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^{1}H-NMR and Hyperpolarized ^{13}C-NMR Assays of Pyruvate-Lactate Exhange: a comparative study

Deborah K. Hill[#1], **Yann Jamin**[#1], **Matthew R. Orton**[1], **Nicolas Tardif**[1], **Harold G. Parkes**[1], **Simon P. Robinson**[1], **Martin O. Leach**[1], **Yuen-Li Chung**[1], and **Thomas R. Eykyn**[1,2]

[1]Cancer Research UK and EPRSC Cancer Imaging Centre, Division of Radiotherapy and Imaging, The Institute of Cancer Research and Royal Marsden NHS Foundation Trust, 15 Cotswold Road, Sutton, Surrey, SM2 5NG. United Kingdom

[2]Division of Imaging Sciences and Biomedical Engineering, Kings College London, St Thomas Hospital, London, SE1 7EH, United Kingdom

[#] These authors contributed equally to this work.

Abstract

Pyruvate-lactate exchange is mediated by the enzyme lactate dehydrogenase (LDH) and is central to the altered energy metabolism in cancer cells. Measurement of exchange kinetics using hyperpolarized ^{13}C NMR has provided a biomarker of response to novel therapeutics. In this study we investigated an alternative *in vitro* ^{1}H assay, using [3-^{13}C]pyruvate, and compared the measured kinetics with a hyperpolarized ^{13}C-NMR assay, using [1-^{13}C]pyruvate, under the same conditions in human colorectal carcinoma SW1222 cells. The apparent forward reaction rate constants (k_{PL}) derived from the two assays showed no significant difference, and both assays had similar reproducibility (k_{PL} = 0.506 ± 0.054 and k_{PL} = 0.441 ± 0.090 nmol/s/10^{6} cells, (mean ± standard deviation, n = 3); ^{1}H, ^{13}C assays respectively). The apparent backward reaction rate constant (k_{LP}) could only be measured with good reproducibility using the ^{1}H-NMR assay (k_{LP} = 0.376 ± 0.091 nmol/s/10^{6} cells, (mean ± standard deviation, n = 3)). The ^{1}H-NMR assay has adequate sensitivity to measure real-time pyruvate-lactate exchange kinetics *in vitro*, offering a complementary and accessible assay of apparent LDH activity.

Keywords

^{1}H NMR; ^{13}C NMR; cancer; hyperpolarized pyruvate; lactate; lactate dehydrogenase activity

Introduction

There is a resurgence of interest in the area of cancer metabolism, including understanding deregulated metabolic pathways, which confer growth advantages to tumors (1). One metabolic adaptation to energy metabolism in cancer is known as the Warburg Effect (2), where glycolysis readily occurs even in the presence of oxygen; this classical hallmark of cancer has been associated with the activity and expression of the key enzyme lactate

thomas.eykyn@kcl.ac.uk; martin.leach@icr.ac.uk

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dehydrogenase (LDH), where increased enzyme activity has been associated with more aggressive disease (3-5). There is a need, therefore, for robust assays of enzyme kinetics, historically carried out by spectrophotometric analysis (6); however, the spectrophotometric assays cannot be performed on whole, viable cells and are consequently an indirect method of evaluating response to targeted therapies (7,8). In contrary to the spectrophotometric technique, NMR has facilitated measurements of real-time enzyme kinetics from viable, whole cells both *in vitro* and *in vivo*, and has been used to identify putative therapeutic metabolic targets in cancers (2,9-12).

In this study we have expanded upon an observation by Day *et al.* (13) and evaluated a ^{1}H-NMR assay of apparent LDH activity, and qualified it against a hyperpolarized ^{13}C NMR measurement using Dynamic Nuclear Polarization (DNP) in the same tumor cell line.

Methods

Cell Preparation for NMR

SW1222 cells were seeded at a density of 3×10^{4} cm^{-2} and cultured in DMEM medium (Invitrogen, UK) with the addition of 10% foetal bovine serum (FBS, Invitrogen, UK), 1% penicillin and streptomycin (Invitrogen, UK) and 1% L-glutamine (Invitrogen, UK) at 37°C in an atmosphere containing 5% CO_2. The floating cell fraction was discarded, and cells were rinsed twice with phosphate buffered saline (PBS). Cells were trypsinised and centrifuged for five minutes at 190 g. Cell pellets were immediately resuspended in 500 µl serum-free medium and NMR studies were carried out within 10 minutes of cell harvesting.

