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Comparison of isotopic fractionation in lactic acid and ethanol fermentations

Ben-Li Zhang,[a,*] Valerie Fallourd,[a] Catherine Role,[b] and Gérard J. Martin[c]

[a] *LAIEM, Université de Nantes, CNRS UMR6006, 2 rue de la Houssinière, B.P. 92208, 44322 Nantes Cedex 03, France*
[b] *Rhodia, Usine de Melle, BP30, 79500 Melle, France*
[c] *Eurofins Scientific, rue P.A. Bobierre, BP 42301, 44323 Nantes Cedex 03, France*

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Abstract

Pure D(−) and L(+) enantiomers of lactic acid were prepared by fermentation reactions with specific bacteria. In addition, naturally deuterated ethanol was prepared and converted into diastereoisomers using mandelic acid. Various sugars and nutrients were fermented into lactic acid in water having different deuterium contents and ethanol samples were obtained from yeast fermentation of sugars from different botanical origins. The methine and methylene groups in lactic acid and ethanol respectively show similar deuterium contents which are related to that found in the fermentation water. However, the methyl groups of both molecules are significantly different whatever the botanical origin of the carbon source in the fermentation medium.

Keywords: SNIF NMR; IRMS; Chirality; Hydrogen transfer; Lactate enantiomers; Deuterated ethanol enantiomers

1. Introduction

Isotopic labeling has been extensively used for studying biosynthetic mechanisms, most notably glycolysis. Stable (e.g., ^{2}H, ^{13}C) or radioactive (e.g., ^{3}H, ^{14}C) isotopes

* Corresponding author. Fax : +33-2-51-12-57-12.
E-mail address: benli.zhang@chimbio.univ-nantes.fr (B.-L. Zhang).

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have provided powerful tracers for investigating the fate of the hydrogen and carbon atoms of both water and carbohydrates in fermentation reactions, resulting in the elucidation of various enzymatic pathways [1,2]. However, only a few studies have been carried out at the natural abundance level, i.e., in tracer competitive conditions. Moreover, since the experimental procedure generally used for determining natural abundance parameters is isotope ratio mass spectrometry (IRMS) which requires the combustion of the sample, only averaged molecular estimates were obtained. Site specific natural isotope fractionation studied by nuclear magnetic resonance (SNIF-NMR) was applied to ^{2}H and ^{13}C NMR spectroscopy [3–5] and appropriate strategies have been developed for elucidating the connections between the molecular positions in the products of glycolysis, such as ethanol, water, and glycerol, and their precursors, carbohydrates and water [6–8]. In addition, the isotopic distribution determined by this method provides an unambiguous fingerprint for characterizing the precursors in terms of natural or synthetic origin, metabolic and botanical type of the plant, and possibly geographical region of production [9,4]. In this respect, ethanol resulting from the glycolysis reaction is a particularly efficient isotopic probe for authenticating numerous food products and beverages. While the methyl group is a rich source of information on the carbohydrate precursors, the methylene site is mainly representative of the aqueous medium [7].

In this work, we have combined the carbon-IRMS and hydrogen-SNIF-NMR methods for investigating lactic acid fermentation, in order to determine its potential for characterizing various types of precursors. Although different kinds of microorganisms are used in lactic acid and ethanol fermentation, the same intermediate, i.e., pyruvate, and the same enzymatic reduction with NADH are involved. Therefore, it is of interest to compare the isotopic fractionation occurring in both processes.

The industrial production of lactic acid is estimated to be about 40,000 tons per year. The product is used largely in the food industry (50% of the market), but also in pharmaceuticals, agrochemicals, and in the manufacture of plastics and polymers. About two-third of the world demand of lactic acid is produced by fermentation. The fermentation procedure has operated on a commercial scale since 1881. The two enantiomers of lactic acid are produced on large scale from different kinds of bacteria including *Leuconostocs* [10,11] and *Lactobacillus* [12,13].

