Kinetic Modeling of Hyperpolarized $^{13}$C Label Exchange between Pyruvate and Lactate in Tumor Cells*

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Measurements of the kinetics of hyperpolarized $^{13}$C label exchange between [1-$^{13}$C]pyruvate and lactate in suspensions of intact and lysed murine lymphoma cells, and in cells in which lactate dehydrogenase expression had been modulated by inhibition of the PI3K pathway, were used to determine quantitatively the role of enzyme activity and membrane transport in controlling isotope flux. Both steps were shown to share in the control of isotope flux in these cells. The kinetics of label exchange were well described by a kinetic model that employed rate constants for the lactate dehydrogenase reaction that had been determined previously from steady state kinetic studies. The enzyme showed pyruvate inhibition in steady state kinetic measurements, which the kinetic model predicted should also be observed in the isotope exchange measurements. However, no such pyruvate inhibition was observed in either intact cells or cell lysates and this could be explained by the much higher enzyme concentrations present in the isotope exchange experiments. The kinetic analysis presented here shows how lactate dehydrogenase activity can be determined from the isotope exchange measurements. The kinetic model should be useful for modeling the exchange reaction in vivo, particularly as this technique progresses to the clinic.

Magnetic resonance spectroscopy is a powerful tool for the non-invasive investigation of cellular metabolism, in systems ranging from isolated cells, through perfused organs (1), and small animals to humans (2, 3). A fundamental limitation of magnetic resonance spectroscopy has been a lack of sensitivity, which limits temporal resolution to the minute time scale and spatial resolution to 1–10 cm$^3$, depending on the magnetic field strength and nucleus detected (4). The recent introduction of dissolution dynamic nuclear polarization (5), which can increase the sensitivity of the magnetic resonance experiment by more than 10,000-fold, has had a significant impact on the field (reviewed in Refs. 6 and 7), dramatically increasing the temporal resolution to the second time scale and the spatial resolution to the millimeter scale. With this technique the $^{13}$C nucleus, in a $^{13}$C-labeled cell metabolite, is hyperpolarized and then injected into the biological system, where this may be an intravenous injection into a small animal or human. The gain in sensitivity, due to hyperpolarization of the $^{13}$C nucleus, means that there is now sufficient signal to image the distribution of the labeled metabolite in the body and, more importantly, its metabolism and conversion into other cellular metabolites. The main limitation is the relatively short lifetime of the polarization, which in the $^{13}$C-labeled metabolites, which have been used to date, is between 10 and 30 s. This means that spectroscopic imaging must be accomplished within 2–3 min and the labeled molecule must be taken up by cells and metabolized very rapidly for a significant labeling of other cell metabolites within the lifetime of the polarization. Although other nuclei can be polarized, attention has been focused on $^{13}$C-labeled molecules because of the relatively long lifetime of the $^{13}$C polarization and the availability of $^{13}$C-labeled cell metabolites that show rapid tissue metabolism.

The most widely used metabolite to date has been [1-$^{13}$C]pyruvate, which in most tissues exchanges the hyperpolarized $^{13}$C label with endogenous lactate and alanine, in the reactions catalyzed by lactate dehydrogenase (LDH) and alanine aminotransferase, respectively. Evidence that the observed lactate labeling is predominantly due to exchange, rather than net conversion of pyruvate to lactate, is summarized in Refs. 8 and 9. Lactate labeling is particularly high in those tissues with a large endogenous lactate pool and high LDH activity, such as tumors (10). In those tissues with a high mitochondrial content, such as heart muscle, there is also production of hyperpolarized $^{13}$C-labeled CO$_2$, due to the oxidative decarboxylation of pyruvate catalyzed by pyruvate dehydrogenase (1, 11).

Acquisition of a dynamic series of $^{13}$C spectra or images can be used to probe the kinetic properties of individual enzymes in the cell. In the case of [1-$^{13}$C]pyruvate, lactate labeling in tumors was originally analyzed using a simple two-site exchange model (8), where the measured $^{13}$C label flux was determined by the rate of pyruvate delivery to the tumor, the kinetics of pyruvate transport into the cell, as well as the kinetic properties of LDH. More recent studies have used a kinetic model, which includes the rate of pyruvate delivery to the tumor (12). However, it is not yet clear how to determine the individual kinetic properties of the membrane transporter and LDH from the measured hyperpolarized $^{13}$C label flux. A recent study, in a human breast cancer cell line, analyzed the pyruvate concentration dependence of the observed flux and concluded

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* The work was supported in part by Cancer Research UK Programme Grant C197/A3514 (to K. M. B.) and by a Translational Research Program Award from The Leukemia and Lymphoma Society. The research was conducted under a research agreement with GE Healthcare, who provided the polarizer and related materials. K. M. B. holds two patents with GE Healthcare.

