A Fast and Sensitive ^{1}H NMR Method to Measure the Turnover of the H2 Hydrogen of Lactate

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A fast and sensitive procedure to determine the turnover of the H2 hydrogen of lactate and quantify its ^{2}H-enrichment by ^{1}H NMR is illustrated using C6 cells metabolizing (3-^{13}C) lactate in 50% ^2H_2O (vol/vol). ^{2}H substitution of the lactate H2 hydrogen resulted in two easily detectable transformations of the vicinal H3 doublet resonance: 1) the formation of an H3 singlet due to the disappearance of the homonuclear coupling to H2 ($^3J_{\beta H\text{-}\alpha H}$ = 7.0 Hz), and 2) an upfield isotopic shift derived from the vicinal ^{2}H2 substitution (Δ_3 = −0.007 ppm). Only those lactate molecules that have passed through the cell cytosol experience these effects, since H2 deuteration involves lactate dehydrogenase activity and NAD(^{2}H). Thus, analysis of the observed shifted and unshifted H3 lactate resonances from the incubation medium allows the discrimination of the perprotonated (3-^{13}C) lactate added as substrate, and the (3-^{13}C, 2-^{2}H) lactate recycled to the incubation medium after passage through the cytosol. Magn Reson Med 54:1014–1019, 2005. © 2005 Wiley-Liss, Inc.

Key words: hydrogen turnover; lactate recycling; C6 glioma cells; ^{1}H NMR; ^{2}H-enrichment

Lactate plays a fundamental role in cellular metabolism and bioenergetics (1–4). It is believed that in the central nervous system (CNS) under physiological conditions, lactate is produced mainly in astrocytes and is exported to neurons, where it is oxidized, constituting an energy shuttle that supports glutamatergic neurotransmission (2,3,5). It has been proposed that in ischemic zones lactate plays a crucial role in cellular survival, and approaches more the nature of a vital substrate than that of a lethal product (6–8). In addition, lactate is classically known to be produced from glucose in tumor cells, in larger amounts than in the surrounding nontumoral tissues. In all of these cases, lactate molecules are produced intracellularly by glycolysis and extruded from the cell to the extracellular space. Under the reduced vascular clearance conditions that occur in cerebral excitation, ischemic episodes, or even in tumors, lactate molecules have been reported to accumulate in the extracellular space, and to reach concentrations that exceed those found in the cell cytosol (6,8). In these situations, extracellular lactate molecules may return to the cytosol through the reversible monocarboxylate transporter, which is located in the plasma membrane of all normal and transformed cells (9,10). Up to now, however, it has been impossible to investigate the inward and outward fluxes of lactate simultaneously, most probably because of the difficulty of distinguishing between the lactate molecules that move in or out of the cell.

We previously proposed several hydrogen turnover methods to investigate the trafficking of various (^{13}C, ^{2}H) labeled isotopomers, including lactate, between different compartments in cell cultures, perfused liver, and brain (11–13). These methods are able to monitor, by ^{13}C NMR, the incorporation of deuterium into ^{13}C-labeled metabolites because covalent binding of ^{2}H results in characteristic ^{13}C-^{2}H heteronuclear couplings and geminal or vicinal isotopic shifts of the corresponding ^{13}C resonances (14,15). The methodology combines the inherent advantage of detecting deuterium incorporation with the positional selectivity and increased sensitivity of ^{13}C NMR, as compared to the direct ^{2}H NMR detection (16,17). However, it requires the use of relatively expensive ^{13}C-labeled substrates, as well as sufficiently long ^{13}C NMR acquisitions.