In order to improve our ability to compare lactic acid and ethanol fermentation it is desirable to distinguish the two deuterated methylene enantiomers of ethanol, CH_3CHDOH (*R*) and (*S*), present at natural abundance. Since the hydrogen atoms at the enantiomeric positions are introduced by stereo-differentiating reactions at different steps of the glycolytic pathway, the amounts of *R* and *S* monodeuterated isotopomers may be different. In the usual achiral media the only accessible parameter, $(D/H)_{CH_2}$, corresponds to a value averaged over the two enantiomeric positions. However, by reacting ethanol with optically pure mandelic acid the methylene protons are rendered diastereotopic and the two isotope ratios, $(D/H)_{CH_2R}$ and $(D/H)_{CH_2S}$, can be determined [14]. By using this strategy, the isotopic distribution in enantiomeric forms of lactic acid produced by different microorganisms can be analyzed in reference to the behavior of stereospecifically defined positions in ethanol.

2. Material and methods

2.1. Preparation of the compounds

2.1.1. Ethanol fermentation and preparation of diastereomer derivatives of ethanol

Five samples of beet sugar were fermented in standard conditions as described previously [15] and ethanol was quantitatively recovered from the medium by spinning band distillation. Ethyl mandelate was synthesized from *R*(−)-mandelic acid (3.04 g, 0.020 mol) and ethanol (2.4 ml, 0.041 mol) in C_6H_6 (20 ml) containing 0.04 ml of sulfuric acid in the presence of pumice. The flask was equipped with a Soxhlet extractor containing molecular sieves impregnated with benzene. The mixture was heated to the boiling point (83 °C) and refluxed. When full, the Soxhlet extractor was emptied and the mixture was poured back into the flask. This operation was repeated five times. The reaction product was then washed, first with 50 ml of distilled water, followed by distilled water saturated with K_2CO_3, and finally with more distilled water. At every step, the aqueous phase was separated from the organic phase in a decanting flask. The organic phase was then dried over anhydrous Na_2SO_4 and filtered under a vacuum. The solvent was removed by evaporation and the ester was distilled at 95 °C under a vacuum (0.5 bar). The yield of the synthesis after distillation was 89%.

2.1.2. Lactic acid fermentation and extraction from the medium

Lactic acid can be produced by numerous micro-organisms [16,17], with *Lactobacillus* being the preferred one [16,18,19]. The micro-organisms used throughout this study were *Lactobacillus delbrueckii* subsp. *lactis* (ATCC 12314) [18,19] which produces the D(−) isomer, and *L. delbrueckii* (BM102) which produces the L(+) isomer, by fermentation routes. Since these *Lactobacillus* strains carry out homolactic fermentation, the only product of glycolysis is lactic acid, with the theoretical conversion of 1 mol of glucose to 2 mol of lactic acid. This situation contrasts with heterolactic fermentation, which gives one mole for each of the three end-products, lactic acid, ethanol, and carbon dioxide and would involve specific isotopic fractionation processes. Lactic acid fermentation has been widely described and a number of different carbon substrates have been considered [17], such as sucrose [18,19], glucose [18,20], lactose [21], starch [19], and glycerol [22]. The fermentation media used in our experiments contain the carbon sources sucrose (from sugar beet or from cane) or glucose (from corn), salts, and nitrogen sources such as corn steep liquor (CSL), yeast extracts or peptone [18,19]. Lactic acid bacteria exhibit complex nutritional requirements, especially for B vitamins contributed by yeast extracts. Fourteen different lactic acid samples were prepared by batch fermentation. These experiments involved different sugars (sucrose or dextrose), different nutrients (CSL based nutrients, designated N1, or yeast extract based nutrients, designated N2) and either tap water or deuterium enriched water. The sugar concentration in the medium was initially adjusted to 12% and the nutrient source (N1 or N2) to 0.50%. The normal water used in most experiments was characterized by an isotope ratio $(D/H)_{W_0} = 150.9$ ppm, but

slightly enriched media, $(D/H)_{W_e} = 220$ ppm, were also used. The inoculums were transferred from a culture tube to a 500 ml flask, and then to a culture tank containing 1.2 L of culture media. The inoculum's volume was 5% of the fermentation broth volume.