All NMR experiments were performed on an 11.7 T Bruker DMX spectrometer (Bruker Biospin, Germany).

^{1}H-NMR assay

100 µl D_2O and 100 µl of a solution containing 50 mM [3-^{13}C]pyruvate (99% isotopically enriched, Sigma-Aldrich, UK) and 50 mM unlabelled, [^{12}C]lactate (in PBS, pH 7) was mixed with a 500 µl cell suspension of $37 \pm 5 \times 10^{6}$ SW1222 cells (n=3) in a 5 mm NMR tube. Proton spectra were recorded at 500 MHz, 37°C, with 128 spectra acquired sequentially every 5 s using a 60° pulse-and-acquire sequence (1 transient, 16k time domain points, 10 kHz spectral width, 5 s recovery time). A presaturation pulse (noesygppr1d) was used to achieve good water suppression and a flat spectral baseline.

Hyperpolarized ^{13}C-NMR assay

18 mg [1-^{13}C]pyruvic acid (99% isotopically enriched, Sigma-Aldrich, UK) containing 15 mM trityl free radical OX63 (Oxford Instruments, UK) was polarised in a HyperSense® DNP polariser (Oxford Instruments Molecular Biotools Ltd, UK) for 1 hour; average polarization is typically of the order P = 10%, corresponding to an enhancement factor of ~8000 compared to the thermal signal acquired on the same sample at 11.7 T. The hyperpolarized sample was dissolved in 4 ml aqueous buffer (50 mM sodium lactate, 50 mM sodium hydroxide, 1 mM EDTA) resulting in a 46 ± 3 mM pyruvate solution at pH 7. A volume of this solution (100 µl) was mixed with a 500 µl cell suspension of $35 \pm 2 \times 10^{6}$

SW1222 cells (n = 3) in a 5 mm NMR tube. The reaction was monitored at 125 MHz, 37°C, with 128 spectra acquired sequentially every 2.0 s using a 10° pulse-and-acquire sequence (1 transient, 16k time domain points, a 19 kHz spectral width).

Kinetic Modeling

Lactate dehydrogenase activity can be described by a two-way chemical exchange model (scheme 1), where forward and backward apparent reaction rate constants are given by k_{PL} and k_{LP} respectively. The exchange reaction can be separated into two pools containing either the ^{13}C or ^{12}C spins, the sum of which represents the total pool sizes e.g. $P(t) = {}^{13}P(t) + {}^{12}P(t)$ and $L(t) = {}^{13}L(t) + {}^{12}L(t)$. For the ^{1}H assay, the exchange reaction can be written as a set of differential equations:

$$\frac{d}{dt}\begin{bmatrix} {}^{13}P(t) \\ {}^{13}L(t) \\ {}^{12}P(t) \\ {}^{12}L(t) \end{bmatrix} = \begin{bmatrix} -k_{PL} & k_{LP} & 0 & 0 \\ k_{Pl} & -k_{LP} & 0 & 0 \\ 0 & 0 & -k_{PL} & k_{LP} \\ 0 & 0 & k_{PL} & -k_{LP} \end{bmatrix} \begin{bmatrix} {}^{13}P(t) \\ {}^{13}L(t) \\ {}^{12}P(t) \\ {}^{12}L(t) \end{bmatrix} \quad (1)$$

Where $P(t)$ and $L(t)$ represent the ^{1}H z-magnetizations of pyruvate and lactate signals respectively within the ^{13}C and ^{12}C pools. Whilst the above matrix appears block-diagonal owing to the absence of interconversion between the ^{13}C and ^{12}C they are connected via the presence of the common cofactor NAD(H). At the time-point of the injection of [3-^{13}C]pyruvate (t = 0) the only non-zero pools are ${}^{13}P(0)$ and ${}^{12}L(0)$ and can be solved to yield time dependencies for [^{13}C] and [^{12}C]pyruvate and lactate signals as follows:

$${}^{13}P(t) = \frac{{}^{13}P(0)\left(k_{LP} + k_{PL}e^{-t\left(k_{PL}+k_{LP}\right)}\right)}{\left(k_{PL}+k_{LP}\right)} \quad (2.1)$$

$${}^{13}L(t) = \frac{{}^{13}P(0)\,k_{PL}\left(1 - e^{-t\left(k_{PL}+k_{LP}\right)}\right)}{\left(k_{PL}+k_{LP}\right)} \quad (2.2)$$

$${}^{12}P(t) = \frac{{}^{12}L(0)\,k_{LP}\left(1 - e^{-t\left(k_{PL}+k_{LP}\right)}\right)}{\left(k_{PL}+k_{LP}\right)} \quad (2.3)$$

$${}^{12}L(t) = \frac{{}^{12}L(0)\left(k_{PL} + k_{LP}e^{-t\left(k_{PL}+k_{LP}\right)}\right)}{\left(k_{PL}+k_{LP}\right)} \quad (2.4)$$

In the hyperpolarized ^{13}C experiments, the observable magnetization is restricted to only the ^{13}C subspace and the rate equations can be written:

$$\frac{d}{dt}\begin{bmatrix} {}^{13}P(t) \\ {}^{13}L(t) \end{bmatrix} = \begin{bmatrix} -k_{PL} - r_P & k_{LP} \\ k_{PL} & -k_{LP} - r_L \end{bmatrix} \begin{bmatrix} {}^{13}P(t) \\ {}^{13}L(t) \end{bmatrix} \quad (3)$$

where $r_{P,L} = 1/T_{1(P,L)}$ denote the relaxation rates of hyperpolarized ^{13}C signals of pyruvate and lactate, respectively. The above equation is frequently encountered in NMR relaxation experiments (14) and readily solved by taking the matrix exponential:

$$\frac{d}{dt}\begin{bmatrix}{}^{13}P(t)\\ {}^{13}L(t)\end{bmatrix} = e^{\mathrm{L}t}\begin{bmatrix}{}^{13}P(0)\\ {}^{13}L(0)\end{bmatrix} = \mathbf{U}^{-1}e^{\mathbf{D}t}\mathbf{U}^{1}\begin{bmatrix}{}^{13}P(0)\\ {}^{13}L(0)\end{bmatrix} \quad (4)$$

Where **L** is the relaxation matrix in equation (3), **D** is a diagonal matrix containing the eigenvalues of **L**, and **U** is a unitary matrix defined by $\mathbf{D} = \mathbf{ULU}^{-1}$. For the case $r_P = r_L$ the solution is analogous to equations (2.1-2.2) convoluted with an additional exponential decay owing to the loss of magnetization. For the general case $r_P \neq r_L$ the above can be solved to give a full analytical expression (13).

Spectral Analysis

Spectra were apodized to 0.5 Hz (^1H) or 3 Hz (^{13}C), phase and baseline corrected, and peaks of interest selected and integrated over the time-course of the experiment. The doublet ^1H resonance from [3-^{13}C]pyruvate and the ^1H resonance from [^{12}C]pyruvate were integrated and the $^1H(^{13}C)$ integrals were summed. Integrals were fitted simultaneously according to the two-way chemical exchange model, equations 2.1 and 2.3, using Matlab (MathWorks, UK) to determine apparent forward k_{PL} and backward k_{LP} rate constants. Rate constants were normalized to cell count for each sample. Integrals of the hyperpolarized [1-^{13}C]-resonances of pyruvate and lactate were flip-angle corrected and simultaneously fitted using Matlab to the full analytical solution (13) of the modified Bloch Equations for two-site exchange.

Statistics

Data are presented as mean ± standard deviation. Differences between rates were identified using a Student's unpaired t-test with a 5% confidence interval.

