Non-equivalence of Hydrogen Transfer from Glucose to the pro-R and pro-S Methylene Positions of Ethanol during Fermentation by *Leuconostoc mesenteroides* Quantified by $^2$H NMR at Natural Abundance*

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The anaerobic fermentation of glucose by *Leuconostoc mesenteroides* via the reductive pentose phosphate pathway leads to the accumulation of lactic acid and ethanol. The isotope redistribution coefficients ($a_{ij}$) that characterize the specific derivation of each hydrogen atom in ethanol in relation to the non-exchangeable hydrogen atoms in glucose and the medium water have been determined using quantitative $^2$H NMR. First, it is confirmed that the hydrogens of the methylene group are related only to the 1 and 3 positions of glucose via the NAD(P)H derived from the reductive pentose phosphate pathway (RPP) and not to the 5 position, in contrast to ethanol produced in the course of this fermentation. The hydrogen atoms of glucose are redistributed among the hydrogen atoms of the medium and the hydrogen atoms in the substrate, the intermediates and the products, and the kinetic isotope effects associated with the biochemical reactions involved in the fermentation pathway. These influences lead to a non-statistical distribution of $^2$H in the population of monodeuterated isotopomers within the final product.

During the anaerobic fermentation of glucose by the reductive pentose phosphate (RPP) pathway, each mole of glucose is converted to 1 mol of CO$_2$, 1 mol of d-lactate, and 1 mol of ethanol. In the course of this fermentation, the hydrogen atoms of glucose are redistributed among the hydrogen atoms of the d-lactate and ethanol that accumulate, or lost to the medium. Hydrogen may be transferred directly in a C-H bond that remains intact, or via the reduced nucleotide pool NAD(P)-H. Further hydrogens are introduced from water. The relationship between the substrate hydrogens and the products has been largely established by using $^3$H or highly enriched $^2$H tracer studies (Fig. 1).

However, such studies mostly show only the qualitative relationships. By using natural abundance and very low $^2$H enrichment, the isotopic redistribution of $^2$H into the products of fermentation can be quantitatively measured using $^2$H NMR (1–3). Thus,

$$\left(\frac{^2H}{^1H}\right)_i = a_{im}(^2H/^1H)_m + \sum_j a_{ij}(^2H/^1H)_j$$

(Eq. 1)

where $(^2H/^1H)_i$ is the isotopic ratio at position $i$ of the product, $(^2H/^1H)_m$ is the isotopic ratio of the medium, and $(^2H/^1H)_j$ is the isotopic ratio at site $j$ of the substrate. The terms $a_{im}$ and $a_{ij}$ are the isotope redistribution coefficients that give the quantitative description of the link between the substrate and the products (2, 4, 5). The value of these parameters is principally defined by the exchange reactions that can occur, directly or indirectly, between the water of the medium and the hydrogen atoms in the substrate, the intermediates and the products, and the kinetic isotope effects associated with the biochemical reactions involved in the fermentation pathway. These influences lead to a non-statistical distribution of $^2$H in the population of monodeuterated isotopomers within the final product (3).

The technique of quantitatively describing the hydrogen isotope transfer coefficient ($C_j$) values using $^2$H NMR has been used to show that it is possible to distinguish the pathway used to ferment glucose to lactic acid by the lactic acid bacteria *Lactococcus (Lc.) lactis* (glycolysis) and *Leuconostoc (Ln.) mesenteroides* (RPP) (5).

In those cases where a prochiral center exists, the stereospecificity of the reductase that transfers hydride from NAD(P)H is a key factor influencing the value of $C_j$, because H is introduced stereospecifically. In ethanol production by the anaerobic fermentation of glucose with *Saccharomyces (S.) cerevisiae*, for example, the $^2$H of NAD$^+$ is introduced to the $\textit{re}$ face of acetaldheyde by the NADH-dependent alcohol dehydrogenase (ADH), leading exclusively to the formation of (R)-[1-$^2$H]ethanol (2).

In *Ln. mesenteroides*, the C2–C3 moiety of glucose is fermented in anaerobic conditions to ethanol. It is evident from the RPP pathway (Fig. 1) that both the (1S)- and (1R)-hydrogens are introduced from the NAD(P)H pool by the terminal steps of...
ethanol biosynthesis, the acetaldehyde dehydrogenase (ALDH) and ADH. Thus, a similar situation should exist as described (5) for lactic acid: the C₆ values for ethanol produced by \textit{Ln. mesenteroides} and \textit{S. cerevisiae} should differ.

However, the situation in \textit{Ln. mesenteroides} is more complex than in \textit{S. cerevisiae}. First, \textit{Ln. mesenteroides} contains two ADHs, one NADH-dependent, the other NADPH-dependent (6). Because the stereochromy varies for different ADH activities, the C₆ values could be strongly influenced by the stereochromy of these two ADHs. For example, the broad specificity NADPH-dependent secondary ADH of \textit{Leuconostoc kefir} has been shown to introduce H to the si face of higher aldehydes or ketones, leading to the formation of the R-isomer (7), whereas the ADH from \textit{Aeropyrum pernix} is NADH-dependent and produces (S)-alcohols from the corresponding ketones (8). As far as we are aware, the stereospecificities of the \textit{Ln. mesenteroides} ADHs have not been defined. Second, one hydrogen is introduced by an ADH and the final prochirality of this hydrogen will depend on the stereospecificity of the subsequent ADH.