The fermentation was carried out at 40 °C and the temperature was maintained by circulating water through the jacket of the culture tank. Lactic acid fermentation is anaerobic, but the microorganisms used are facultative aerobes. The pH of the culture broth was kept in the range of 5.6–6.0 (the preferred pH value is 5.8) by neutralization of the acid produced. Continuous control of the pH is advantageous because it results in increased yields and rates. If the lactic acid formed is not neutralized, high acidity develops which the bacteria cannot tolerate, and the fermentation does not go to completion. The broth was gently stirred to ensure homogeneous mixing between acid and base. The fermentation was considered to be complete when the reducing sugar content dropped to less than 0.1%. Usually complete fermentation of 12% sugar was achieved in 70–80 h. At the end of fermentation, the medium was concentrated by evaporation. Then the pH of the concentrated medium was adjusted to 2.15 and extracted with a mixture of solvents (butan-2-one/isopropylic ether, 80/20). After evaporation of the solvents at low temperature (<50 °C), lactic acid was obtained. The fermentation yields were greater than 95% and the optical purity of the enantiomers, either D(−) or L(+), was always greater than 95% (Table 1).

Table 1
Enantiomeric composition and isotopic values of lactic acids obtained by fermentation

Case No.	Bacteria[a]	% D(−)[a]	% L(+)[a]	Sugar	Water[b]	Nutrient[c]	$(D/H)_{CH}$ (ppm)	$(D/H)_{CH_3}$ (ppm)	$\delta^{13}C$ (‰)
1	A	98.8	1.2	Beet molasses	W_0	N1	119.3	98.8	−24.9
2	A	97.9	2.1	Beet molasses	W_0	N1	123.5	99.9	−24.2
3	A	98.3	1.7	Beet sucrose	W_0	N1	116.1	102.8	−24.5
4	A	98.0	2.0	Beet molasses	W_0	N2	115.5	109.6	−23.6
5	A	97.7	2.3	Beet sucrose	W_0	N2	113.7	100.2	−23.3
6	A	97.5	2.5	Beet sucrose	W_e	N2	144.0	113.0	−25.2
7	B	0.8	99.2	Beet sucrose	W_0	N1	121.5	97.2	−24.0
8	B	1.9	98.1	Beet sucrose	W_0	N2	123.7	99.3	−23.2
9	B	0.7	99.3	Beet sucrose	W_e	N1	137.8	102.6	−23.9
10	B	1.9	98.1	Beet sucrose	W_e	N2	139.0	107.4	−23.0
11	A	97.8	2.2	Cane sucrose	W_0	N1	120.7	116.2	−10.8
12	A	98.7	1.3	Corn glucose	W_0	N1	115.2	119.6	−11.6
13	A	98.8	1.2	Corn syrup	W_0	N1	113.1	120.3	−10.5
14	B	0.8	99.2	Corn syrup	W_0	N1	120.1	117.7	−10.4

[a] The *Lactobacillus* strains A and B are defined in Materials and methods.

[b] W_0 is tap water characterized by an isotopic ratio 150.9 ppm, W_e denotes slightly enriched water (220 ppm).

[c] The nutrient compositions N_1 and N_2 are described in Materials and methods. N_1 is richer in nitrogen than N_2.

2.2. Measurements of the isotope ratios

Tetramethylurea (TMU) with a calibrated isotope content was used as an intermolecular reference. Precisely weighed quantities of TMU were added to the sample contained in a 10 mm (o.d.) NMR tube. The ^{2}H-NMR spectra were obtained at 61.4 MHz (or 76.7 MHz) with a Bruker AM 400 (or AM 500) spectrometer equipped with a fluorine locking device. The following conditions were used: broadband proton decoupling, frequency window: 4800 Hz, memory size 32K, and exponential multiplication associated with a line broadening of 1 Hz. In the case of ethanol, lactic acid, and ethyl mandelate, 304, 1024, and 3000 scans, respectively, were accumulated. Quantitative analyses were performed with dedicated software (Eurospec from Eurofins-Scientific) which automatically adjusts all phases and base line parameters and fits the experimental complex spectrum (real and imaginary parts) to a theoretical model [23]. Mean values of the isotopic parameters were computed from lorentzian 8 or 3 spectra obtained successively for every ethanol or lactic acid and ethyl mandelate sample. The standard deviation was about 0.2 ppm for ethanol and 1 ppm for mandelate and lactic acid.

