Real-Time Pyruvate Chemical Conversion Monitoring Enabled by PHIP

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ABSTRACT: In recent years, parahydrogen-induced polarization side arm hydrogenation (PHIP-SAH) has been applied to hyperpolarize [1-13C]pyruvate and map its metabolic conversion to [1-13C]lactate in cancer cells. Developing on our recent MINERVA pulse sequence protocol, in which we have achieved 27% [1-13C]pyruvate carbon polarization, we demonstrate the hyperpolarization of [1,2-13C]pyruvate (~7% polarization on each 13C spin) via PHIP-SAH. By altering a single parameter in the pulse sequence, MINERVA enables the signal enhancement of C1 and/or C2 in [1,2-13C]pyruvate with the opposite phase, which allows for the simultaneous monitoring of different chemical reactions with enhanced spectral contrast or for the same reaction via different carbon sites. We first demonstrate the ability to monitor the same enzymatic pyruvate to lactate conversion at 7T in an aqueous solution, in vitro, and in-cell (HeLa cells) via different carbon sites. In a second set of experiments, we use the C1 and C2 carbon positions as spectral probes for simultaneous chemical reactions: the production of acetate, carbon dioxide, bicarbonate, and carbonate by reacting [1,2-13C]pyruvate with H2O2 at a high temperature (55 °C). Importantly, we detect and characterize the intermediate 2-hydroperoxy-2-hydroxypropanoate in real time and at high temperature.

INTRODUCTION

Nuclear magnetic resonance (NMR) is a non-invasive and quantitative analytical technique with applications in structural biology, drug discovery, and biomedicine. The limited NMR sensitivity has been tackled by hyperpolarization techniques to enhance signals over 10,000-fold with a large focus on metabolic studies. Dynamic nuclear polarization (DNP), parahydrogen-induced polarization (PHIP), and SABRE-signal amplification by reversible exchange (SABRE) have been intensively researched with this respect. SABRE succeeded in hyperpolarizing [1-13C]-acetate and [1-13C]pyruvate at >10% in methanol and very recently in water–methanol solution. PHIP is relatively inexpensive and achieves carbon polarization levels similar to those of dissolution DNP on specific targets. Inter alia, the PHIP side-arm hydrogenation (PHIP-SAH) method by Reineri et al. can in principle be applied to any molecule containing a carboxylic group. In essence, a precursor molecule formed by an unsaturated moiety linked through an ester bond to the substrate of interest is hyperpolarized by PHIP, and upon hydrolysis via base injection (NaOH or Na2CO3), the hyperpolarized metabolite is retained. The heteronuclear polarization transfer from parahydrogen is realized by magnetic field cycling (MFC) or pulsed NMR methods.

Among the various molecular systems, pyruvate plays a crucial role in deregulated glycolytic pathways in diseases associated with inflammation, neurodegeneration, and cancer. As the end product of glycolysis, pyruvate might be converted into alanine via alanine transaminase (ALT) or lactate via lactate dehydrogenase (LDH), or it can enter the tricarboxylic acid cycle (TCA) via the catalysis of the pyruvate dehydrogenase complex (PDH). The metabolic conversion of [1-13C]pyruvate-to-[1-13C]lactate (P–L) has shown potential in clinical trials of prostate cancer patients and we have recently used it to produce the first mouse tumor imaging by PHIP-SAH. However, by approaching TCA, [1-13C]pyruvate is converted via PDH into 13CO2, thereby preventing the direct detection of downstream TCA metabolites. Instead, [2-13C]-pyruvate enters TCA and can potentially be used to access, for example, [5-13C]glutamate, thus broadening the range of accessible metabolic information. [1,2,13C]Pyruvate combines the advantages of [1-13C] and [2-13C]pyruvates. It has been explored in the context of in vitro and in vivo DNP and in that of nuclear long-lived spin states. SABRE succeeded in [1,2,13C]pyruvate 13C hyperpolarization at <2% albeit in methanol-d4. In addition to its central role in cellular energy production, pyruvate plays a...
crucial part in shielding neurons and other cell types from the harmful effects of hydrogen peroxide (H₂O₂). The primary cause of the neuroprotective effect appeared to be related to the non-enzymatic decarboxylation of α-ketoacids rather than to an improvement of energy metabolism.53

Here, we report on [1,2-13C]pyruvate hyperpolarization via PHIP-SAH in combination with our maximizing insensitive nuclei enhancement reached via para-hydrogen amplification (MINERVA) method at 7T.54 We control the phase and polarization levels at C1 and C2 carbon positions by only adjusting one sequence parameter: the final β pulse (see Figure 2). Hyperpolarized [1,2-13C]pyruvate in an aqueous solution is used to monitor the real-time P–L conversion (Figure 1b) in

![Figure 1](image)