A further complication is that, whereas in \textit{S. cerevisiae} only the NAD⁺/NADH pool is involved in ethanol biosynthesis, in \textit{Ln. mesenteroides} both NAD⁺ and NADP⁺ participate in the hydride abstraction and hydride donor reactions. Reductant power in the form of NAD(P)H is produced by two oxidase reactions: glucose-6-phosphate-1-dehydrogenase (Glu-6-PDH, EC 1.1.1.49), 6-phosphogluconate dehydrogenase (6-PDG, EC 1.1.1.44), and glyceraldehyde-3-phosphate dehydrogenase (phosphorylating) (GAPDH, EC 1.2.1.12) (Fig. 1). Redox balance in the NAD⁺/NADH and NADP⁺/NADPH ratios is achieved via three terminal reductase reactions: d-lactate dehydrogenase (d-LDH, EC 1.1.1.28), acetaldehyde dehydrogenase (EC 1.2.1.10), and alcohol dehydrogenase (EC 1.1.1.1 and EC 1.1.1.2). In \textit{Ln. mesenteroides}, Glu-6-PDH and 6-PDG are both capable of transferring a hydrogen atom to either NAD⁺ or NADP⁺, although with different affinities for the two cofactors (9, 10). In contrast, GAPDH in \textit{Leuconostoc oenos} is NAD⁺-specific (11). In the regeneration steps, both d-LDH (12) and ADH are NADH-dependent (13), whereas \textit{Ln. mesenteroides} ADH uses both NADH and NADPH (6).

With the aim of describing quantitatively the origins of all the non-exchangeable hydrogens in ethanol derived by glucose fermentation with \textit{Ln. mesenteroides}, we have fermented glucose slightly enriched with \([1-^2H]_\text{-}, [2-^2H]_\text{-}, \text{or } [3-^2H]_\text{-}\)glucose and measured the isotopic incorporation into ethanol. We have observed that isotopic transfer to the pro-(1S)- and pro-(1R)-hydrogen positions of ethanol is not equivalent. To explain this observation, we have determined the stereospecificities of the NADH-dependent and NADPH-dependent ADH activities and the cofactor selectivities of the enzymes that participate in the nucleotide cofactor cycle. This article presents the isotopic transfer data and interprets this in relation to the input to and use of the NAD(P)H pools.

**EXPERIMENTAL PROCEDURES**

**Materials**—d-Glucose was obtained from Prolabo. Specifically labeled glucose (all at 98 atom %) was obtained from the following sources: \([1-^2H]_\text{-glucose and } [6,6-^2H_2]_\text{-glucose from Euriso-Top}; [2-^3H]_\text{-glucose from Cambridge Isotope Labs, [3-^2H]_\text{-glucose from OMICRON Biochemicals Inc. Sodium [1-^2H]_\text{-formate was obtained from Sigma and } {^2H_2}O (99.95 \text{ atom } %) \text{ from Euriso-Top}. \text{Components of the culture medium were obtained as follows: tryptone, yeast extract (autolytic) from Biokar; casein-meal peptone from Laborgros; soya-flour peptone (prepared using papain) and sodium glycerophosphate from Merck; ascorbic acid and } \text{MgSO}_4 \text{ from Sigma. The same supply of each component was used for all fermentations. } NAD^+/Na was obtained from Roche Applied Science GmbH, } \text{NADP}^+/Na (97%) \text{ and glucose dehydrogenase (382 units/ml) from Sigma, and formate dehydrogenase (176 units/ml) from Fluka.}

**Bacterial Cultures**—\textit{Ln. mesenteroides mesenteroides} strain 19D cit⁺ was obtained from the collection of the Laboratoire de Microbiologie Alimentaire Industrielle (ENITIAA, Nantes, France) and was stored at \(-80^\circ \text{C in M17 medium (14) with 15% (v/v) glycerol.}

**Culture Conditions for Ethanol Production**—Culture was carried out as described previously (5) and was ended when constant weight was attained (typically \(-72 \text{ h). The complete utilization of glucose was verified by } ^1H \text{ NMR (15). Supernatant was recovered by centrifugation (4250 } \times \text{ g for 10 min at 4 }^\circ \text{C) and kept at \(-20^\circ \text{C.}

To follow the isotopic relationship between each position in glucose and each position in ethanol, a trace quantity of specifically enriched glucose was added to the fermentation medium. To 30.01 g (± 1 mg) of d-glucose was added 7.8 mg (±0.1 mg) of \([1-^2H]_\text{-glucose, or } 7.8 \text{ mg (±0.1 mg) [2-^2H]_\text{-glucose, or } 8.1 \text{ mg (±0.1 mg) [3-^2H]_\text{-glucose, or } 7.9 \text{ mg (±0.1 mg) of [6,6-^2H_2]_\text{-glucose. These quantities gave site-specific enrichments of } \sim 400 \text{ ppm, } \sim 3\text{-fold natural abundance (}\sim 150 \text{ ppm). At this level, the NMR properties of the molecule remain unaltered. The resulting enrichment was obtained by calculation and is given in Table 1. Similarly, to establish the isotopic connection with water, 10004.6 g of tap water was enriched with 2.1336 g of \(^2H_2O (99.95 \text{ atom } %), \text{giving a fermentation medium enriched at 341.0 ppm (natural abundance } = 149.1 \text{ ppm).}

**Culture Conditions for Enzyme Preparations**—For the preparation of enzyme extract, static anaerobic fermentation was carried out in 500 ml of M17 medium (14) (pH 7.4) containing 4.5 g of glucose (25 mmol) in a 0.5-liter closed Duran bottle. Following inoculation with \textit{Ln. mesenteroides} recovered from \(-80^\circ \text{C storage, the culture was left for } \sim 36 \text{ h at 30 }^\circ \text{C until the optical dispersion at 550 nm was } \sim 1.3.