Figure 1 illustrates the process of lactate recycling through the plasma membrane of a representative tumor cell. Once lactate molecules reach the cytosolic space, they participate in the lactate dehydrogenase equilibrium, incorporating one deuteron in their methine carbon and eventually returning to the extracellular space. This circumstance is easily observed by ^{1}H NMR because of the vicinal isotopic effect of ^{2}H2 on the chemical shifts of the methyl resonance of lactate in the incubation medium. This makes it possible to discriminate lactate molecules that have passed through the cell cytosol from those that have not, because only the former become deuterated by cytosolic lactate dehydrogenase in incubation media that contain ^2H_2O. Notably, the proposed methodology allows the detection of H2 deuteration with the increased sensitivity of ^{1}H NMR, and thus avoids the necessity of using ^{13}C-labeled substrates. Even if these are used, however, our method allows the turnover of the H2 hydrogen in both ^{13}C-labeled and ^{12}C lactate molecules to be measured simultaneously, and thus provides a robust method to investigate ^{2}H and ^{13}C isotopic effects simultaneously on lactate turnover. Finally, the protocols described herein may be easily extendable to other molecules and exchange pathways between intra- and extracellular spaces, where a deuterium exchange site is present only in the intracellular milieu.

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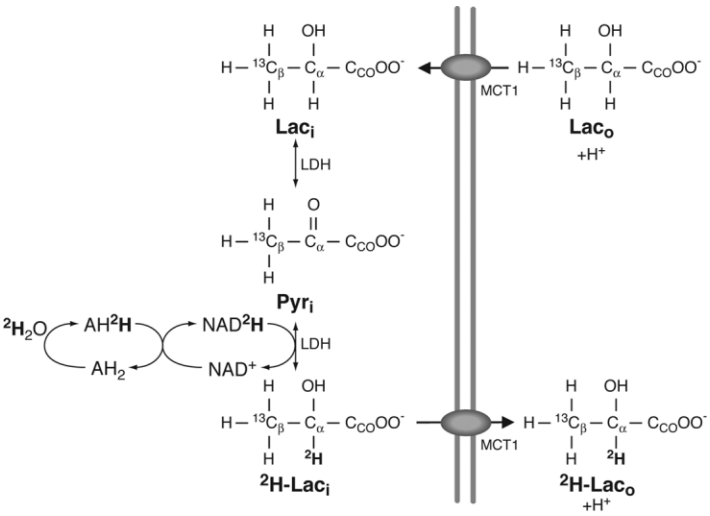

FIG. 1. Mechanism of lactate recycling during metabolism of 5 mM (3-^{13}C) lactate in Krebs-Henseleit buffer containing 50% ^2H_2O (vol/vol). Extracellullar (3-^{13}C) lactate (Lac$_o$, unshifted) enters the cytosol (Lac$_i$) through the MCT1 transporter. (3-^{13}C) Lac$_i$ loses its H2 hydrogen and incorporates a deuteron from NAD(^{2}H) in the cytosolic lactate dehydrogenase (LDH) equilibrium. (3-^{13}C, 2-^{2}H) Lac$_i$ abandons the cell through the reversible MCT1 transporter originating the shifted singlet (^{2}H-Lac$_o$) in the incubation medium (cf., Figs. 2 and 3). Deuterons are transferred to NAD$^+$ through a combination of hydration-dehydration (formation of A^{2}H) and red-ox reactions (formation of NAD^{2}H). Lac: lactate, LDH: lactate dehydrogenase, MCT1: monocarboxylate transporter type 1, Pyr: pyruvate.

MATERIALS AND METHODS

Cell Culture and Preparation

C6 glioma cells were purchased from the local representative of the American Tissue Culture Collection (LGC Promochem, Barcelona, Spain). Briefly, C6 glioma cells were grown to confluence in Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal bovine serum (FBS; 5%), 100 μg/mL streptomycin, 25 μg/mL gentamycin, and 100 U/mL of penicillin and fungizone (1% vol/vol) in sterile Petri dishes (10-cm diameter) at 37°C in a humidified atmosphere containing 5% CO_2-95% O_2. Four independent cultures of C6 cells were used. Confluent cells (three cultures) were incubated (3–30 hr, 37°C) in Krebs-Henseleit buffer (NaCl, 119 mM; KCl, 4.7 mM; $CaCl_2$, 1.3 mM; $MgSO_4$, 1.2 mM; HEPES, 15 mM; and KH_2SO_4, 1.2 mM) containing 50% (vol/vol) ^2H_2O with 5 mM (3-^{13}C) lactate. Aliquots from the medium (1 mL) were collected for increasing incubation periods, lyophilized, and resuspended in 0.5 mL ^2H_2O (99.9% ^{2}H) prior to high-resolution ^{1}H NMR analysis. Appropriate control experiments were carried out with cell-free incubations or incubations carried out under similar cellular conditions (one culture) in the absence of ^2H_2O.