Figure 1. (a) PHIP-SAH steps: [1,2-13C]perdeuterated vinyl pyruvate precursor upon para-hydrogen addition converts into hyperpolarized ethyl [1,2-13C]pyruvate precursor. The MINERVA sequence transfers the polarization to the 13C, and/or 12C, of the pyruvate moiety. Upon Na₂CO₃-induced hydrolysis, free [1,2-13C]pyruvate is obtained. (b) Hyperpolarized (HP) P–L conversion triggered by the lactate dehydrogenase (LDH) enzyme. Circles indicate HP nuclei.

vitro and in-cell. Furthermore, we show that we can track multiple chemical reactions simultaneously through the different carbon-13-tagged sites by investigating the H₂O₂-induced pyruvate decarboxylation pathway. Importantly, we report the transient real-time formation of the intermediate 2-hydroperoxy-2-hydroxypropanoate (I) at high temperature (55 °C) and at different pH conditions.

## RESULTS AND DISCUSSION

**PHIP-SAH.** For all our studies, we used [1,2-13C]-perdeuterated vinyl pyruvate which is synthesized as described in the Supporting Information54 and used as a [1,2-13C]-pyruvate PHIP-SAH precursor (see Figure 1a). Experimentally, parahydrogen at 7 bars is supplied to a degassed 100 µL acetone-d₆ solution containing 5 mM precursor and 10 mM of [1,4-Bis(diphenylphosphino)butane](1,5-cyclooctadiene)-rhodium(I) tetrafluoroborate catalyst. The duration of parahydrogen supply was 20 s at 55 °C and 7T. The subsequent application of MINERVA transfers the parahydrogen polarization to the target carbon nuclei on the precursor molecule at C1, C2, or both positions depending on the β angle used (see below). In the following 5 s, the pressure is released, and 100 µL of a 50 mM solution of Na₂CO₃ in D₂O is injected into the NMR tube via a plastic cannula (i.d. 1 mm) coupled externally to a 1 mL syringe. Upon injection of the aqueous solution, the drop in catalyst’s solubility initiates the catalyst’s precipitation. Following the base injection, a vacuum pump connected to the NMR tube is activated for 15 s to evaporate the acetone from the acetone–D₂O mixture present in the NMR tube (see Supporting Information 3). A further 5 s delay is needed to inject 100 µL of buffer solution to adjust the aqueous pH to circumneutral values and obtain isotonicity. In the last step, a volume of 200 µL of H₂O₂, the enzymatic, or the cell solution—in different experiments—is injected through a different plastic cannula. The carbon spectrum is acquired via the subsequent application of 20° (45° for HeLa cell experiments) flip angle pulses every 2 s.

**MINERVA.** We describe the hyperpolarized precursor molecule as a four-spin system with two parahydrogen ¹H, I₁ and I₂, and two ¹³C nuclear spins S₁ and S₂ with the relevant J-coupling network reported in Figure 1a. The initial state upon hydrogenation at 7T is incoherently averaged to ρ₁ = 2 I₁I₂S₁S₂. The first block of the sequence (1–2 in Figure 2a) converts ρ₁

![Figure 2](image)

Figure 2. (a) MINERVA for the transfer of longitudinal spin order of parahydrogen into magnetization. The filled and empty rectangles are flip angle pulses every 2 s. (b) Simulated spectra with β = π/2 (left) and β = π/4 (right).
170.5 ppm for C1 and $\delta_{P_2} = 193.2$ ppm to $\delta_{P_2} = 205.6$ ppm for C2 (see Figure 3a). Each peak is further split by $J_{CC} \sim 68$ Hz. The $^{13}$C polarization levels (Pol) at each steps go from Pol $\sim 24 \pm 1.6$% before cleavage to Pol $\sim 7 \pm 1.0$% after cleavage, Pol $\sim 2.5 \pm 1.0$% after solvent evaporation and buffer pH adjustment, to the final average Pol $\sim 0.7 \pm 0.3$% at the moment of $\text{H}_2\text{O}_2$ or enzymatic/cell solution injection (Figure 3a). The polarization levels are similar at C1 and C2. The polarization levels are similar at C1 and C2. The polarization levels are similar at C1 and C2.

### In Vitro Enzymatic Reaction Monitoring

In the final step, 200 $\mu$L of a buffer solution containing 100 units of rabbit muscle LDH and 20 mM NADH, thermalized at 37 °C, is injected at 7T, and the P–L conversion was monitored by $^{13}$C pulses every 2 s (Figure 3b). For lactate, the resonances $\delta_{L_2} = 183$ ppm and $\delta_{L_2} = 69$ ppm are observed. In Figure 3b, MINERVA is adapted to retain the signal primarily on C1 (red spectrum using the first block of the sequence, i.e., 1–2 in Figure 2a), on C2 (green spectrum by $\beta = \pi/2$), and on both (blue spectrum by $\beta = \pi/4$). The expansion in Figure 3c shows the $\{^2H\}$ spectrum at the final step, where $J_{CH} = 141$ Hz.