In order to obtain a complete balance of the deuterium distribution during the fermentation, the ^{2}H contents of the raw materials (sugars and water) were determined by isotope ratio mass spectrometry (IRMS) (VG SIRA9) and the origin of the sugars (C_3 or C_4 photosynthetic metabolism) was checked by ^{13}C IRMS.

The hydrogen isotope parameters, measured with respect to the international standard Vienna Standard Mean Ocean Water (V.SMOW) [24], are expressed as absolute D/H ratios in ppm and the carbon isotopic deviations, δ^{13}C, are given in ‰ with respect to the international reference V.PDB (Vienna Pee Dee Belemnite) [25].

3. Results and discussion

3.1. Isotopic characterization of lactic acid

The different lactic acids studied were prepared by fermentation using well defined isotopic conditions. Two series of C_3 (pure beet sucrose, beet molasses) and C_4 (pure cane sucrose, corn glucose syrup) sugars were investigated. The isotope ratios of these starting materials are in the following ranges (where nex stands for the non-exchangeable sites of the carbohydrates [26]:

$$C_3 \text{ sugars} : \delta^{13}C = -24.2 \text{ to } -25.2‰, \quad (D/H)_{nex} = 130 \text{ to } 132 \text{ ppm},$$

$$C_4 \text{ sugars} : \delta^{13}C = -10.2 \text{ to } -10.8‰, \quad (D/H)_{nex} = 148 \text{ to } 150 \text{ ppm}.$$

The fermentation reactions were studied at natural isotopic abundance in tap water characterized by an isotope ratio $(D/H)_{W_0} = 150.9$ ppm. However, in order to determine precisely the role of the aqueous medium, some experiments were carried out in water slightly enriched in deuterium: $(D/H)_{W_e} = 220$ ppm. The

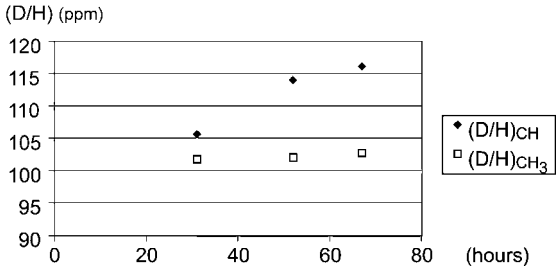


Fig. 1. Variation of the isotope ratios of D(−) lactic acid in the course of a fermentation reaction.

concentration of the sugars before fermentation was always maintained at 120 g/L. As shown in Table 1, the optical purity of the lactic acid enantiomers obtained, either D(−) or L(+), was always greater than 95%. The conversion rate of sucrose or glucose into lactic acid was greater than 95% after recycling of the residual sugars. The overall recovery of pure lactic acid from the fermentation medium, which was on the order of 75%, somewhat depended on the fermentation conditions. Since the quantity of sugars remaining at the end of the fermentation was negligible and the quantity of biomass formed was always less than a few percent, most of the lactic acid not recovered after the extraction procedure is assumed to remain in the mother liquor. Fortunately, from the isotopic point of view, the solvent extraction procedure used is known to induce only very small, if any, fractionation on the non-exchangeable sites because the extraction yield is high.

The evolution of the hydrogen isotope ratios of D(−) lactic acid as a function of the time course of fermentation is shown in Fig. 1. Whereas the variation of $(D/H)_{CH_3}$ with time remains very small, significant variations are observed at position 2 of lactic acid. Thus, obtaining a high conversion rate is an important requirement for the isotopic characterization.

3.2. Influence of different factors on the isotope ratios of lactic acid

The influence of the nature of the microorganism, the carbon source and the fermentation medium on the isotope ratios of lactic acid is illustrated in Table 1. Table 1 also gives the carbon deviation and the methylene and methine hydrogen isotope ratios for the (*R*) or D(−) and (*S*) or L(+) enantiomers predominantly produced by a given kind of microorganism. It is observed that the nature and concentration of the usual nutrients have only a small influence on the isotopic contents as was the case in the ethanolic fermentation [14]. Nevertheless, for analytical experiments, a highly reproducible composition of the medium must be maintained.