1 Supported by a GE Healthcare-Biotechnology and Biosciences Research Council CASE studentship.

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3 The abbreviations used are: LDH, lactate dehydrogenase; MCT, monocarboxylate transporter.
that the membrane transporter was rate-limiting (13). We show here, both by determining the effect of removing the membrane permeability barrier and by varying cellular LDH content on the measured label flux, that the kinetic analysis employed in this previous study does not necessarily indicate that membrane transport is rate-limiting. We show in a murine lymphoma cell line that control of label flux is shared between the membrane transporter and LDH and demonstrate how their individual kinetic properties can be determined. We also show that the kinetics of lactate-pyruvate $^{13}$C label exchange are well described using a Theorell-Chance mechanism and previously published rate constants determined for the rabbit muscle enzyme using steady state kinetic measurements.

EXPERIMENTAL PROCEDURES

Materials—Tissue culture reagents, SYTOX® Red dead cell stain, and annexin V-Pacific Blue™ were from Invitrogen. LY294002 was from Cayman Chemical (Ann Arbor, MI). Anti-rabbit Akt, phospho-Akt (Ser473), and phospho-Akt (Thr308) antibodies were from Cell Signaling (Danvers, MA). Rabbit anti-LDH-A antibody was from Santa Cruz Biotechnology Inc. Both rabbit anti-MCT-1 and MCT-4 antibodies were a kind donation from Professor Andrew Halestrap (Department of Biochemistry, University of Bristol, UK). Peroxidase-conjugated donkey anti-rabbit IgG antibody was from Jackson ImmunoResearch Europe Ltd., Newmarket, UK. All other enzymes and chemicals were of analytical grade from Sigma. Murine lymphoma (EL-4) cells were from the European Collection of Cell Cultures (Salisbury, Wiltshire, UK).

Cell Culture—EL-4 cells were grown in RPMI 1640 media, supplemented with 10% fetal calf serum, 2 mm l-glutamine, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO$_2$. Cell number and viability were assessed following trypan blue staining. For PI3K inhibition studies, cells were treated with either solvent vehicle (dimethyl sulfoxide) or LY294002 (50 μM final concentration) for 24 h.

Detection of Cell Death—Apoptosis and necrosis were visualized by staining with acridine orange (AO; 5 μg/ml) and propidium iodide (25 μg/ml) (14). For measurements of cell death by flow cytometry, cells were stained with annexin V-Pacific Blue and SYTOX Red and then analyzed on an LSRII cytometer (BD Biosciences) as described previously (15).

Lactate Dehydrogenase Assay—LDH activity was determined by following the initial rate of pyruvate reduction to lactate, as described in Ref. 16. Briefly, a lysate was prepared from 10$^5$ cells using modified RIPA lysis buffer (50 mM Tris, pH 7.8, 150 mM NaCl, 5 mM EDTA, 15 mM MgCl$_2$, 1% Nonidet P-40, 0.5% sodium deoxycholate) and added to an assay mixture containing 0.22 mM reduced β-nicotinamide adenine dinucleotide (NADH) in 0.2 M KC1 buffer containing 40 mM HEPES, pH 7.1, pre-warmed to 37 °C. The assay was initiated by the addition of sodium pyruvate and the decrease in absorbance at 340 nm was recorded at 37 °C over 20 min. For steady state kinetic studies, sodium pyruvate was added at final concentrations from 0.05 to 20 mM. In a further series of experiments, LDH activity was determined at pyruvate concentrations of 2 and 20 mM in the presence of 0, 20, or 40 mM lithium lactate. For determination of LDH activity following PI3K inhibition, a pyruvate concentration of 30 mM was used and the specific activity of LDH was expressed in units per milligram of protein, where 1 unit of enzyme activity is defined as the amount of enzyme that oxidizes 1 μmol of NADH in 1 min at 37 °C, pH 7.1. Protein concentrations were determined using Bradford’s reagent (Bio-Rad) (17).

Western Blots—LY294002-mediated changes in protein expression were assessed by Western blotting. Plasma membrane proteins were fractionated using a kit from Biovision (Mountain View, CA), with the protein concentration determined using a Non-Interfering Protein Assay kit (Merck Chemicals Ltd., Darmstadt, Germany). Whole cell lysates were prepared as described above. Protein concentrations were determined using Bradford’s reagent (Bio-Rad) (17). Proteins were resolved by SDS-PAGE and subjected to Western blotting using standard techniques (18). Changes in protein expression were visualized using the ECL Plus kit (GE Healthcare). Blots were scanned (PowerLook III, Umax Systems GmbH, Willich, Germany) and quantitation of the signals was performed by densitometry, using scanning analysis software (TotalLab TL120, Nonlinear Dynamics, Newcastle upon Tyne, UK).