### In Cell Real-Time P–L Conversion

Figure 4a shows that the P–L conversion can be followed through both carbon signatures C1 and C2, also in-cells. HeLa cancer cells are prepared as described in the Supporting Information. After the $\text{H}_2\text{O}_2$ or enzymatic/cell solution injection (Figure 3a). The polarization levels are similar at C1 and C2.

### Factors Influencing $^{13}$C Polarization

At every step in Figure 3, the polarization is affected by many parameters such as $\text{H}_2$ solubility, the solvent, the catalyst, and others. Acetone has a relative low toxicity and guarantees a good $\text{H}_2$ solubility and a weak binding affinity to the Rh(1) catalyst used. This, in turn, favors an improved efficiency and better polarization levels as the fast displacement of the product molecule from the metal center limits the singlet/triplet mixing on the
injection. (b) Detection in HeLa cells (polarization.
intermediate reaction products that has been linked to loss of
Table 1.
Figure 4. (a) Pseudo-2D $^{13}$C NMR experiments with P−L conversion
detected in HeLa cells (~25 M) at 7T, with cells kept at 37 °C until injection. (b) $^{13}$C hyperpolarized NMR spectrum (ns = 1, 45° recording angle) and thermal spectrum (ns = 230, 90°, ×2000-fold) acquired from the Hela cell sample.

Table 1. $^{13}$C Chemical Shifts for the In-Cell Real-Time P−L Conversion at pH 7, T = 37 °C

<table>
<thead>
<tr>
<th></th>
<th>$^{13}$C1</th>
<th>$^{13}$C2</th>
</tr>
</thead>
<tbody>
<tr>
<td>pyruvate</td>
<td>171.0</td>
<td>206.0</td>
</tr>
<tr>
<td>hydrate</td>
<td>179.3</td>
<td>94.6</td>
</tr>
<tr>
<td>dimer</td>
<td>177.3</td>
<td>73.0</td>
</tr>
<tr>
<td>lactate</td>
<td>183.2</td>
<td>69.2</td>
</tr>
</tbody>
</table>

Real-Time Pyruvate Decarboxylation Monitoring. As explained in the Introduction, pyruvate protects neurons and other cell types from the toxic hydrogen peroxide (H$_2$O$_2$).$^{53,62}$ H$_2$O$_2$ is stable in abiotic environments at ambient temperature and neutral pH, yet rapidly kills any type of cells by producing highly reactive hydroxyl radicals. Although catalases are commonly deployed by cells as powerful H$_2$O$_2$ scavengers,$^{63}$ pyruvate is also used to quench H$_2$O$_2$ by reacting quickly and irreversibly to yield acetate and carbon dioxide. According to a proposed mechanism, supported by experimental results with H$_2$15O$_2$, the product formation in the H$_2$O$_2$-induced pyruvate decarboxylation occurs through the intermediate 2-hydroxyperoxo-2-hydroxypropanoate (I)$^{64−66}$ (see Figure 5a). Low-temperature $^{13}$C NMR and UV spectrophotometry have been used to capture I’s presence.$^{64,65}$ However, the real-time monitoring of I at high temperature by NMR has been hampered so far by the low sensitivity and limited lifetime. In Figure 5b, we report the stacked plot showing the $^{13}$C NMR for the non-enzymatic decarboxylation of [1,2-$^{13}$C]pyruvate according to the reaction in Figure 5a. The experiment has been conducted at 55 °C following the steps already described in Figure 3a. In short, we applied MINERVA to hyperpolarize 5 mM [1,2-$^{13}$C]pyruvate as per Figure 1a. After injecting the buffer solution to bring the pH to 7 (pH 2 and 9 in other experiments, see Supporting Information 6.1), we waited 4 s before applying a series of 20-degree flip angle pulses to track the $^{13}$C NMR signal. The $^{13}$C NMR spectra at the bottom of Figure 5b,c show the presence of pyruvate, the corresponding hydrated form, and the dimer signals.$^{65}$ In the final step, after the injection of 200 μL of H$_2$O$_2$ at 326 mM (H$_2$O$_2$ 1%), the $^{13}$C NMR spectrum drastically changes. The middle spectrum in Figure 5c shows a negative and a positive signal both separated into two peaks 64 Hz apart (squared dashed box). The spectral positions are compatible with the previously reported values for I in methanol/water in ref 65. These signals are relatively short-lived as they vanish 12 s after the H$_2$O$_2$ injection, as shown in the top transient (20 s) in Figure 5c. We attribute these two signals to the C1 and C2 signatures of [1,2-$^{13}$C]I due to their opposite phases, identical 64 Hz splitting, short lifetime, and compatibility with previously reported values.$^{65}$ In addition to the [1,2-$^{13}$C]I formation, we observe the presence of all of the species detailed in Figure 5a−c, whose chemical shift is reported in Table 2 for clarity. The carbon polarization is sufficiently high to investigate the formation of acetate and CO$_2$ in a single experiment using the intermediate [1,2-$^{13}$C]I and the C2 and C1 carbon signatures of [1,2-$^{13}$C]pyruvate, respectively. Conveniently, the positive and negative NMR signals correspond to the carbon spins at C1 and C2, respectively. Therefore, different carbon sites are useful for investigating different simultaneous chemical processes.
By using the pathway in Figure 5a as a guide, a fitting model for the reaction rates $k_1$ and $k_2$ was developed. The model (see Supporting Information 6) is oversimplified because (i) we omit the pyruvate equilibria of its hydrate and dimer forms as they have the weakest signal in our spectra; (ii) we treat carbon dioxide, bicarbonate, and carbonate as a single product since the equilibria among them is irrelevant for the intermediate formation; and (iii) we assume unidirectional reaction rates. From the analysis detailed in the Supporting Information, assuming a $T_1 = 50$ s for pyruvate (the same at C1 and C2, as per conventional NMR under similar conditions), $T_1 = 3.0$ s for I (the same at C1 and C2, as different values seem to be less compatible with our experimental data), we find that $k_1 = 0.18$ s$^{-1}$ and $k_2 = 0.23$ s$^{-1}$ with a $T_1 = 25$ s for acetate at 7T.