^{1}H NMR Spectroscopy

^{1}H NMR spectra from 0.5-mL samples of the incubation medium were obtained at 11.7 Tesla (500.130 MHz, 25°C, pH 7.2) with a Bruker AVANCE 500WB NMR spectrometer using a commercial (5-mm) triple resonance probe (^{1}H, ^{13}C, ^{2}H). The acquisition conditions were as follows: π/3 pulses, 10.3 kHz spectral width, 3.17 s acquisition time, 64 k words data table, and an interpulse delay of 3.0 s, resulting in a repetition time (TR) of 6.17 s. Fully relaxed ^{1}H NMR spectra were acquired under the same conditions using a π/3 flip angle and a relaxation delay of 20 s. T_1 measurements were obtained and analyzed with the Bruker analysis software (Bruker BioSpin GmbH, Rheinstetten, Germany) using inversion recovery experiments with at least 15 different τ values. The T_1 values of the (3-^{13}C) and (3-^{12}C) lactate methyl multiplets ranged from 114 to 128 ms, indicating an almost complete relaxation under our acquisition protocol. Under these conditions it is not necessary to introduce correction factors to account for partial saturation of any multiplet resonance.

The ^{1}H spectra were line-broadened (0.2 Hz), zero-filled, Fourier-transformed, phased, and referenced to external TSP (2, 2′, 3, 3′-tetradeutero trimethylsilyl propionate sodium salt at 0.0 ppm). Selectively ^{13}C-decoupled ^{1}H NMR spectra of the methyl resonance of lactate were obtained with low-power single-frequency irradiation over the ^{13}C3 lactate resonance (20.9 ppm), using the globally optimized alternating-phase rectangular pulses (GARP) technique (18). Simulated spectra were generated with the program NUTS™ (Acorn, Freemont, CA, USA) to investigate the isotopic shifts and quantify relative resonance areas.

The ^{1}H NMR time courses $M_{(t)}$ were fitted to single exponentials of the type $M_{(t)} = M_{(\infty)}$ (1-exp(-k.t)) to obtain the values of the rate constant k and the asymptote $M_{(\infty)}$. For this purpose we used the nonlinear least-squares routines of the Sigma Plot program (SPSS Inc., Chicago, IL, USA) as implemented on an Intel PC platform. The statistical significance in the comparisons of values of k and $M_{(\infty)}$ was assessed using Student's *t*-test.

Determination of Lactate Concentration

The lactate concentration in the incubation media was determined spectrophotometrically using classic enzymatic end-point methods coupled to the increase in NADH absorption at 340 nm. Conventional methods were adapted to become operative using 96-well microplates (0.25 mL) and a vertical microplate reader (Molecular Devices, Spectramax, Sunnyvale, CA, USA).

(3-^{13}C) lactate (98% ^{13}C) and deuterated solvents were obtained from Cambridge Isotope Laboratories (Andover, MA, USA). ^2H_2O (99.9% ^{2}H) was obtained from Apollo Scientific Ltd. (Stockport, Cheshire, UK). Auxiliary enzymes and cofactors were obtained from Boehringer Mannheim (Germany). FBS and DMEM were purchased from GIBCO BRL (Gent, Belgium). Other items were of the highest purity available commercially from Sigma Chemical Co. (St. Louis, MO, USA).