$^{[1-13]}$CPyruvate Hyperpolarization—A 44-mg sample of 91% $^{[1-13]}$Cpyruvic acid, containing 15 mM of the trityl radical OXO63 (GE Healthcare), was hyperpolarized as described previously (8). The frozen sample was dissolved at 180 °C in 6 ml of buffer containing 40 mM HEPES, 94 mM NaOH, 30 mM NaCl, 50 mg/liter of EDTA, pH 12.5. The sample was dissolved in less than 10 s and polarization levels were between 25 and 32%.

Nuclear Magnetic Resonance Spectroscopy—Cells (10$^5$) were washed once in RPMI 1640 medium at 37 °C and then resuspended in 2 ml of the same medium before being transferred to a 10-mm NMR tube. Cells were examined using a broadband probe (Varian NMR Instruments, Palo, CA) in a 9.4-T vertical wide-bore magnet (Oxford Instruments, Abingdon, Oxfordshire, UK). The sample temperature was maintained at 37 °C. Hyperpolarized $[1^{-13}C]$pyruvate (0.5 to 20 mM final concentration) and non-hyperpolarized, unlabeled lithium lactate (10 mM) were injected simultaneously into the cell suspension. The effect of lactate addition was assessed using three further lactate concentrations, 0, 20, and 40 mM, over a range of hyperpolarized pyruvate concentrations. Lysed cell preparations were prepared by freeze-thawing the cell pellets twice in liquid nitrogen before resuspending them in a 0.2 M KCl buffer containing 40 mM HEPES, 10 mM nicotinamide, 0.4 mM NAD$^+$, pH 7.1, at 37 °C. For experiments in which the cells had been treated with the PI3K inhibitor, LY294002, 40 mM hyperpolarized $[1^{-13}C]$pyruvate, and 40 mM non-hyperpolarized, unlabeled lactate were injected into the cell suspension. For all experiments, single transient $^{13}$C spectra were acquired every second for 180 s, using a 6° flip angle pulse and a spectral width of 32 kHz. At the end of each experiment, the cells were counted with a hemocytometer, and cell viability was assessed using trypan blue dye exclusion (0.4% in 0.81% sodium chloride and 0.06% potassium phosphate). Pyruvate-lactate exchange rate constants ($k_p$) were derived by fitting the pyruvate and lactate signal intensities to the modified Bloch equations for two-site exchange (8). As has been observed previously (9), similar values for $k_p$ were obtained regardless of whether $p_l$ was
assumed to be equal to \( p_L \) or whether \( p_L \) and \( p_R \) were allowed to vary independently or whether \( k_t \), the rate constant for the reverse reaction, was assumed to be zero. Label flux was calculated by multiplying the rate constant by the concentration of hyperpolarized pyruvate.

**Statistics**—Results are expressed as the mean ± S.D. Significant differences between mean values were determined using a two-tailed t test. Differences between treatment groups were considered significant if \( p < 0.05 \).

**RESULTS**

Dependence of the Rate of \(^{13}\text{C}\) Label Flux between \([1-^{13}\text{C}]\) Pyruvate and Lactate on Pyruvate Concentration in Intact and Lysed Murine Lymphoma (EL4) Cells—The pyruvate concentration dependence of hyperpolarized \(^{13}\text{C}\) label flux between \([1-^{13}\text{C}]\) pyruvate and lactate in intact EL-4 cells was fit to the Michaelis-Menten equation (Equation 1; Fig. 1) to give an apparent velocity in intact cells (Fig. 1), suggesting that the transporter activity is rate-limited for \(^{13}\text{C}\) label exchange in this murine lymphoma cell line. However, lysis of the cells and thus removal of the membrane permeability barrier, produced only a modest increase in the isotope exchange velocity (Fig. 1), suggesting that whereas the transporter activity has some effect on \(^{13}\text{C}\) label exchange it is not entirely rate-limiting.

For a coupled series of isotope exchange reactions the overall exchange velocity \( V_{\text{obs}} \) is equal to the sum of the reciprocals of the individual exchange velocities. Therefore, for the exchange of \(^{13}\text{C}\) label between pyruvate and lactate in intact cells,

\[
\frac{1}{V_{\text{obs}}} = \frac{1}{V_{\text{LDH}}} + \frac{1}{V_{\text{MCT}}} \tag{Eq. 2}
\]

where \( V_{\text{LDH}} \) is the equilibrium isotope exchange velocity for cell viability during these measurements, as determined by trypan blue staining, and analysis of previous \(^{31}\text{P}\) magnetic resonance spectroscopy measurements on cell extracts (8) showed that the NTP/NDP ratio was in excess of 3 following the \(^{13}\text{C}\) magnetic resonance spectroscopy measurements. The similarity of the \( K_m \) determined here with a \( K_m \) of 0.72 mM determined for pyruvate transport in Ehrlich-Lettre tumor cells (19) would indicate, according to the analysis of Harris et al. (13), that the membrane transporter must be rate-limiting for \(^{13}\text{C}\) label exchange in this murine lymphoma cell line. However, lysis of the cells and thus removal of the membrane permeability barrier, produced only a modest increase in the isotope exchange velocity (Fig. 1), suggesting that whereas the transporter activity has some effect on \(^{13}\text{C}\) label exchange it is not entirely rate-limiting.