With regard to the nature of the starting materials, the unambiguous differentiation of the C_3 and C_4 photosynthetic metabolisms is straightforward by considering the hydrogen isotope ratios of the methyl site of the D(−) or L(+) lactic acid enantiomer and the overall $\delta^{13}C$ values. The C3 products have always a lower content

in the heavy isotopes than the C4 derivatives: (the standard deviations over the populations considered in Table 1 are given within parentheses):

C_3 (beet root)

D(−)	$(D/H)_{CH_3} = 102.3(4.4)$ ppm	$\delta^{13}C = -24.1(0.7)‰$
L(+)	$(D/H)_{CH_3} = 98.3(1.5)$ ppm	$\delta^{13}C = -23.6(0.6)‰$

C_4 (cane, maize)

D(−)	$(D/H)_{CH_3} = 118.7(2.1)$ ppm	$\delta^{13}C = -11.0(0.5)‰$
L(+)	$(D/H)_{CH_3} = 117.7$ ppm	$\delta^{13}C = -10.4‰$

It can be noted that the ^{13}C contents of the lactic acid product remain very close to those of the starting sugars. Whereas a systematic depletion of about −1.4‰ accompanies the loss of two carbon atoms of glucose in its bioconversion to ethanol by *Saccharomyces cerevisiae* no such fractionation is expected in complete homolactic fermentation which transfers all carbon atoms.

Although different microorganisms, characterized by different enzymatic systems, are involved in the production of L(+) and D(−) lactic acid, the nature of the microorganism exerts only a minor role on the isotopic distribution. Some influence of the medium or of the isotope ratios of the starting carbohydrates could also be invoked to explain the small differences exhibited by the methyl sites of the two enantiomers.

As expected from the similarity of the glycolytic pathways, the isotopic behavior of the methyl hydrogen atoms of lactic acid as a function of the reactants parallels that of the methyl site of ethanol. However, the mean values of $(D/H)_{CH_3}$ calculated for a C3 or a C4 biomass (Table 2) is significantly higher in the case of lactic acid. Although the methyl hydrogen atoms are strongly connected to positions 1,2, and 6,6′ of glucose [7], they also involve a participation of the aqueous medium which may be different in the two types of bioconversion.

In contrast to the methyl parameter, the isotope ratio of the –CH(OH)– fragment of lactic acid is nearly independent of the nature of the starting materials. Moreover it varies only to a small extent with the nitrogen content of the nutrients. A mean value $(D/H)_{CH} = 118.4$ ppm, associated with a relatively small value of the standard deviation (3.3 ppm), is calculated for the fermentation reactions carried out in normal water.

Table 2
Mean values in ppm of the site-specific isotope ratios of lactic acid and ethanol obtained by fermentation or chemical synthesis[a]

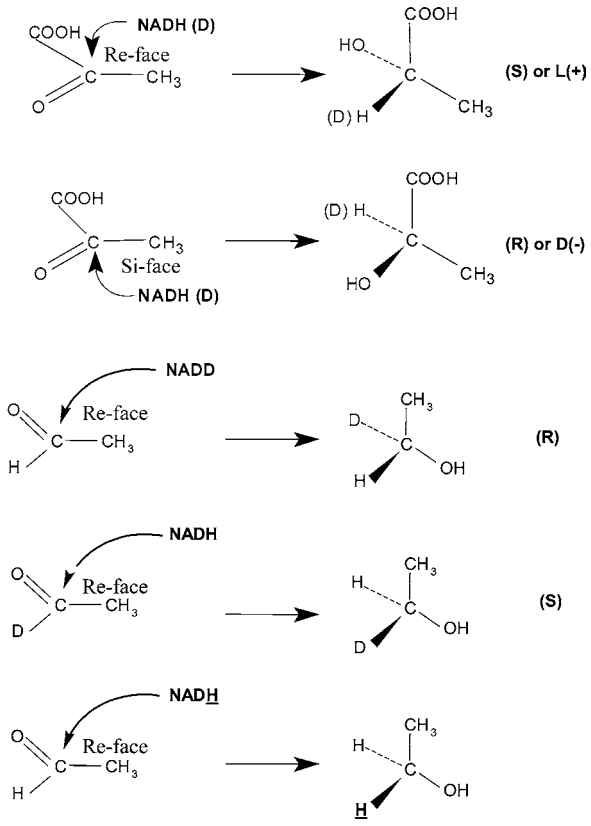
Starting material	$(D/H)_{CH_3}$ Lactic acid	$(D/H)_{CH_3}$ Ethanol	$(D/H)_{CH}$ Lactic acid	$(D/H)_{CH_2}$ Ethanol
C_3 biomass	103.5 (2)	92.5 (2)	120.1 (3)	122 (2)
C_4 biomass	118.2 (1)	110.5 (2)	121.3 (3)	123 (2)
Fossil	148 (3)	142 (2)	135 (4)	125 (2)