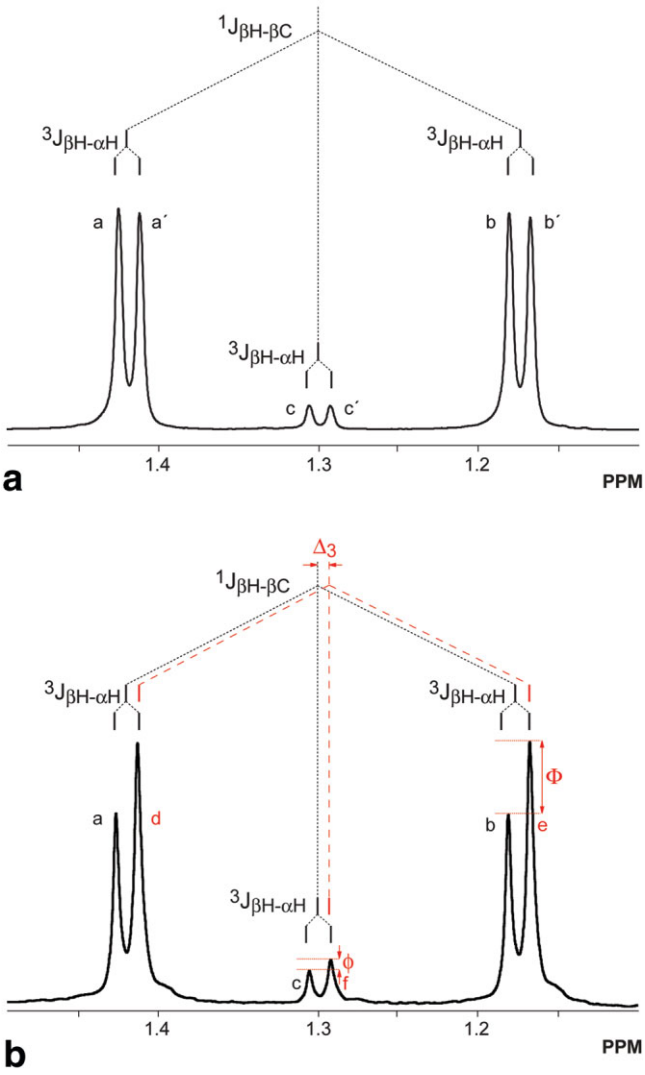

FIG. 2. ^{1}H NMR resonances (500.13 MHz, 25°C, pH 7.2) of the methyl signals of lactate from the medium of C6 glioma cells incubated with 5 mM (3-^{13}C) lactate in Krebs-Henseleit buffer containing 50% ^2H_2O (vol/vol). **a:** Immediately after the addition of the ^{13}C label. (3-^{13}C) and (3-^{12}C) lactate originate an isointense doublet of doublets a,a′-b,b′ ($^1J_{\beta H-\beta C}$ = 128.0 Hz + $^3J_{\beta H-\alpha H}$ = 7.0 Hz) and a doublet c,c′ ($^3J_{\beta H-\alpha H}$ = 7.0 Hz), respectively. **b:** Thirty hours later, (3-^{13}C, 2-^{2}H) and (3-^{12}C, 2-^{2}H) originate an isotopically shifted doublet (Δ_3 = −0.007 ppm, $^1J_{\beta H-\beta C}$ = 128.0 Hz) and a singlet (Δ_3 = −0.007 ppm), respectively. The shifted resonances are superimposed over those of the perprotonated multiplet, originating an asymmetric increase in the intensity of the upfield transitions of the multiplets d,e (Φ) and f (ϕ).

RESULTS AND DISCUSSION

Figure 2a and b show the ^{1}H NMR resonances of the methyl group of (3-^{13}C) and (3-^{12}C) lactate from the incubation medium of C6 cells, immediately after the addition of (3-^{13}C) lactate in Krebs-Henseleit buffer containing 50% ^2H_2O (vol/vol) (Fig. 2a), or 30 hr later (Fig. 2b). At the beginning of the incubation (Fig. 2a), the ^{1}H NMR multiplet from the methyl group of lactate consists of six resonances: 1) a central doublet originating from the vicinal homonuclear coupling $J_{\beta H-\alpha H}$ (7.0 Hz) of the methyl group from (3-^{12}C) lactate molecules (Fig. 2a: resonances c-c′), and 2) a doublet of doublets derived from the combination of the larger geminal heteronuclear coupling $J_{\beta H-\beta C}$ (128.0 Hz) (Fig. 2a: a-b, a′-b′) with the smaller vicinal homonuclear $J_{\beta H-\alpha H}$ coupling (7.0 Hz) (Fig. 2a: a-a′, b-b′) from the methyl group of (3-^{13}C) lactate molecules. The ratio of intensities of the external doublet of doublets to the internal doublet allows the fractional ^{13}C enrichment in the lactate C3 carbon to be determined (19).