**Enzyme Extracts**—Bacterial cells were recovered from culture by centrifugation (4250 \times \text{ g for 10 min at } 4^\circ \text{C) and the pellet was resuspended in 20 ml of ice-cold sodium phosphate buffer (100 mm, pH 7.1). This suspension was transferred to a 30-ml centrifuge tube and repelleted (4250 \times \text{ g for } 10 \text{ min at } 4^\circ \text{C). The pellet was resuspended in 10 ml of ice-cold sodium phosphate buffer (100 mm, pH 7.1, 0.5 mg/ml dithiothreitol) and the cells disrupted by ultrasonication (Vibracell: 60% amplitude, 5 min, pulse sequence 5-s on/off, power } \sim 23 \text{ W, } 4^\circ \text{C). The debris was removed by centrifugation (4250 \times \text{ g for 10 min at } 4^\circ \text{C) and the super-
Isotopic Relationship in Glucose Fermentation

Enzyme preparation. Protein content was determined from the OD$_{280}$/OD$_{260}$ ratio.

**Activity**

Enzyme activities were measured spectrophotometrically (rate of change of OD at 343 nm) at 30 °C in 100 mM sodium phosphate (pH 7.1) with 2.5 mM MgCl$_2$. Enzyme concentration was adjusted to give pseudo-first order kinetics over a ~5 min initial period. Cofactors were used at saturating concentration (0.5 mM). It was confirmed that the concentrations of substrates used were saturating. These were: for Glu-6-PDH, 6 mM glucose 6-phosphate; for 6-PDG, 6 mM 6-phosphogluconate; for GAPDH, 5.9 mM glyceraldehyde 3-phosphate; for d-LDH, 1.5 mM pyruvate; for ADH, 0.28 mM acetaldehyde; for AIDH, 0.5 mM acetyl coenzyme A. All activities were correct for non-substrate blanks.

**Formation of [1-2H]Ethanol by NADH-dependent ADH Activity**—Ln. mesenteroides crude extract was incubated under conditions in which NADPH was constantly regenerated using the [1-2H]formate/formate dehydrogenase system (16). In 6 replicate 2-ml Eppendorf tubes was mixed: 50 µl of NADPH (7.8 mM, 6 mg/ml of NADPH/Na freshly prepared in H$_2$O), 100 µl of [1-2H]formate (0.68 mM, 47 mg/ml sodium [1-2H]formate in 0.3 M sodium phosphate buffer, pH 7.5), and 50 µl of formate dehydrogenase (17.6 units/ml in 0.3 M sodium phosphate buffer, pH 7.5). After 15 min at 30 °C, 1.0 ml of Ln. mesenteroides crude extract and 100 µl of acetaldehyde (1.78 µl, 100 µl ml H$_2$O) were added, and the mixture was left to incubate for ~18 h at 30 °C. One tube was used to verify that ethanol production had occurred: the other 5 were used to extract ethanol for $^2$H NMR analysis.

**Formation of [1-2H]Ethanol by NADPH-dependent ADH Activity**—Ln. mesenteroides crude extract was incubated under conditions in which NADPH was constantly regenerated using the [1-2H]glucose/glucose dehydrogenase system (17). In 6 replicate 2-ml Eppendorf tubes was mixed: 50 µl of NADPH (11.2 mM, 7.7 mg/ml of NADPH/Na freshly prepared in H$_2$O), 100 µl of [1-2H]glucose (0.5 mM, 90 mg/ml [1-2H]glucose freshly prepared in H$_2$O), and 20 µl of glucose dehydrogenase (3.8 units/ml in 0.3 M sodium phosphate buffer, pH 7.5). After 15 min at 30 °C, 1.0 ml of Ln. mesenteroides crude extract and 100 µl of acetaldehyde (1.78 µl, 100 µl ml H$_2$O) were added, and the mixture was left to incubate for ~18 h at 30 °C. One tube was used to verify that ethanol production had occurred: the other 5 were used to extract ethanol for $^2$H NMR analysis.

**Extraction of Ethanol from Fermentation Medium**—The ethanol was recovered from the fermentation medium or from enzyme incubation by distillation using a Cadiac column equipped with a Teflon turning band. The water titers, as determined by Karl-Fischer titration, was ~5%.

**Quantification of Ethanol**—Ethanol production was quantified by gas chromatography. For enzyme incubations, 0.4 g of NaCl and 0.5 ml of ethyl acetate were added to the incubation mixture. After vigorous agitation (3 min), the phases were separated by centrifugation (13,000 g for 1 min) and the ethyl acetate phase recovered. Ethanol was quantified by gas chromatography on a Hewlett Packard 6830 gas chromatograph fitted with a PTA-5 column (30 m x 0.32 mm, film thickness 0.5µm) with helium as carrier gas at a constant flow (1.2 ml/min) and an injector temperature of 250 °C. Elution conditions were as follows: 35 °C for 4 min, 40 °C/min to 200 °C, and 200 °C for 1 min. Detection was by flame ionization detection at 250 °C. Under these conditions, the retention times were: ethanol at 2.99 min and acetaldehyde at 3.28 min. Calibration was with known concentrations of ethanol (15 to 190 mM) treated exactly like the samples.

**Esterification of Ethanol**—To resolve the (1S)- and (1R)-deuterium positions of ethanol in the NMR spectrum, the ethanol was converted to ethyl mandelate, essentially as described in Ref. 18. To an ethanol sample of 3.5–5.0 ml (56–80 mmol) was added 20 ml of dry benzene in a 50-ml round-bottomed flask. Residual water was eliminated by slow azeotropic distillation on a Dean-Stark apparatus using 3-Å molecular sieves and gentle warming. Distillation was repeated about 15 times, replacing the molecular sieves as required, until <0.2% water remained, as determined by Karl-Fischer titration. Then, 3.04 g (20 mmol) of S(-)-mandelic acid and 50 µl of 18 m sulfuric acid were added. The reaction mixture was gently warmed and water eliminated as described using the Dean-Stark apparatus. After about 20 cycles, heating was increased to remove the benzene by distillation and the ethyl mandelate was recovered by distillation in vacuo at 120 °C. Yield was typically 58–60%.