![Figure 1](image-url)

**FIGURE 1. Measurements of hyperpolarized \(^{13}\text{C}\) label flux between pyruvate and lactate in intact cells and cell lysates.** A, measured isotope exchange velocity in intact (○) and lysed EL-4 cells (●) at the indicated pyruvate concentrations. Hyperpolarized \(^{13}\text{C}\) label flux was measured following the addition of 10 mM unlabeled lactate and the indicated hyperpolarized \([1-^{13}\text{C}]\) pyruvate concentrations to an EL-4 cell suspension at 37 °C (10^6 cells in 2 ml of RPMI 1640 medium) or a cell lysate that had been prepared by freeze-thawing the same number of cells in 0.2 M KCl buffer containing 40 mM HEPES, 10 mM nicotinamide, 0.4 mM NAD^+, pH 7.1. The solid line for the lysed cell data were obtained by fitting these data to the Michaelis-Menten equation (Equation 1). Because there was insufficient data at low pyruvate concentrations the apparent \( V_{\text{max}} \) could not be determined and the data were fit with \( K_m = 0 \). The solid line for the intact cell data were obtained by fitting these data to Equation 3, assuming that \( V_{\text{LDH}} \) was the exchange velocity measured in the lysed cells. B, exchange velocities calculated using Equation 8 (curve i) and Equation 9 (curve ii), the latter includes pyruvate inhibition of the enzyme. The rate constants used in these calculations are given in Table 1. The enzyme concentration per cell \((\text{Eq. 1; see text})\) and the pyruvate inhibition constant \((K_p, 9.4 \text{mM})\) were determined by fitting the steady state kinetic data shown in Fig. 2 to Equation 7, using the rate constants in Table 1 and a lactate inhibition constant \((K_L, 0.209 \text{mM})\). The theoretical curve for the cells (iii) was calculated using Equation 3, assuming a \( K_m \) for pyruvate transport of 3.0 mM and the calculated theoretical value for \( V_{\text{LDH}} \) (curve i). C, flux control coefficients for LDH \((U_{\text{LDH}})\) and the monocarboxylate transporters \((U_{\text{MCT}})\) were calculated numerically using Equation 10 by determining the effect on the observed isotope exchange flux \((V_{\text{obs}})\) of a 1/1000th increase in \( V_{\text{LDH}} \) or \( V_{\text{MCT}} \) (Equation 2). D, expansion of the plot shown in C. The numbers next to the lines indicate lactate concentration (mM).
LDH and $V_{MCT}$ is the equilibrium exchange velocity for the monocarboxylate transporters. Although this equation was derived for trace isotope exchange (20) it has also been shown to apply to bulk isotope exchange (21), as is the case here. The reaction is expected to be near to chemical equilibrium because only a very small fraction of pyruvate needs to undergo net conversion to lactate to achieve chemical equilibrium, whereas the whole pyruvate pool must exchange to achieve isotopic equilibrium. The arguments for near chemical equilibrium in EL-4 cells suspensions have been discussed in detail in Ref. 8 (and in the supplementary information to this earlier paper), and has been demonstrated experimentally for EL-4 tumors in vivo using magnetization transfer measurements (9). Because in lysed cells $1/V_{obs} = 1/V_{LDH}$, we can use Equation 2 to calculate the individual isotope exchange velocities. This gives a value for $V_{LDH}$ of 64 fmol min$^{-1}$ cell$^{-1}$ and, assuming that $V_{LDH}$ in the cell lysate is equivalent to that in the cell, a value for $V_{MCT}$ in the intact cell of 114 fmol min$^{-1}$ cell$^{-1}$. The pH, ionic strength, and NAD$^+$ concentration in the lysate were chosen to simulate the conditions in the cell (8). Thus the monocarboxylate transporters are not rate-limiting for the exchange in these cells and both steps play a role in determining the isotope exchange flux. The isotope exchange velocities for the intact cells shown in Fig. 1A were fit to Equation 3,

$$V_{obs} = \frac{1}{V_{LDH}} + \frac{1}{K_m[MCT-Pyr]} \left( \frac{1}{K_{MCT-Pyr} + [Pyr]} \right) \tag{Eq. 3}$$

where $K_m[MCT-Pyr]$ is the $K_m$ of the monocarboxylate transporter for pyruvate. The data were fit using a value for $V_{LDH}$ of 64 fmol min$^{-1}$ cell$^{-1}$ and a value for $V_{MCT}$ of 114 fmol min$^{-1}$ cell$^{-1}$. This gave a $K_m[MCT-Pyr]$ of 3.0 mM, which is much higher than the value of 0.88 mM determined by fitting to the Michaelis-Menten equation (Equation 1).

