPP, Reductive Pentose-Phosphate pathway; KIE, Kinetic isotope effect. Abbreviations specific to figures are given in the figure legends.
FIGURE LEGENDS

FIGURE 1. Summary of the three metabolic pathways evaluated. A, The metabolic routes from glucose to ethanol following the Embden-Meyerhof-Parnas (EMP), the Entner-Doudoroff (ED), and the Reductive Pentose-Phosphate (RPP) pathways. B, the affiliation between the carbon positions in glucose and the carbon positions in ethanol and other fermentation products of these pathways. Enzymes indicated are: 1, glucose-6-phosphate (G6P) dehydrogenase (EC 1.1.1.49); 2, glucose-6-phosphate isomerase (EC 5.3.1.9); 3, phosphogluconate dehydrogenase (decarboxylating), (EC 1.1.1.44); 4, phosphogluconate dehydratase (EC 4.2.1.12); 5, 2-dehydro-3-deoxy-phosphogluconate aldolase (EC 4.1.2.14); 6, fructose-1,6-bisphosphate aldolase (EC 4.1.2.13); 7, triose phosphate isomerase (EC 1.2.1.12); 8, phosphoketolase (EC 4.1.2.9); 9, enolase (EC 4.2.1.11); 10, pyruvate kinase EC 2.7.1.40); 11, pyruvate dehydrogenase (EC 1.8.1.4); 12, alcohol dehydrogenase (EC 1.1.1.1). Abbreviations in compound names are: P, phosphate; KDGP, 2-keto-3-deoxy-6-phosphogluconate; DHAP, dihydroxyacetone phosphate.

FIGURE 2. $^{13}$C NMR spectrum of ethanol in DMSO-$d_6$ acquired under quantitative conditions. See Experimental for the acquisition conditions.

FIGURE 3. Enzymatic reactions of the Embden-Meyerhof-Parnas indicating possible associated isotopic fractionation sites. A, Steps unique to the EMP pathway potentially affecting fractionation at the C-2 position; B, steps common to the EMP, ED and RPP pathways, potentially affecting the C-1, C-2, C-5 and C-6 positions. Enzymes involved are numbered as in Figure 1.

FIGURE 4. Enzymatic reactions unique to the Enterer-Doudoroff pathway indicating possible associated isotopic fractionation steps. Enzymes involved are numbered as in Figure 1.

FIGURE 5. Enzymatic reactions unique to the reductive pentose-phosphate pathway indicating possible associated isotopic fractionation steps. Enzymes involved are numbered as in Figure 1, except: 12, alcohol dehydrogenase (NAD$^+$) (EC 1.1.1.1) + alcohol dehydrogenase (NADP$^+$) (EC 1.1.1.2); 13, acetyl-CoA:phosphate acetyltransferase (EC 2.3.1.8); 14, acetaldehyde dehydrogenase (EC 1.2.1.10). Compounds are: G6P, glucose 6-phosphate; 6PG, 6-phosphogluconate; R5P, ribulose-5-phosphate; X5P, xylulose-5-phosphate; G3P, glyceraldehyde 3-phosphate.
### Ethanol from *Saccharomyces cerevisiae*

| Fermentation | Sample N° | N° spectra | fi/Fi CH₂ | fi/Fi CH₃ | δₕ (‰) | feth (%/100) | δᵢ CH₂ (%o) Mean | δᵢ CH₂ (%o) SEM | δᵢ CH₃ (%o) Mean | δᵢ CH₃ (%o) SEM |
|--------------|-----------|------------|-----------|-----------|-------|-------------|------------------|-----------------|-----------------|----------------|
| F12          | P1        | 5          | 1.0001273 | 0.9998727 | -10.71| 0.113       | -10.59           | 0.27             | -10.84          | 0.27            |
|              | P2        | 5          | 1.0000535 | 0.9999465 | -10.02| 0.491       | -9.97            | 0.22             | -10.08          | 0.22            |
|              | P3        | 5          | 1.0001331 | 0.9998669 | -9.90 | 0.553       | -9.77            | 0.21             | -10.04          | 0.21            |
|              | P1        | 5          | 1.0006155 | 0.9993845 | -10.31| 0.091       | -9.69            | 0.20             | -10.04          | 0.20            |
|              | P2        | 5          | 1.0004020 | 0.9995980 | -10.14| 0.116       | -9.73            | 0.20             | -10.04          | 0.20            |
|              | P3        | 5          | 0.9999736 | 1.0000264 | -10.31| 0.373       | -10.33           | 0.19             | -10.37          | 0.17            |
| F13          | P1        | 5          | 0.9997986 | 1.0002104 | -10.50| 0.094       | -10.63           | 0.14             | -10.28          | 0.07            |
|              | P2        | 5          | 1.0002392 | 0.9997608 | -10.06| 0.526       | -9.88            | 0.14             | -10.37          | 0.17            |
|              | P3        | 5          | 0.9996871 | 0.9996871 | -9.76 | 0.602       | -9.42            | 0.20             | -10.11          | 0.20            |