**$^2$H NMR Spectroscopy**—The $^2$H NMR spectra of ethanol were recorded with a Bruker DPX 400 spectrometer operating at 61.4 MHz and fitted with a $^{19}$F field frequency locking device. Acquisition parameters for ethanol from fermentations were: number of scans, 400; sweep width, 1197 Hz; acquisition time, 2.45 s; delay, 4.45 s; pulse (90°), 18.8 µs; T = 303 K; broadband $^1$H decoupling; 5 repetitions. Acquisition parameters for ethanol from enzyme incubations were: number of scans, 200; sweep width, 1197 Hz; acquisition time, 6.8 s; delay, 0.2 s; pulse (90°), 19.5 µs; T = 303 K; broadband $^1$H decoupling; 10 repetitions. The $^2$H NMR spectra of ethyl mandelate were recorded with a Bruker DRX 500 spectrometer operating at 76.7 MHz and fitted with a $^{19}$F field frequency locking device. Acquisition parameters were: number of scans, 5000; sweep width, 1502 Hz; acquisition time, 3.4 s; delay, 0.1 s; pulse (90°), 13 µs; T = 315 K; broadband $^1$H decoupling; 5 repetitions.

The sample preparation for ethanol was ~1.8 g of ethanol and 1.2 g of N,N,N',N’-tetramethylurea (internal reference) mixed in a sample vial with 100 µl of C$_6$F$_6$ (lock). Exact quantities were recorded. The sample was filtered and introduced into a 10-mm NMR tube fitted with an anti-vortex. Five or 10 spectra were recorded for each sample and an
exponential multiplication associated with a line broadening of 2 Hz was used.

The sample preparation for ethyl mandelate was ~1.7 g of ethyl mandelate and 1.8 g of benzene mixed in a sample vial with 100 μl of C₆F₆ (lock). Exact quantities were recorded. The sample was filtered and introduced into a 10-mm NMR tube fitted with an anti-vortex. Five spectra were recorded for each sample and an exponential multiplication associated with a line broadening of 0.5 Hz was used. The quantitative measurement of the areas under the peaks and the calculation of the (2H/1H) ratios were carried out as described previously (5).

The isotopic ratios (2H/1H)CH-R and (2H/1H)CH-S were obtained from Equations 2 and 3, respectively,

\[
(2H/1H)_{CH-R} = 2 \times (2H/1H)_{CH} \times \frac{A_R}{A_R + A_S} \quad (Eq. 2)
\]

\[
(2H/1H)_{CH-S} = 2 \times (2H/1H)_{CH} \times \frac{A_S}{A_R + A_S} \quad (Eq. 3)
\]

where (2H/1H)CH is the area under the CH peak in the ethanol spectrum and A₀ and A₂ are the areas under the peaks for the CH-R and CH-S, respectively, obtained by integration of the NMR spectrum of ethyl mandelate.

The enantiomeric excess was calculated from Equation 4.

\[
EE = \frac{(2H/1H)_{CH-R} - (2H/1H)_{CH-S}}{(2H/1H)_{CH-R} + (2H/1H)_{CH-S}} \quad (Eq. 4)
\]

**RESULTS**

Ethanol has five non-exchangeable hydrogen atoms: three in the methyl (CH₃) group and two in the methylene (CH₂OH) group. The former are equivalent, whereas the latter are prochiral. Thus, both (1S)-2H- and (1R)-2H-ethanol can occur naturally, making three monodeuterated isotopomers possible. To establish the isotopic C value between different positions of glucose and these three monodeuterated isotopomers in ethanol biosynthesized by *Ln. mesenteroides*, fermentation was carried out in the presence of glucose slightly deuterated at sites 1, 2, or 3. Glucose slightly bi-deuterated at 6R6S (6,6 designates the average of these positions) was used to test that no ethanol was produced by glycolysis. Glucose from *Zea mays* was used as a reference, the natural abundance (2H/1H) having been previously determined for each hydrogen position j (19). Fermentation was also performed with slightly deuterated water to determine the extent to which this was a source of hydrogen in ethanol.

As Equation 1 is a linear function, isotope redistribution coefficients aᵢ are can be established using only two (2H/1H) values and their corresponding (2H/1H)₀ values. Therefore, the isotopic correlation was measured at natural abundance and at ~3-fold site-specific enrichment in glucose or water.

For each fermentation, it was confirmed by HPLC and/or ¹H NMR that all the glucose had been consumed. The molar yield of ethanol was 0.85 in relation to glucose and 0.95 in relation to lactate, in close agreement with literature values (20). The deuterium content of ethanol isolated from fermentation with water or glucose at different slight enrichments is summarized in Table 1.

**Deuterium Transfer from Medium Water to Ethanol**—The data in Table 1 show that ²H is incorporated from water into both the CH₂OH and CH₃ positions of ethanol. It is also clear that incorporation occurs in both hydrogen positions of the CH₂OH. Equations 5–8 are the solutions to Equation 1 for the fermentation of natural abundance glucose in the presence of slightly deuterated water, showing the aᵢ for each potential isotopomer.

\[
(2H/1H)_{CH} = 0.28(2H/1H)_{m} + 53.2 \quad (Eq. 5)
\]

\[
(2H/1H)_{CH₂} = 0.26(2H/1H)_{m} + 109.1 \quad (Eq. 6)
\]

\[
(2H/1H)_{CH-R} = 0.22(2H/1H)_{m} + 128.8 \quad (Eq. 7)
\]

\[
(2H/1H)_{CH-S} = 0.29(2H/1H)_{m} + 89.8 \quad (Eq. 8)
\]