Comparison of Steady State Enzyme Kinetics with the Kinetics of Equilibrium Isotope Exchange—The reaction kinetics for rabbit muscle lactate dehydrogenase can be explained in terms of an ordered ternary complex mechanism in which the coenzymes bind first (Equations 4–6) (22). This enzyme is expected to show similar kinetic properties to the LDH-A isoform found in EL-4 cells (see below).

$$E + \text{NAD}^+ \xrightarrow{k_1} E \cdot \text{NAD}^+ \tag{Eq. 4}$$

$$E \cdot \text{NAD}^+ + \text{Lac} \xrightarrow{k_2} E \cdot \text{NADH} + \text{Pyr} \tag{Eq. 5}$$

$$E \cdot \text{NADH} \xrightarrow{k_3} E + \text{NAD}^+ \tag{Eq. 6}$$

The steady state rate equation for the reverse reaction (pyruvate $\rightarrow$ lactate) was derived by Zewe and Fromm (22). Their equation included the effects of lactate on the initial rate of the reverse reaction, which will have two effects: it will cause reversal of the reaction in which it is produced from pyruvate and it will form an abortive ternary complex with the $E$-NADH complex. We have further modified the equation derived by Zewe and Fromm (22) to include pyruvate inhibition of the enzyme,

$$v_r = \frac{E_0}{1 + \frac{[P]}{K_i}} + \frac{1 + \frac{[L]}{K_i}}{1 + \frac{[L]}{K_i}} + \frac{k_3(1 + \frac{[L]}{K_i})}{k_2} + \frac{k_2}{k_1k_2[H][P]} \tag{Eq. 7}$$

where $v_r$ is the initial rate of the reverse reaction, $L$ is lactate, $P$ is pyruvate, $H$ is NADH, $E_0$ is total enzyme concentration, and the rate constants are those indicated in Equations 4–6. $K_i$ is the binding constant for lactate to the $E$-NADH complex and $K_i$ is the binding constant for pyruvate to the $E$-NAD$^+$ complex. Zewe and Fromm (22) determined the rate constants for rabbit muscle LDH, although because they did not know the enzyme concentration they were unable to determine their absolute values. We have calculated these (see Table 1) by assuming that the on-rate constant for NADH binding to the bovine heart enzyme at 28 °C, calculated from the data given by Borgmann et al. (23), was the same for the rabbit muscle enzyme. The steady state kinetic data shown in Fig. 2 were fit to Equation 7 to estimate an enzyme concentration ($E_0$) in the cell and an inhibition constant for pyruvate ($K_i$). The rate constants used were those given in Table 1 and the inhibition constant for lactate ($K_i$) was assumed to be 0.209 mM (22). The estimated enzyme concentration from this fit was $1.2 \times 10^{-14}$ M, which would be the concentration in the spectrophotometer cuvette (volume 1 ml) if 1 cell were lysed. Because the extract was prepared from 10$^5$ cells the total enzyme concentration in the cuvette was $1.2 \times 10^{-9}$ M. The data clearly show that there is pyruvate inhibition of the enzyme (Fig. 2) and the data were fitted with a $K_i$ of 9.4 mM.

The equilibrium isotope exchange velocity for exchange of the $^{13}$C label between [1-$^{13}$C]pyruvate and [1-$^{13}$C]lactate ($V_{eq}$) is given by Equation 5. The following expression (20), gives the pyruvate-lactate exchange velocity as a function of the kinetic properties of the enzyme and the concentrations of the substrates of the enzyme,

**Table 1**

| Rate constants | $k_1$ | $k_2$ | $k_3$ | $K_i$ |
|---------------|-------|-------|-------|-------|
|                | 9.12 s$^{-1}$ | 8.82 s$^{-1}$ | 3.44 s$^{-1}$ | 6.58 s$^{-1}$ |
|                | 5.49 s$^{-1}$ | 1.03 s$^{-1}$ | 6.72 s$^{-1}$ | 3.52 s$^{-1}$ |

These rate constants were calculated from data given in Ref. 22, assuming that the on-rate constant for NADH binding to the bovine heart enzyme at 28 °C, calculated from the data given by Borgmann et al. (23), was the same for the rabbit muscle enzyme. Stinson and Holbrook (34) measured a $K_i$ for NADH binding to the bovine heart enzyme of 1.4–2.0 μM and for binding to the rabbit muscle enzyme of 3.5 μM, at pH 7.2, and 20 °C. These values are comparable with values of 1.99 μM for the bovine heart enzyme from the kinetic data of Borgmann et al. (23) and 5.32 μM for the rabbit muscle enzyme from the kinetic data of Zewe and Fromm (22).
Kinetic Studies with Hyperpolarized [1-13C]Pyruvate