[a] The standard deviations (in parentheses) have been estimated on the basis of the results shown in Table 1 for lactic acid and a larger number of samples (databank) for ethanol. C3 and C4 biomasses are, respectively, obtained from sugar beet and cane or maize.

3.3. Stereochemical isotopic comparison of lactic acid and ethanol fermentation

The isotopic distribution in ethanol was previously discussed in terms of the botanical and environmental conditions which governed the growth of the carbohydrate precursors [9,5,27] and the mechanism of the hydrogen transfers occurring during fermentation was carefully studied [6,7]. Consequently, it is interesting to compare the stereochemical isotopic properties of both lactic acid and ethanolic fermentation processes.

The results corresponding to fermentation experiments conducted in slightly enriched water (Table 1) corroborate a connection between the methine hydrogen of lactic acid with water similar to that which was observed for the methylene group of ethanol [7]. This behavior is again in agreement with the mechanistic similarities of the ethanol and lactic bioconversions. Indeed the biosyntheses of both ethanol and lactic acid involve the stereospecific reduction of a carbonyl group (see Scheme1).



Scheme 1. The different ways of formation of the *R* and *S* enantiomers of D2-lactic acid and D1-ethanol at the natural abundance.

In the case of lactic acid, either an *R*(−) or *S*(+) enantiomer is produced by incorporation of a hydrogen isotope from site 4 pro-*R* of NADH into the carbonyl fragment of pyruvate. In the case of ethanol fermentation by *S. cerevisiae,* the pro-*S* methylene hydrogen is initially transferred from water to site 2 of pyruvate whereas the pro-*R* hydrogen results from a subsequent stereospecific reduction of acetaldehyde by NADH, mediated by alcohol dehydrogenase. In both cases NADH involved in the reduction results theoretically from the dehydrogenation of glyceraldehyde 3-phosphate. However, it has been shown that the methylene site of ethanol is predominantly connected with the aqueous medium and exhibits only a loose connection with site 4 of glucose [7].

In order to further compare isotopic fractionation occurring in lactic and ethanol fermentation, five fermentation experiments of beet sugar by *S. cerevisiae* were carried out in Nantes tap water (150 ppm) and the (*R*) and (*S*) diastereotopic methylene hydrogen atoms were observed by reacting the ethanol samples extracted from the fermentation medium with optically pure mandelic acid [14]. The following mean values of the isotope ratios were determined on the mandelate samples synthesized in this reaction:

$$(D/H)_{CH_2D} = 92.9(0.5), \quad (D/H)_{CHD(R)} = 133(2)$$
$$\text{and } (D/H)_{CHD(S)} = 112.5(0.5).$$

Although significant enantiomeric excess in favor of the (*R*) CH_3CHDOH isotopomer is detected, these results show that the reduction of acetaldehyde by NADH is associated with important deuterium depletion with respect to water. A similar, and even more pronounced, behavior is observed in lactic fermentation by both types of microorganisms. The limit value of the fractionation factor estimated in the hypothesis of an exclusive connection with water is on the order of 1.25.

4. Conclusion

It is concluded that isotope fractionation phenomena occurring in the glycolytic pathway are satisfactorily reproducible for lactic acid fermentation performed in an industrial context. In comparable experimental conditions the nature of the microorganism which produces either L(+) or *S*(−) lactic acid has only a small influence on the isotopic distribution. The hydrogen and carbon isotope ratios of lactic acid may therefore be considered as reproducible probes of the isotopic distribution in the precursors. The typical mean values and dispersion ranges of the isotopic parameters computed for lactic acid and for ethanol obtained by fermentation of C3 and C4 materials or synthesized from fossil sources (Table 2) illustrate the analytical potential of the isotopic methods.

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