These coefficients indicate the magnitude of isotope redistribution between medium water and position i of ethanol, whereas the residue shows the extent to which ²H in position i of the

| Medium, (2H/1H)ᵢ | Glucose | Ethanol |
|------------------|---------|---------|
| (2H/1H)ᵢ | (2H/1H)ᵢ | (2H/1H)ᵢ | (2H/1H)ᵢ |
| ppm | ppm | ppm | ppm |
| --- | --- | --- | --- |
| 149.5 | Ref² | 94.3 (0.3)⁸ | 147.3 (0.5)⁵ |
| 341.0 | Ref | 147.0 (0.2)⁵ | 196.1 (1.1)⁵ |
| 149.5 | 1 | 173.8 | 428.6 |
| 149.5 | 2 | 156.7 | 413.1 |
| 149.5 | 3 | 146.4 | 410.9 |
| 149.5 | 6.6 | 147.2 | 405.2 |

² Ref = commercial corn glucose, for which the (2H/1H) values are taken from Ref. 19.

⁸ The (2H/1H) values of the enriched glucose at site j, by calculation.

⁹ Mean values ± S.D. (in parentheses) are given for n = 3 to n = 5 spectra depending on the sample.

⁵ Calculated with (2H/1H)₀ = 149.1 ppm (NTW) and (2H/1H)OH of glucose = 159.4 ppm.

⁶ (2H/1H) values for ethanol obtained from reference glucose in reference medium.

⁷ By calculation.

⁸ Because the labeled glucose is a bi-deuterated molecule, mono- and bi-deuterated isotopomers are present. The given value is corrected to take this into account: – 0.5(S₆₆ – S₅₆) + S₅₆, where S₆₆ is the measured surface area under the peak with [6,6-²H₂]glucose and S₅₆ is the measured surface area under the peak with unenriched glucose (line 1).
ethanol is derived from other sources (principally the different positions of glucose). From Equations 5 and 6 it can be concluded that hydrogen is introduced into the CH₃ or CH₂OH of ethanol from the medium to a very similar extent. However, it is also clear from the residues that the contribution of the non-exchangeable sites in glucose is greater for the CH₂OH than for the CH₃. Furthermore, Equations 7 and 8 show that this contribution is greater for the CH₂OH than for the CH₃. Conversely, enrichment at the 2H position does not contribute to the enrichment of ethanol. Hence, Equation 17 represents the number of hydrogens at a given site in glucose of the non-exchangeable sites in glucose.

From Table 1, it is evident that the CH₃ is only enriched from the 2 position of glucose. Together with the lack of enrichment from either the 1 or the 6,6 positions, this confirms that there is no glycolytic contribution to ethanol biosynthesis in *L. mesenteroides*, as expected. Conversely, enrichment at the 2 position does not contribute to the enrichment of ethanol in the 1 or 3 position in glucose enriches both the (2H/1H)CH₃ and (2H/1H)CH₂OH positions in ethanol: again the predicted correlation based on an exclusively RPP pathway metabolism (Fig. 1). In contrast, the 1 or 3 position in glucose enriches both the (2H/1H)CH₃ and (2H/1H)CH₂OH positions in ethanol: again the predicted correlation based on an exclusively RPP pathway metabolism.

The data used to obtain Equations 9–15 are quantitative and obtained under uniform conditions. Therefore, the conversion factor (Cᵢ,j), which represents quantitatively the extent to which each ²H of glucose is retained in the ethanol produced, can be calculated (2, 5). This factor is dependent on the stoichiometry of the overall bioconversion (Reaction 1).

Glucose(H) → 1 lactic acid(H) + 1 ethanol + 1 CO₂

**REACTION 1**

Because during RPP metabolism, nᵣᵢ expressions are converted to only nᵣᵢ molecules of lactic acid and ethanol, then nᵣᵢ site j monodeuterated glucose molecules are converted to Cᵢμᵢ site i monodeuterated ethanol molecules. Cᵢ can be expressed as Equation 16,

\[
\frac{(2H/1H)_i}{(2H/1H)_j} = a_i \frac{(2H/1H)_i}{(2H/1H)_j} = C_i
\]

where ²Hᵢ represents uniquely monodeuterated molecules of ethanol in which the ²H is derived from site j of glucose and Pᵢ represents the number of hydrogens at a given site i in the product, hence Equation 17.

\[
C_i = a_i \times P_i
\]

If there is no loss to the medium or transfer to non-identified positions in other molecules, then the sum of the Cᵢ,j values for a given position in glucose is 1.00. The Cᵢ,j values for ethanol are presented in Table 2, together with those previously determined for lactic acid (5) to enable a complete explanation of the transfers.
It is shown that the hydrogens at positions 1, 2, and 3 of glucose are substantially retained in the ethanol produced. The position 2 $^2$H is highly (82%) retained in the CH$_3$ as would be expected, because the C–H bond remains intact throughout the pathway (Fig. 1). This retention is greater than that observed for the transfer of the 2-2H to lactic acid in Lc. lactis (62%), in which an intramolecular transfer is involved (5).

Moreover, the hydrogens at the 1 and 3 positions of glucose, from which the $^2$H is transferred to the reduced cofactor pool NAD(P)H, are strongly correlated with the CH$_2$OH. Again this is as predicted, because this position receives both hydrogen atoms from this source during the final two reductase reactions of ethanol formation (Fig. 1B). Thus, 72% of the position 1 $^2$H and 56% of the position 3 $^2$H are transferred to the CH$_2$OH, a further 18 and 38%, respectively, being transferred to the hydroxymethyl of lactic acid (Table 2). Hence, ~90% of these hydrogen atoms can be accounted for in the terminal products lactic acid and ethanol, a level comparable with that seen for the $^2$H that are directly incorporated: 95% for the 6,6 into lactic acid and 82% for the 2 into ethanol. (The apparent transfer of 21% of position 1 $^2$H to the CH$_3$ of lactic acid is discussed in Ref. 5).