FIGURE 2. Effect of pyruvate concentration on the initial reaction velocity determined spectrophotometrically in cell lysates. Initial reaction velocity was determined by measuring the decrease in NADH absorbance at 340 nm. The results were fitted to Equation 7 (solid line) by varying the value for \( v_0 \), the enzyme concentration, and \( K_p \) the pyruvate inhibition constant, and by using the rate constants shown in Table 1. The assay system contained 0.22 mM NADH in 40 mM HEPES buffer, pH 7.1, containing 0.2 M KCl, pre-warmed to 37 °C.

\[
V_{LDH} = \frac{E_0 k_6 k_0[H][P]}{k_5 \left( 1 + \frac{k_5[H]}{k_s} + \frac{k_5 k_0[H][P]}{k_s k_6[L]} \right)}
\]

(Eq. 8)

where \( E_0 \) is total enzyme concentration, \( H \) is NADH, \( P \) is pyruvate, and \( L \) is lactate. Pyruvate inhibition of the exchange velocity, through the formation of an abortive ternary complex with \( E-NAD^+ \) (24), is given by,

\[
V_{LDH} = \frac{E_0 k_6 k_0[H][P]}{k_5 \left( 1 + \frac{k_5[H]}{k_s} + \frac{k_5 k_0[H][P]}{k_s k_6[L]} \right)}
\]

(Eq. 9)

where \( K_p \) is the binding constant for pyruvate to the \( E-NAD^+ \) complex. Equations 8 and 9 were used to calculate the \( ^{13}C \) isotope exchange velocities, as a function of pyruvate concentration. These calculations assume that the endogenous lactate concentration has little or no effect on the kinetics of \( LDH \) (using Equation 3) because at a tumor pyruvate concentration of 0.5 mM and an endogenous lactate concentration of 1.0 mM, \( I_{LDH} \) decreases with increases in lactate concentration.

The relative importance of \( LDH \) and the monocarboxylate transporters in determining the observed isotope exchange fluxes can be determined quantitatively by applying metabolic control analysis (25) to the exchange velocities calculated using the kinetic model. The flux control coefficient, which is the fractional change in flux due to a fractional change in enzyme concentration (Equation 10), defines the degree to which an enzyme or transporter controls flux, where this is unity for a step that is entirely rate-limiting and near zero for a step that has little or no control.

\[
\frac{\delta F/F}{\delta E/E} = J
\]

(Eq. 10)

where \( F \) is flux, \( E \) is enzyme concentration, and \( J \) is the flux control coefficient. For a simple linear pathway the flux control coefficients of the individual steps sum to unity. In general, application of metabolic control analysis has shown that flux control is distributed (25). The flux control coefficients, calculated for \( LDH \) \((J_{LDH})\) and the monocarboxylate transporters \((J_{MCT})\) at different pyruvate and lactate concentrations are shown in Fig. 1, C and D. These show, as one would expect intuitively, that \( J_{MCT} \) decreases and \( J_{LDH} \) increases with increases in pyruvate concentration and, conversely, that \( J_{MCT} \) increases and \( J_{LDH} \) decreases with increases in lactate concentration. These calculations assume that the endogenous lactate concentration has little or no effect on the kinetics of hyperpolarized [1-13C]pyruvate transport. Thus at a tumor pyruvate concentration of 0.5 mM and an endogenous lactate concentration of 1.0 mM, \( J_{LDH} \) is 0.71 \((J_{MCT} = 0.29)\), but this drops with increasing lactate concentration to 0.20 at 10 mM lactate \((J_{MCT} = 0.80)\).

The results of experiments examining the effect of lactate concentration on the isotope exchange velocities and steady state kinetics of the enzyme in cell lysates are shown in Fig. 3, A and C, respectively. The corresponding theoretical curves, which were calculated using Equations 8 and 9 for the isotope exchange velocities and Equation 7 for the steady state rates are shown in Fig. 3, B and D, respectively. The steady state kinetic rates were best fit using an enzyme concentration of 1.0 \( \times 10^{-14} \) M, as compared with 1.2 \( \times 10^{-14} \) M for the data shown in Fig. 2, where this is the enzyme concentration that would result from lysing a single cell in 1 ml. These curves were drawn using the rate constants given in Table 1 and the same inhibition constants for pyruvate and lactate as were used to draw the curve in Fig. 2 \((K_p = 9.4 \text{ mM}, K_{l} = 0.209 \text{ mM})\). The calculated exchange velocities showed similar effects of changes in lactate and pyruvate concentrations as observed experimentally. However, the discrepancy between the calculated and observed LDH exchange velocities increased with increasing lactate concentration. At 20 mM pyruvate and 10 mM lactate the calculated
value was 62 and the experimentally determined value 63 fmol/min/cell. At 20 mM lactate these values had increased to 125 and 105 fmol/min/cell, respectively, and at 40 mM lactate they were 249 and 154 fmol/min/cell, respectively. Curves in Fig. 3B were calculated for 20 and 2 mM pyruvate (solid and dashed lines, respectively), with pyruvate inhibition (Equation 9 and Fig. 3, lines labeled with a #) and without pyruvate inhibition of the enzyme (Equation 8). Comparison of the experimental data with these curves again shows that there is no pyruvate inhibition of the enzyme in the lysates used for the isotope exchange experiments, although there was clearly pyruvate inhibition of the enzyme in the more dilute cell lysates used for the steady state kinetic measurements (Fig. 3C).