The substitution of the H2 hydrogen of (3-^{13}C) lactate by one deuteron introduces remarkable changes in the multiplet structure of the H3 resonance: 1) the transformation of the original doublet of doublets into an apparent doublet because of the disappearance of the homonuclear vicinal $J_{\beta H-\alpha H}$ coupling (7.0 Hz), and 2) the upfield shift of this doublet caused by the vicinal heavy atom isotope effect (Δ_3 = −0.007 ppm). This upfield isotopic shift has a very similar value to the ½ $J_{\beta H-\alpha H}$ coupling constant, a situation that results in the superposition of the shifted doublet over the higher-field components of the doublet of doublets, causing an apparent doublet of doublets with dissymmetric intensities (Fig. 2b, dotted lines, e-b = Φ).

Similar effects of H2 deuteration are observed in the central (3-^{12}C) lactate doublet, which loses the vicinal, $J_{\beta H-\alpha H}$ coupling (7.0 Hz) to originate a vicinal and isotopically shifted singlet (Fig. 2b: f) that superimposes on the high-field component of the doublet. This superposition causes a difference in intensity between the otherwise isointense transitions of the (3-^{12}C) lactate doublet (see c-c′ in Fig. 2a), the imbalance being due to superposition of the (3-^{12}C, 2-^{2}H) singlet over the high-field component of the doublet (Fig. 2b: dotted lines, f-c = ϕ). Notably, H2 deuteration of lactate was not observed in the same experimental time when the cell-free incubations with (3-^{13}C) lactate in Krebs-Henseleit buffer containing 50% ^2H_2O (vol/vol) were used, revealing the intracellular origin of the deuteration process.

Figure 3a–c illustrate these changes in more detail by showing the simulated deconvolution of the heteronuclear multiplet from the methyl group of (3-^{13}C) lactate in the corresponding components. Figure 3a depicts the structure of the doublet of doublets derived from (3-^{13}C) lactate. Figure 3b shows the methyl resonance from (3-^{13}C, 2-^{2}H) lactate, in which the vicinal homonuclear $J_{\beta H-\alpha H}$ coupling (7.0 Hz) is lost because of the ^{2}H2 substitution. A residual heteronuclear coupling $J_{\beta H-\alpha^2 H}$ remains resolved (ca. 1.07 Hz) in the simulation. This residual doublet is approximately 6.25 times smaller than the corresponding doublet derived from homonuclear coupling (ca. 7 Hz; Fig. 3a), as would be expected from the 6.25 smaller value of the gyromagnetic ratios of ^{2}H vs. ^{1}H (20). Unfortunately, the small vicinal heteronuclear ^{1}H-^{2}H scalar coupling constant (ca. 1.07 Hz) observed in the simulation is smaller than the natural linewidth of the H3 proton doublet in extracts or in vivo, which makes this heteronuclear $J_{\beta H-\alpha^2 H}$ coupling virtually unobservable experimentally. Figure 3c shows the combined multiplet structure of the methyl resonances from a mixture of (3-^{13}C) and (3-^{13}C, 2-^{2}H) lactate isotopomers, after the linewidth of the latter is increased to the natural linewidths observed in extracts (2–2.5 Hz). The same effect occurs in (3-^{12}C) lactate (not shown). In this case, ^{2}H binding to C2 transforms and