These $C_f$ values for ethanol can be compared with those previously measured for the ethanolic fermentation of glucose by a mixed culture of S. cerevisiae and Saccharomyces bayanus (2). In this case, where ethanol is produced via glycolysis and accumulates as the sole major product of fermentation, sites 1 (84%), 2 (54%), and 6,6 (96%) are all strongly correlated to ethanol, in marked contrast to the situation in Ln. mesenteroides (Table 2). The position 3, on the contrary, has zero correlation, whereas in Ln. mesenteroides it is a major source of the hydrogen in the CH$_2$OH group (Table 2). Hence, from a simple analysis of the pattern of the $^2$H transfers in these two organisms, it is easy to determine whether the ethanol has been derived by the glycolytic or the RPP pathway. This mirrors the way that lactic acid produced by the same pathways has been distinguished (5).

### Stereospecificity in the Transfer of Deuterium from Glucose to Ethanol—An analysis of the transfer of hydrogen to the pro-R and pro-S positions of the CH$_2$OH group in ethanol was presented in the final two columns of Table 1. It is clear that the enhancements observed in these two non-exchangeable positions are markedly different. Transfer of $^2$H to the pro-R from the 1 position in glucose is not the same as from the 3 position, leading to a $C_f$-1 (i.e. the transfer coefficient from the 1 position of glucose) that is twice the $C_f$-3. Similarly, the enrichment in the pro-S from the 1 and 3 positions differs, but in this case slightly in favor of the 3 position. Furthermore, not only do the $C_f$-1 and $C_f$-3 values differ, but the transfer of hydrogen from each position to the pro-S and pro-R of the methylene group is not equivalent: the $C_f$-1-R:$C_f$-1-S ratio is 2.1, whereas the $C_f$-3-R:$C_f$-3-S ratio is 0.8.

In addition, the (2H/1H)$_{CH_3}$ is enhanced significantly more (55.4 ± 2.7 ppm) from water than the (2H/1H)$_{CH_2}$ position (42.2 ± 2.9 ppm) (Table 1). There are a number of possible explanations for these observations, which are presented and assessed in the following sections.

### Deuterium Exchange in the Reduced Nucleotide Pools—Deuterium exchange between NAD(P)H and water can potentially occur due to the action of flavin/diaphorase (see (18)). In S. cerevisiae, ~8% post-fermentation exchange between NAD$^2$H and water was detected (2). If differential exchange of $^2$H in NAD$^2$H and NADP$^2$H with water occurred, this could lead to non-equivalent dilution from the [1-2H]- and [3-2H]glucose and to enrichment from $^2$H$_2$O. This would impact differentially on incorporation into the pro-R and pro-S methylene positions.

To test whether such an exchange occurs in Ln. mesenteroides, a fermentation was carried out with natural abundance glucose and water until the glucose was used up. Then, $^2$H$_2$O was added and the culture left for a further 48 h. As can be seen from the data in Table 3, no detectable enzymatic exchange of the hydrogens of ethanol and the medium was found, eliminating this possible cause. This conclusion is supported by previous workers, who were unable to detect NAD(P)H oxidase activity in Ln. mesenteroides enzyme extracts (13).

### Stereospecificity in the NADH- and NADPH-dependent ADH Activities—As already indicated in the Introduction, ADH can potentially insert hydride at either the re or the si face of an aldehyde. If the NADH-dependent and NADPH-dependent ADH activities in Ln. mesenteroides were of opposing stereospecificities, hydride would be introduced at both the re and si faces of the aldehyde, causing different levels of enrichment at the CH-R and CH-S positions. A cumulative effect is caused by the hydride already introduced by the AldH adopting a differ-

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### Table 2

| Deuterated site $i$ of product | Deuterated site $j$ of glucose | Conversion factor $C_f$ |
|-------------------------------|-------------------------------|-------------------------|
| CH$_3$                        | 1                             | 0.21                    |
| CH$_2$                        | 2                             | 0.82                    |
| CH$_3$                        | 3                             | 0                       |
| CH$_3$                        | 6,6                           | 0.95                    |
| CH$_3$                        | 1                             | 0.18                    |
| CH$_3$                        | 2                             | 0.9                    |
| CH$_3$                        | 6,6                           | 0.38                    |
| CH$_3$                        | 3                             | 0.49                    |
| CH$_3$                        | 3                             | 0.25                    |
| CH$_3$                        | 1                             | 0.23                    |
| CH$_3$                        | 3                             | 0.31                    |

$^a$ Data from Ref. 5.

$^b$ O = any small difference in the (2H/1H)$_{CH_3}$ values between the $^2$H-enriched glucose and natural abundance samples was not significant at 2 S.D.

$^c$ $n = 2$ for ethanol and $n = 1$ for lactic acid.

$^d$ Indicates no data.

### Table 3

Effect of adding deuterated water on post-fermentation deuterium incorporation into ethanol

| Medium water (2H/1H)$_{H_2}O$ | Ethanol |
|-------------------------------|---------|
| During fermentation | After fermentation | (2H/1H)$_{CH_3}$ | (2H/1H)$_{CH_2}$ | (2H/1H)$_{CH-R}$ | (2H/1H)$_{CH-S}$ |
| ppm  | ppm  | ppm   | ppm   | ppm         | ppm         |
| 149.5 | 149.5 | 94.3 (0.3) | 147.3 (0.5) | 161.7 (0.9) | 132.9 (0.8) |
| 149.5 | 640.0 | 92.8 (0.7) | 148.6 (2.1) | 159.8 (2.5) | 137.4 (2.2) |
ent prochiral position depending on which face is attacked by the ADH.