Results

Following the addition of an equimolar solution of [3-^{13}C]pyruvate and [^{12}C]lactate to a suspension of SW1222 cells, a time-dependent decrease in the ^1H resonances of both [3-^{13}C]pyruvate (2.4ppm, $^1J_{C3\text{-}H3}$ = 128.7Hz) and [^{12}C]lactate (1.3ppm) was observed with a concomitant increase in the ^1H resonances of [^{12}C]pyruvate (2.4ppm) and [3-^{13}C]lactate (1.3ppm, $^1J_{C3\text{-}H3}$ = 128.7Hz), reflecting LDH-mediated pyruvate-lactate exchange (Fig. 1A). The signal-to-noise of the ^1H [3-^{13}C]pyruvate peaks at time t = 0 was 31.5 ± 2.8 (n = 3). The ^1H-NMR assay allowed the detection of both forward and reverse reactions with apparent reaction rates k_{PL} = 0.506 ± 0.054 nmol/s/10^6 cells and k_{LP} = 0.376 ± 0.091 nmol/s/10^6 cells (n = 3), derived from kinetic modeling of the ^1H data. There was no significant difference between k_{PL} and k_{LP} (p = 0.10). The sum of the $^1H(^{13}C)$ and $^1H(^{12}C)$ signals was constant over the time series.

Following the addition of an equimolar solution of hyperpolarized [1-^{13}C]pyruvate and [^{12}C]lactate to a suspension of SW1222 cells, a decrease in the hyperpolarized ^{13}C resonance of pyruvate is observed owing to T_1 loss of polarization as well as metabolic

conversion, with a concomitant increase in the ^{13}C resonance of [1-^{13}C]lactate, again reflecting LDH-mediated pyruvate-lactate exchange (Fig. 1A). The signal-to-noise of the [^{13}C]pyruvate peak at time t = 0 was measured to be 2186 ± 390 (n=3). Apparent reaction rates k_{PL} = 0.441 ± 0.090 nmol/s/10^6 cells and k_{LP} = 0.289 ± 0.208 nmol/s/10^6 cells were derived from kinetic modeling of the ^{13}C data (p = 0.91) (n = 3). A significant uncertainty was associated with the measurement of the reverse reaction rate constant.

There was no significant difference between k_{PL} (p = 0.35) and k_{LP} (p = 0.82), measured from the ^{1}H and ^{13}C assays.

Discussion

In this study we demonstrated that the use of [3-^{13}C]pyruvate in combination with ^{1}H-NMR provides a robust assay to monitor pyruvate-lactate exchange kinetics *in vitro*. This was compared with the equivalent hyperpolarized ^{13}C-NMR assay. While the signal-to-noise ratio of the hyperpolarized ^{13}C-NMR assay was, at most, 69 times greater than the ^{1}H-NMR assay, the ^{1}H assay provided adequate signal to monitor pyruvate-lactate exchange kinetics in real time. Furthermore, the transient hyperpolarized signal restricts the acquisition window to a few minutes, whereas the ^{1}H assay allows continuous observation of the enzymatic process.

The ^{1}H-NMR assay allows direct, and therefore more accurate, measurement of k_{LP} than the hyperpolarized ^{13}C assay. Accurate determination of k_{LP} from the kinetic modeling of ^{13}C-NMR data is challenging because of the initial condition of an empty [^{13}C]lactate pool coupled with the decay of all the signals owing to relaxation, and the greater number of model fitting parameters in the hyperpolarized assay. This translates into greater uncertainty in k_{LP} estimates from the kinetic modeling of ^{13}C-NMR compared with the ^{1}H assay. The accurate determination of k_{LP} using the hyperpolarized assay has been addressed recently by inverting the magnetization of one of the hyperpolarized metabolite pools, which allows the measurement of forwards and reverse exchange rates (15). A potential solution to allow the direct measurement of k_{LP} using the hyperpolarized assay is to co-polarize [2-^{13}C]pyruvate with [1-^{13}C]lactate, allowing simultaneous detection of both reactions.

Additionally, the measured rate of pyruvate-lactate exchange not only reflects LDH activity (16) but is also dependent on the level of LDH cofactors (NAD/NADH), providing information on cellular energetics, the pool sizes of the injected ^{13}C species, and the pool sizes of (either endogenous or exogenous) ^{12}C metabolites (13,15), as well as the activity and expression of the monocarboxylate transporters, MCT1 and MCT4, that respectively mediate the transport of hyperpolarized pyruvate and lactate into and out of the cells (17). These parameters are often deregulated in cancer, and both assays could be useful to study these important factors, and their modulation, *in vitro*.