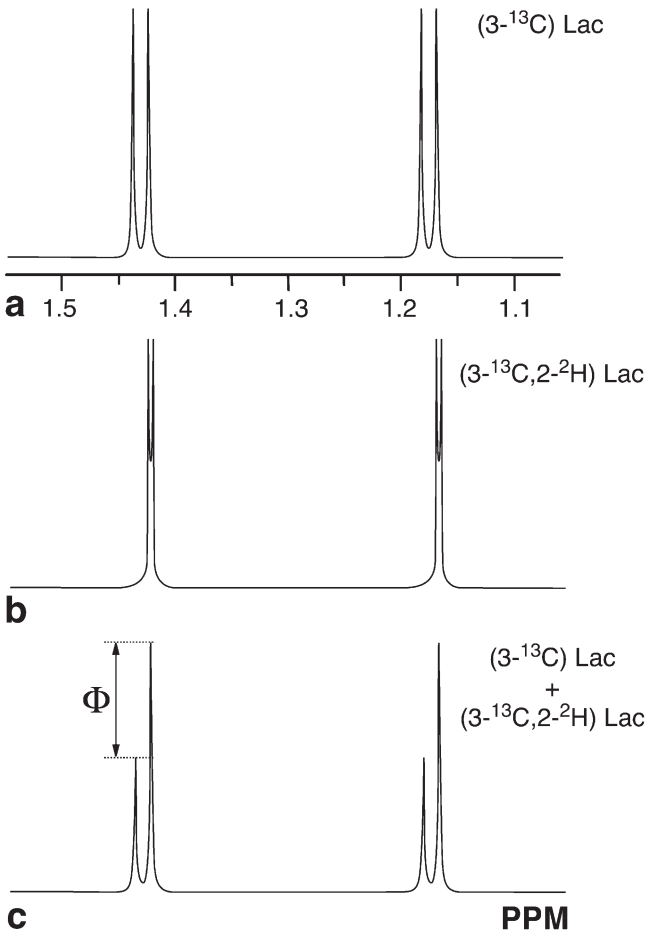

FIG. 3. Representative ^{1}H NMR simulations of the methyl resonances of (**a**) (3-^{13}C) lactate ($^1J_{\beta H\text{-}\beta C}$ = 128.0 Hz, $^3J_{\beta H\text{-}\alpha H}$ = 7.0 Hz), (**b**) (3-^{13}C, 2-^{2}H) lactate ($^1J_{\beta H\text{-}\beta C}$ = 128.0 Hz, $^1J_{\beta H\text{-}\alpha 2H}$ = 1.07 Hz; Δ_3 = −0.007 ppm), and (**c**) a mixture of (3-^{13}C, 66%) and (3-^{13}C, 2-^{2}H, 33%) lactate with natural linewidth of 2.0 Hz. Simulations were performed with the NUTS™ program. Φ: increase in intensity due to the appearance of (3-^{13}C, 2-^{2}H) lactate.

shifts the doublet resonance from the methyl group of (3-^{12}C) lactate into an apparent singlet, which appears superimposed on the high-field portion of the doublet, resulting in an increase in intensity that depends on the amount of ^{2}H incorporated (c.f., Fig. 2a, central multiplet).

Therefore, analysis of the structure of the ^{1}H NMR multiplets from (3-^{13}C) and (3-^{12}C) lactate allows the determination of the relative amounts of (3-^{13}C) and (3-^{12}C) lactate vs. (3-^{13}C, 2-^{2}H) and (3-^{12}C, 2-^{2}H) lactate, respectively. The differences in multiplet structure of the methyl resonances (3-^{13}C) and (3-^{12}C) lactate and those of (3-^{13}C, 2-^{2}H) and (3-^{12}C, 2-^{2}H) lactate allow the determination of the fractional deuterium enrichment in H2 of (3-^{13}C) and (3-^{12}C) lactate, as indicated by Eqs. [1] and [2], respectively:

Fractional ^{2}H2 enrichment in (3 − ^{13}C) lactate

$$= \frac{[^2H2]}{([^2H2] + [^1H2])} = \frac{(d + e) - (a + b)}{a + b + d + e} = \frac{2\Phi}{a + b + c + d} \quad [1]$$

Fractional ^{2}H2 enrichment in (3 − ^{12}C) lactate

$$= \frac{[^2H2]}{([^2H2] + [^1H2])} = \frac{f - c}{c + f} = \frac{\phi}{c + f} \quad [2]$$

The intensities of resonances *a–f* are illustrated in Fig. 2b.