Therefore, the stereospecificities of the NADH-dependent ADH and NADPH-dependent ADH in *Ln. mesenteroides* were determined. This was done using a crude enzyme preparation incubated with acetaldehyde under conditions in which NAD\(^+\)H or NADP\(^+\)H were regenerated enzymatically. Following analysis of the \(^2\)H\)ethanol produced as ethyl mandelate, the stereospecificity of each ADH activity could be independently determined. As a control for the methodology, commercial ADH from *S. cerevisiae* was similarly treated. The results are presented in Table 4.

The data shown in Table 4 confirm that the NADH-dependent ADH of *S. cerevisiae* enriches the CH\(-R\) position of ethanol. This is in accord with the transfer of hydride from NADH to the \(\text{re}\) face of acetaldehyde, which is the same stereospecificity as demonstrated for ethanol produced by fermentation (2). Hence, this control shows that the *in vivo* and *in vitro* measurements are in agreement.

An identical analytical approach to the NADH- and NADPH-dependent ADH activities in the extract of *Ln. mesenteroides* shows the enrichment of the CH\(-R\) position of ethanol from both enzymes (Table 4). Thus, both enzymes show the same stereochemistry, and with an enantiomeric excess close to unity (Table 4). Hence, it can be concluded unequivocally that none of the observed transfer of hydrogen to the pro-\(-S\) position in the CH\(_2\)OH of ethanol generated during fermentation of \([1-2\text{H}]\)glucose is due to the activity of either NADH- or NADPH-ADH. The converse implication is that all of the hydrogen transfer to the pro-\(-S\) position is due to the action of ADH on acetyl-CoA.

**Cofactor Specificities of the Enzymes in the Reduced Nucleotide Cycle**—It has previously been shown that Glu-6-PDH and 6-PDG in *Ln. mesenteroides* have different specificities for NAD\(^+\) and NADP\(^+\) (9) and that the ADH activity favors NADPH as cofactor (21). Notably, Glu-6-PDH was reported to favor NADP\(^+\) slightly (NAD\(^+\)/NADP\(^+\) = 0.67) and 6-PDG to favor NAD\(^+\) strongly (NAD\(^+\)/NADP\(^+\) = 25). These specificities suggest that the NADP\(^+\)H pool should be slightly more enriched than the NAD\(^+\)H pool from [1-\(^2\)H]glucose, whereas NAD\(^+\)H should be much more enriched than NADP\(^+\)H from [3-\(^2\)H]glucose.

This implies that the relative deuteration of the pools of NADH and NADPH from [1-\(^2\)H]- or [3-\(^2\)H]glucose will have a strong influence on the transfer coefficients for the introduction of hydrogen into the pro-\(-R\) and pro-\(-S\) positions in the CH\(_2\)OH, and that this influence will be modulated by the relative affinities of the ADH and AIDH for NADH and NADPH. A further influence will be the introduction of hydride into the NAD(P)H pool by GAPDH and the consumption of NAD(P)H by LDH (Fig. 1).

It has previously been shown that these various enzymes have different specificities for cofactor (summarized in the Introduction), but all the cofactor usages have not been measured in *Ln. mesenteroides*, and all six enzyme activities have not been examined in the same strain grown under the same conditions. Therefore, the selectivity ratios in the strain used for the present experiments were determined (Table 5). From the data presented in Table 5, it is possible to draw a number of conclusions as to how the cofactor selectivity is responsible for the observed \(C_f\) values.

In the CH\(_2\)OH group, the pro-\(-S\) position has now been shown to be introduced exclusively by AIDH. From Table 5, it is seen that this enzyme is primarily NADH-dependent, as previously reported (13). On this basis, therefore, the incorporation of \(^2\)H into the pro-\(-S\) position is predicted to be greater from [3-\(^2\)H]glucose than from [1-\(^2\)H]glucose. This is confirmed experimentally: the \(C_f\)-3 value is higher than the \(C_f\)-1 value (Table 2).

In the CH\(_3\)OH group, the pro-\(-R\) position has now been shown to be introduced exclusively by ADH. Analyzing incorporation into the pro-\(-R\) is more difficult than into the pro-\(-S\), as hydride transfer from both NADPH and NADH occurs simultaneously. However, as shown in Table 5, the NADPH-dependent ADH has a 4.3-fold higher activity than the NADH-dependent ADH. This will favor introduction from NADPH into the pro-\(-R\). On the basis of the cofactor specificities for Glu-6-PDH and 6-PDG, the NADPH pool will be more strongly replenished from [1-\(^2\)H]glucose, which should lead to \(C_f\)-1 \(>\) \(C_f\)-3. This is again exactly as found experimentally, with the measured \(C_f\)-1 being double the \(C_f\)-3.

**DISCUSSION**

The transfer coefficients for the redistribution of non-exchangeable hydrogens during the fermentation of glucose to ethanol by *Ln. mesenteroides* are shown to be clearly distinct from those for the fermentation of glucose to ethanol by *S. cerevisiae*. The observed differences can be fully explained by the divergent fermentation pathways used by these two micro-