While the ^{1}H-NMR assay demonstrates several attractive features *in vitro*, hyperpolarized ^{13}C-NMR remains the technique of choice for *in vivo* measurements, owing to the necessary signal enhancements and large chemical shift range of ^{13}C; it also allows detection of the other metabolic fates of pyruvate, such as the formation of alanine and

bicarbonate, biomarkers of alanine transaminase activity and pyruvate dehydrogenase (PDH) flux respectively (18-20).

Conclusions

The ^{1}H-NMR assay of pyruvate-lactate exchange offered a complementary and accessible measurement of apparent LDH activity in live cancer cells *in vitro*, with the added value of directly measuring the backward reaction.

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Abbreviations

DNP	Dynamic Nuclear Polarization
LDH	lactate dehydrogenase
MCT	monocarboxylate transporter

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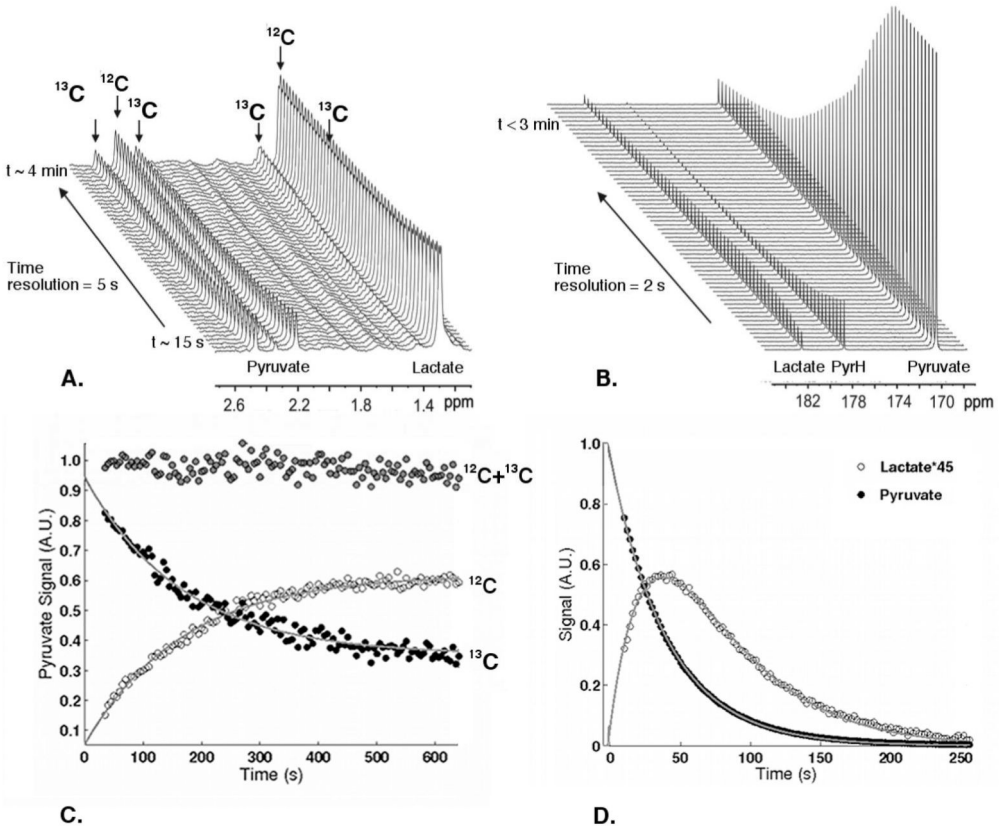

Figure 1.

(A) Real-time ^{1}H-NMR and (B) hyperpolarized ^{13}C-NMR assays of pyruvate-lactate exchange in colorectal SW1222 cancer cells and the corresponding time evolution of the spectral integrals (C, D). (C) Integrals of the ^{1}H(^{12}C) peak, open symbols, and ^{1}H(^{13}C) pyruvate peaks, closed symbols, from the ^{1}H assay. (D) Integrals of the hyperpolarized [1-^{13}C]pyruvate peak, closed symbols, and [1-^{13}C]lactate peaks, open symbols, from the ^{13}C assay. The solid lines correspond to the fits from the kinetic models described in the text.