### Ethanol from *Zygomonas mobilis*

| Fermentation | Sample N° | N° spectra | fi/Fi CH₂ | fi/Fi CH₃ | δₕ (‰) | feth (%/100) | δᵢ CH₂ (%o) Mean | δᵢ CH₂ (%o) SEM | δᵢ CH₃ (%o) Mean | δᵢ CH₃ (%o) SEM |
|--------------|-----------|------------|-----------|-----------|-------|-------------|------------------|-----------------|-----------------|----------------|
| F22          | P2        | 5          | 1.0016266 | 0.9983734 | -11.34| 0.14        | -9.71            | 0.24             | -12.97          | 0.24            |
|              | P3        | 5          | 1.0014688 | 0.9985312 | -11.31| 0.30        | -9.85            | 0.15             | -12.78          | 0.15            |
|              | P4        | 5          | 1.0016785 | 0.9983215 | -11.14| 0.430       | -9.46            | 0.18             | -12.81          | 0.18            |
|              | P2        | 5          | 1.0015324 | 0.9984676 | -11.77| 0.120       | -10.24           | 0.15             | -13.31          | 0.15            |
|              | P3        | 5          | 1.0015425 | 0.9984575 | -11.65| 0.175       | -10.11           | 0.15             | -13.20          | 0.15            |
|              | P4        | 5          | 1.0013188 | 0.9986812 | -11.40| 0.300       | -10.08           | 0.14             | -13.22          | 0.11            |
|              | P2        | 5          | 1.0011835 | 0.9986815 | -12.04| 0.081       | -10.40           | 0.21             | -13.01          | 0.34            |
|              | P3        | 5          | 1.0013011 | 0.9986989 | -11.70| 0.149       | -10.11           | 0.34             | -13.17          | 0.34            |
|              | P4        | 5          | 1.0015300 | 0.9984700 | -11.64| 0.261       | -11.64           | 0.24             | -13.17          | 0.24            |
### Ethanol from *Leuconostoc mesenteroides*

| Sample N° | F26 | F28 |
|-----------|-----|-----|
| P1        | 5   | 5   |
| P2        | 5   | 5   |
| P3        | 5   | 5   |
| N° spectra| 5   | 5   |
| $f_i/F_i$ CH$_2$ | 0.9999476 | 1.0007782 |
| $f_i/F_i$ CH$_3$ | 1.0000524 | 0.9992218 |
| $\delta_{g}$ (‰) | -13.77 | -12.47 |
| $f_{eth}$ (%/100) | 0.242 | 0.581 |
| $\delta_{i}$ CH$_2$ (‰) Mean | -13.82 | -11.69 |
| $\delta_{i}$ CH$_2$ (‰) SEM | 0.44 | 0.26 |
| $\delta_{i}$ CH$_3$ (‰) Mean | -13.71 | -13.24 |
| $\delta_{i}$ CH$_3$ (‰) SEM | 0.44 | 0.26 |

For definitions of terms, see the Experimental. All fermentations were made using the same batch of D-glucose obtained from Sigma-Aldrich (batch # 071M014552V).
Table 2. Apparent Kinetic Isotope Effects obtained for the Methylene (CH$_2$) and Methyl (CH$_3$) Positions of Ethanol produced by the Fermentation of D-Glucose.

| Fermentation N° | $S.\ cerevisiae$/S. bayanus | Z. mobilis | L. mesenteroides |
|-----------------|-----------------------------|------------|-----------------|
|                 | $f_{\text{eth}}$ (%) | CH$_2$ | CH$_3$ | $f_{\text{eth}}$ (%) | CH$_2$ | CH$_3$ | $f_{\text{eth}}$ (%) | CH$_2$ | CH$_3$ |
| 1               | 11.3 | 1.0000 | 1.0017 | 14.3 | 0.9991 | 1.0033 | 24.2 | 1.0068 | 1.0059 |
| 49.1            | 0.9992 | 1.0012 | 30.1 | 0.9991 | 1.0034 | 58.1 | 1.0059 | 1.0073 |
| 55.3            | 0.9988 | 1.0012 | 43.0 | 0.9985 | 1.0039 |        |        |        |
| 2               | 9.1 | 0.9991 | 1.0018 | 12.0 | 0.9997 | 1.0036 | 30.4 | 1.0055 | 1.0049 |
| 11.6            | 0.9991 | 1.0014 | 17.5 | 0.9995 | 1.0036 | 57.1 | 1.0049 | 1.0044 |
| 37.3            | 0.9997 | 1.0013 | 30.1 | 0.9994 | 1.0034 | 73.7 | 1.0057 | 1.0060 |
| 3               | 9.4 | 1.0001 | 1.0012 | 8.1 | 1.0003 | 1.0035 |        |        |        |
| 52.6            | 0.999 | 1.0015 | 14.9 | 0.9998 | 1.0034 |        |        |        |
| 60.2            | 0.9981 | 1.0014 | 26.2 | 0.9995 | 1.0038 |        |        |        |
| Mean            | 0.9992 | 1.0014 | 0.9994 | 1.0035 | 1.0058 | 1.0057 |        |        |
| S.D.            | 0.0006 | 0.0002 | 0.0005 | 0.0002 | 0.0007 | 0.0011 |        |        |
FIGURE 1

13C isotopic relationship in glucose fermentation

A

B

Embden–Meyerhof–Parnas pathway

Entner–Doudoroff pathway

Reductive Pentose Phosphate pathway

S. cerevisiae

Z. mobilis

L. mesenteroides
$^{13}$C isotopic relationship in glucose fermentation

FIGURE 2

[Chemical shift diagram with isotopic labels and DMSO-d$_6$]
FIGURE 3

$^{13}$C isotopic relationship in glucose fermentation

A

B
FIGURE 4

13C isotopic relationship in glucose fermentation

Upper Entner-Doudoroff

Possible effect on future CH₂

Possible effect on future CH₃

KDPG

Pyruvate

Glyceraldehyde-3-P

6-Phosphogluconolactone

6-Phosphogluconate
FIGURE 5

Possible effect on CH₂

Only possible effect at future CH₃
Non-Statistical $^{13}$C Distribution during Carbon Transfer from Glucose to Ethanol During Fermentation is Determined by the Catabolic Pathway Exploited

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