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**TABLE 4**

Incorporation of \(^2\)H from deuterated cofactors NADH or NADPH into ethanol by cell-free extracts of *Ln. mesenteroides*

| Sample          | Cofactor | Ethanol (H/H)CH\(_3\) | Ethanol (H/H)CH\(_2\)R | Ethanol (H/H)CH-S | Stereoselectivity | \(\Delta(H/H)CH\(_2\)R\) \(a\) | \(\Delta(H/H)CH-S\) \(a\) | Enantiomeric excess \(b\) |
|-----------------|----------|------------------------|------------------------|------------------|-------------------|------------------|------------------|------------------|
| Ethanol added\(c\) |          | 117.3 (0.7)            | 135.9 (0.5)            | 137.3 (0.5)      | 134.5 (0.5)       | 344.7            | 6.8              | 0.96             |
| *S. cerevisiae* | NAD\(^-\)H | 118.1 (0.7)            | 311.6 (1.6)            | 482.0 (1.6)      | 141.3 (1.6)       | 300.9            | 13.8             | 0.91             |
| *Ln. mesenteroides* | NAD\(^-\)H | 118.2 (1.2)            | 293.2 (1.4)            | 438.2 (1.4)      | 148.3 (1.4)       | 345.3            | 2.8              | 0.98             |
| *Ln. mesenteroides* | NADPH    | 119.1 (0.5)            | 309.9 (0.8)            | 482.6 (0.8)      | 137.3 (0.8)       | 345.3            | 2.8              | 0.98             |

\(a\) Difference between the values obtained with unenriched substrate and deuterated substrate.

\(b\) Calculated using Equation 4.

\(c\) Mean of two incubations with enzyme extract and one determination directly on the carrier ethanol used. No significant difference was seen whether the ethanol was measured directly or submitted to the extraction procedure.
organisms: the RPP pathway in *L. mesenteroides* and the glycolytic pathway in *S. cerevisiae*. The most notable distinction is the incorporation of \(^2\text{H}\) into both prochiral positions of the CH\(_2\)OH group of ethanol. The data also confirm that the glycolytic pathway is inactive in *L. mesenteroides*.

The most striking result is that the transfer of \(^3\text{H}\) from \([1-^3\text{H}]\text{glucose}\) and \([3-^3\text{H}]\text{glucose}\) into the *pro-R* and *pro-S* positions is not equal. This disparity is fully explained by the different availabilities of the reduced cofactors predicted from the cofactor preferences of the major enzymes of the reduced nucleotide cycle. Thus, it can be concluded that the non-equivalent introduction of \(^3\text{H}\) from \([1-^3\text{H}]\text{glucose}\) and \([3-^3\text{H}]\text{glucose}\) into the *pro-R* and *pro-S* positions is due to the balance in the inputs to and outputs from the NAD(P)H pool, combined with the greater activity of the NADPH-utilizing ADH. It can also be concluded that the stereochemistry of the ADH enzymes has no influence on this unequal distribution: only the relative levels of activity are important.

Nevertheless, whereas the values for the various transfer coefficients \(C\) are fully in accord with the enzyme parameters, the magnitude of the differences is not as great as might be predicted based on the relative specificities and levels of activity of the enzymes concerned. This can be explained, however, when the influence of the other enzymes involved in the cycle, GAPDH and \(\text{d-LDH}\), is taken into account.

During fermentation, \(\text{d-lactic acid}\) and ethanol accumulate in a 1:1 molar ratio. Because \(\text{d-LDH}\) from *L. mesenteroides* is completely NADH-specific (Table 5) (12), that half of the pool of NAD(P)H required for lactic acid production is only composed of NADH. This explains why \(C\) is exactly as seen for the observed values, with the \(C\) for \(\text{d-LDH}\) is higher \((\text{CH}^\text{S}-\text{3})\) than into the \(\text{CH}^\text{R}-\text{3}\). The observed effect is the summated mean of a number of interacting parameters, including the pathway being used, the substrate and cofactor availabilities, the level of enzyme activity and selectivity, and the isotope effects, and a number of environmental influences. Whereas metabolic flux control studies have made some progress toward describing how glucose metabolism in the lactic acid bacteria is controlled (22, 23), it is hard to predict the repercussions throughout the related metabolism that will result from interference at one specific point in the metabolic network. This is particularly true when the reduced cofactor pool is considered (23, 24).

By analyzing the hydrogen isotope transfer relationships between different positions in a substrate and its products, it is possible to obtain a quantitative measurement of the links between molecules and to interpret this in terms of the properties of the pathways involved. Preliminary data indicate that this approach has potential for the elucidation of metabolic networks and, in particular, perturbations in metabolism caused by genetic modification (25).

### TABLE 5

| Enzyme Substrate | Cofactor (0.5 mm) | Rate* | Contribution to total | Contribution to specific | Ratio NADH/NADPH |
|------------------|------------------|-------|----------------------|------------------------|------------------|
| Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) | 6.00 | NAD* | 1414.8 | 24.0 | 39.9 | 0.679 |
| NADP* | 2862.9 | 205.3 | 35.4 | 58.7 | 6.48 |
| 6-Phosphogluconate dehydrogenase (EC 1.1.1.44) | 6.00 | NAD* | 3166.0 | 42.1 | 5.7 | 8.9 |
| NADP* | 48.8 | 2.9 | 0.8 | 1.4 | |
| Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) | 5.88 | NAD* | 1819.0 | 0.1 | 30.9 | 51.2 |
| NADP* | 202.1 | 0.2 | 3.4 | 5.7 | 9.00 |
| D-Lactate dehydrogenase (EC 1.1.1.28) | 1.50 | NADH | 9842.8 | 448.2 | 71.8 | 92.8 |
| NADPH | 4.4 | 3.2 | 0.03 | 0.14 | 3030.0 |
| Alcohol dehydrogenase (EC 1.1.1.1 and 1.1.1.2) | 0.28 | NADH | 717.5 | 27.7 | 5.2 | 6.8 |
| NADPH | 3084.7 | 48.4 | 22.5 | 99.8 | 0.233 |
| Acetaldehyde dehydrogenase (EC 2.3.1.8) | 0.50 | NADPH | 50.5 | 14.3 | 0.37 | 0.48 |

*a* Percentage of non-substrate blank.

*b* Percentage of total cofactor produced or consumed.

*c* Percentage of specific cofactor produced or consumed.

### Isotopic Relationship in Glucose Fermentation
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