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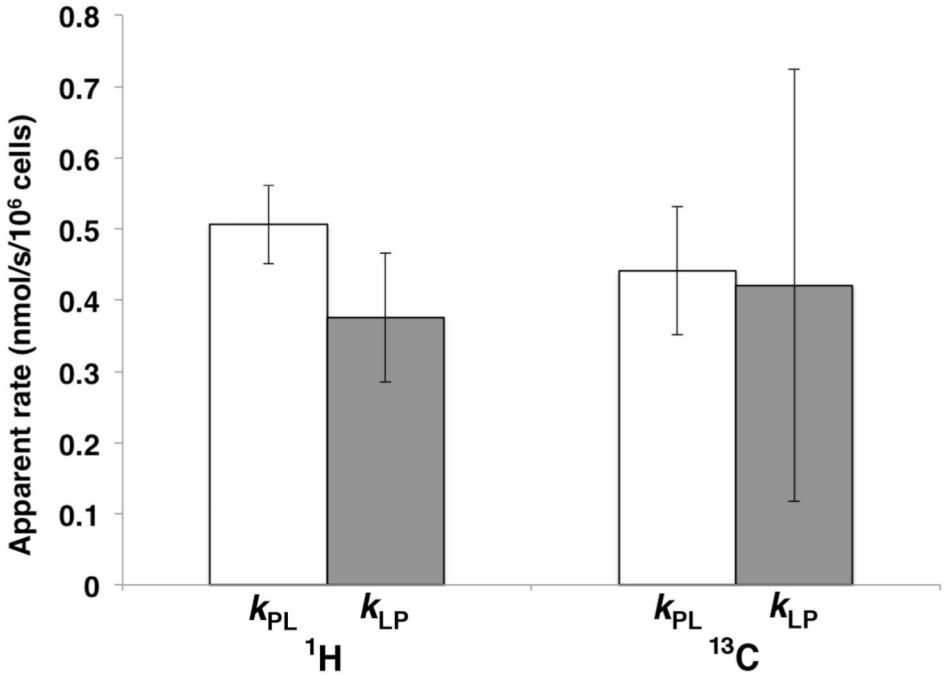

Figure 2.

LDH-mediated pyruvate-lactate exchange rates measured in SW1222 cells. (A) Forward and reverse apparent rate constants k_{PL} and k_{LP} measured from the ^{1}H-NMR assay (B) Forward and reverse rate constants k_{PL} and k_{LP} measured from the hyperpolarized ^{13}C-NMR assay. Rates are normalized to cell number in each sample.

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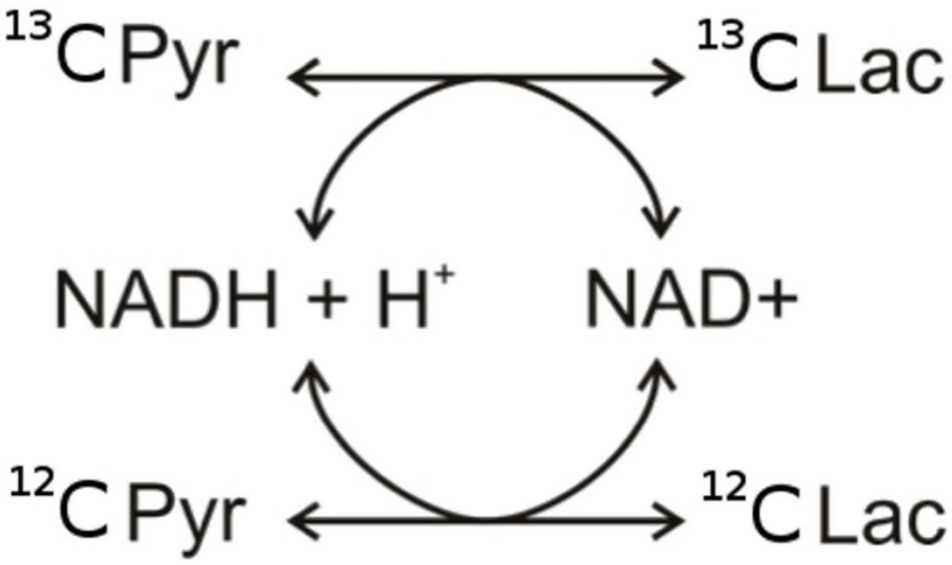

Scheme 1.