The rate constants shown in Table 1 were calculated from the kinetic parameters determined by Zewe and Fromm at 28 °C (22). The experiments performed here were conducted at 37 °C. The relative values of the rate constants are likely to change with temperature, as demonstrated for the bovine heart enzyme (23), and this may contribute to the discrepancy between the calculated and observed LDH exchange velocities at high lactate concentrations. However, this does not under-

![Image](https://via.placeholder.com/150)

**FIGURE 3.** Effect of lactate concentration on the flux of hyperpolarized 13C label between [1-13C]pyruvate and lactate and on the initial rate of pyruvate reduction in cell lysates. A, hyperpolarized 13C label flux was measured following addition of either 2 (C) or 20 mM (D) pyruvate, with the indicated lactate concentrations, to an assay system containing 0.22 mM NADH in 40 mM HEPES buffer, 0.2M KCl, pH 7.1, pre-warmed to 37 °C. The theoretical curves for the enzyme in the more dilute cell lysates used for the steady state kinetic measurements (Fig. 3C).

The rate constants shown in Table 1 were calculated from the kinetic parameters determined by Zewe and Fromm at 28 °C (22). The experiments performed here were conducted at 37 °C. The relative values of the rate constants are likely to change with temperature, as demonstrated for the bovine heart enzyme (23), and this may contribute to the discrepancy between the calculated and observed LDH exchange velocities at high lactate concentrations. However, this does not under-

crepancy cannot be explained by lactate inhibition through binding of lactate to form an abortive E-NADH-lactate complex (data not shown). This is minimal because lactate binding is weak and the concentration of the E-NADH complex very low.

**Determination of the Lactate Dehydrogenase Isotope Exchange Velocity (V_{LDH}) in Intact Cells**—

Determining the isotope exchange velocities of lactate dehydrogenase and the monocarboxylate transporter by cell lysis is, of course, not a practical approach for determining the individual exchange velocities in vivo; however, this could be achieved by specifically modulating LDH expression using an inhibitor of the PI3K pathway and determining the effect on the 13C isotope exchange velocity (26). Treatment of EL-4 cells for 24 h with LY294002 (50 μM), an inhibitor of the PI3K pathway, which is known to control levels of LDH expression, resulted in inhibition of PI3K activity, as assessed by the absence of detectable Akt phosphorylation at Ser473 and Thr308. There was no change in total Akt levels (Fig. 4A) and no significant induction of cell death (n = 3; p > 0.05), as determined by flow cytometry of cells stained with annexin V-Pacific Blue, which labels apoptotic and necrotic cells, and SYTOX Red dead cell stain, which labels necrotic cells. Fluorescence microscopy of cells stained with acridine orange and propidium iodide confirmed that drug treatment had little effect on cell viability. There was also no
Kinetic Studies with Hyperpolarized [1-13C]Pyruvate

A

Phospho-Akt (Ser473)
Phospho-Akt (Thr308)
Akt
Actin

B

Time (s)

0
10
20
30
40
50
60
70

\( \frac{1}{V_{\text{LDH}}} \)

D

P.M. MCT-1
P.M. MCT-4
LDH-A
Actin

FIGURE 4. Changes in LDH expression and activity following treatment of cells with PI3K inhibitor, LY294002. A, inhibition of Akt phosphorylation following treatment of cells with LY294002. Cells were treated with either solvent vehicle (dimethyl sulfoxide) or LY294002 (50 \( \mu \)M final concentration) for 24 h before protein extraction. The figure shows a representative Western blot of phospho-Akt (Ser473), phospho-Akt (Thr308), and total Akt expression in LY294002 and vehicle-treated EL-4 whole cell lysates. Actin was used as a loading control. B, LDH activity was measured from the decrease in NADH absorbance at 340 nm following the addition of a cell extract to an assay system containing pyruvate and NADH. Activity was measured in extracts of cells that had been treated for 24 h with 50 \( \mu \)M LY294002 or the solvent vehicle (dimethyl sulfoxide). The mean absorbance values with time are shown (\( \pm \) S.D.; \( n = 5–6 \)). C, representative Western blot of LDH-A in whole cell lysates and MCT-1 and MCT-4 in plasma membrane fractions following LY294002 treatment. Actin was used as a loading control. D, hyperpolarized [1-13C]lactate peak intensities following addition of 40 mM hyperpolarized [1-13C]pyruvate and 40 mM unlabeled lactate to EL-4 cell suspensions that had been pretreated with either LY294002 (50 \( \mu \)M) or the solvent vehicle (dimethyl sulfoxide). Signal intensities were corrected for cell number and scaled to the initial pyruvate signal intensity to correct for variation in polarization levels. Mean values \( \pm \) S.D. (\( n = 4–5 \)).

change in the levels of cytoplasmic NADH, as assessed by flow cytometric measurements of cellular UV autofluorescence (excitation at 350 nm; emission at 475 nm) (8, 15, 27) (data not shown).