Figure 4 depicts the time dependence of the H2 deuteration process as detected by the changes in the structure of the methyl multiplets of (3-^{13}C) and (3-^{12}C) lactate, during a 30-hr incubation of C6 cells with (3-^{13}C) lactate in Krebs-Henseleit buffer containing 50% ^2H_2O (vol/vol). During this period the total lactate concentrations in the incubation medium decreased from 4.2 ± 0.6 mM at the beginning of the incubation to 3.3 ± 1.2 mM at the end. An exponential increase in the deuterared portion of the (3-^{13}C) and (3-^{12}C) multiplets was detected (arrows). These modifications are not observed in cell-free incubations of Krebs-Henseleit buffer (50% ^2H_2O, vol/vol) with (3-^{13}C) lactate, or in incubations of C6 cells with (3-^{13}C) lactate in undeuterated Krebs-Henseleit buffer (data not shown), confirming that the described modifications are indeed derived from de novo enzymatic production of (3-^{13}C, 2-^{2}H) lactate. In addition, Fig. 4 also shows that it is

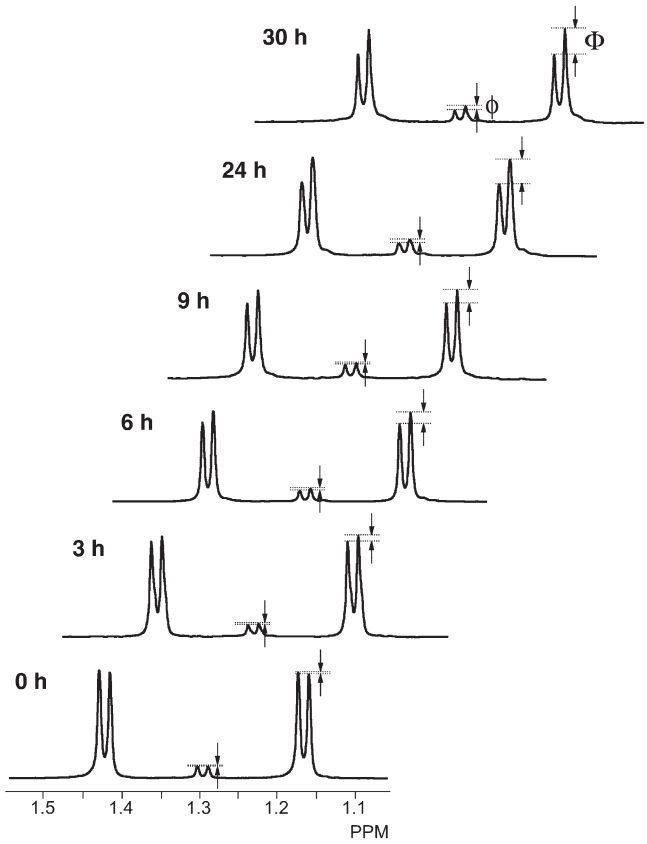

FIG. 4. Representative time course of H2 deuteration as detected by the changes in the relative intensities of the multiplet resonances from the methyl groups of (3-^{13}C) and (3-^{12}C) lactate. C6 glioma cells were incubated for 30 hr with 5 mM (3-^{13}C) lactate in Krebs-Henseleit buffer containing 50% ^2H_2O (vol/vol). Deuteration can be easily followed through the asymmetric increase in the intensity of the upper field transitions, reflecting the increase in concentration of (3-^{13}C, 2-^{2}H) lactate (Φ) or (3-^{12}C, 2-^{2}H) lactate (ϕ).