Furthermore, the signals from CO$_2$ and HCO$_3^-$ in Figure 5b have similar lifetimes, and as can be seen in Figure 5c, they show comparable polarization levels at each transient. Under these premises and taking into account the fast exchange regime between CO$_2$ and HCO$_3^-$, a stable pH is found throughout the chemical reaction by taking the integral ratio of HCO$_3^-$ and CO$_2$ according to the Henderson−Hasselbalch equation (eq. Supporting Information 9) at each transient (Figure S15). Additional experiments were conducted at pH = 2 and pH = 9 (see Supporting Information 6.1). At pH = 2 in particular, we see that HCO$_3^-$ forms from $^{13}$CO$_2$ as the bicarbonate at $\sim$159 ppm signature is only visible after carbon dioxide formation at $\sim$123 ppm (carbon signals in the dashed gray box in Figure S14b). At basic conditions (pH = 9), pyruvate is immediately quenched by reacting with H$_2$O$_2$, and we neither observe the formation of I nor of $^{13}$CO$_2$ (Figure S14a).

### CONCLUSIONS

In conclusion, we succeeded in hyperpolarizing [$1,2^{13}$C]pyruvate via PHIP-SAH. We investigated the P−L metabolic conversion kinetics in aqueous solution in vitro and in-cells at 7T. To the best of our knowledge, although previous reports on doubly labeled pyruvate hyperpolarization exist, no measure of the kinetics via C1, C2, or both was reported so far by PHIP-SAH-based techniques.

We also follow in real-time the non-enzymatic pyruvate decarboxylation through different carbon sites C1 and C2,
confirming the intermediate formation of 2-hydroperoxy-2-hydroxypropanoate (1) that was previously reported only by low-temperature $^{13}$C NMR and not in real time.

In the proposed experiment, the pH of the solution can be monitored in real time by tracking the production of $\text{H}^{13}\text{CO}_3^-$ and $^{13}\text{CO}_3^-$ during the decarboxylation reaction via the correspondent carbon signals.

We think that the possibility to follow various chemical reactions or the same reaction via different carbon sites, as we show here, without the need of numerous metabolic probes via a straightforward and widely accessible PHP-SAH protocol is particularly noteworthy. The NMR pulse strategy used is flexible and allows for the selective or simultaneous hyperpolarization of C1 and C2 with opposite phase by only changing a single pulse in the MINERVA sequence. We show that the kinetics information can be extracted for P−L conversion from either C1 or C2 site. This approach may also be of use to better analyze mixtures of metabolites that are tagged with different phases in the same sample.

**ASSOCIATED CONTENT**

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.2c13198.

Chemical synthesis, enzyme and cell experiments, hyperpolarization experiments, kinetic fitting, and simulations (PDF)

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**Notes**
The authors declare no competing financial interest.

**ABBREVIATIONS**

MFC magnetic field cycling
PHP parahydrogen-induced polarization
SABRE signal amplification by reversible exchange
MINERVA maximizing insensitive nuclei enhancement reached via para-hydrogen amplification
P−L pyruvate to lactate

**REFERENCES**


