A Fast and Sensitive (^{1}H, ^{2}H) NMR Method to Measure the Turnover of the H2 Hydrogen of Lactate

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Introduction. Lactate plays a fundamental role in cellular metabolism and bioenergetics. Under the reduced vascular clearance conditions occurring in ischemic episodes, tumors or even during some phases of cerebral activation, lactate molecules have been reported to accumulate in the extracellular space reaching even higher concentrations than those found in the cytosol. Under these conditions, extracellular lactate molecules may return to the cytosol through the reversible monocarboxylate proton co-transporter, located in the plasma membrane of virtually all normal and transformed cells. It has not been previously possible, however, to investigate simultaneously the inward and outward fluxes of lactate through this transporter, most probably because of the difficulties found in the discrimination between lactate molecules moving inward or outward of the cell, respectively. Here we report a novel ^{1}H NMR method that allows to discriminate easily between lactate molecules that have passed through the cell cytosol and those that have not. This is so because only the former become intracellularly deuterated in incubation media containing ^2H_2O, this deuteration process being conveniently detected by high resolution (^{1}H, ^{2}H) NMR. The present approach allows the detection of H2 deuteration with the increased sensitivity of ^{1}H NMR, avoiding the use of ^{13}C labeled substrates. Moreover, even if ^{13}C precursors are used, our method allows direct measurements of H2 deuteration both in ^{13}C labeled and ^{12}C lactate molecules, respectively.

Methods. C6 glioma cells were grown to confluence in DMEM and incubated (3-30 h, 37 ºC) in KHB medium containing 50% (vol/vol) ^2H_2O with 5 mM (3-^{13}C) lactate. Aliquots from the medium (1 mL) were collected sequentially after increasing incubation periods, prior to high resolution ^{1}H NMR analysis (500.130 MHz, 25 ºC, pH 7.2) with an AVANCE 500WB NMR spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany). Spectral deconvolution and quantification of relative peak areas were performed with NUTS (Acorn, Freemont, CA, USA).

Results. Figures 1A and 1B 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) (A) or 30 h later (B). ^{2}H substitution of the H2 hydrogen of (3-^{12}C) lactate results in two easily detectable transformations of the vicinal H3 doublet resonance (c,c´): (i) the formation of an H3 singlet (f-ϕ) due to the disappearance of the homonuclear coupling to H2 ($^3J_{\beta H\text{-}\alpha H}$ = 7.0 Hz) and (ii) an upfield isotopic shift derived from the vicinal ^{2}H2 substitution (Δ_3 = -0.007 ppm). Only lactate molecules that have passed through the cell cytosol experience these effects, since the H2 deuteration requires lactate dehydrogenase activity and NAD(^{2}H). A similar transformation is observed in (3-^{13}C) lactate, where the original doublet of doublets from the methyl group (aa´-bb´) is transformed in an isotopically shifted doublet (d,e-Φ). Thus, analysis of the observed shifted and unshifted H3 lactate resonances from the incubation medium allows to discriminate between 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. Notably, kinetic experiments indicate that the H2 hydrogens of (3-^{13}C) and (3-^{12}C) lactate depict cytosolic different residence times of 17.9 and 7.19 h, respectively. This represents to our knowledge, the first time that a significant ^{13}C isotopic effect is reported, decreasing to approximately one half the rate of incorporation of ^{2}H, only into the ^{13}C labeled molecule.

Conclusion. We report on a simple, robust and sensitive method to investigate the turnover of the H2 hydrogen of (3-^{13}C) and (3-^{12}C) lactate molecules by (^{1}H, ^{2}H) NMR. The procedure is faster than previous ^{13}C NMR approaches (1), is easily implemented in routine NMR instruments and does not require the use of ^{13}C labeled precursors. Moreover, the proposed strategy is easily extendable to other molecules and pathways of intra- and extracellular trafficking, where a ^{2}H exchange site is present only in the intracellular milieu.

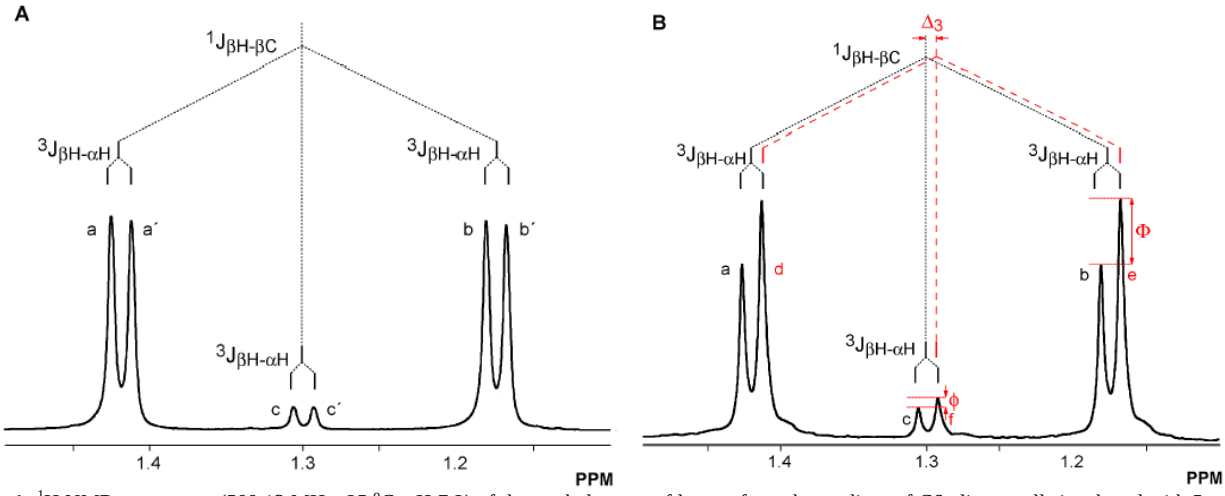

Fig. 1: ^{1}H NMR resonances (500.13 MHz, 25 ^{o}C, pH 7.2) of the methyl group of lactate from the medium of C6 glioma cells incubated with 5 mM (3-^{13}C) lactate in Krebs Henseleit Buffer containing 50% ^2H_2O, immediately after the addition of (3-^{13}C) lactate (Fig.1A) and 30h later (Fig.1B). Note the increased intensity of the higher field portions of the multiplets caused by the overlap of the isotopically shifted doublet (Δ_3) of (3-^{13}C, 2-^{2}H) lactate (Φ) and the singlet of (3-^{12}C, 2-^{2}H) lactate (ϕ). $^1J_{\beta H\text{-}\beta C}$ = 128.0 Hz; $^3J_{\beta H\text{-}\alpha H}$ = 7.0 Hz; Δ_3 = -0.007 ppm.

References. (1) Rodrigues, TB, Gray, HL, Benito, M, Garrido, S, Sierra, A, Geraldes, CF, Ballesteros, P and Cerdán, S; Futile Cycling of Lactate Through the Plasma Membrane of C6 Glioma Cells as Detected by {^{13}C, ^{2}H} NMR. *Journal Neurosci Res* (in press).