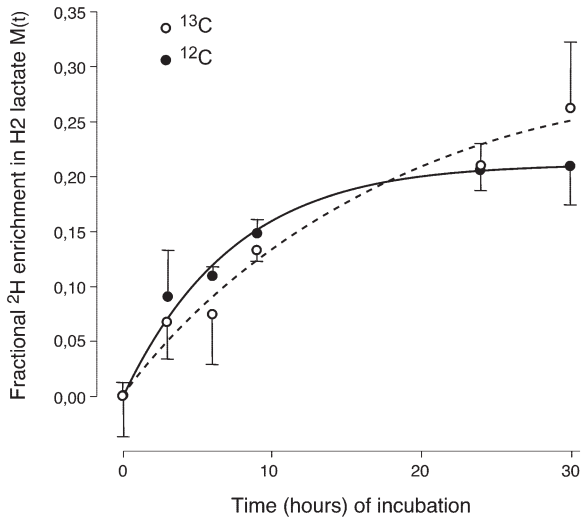

FIG. 5. Turnover of the H2 hydrogen from (3-^{13}C) lactate (empty circles, dotted line) and (3-^{12}C) lactate (filled circles, full line) during incubations with 5 mM (3-^{13}C) lactate in Krebs-Henseleit buffer containing 50% ^2H_2O (vol/vol). Fractional ^{2}H enrichments in H2 lactate for each time point were determined as described in the text. The experimental points $M_{(t)}$ were fitted to a single exponential of the type $M_{(t)} = M_{(\infty)}$ (1-exp(-k.t)) to determine $M_{(\infty)}$ and k. Results are shown as the mean ± SD of three independent experiments with different cell cultures.

possible to analyze the deuteration of (3-^{13}C) and (3-^{12}C) lactate molecules simultaneously, allowing the evaluation of the kinetics of H2 deuteration in both (3-^{13}C) and (3-^{12}C) lactate, as indicated in Fig. 5.

Figure 5 shows the time course of H2 deuteration in (3-^{13}C) and (3-^{12}C) lactate, as obtained from three experiments with different cultures of C6 cells. The kinetics were fitted to a single exponential and gave values for the rate constants k (N = 3, mean ± SD) of 0.06 ± 0.02 h^{-1} and 0.15 ± 0.05 h^{-1}, for the production of (3-^{13}C, 2-^{2}H) lactate and (3-^{12}C, 2-^{2}H) lactate, respectively. Similarly, the asymptote values $M_{(\infty)}$ were 0.30 ± 0.05 and 0.21 ± 0.02 for (3-^{13}C, 2-^{2}H) lactate and (3-^{12}C, 2-^{2}H) lactate, respectively. The differences found between the average rate constants and asymptote values were not statistically significant, as revealed by Student's t-test (P = 0.12 for k, and P = 0.14 for $M_{(\infty)}$).

Finally, the present study shows that it is possible to distinguish lactate molecules that are moving from the cytosol to the incubation medium because they become labeled with ^{2}H in the lactate dehydrogenase equilibrium (c.f., Fig. 1 and Eq. [3]).

$$\text{Pyruvate} + \text{NAD}(^2\text{H}) + \text{H}^+ \leftrightarrow (2 - {}^2\text{H})\ \text{Lactate} + \text{NAD}^+ \quad [3]$$

The required NAD(^{2}H) for this process may be derived from the sequential combination of a hydration-dehydration reaction (Eq. [4]) and a red-ox process (Eq. [5]) as follows:

$$\text{AH}_2 + {}^2\text{H}_2\text{O} \leftrightarrow \text{AH}(^2\text{H}) + (^2\text{H})\text{HO} \quad [4]$$

$$\text{AH}(^2\text{H}) + \text{NAD}^+ + \text{H}^+ \leftrightarrow \text{AH}_2 + \text{NAD}(^2\text{H}) \quad [5]$$

$$\text{Total: } {}^2\text{H}_2\text{O} + \text{NAD}^+ + \text{H}^+ \leftrightarrow \text{H}(^2\text{H})\text{O} + \text{NAD}(^2\text{H}), \quad [6]$$

where A is the metabolite incorporating the ^{2}H from ^2H_2O that will be transferred later to NAD^+ in the red-ox process. Both reactions are stereospecific, and only deuterons incorporated through malic enzyme or isocitrate dehydrogenase activities produce the correct stereochemistry in NAD(^{2}H) to become a cofactor in the lactate dehydrogenase equilibrium (14). Therefore, cytosolic deuteration of lactate in H2 is a complex process that involves several enzymes. The rate-limiting step of this process is currently under investigation in our laboratory.

CONCLUSIONS

In summary, we have reported a simple, robust, and sensitive ^{1}H NMR approach for measuring the turnover of the H2 hydrogen of (3-^{13}C) and (3-^{12}C) lactate. The method is faster than previous ^{13}C NMR approaches and can be easily implemented in virtually all NMR apparatuses. It is important to note that while the measurement of perdeuteration was done in ^{13}C-labeled lactate, with all the demonstrated benefits, it is also applicable to lactate in the absence of ^{13}C labeling.

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