Treatment of EL-4 cells for 24 h with 50 \( \mu \)M LY294002 resulted in a 26% reduction in LDH activity, from 5.2 ± 0.9 to 3.9 ± 0.4 units/mg of protein (\( n = 5–6 \); \( p < 0.01 \)) (Fig. 4B) and a 23 ± 2% reduction in LDH-A expression, as assessed by Western blotting (\( n = 3; \ p < 0.05 \)) (Fig. 4C). The similarity in the relative reduction in enzyme activity and expression of LDH-A implies that most of the LDH in EL-4 cells is the LDH-A isoform. The plasma membrane content of the monocarboxylate transporters, MCT-1 and MCT-4, showed no significant change following drug treatment (Fig. 4C). Drug treatment also resulted in a 19% decrease in the rate constant (\( k_v \)) describing flux of hyperpolarized [1-13C]pyruvate and lactate, with the calculated isotope exchange flux falling from 52.5 ± 6.0 to 42.2 ± 3.5 fmol min\(^{-1}\) cell\(^{-1}\) (\( p = 0.019 \); Fig. 4D).

From Equation 3 we can see that a plot of \( 1/V_{\text{obs}} \) versus 1/(LDH activity) has a slope of \( 1/\alpha \), where \( \alpha \) is the specific exchange velocity of the enzyme (21). This gives a value for \( V_{\text{LDH}} \) in the intact cell of 72 fmol min\(^{-1}\) cell\(^{-1}\) and for \( V_{\text{MCT}} \) a value of 196 fmol min\(^{-1}\) cell\(^{-1}\) and demonstrates again that control of isotope flux is shared between LDH and the membrane transporter.

DISCUSSION

Imaging measurements of hyperpolarized 13C label flux between [1-13C]pyruvate and lactate have been used to assess tumor grade and treatment response in a variety of tumor types (7) and the technique has recently entered Phase I/II clinical trials in prostate cancer. The observed label flux will depend on the delivery of pyruvate to the tumor via the vasculature, on the rate of pyruvate transport across the tumor cell plasma membrane and subsequently on the concentration of tumor cell LDH and its substrates. Therefore interpreting changes in lactate labeling will require an understanding of the relative importance of these steps in determining the observed kinetics of label exchange.

We showed previously in EL-4 tumors, in animals injected intravenously with hyperpolarized [1-13C]pyruvate, that the exchange rate constants determined over periods of 2–10 s during the exchange time course, using magnetization transfer measurements, were similar to those obtained by fitting the entire hyperpolarized 13C isotope exchange time course over a period of 30–40 s (9). This implies that there is little change in the steady-state lactate and pyruvate concentrations over this 30–40 s of the observable exchange time course, or if there are changes in concentration that they have little effect on the kinetics of LDH. The further implication of this observation, which is important for this discussion, is that the rate of pyruvate delivery to this EL-4 tumor appears to have little effect on the measured rate of label exchange, although this may not be the case for more poorly perfused tumors.

Harris et al. (13) attempted to determine the importance of membrane transport for the observed isotope exchange kinetics by measuring the pyruvate concentration dependence for hyperpolarized 13C label flux between [1-13C]pyruvate and lactate in an immobilized human breast cancer cell preparation. They determined an apparent \( K_m \) for 2.14 ± 0.03 mM and a \( V_{\text{max}} \) for 27.6 ± 1.1 fmol min\(^{-1}\) cell\(^{-1}\), assuming simple Michaelis-Menten kinetics (Equation 1). Because this \( K_m \) was much higher than the \( K_m \) of ~0.2 mM determined from steady state kinetic studies for the net conversion of pyruvate to lactate in a permeabilized cell preparation (28) and more similar to the \( K_m \) values reported for pyruvate transport by the monocarboxylate transporters (MCTs) (29, 30) they concluded that membrane transport must be rate-limiting for hyperpolarized 13C label exchange between [1-13C]pyruvate and lactate. We have shown here that this analysis is not strictly correct and that in EL-4 cells control of 13C isotopic exchange flux is shared between the membrane transporters and LDH, with the flux control coefficients of LDH and the transporters varying with lactate and pyruvate concentrations (Fig. 1, C and D). Although the \( K_m \) of the exchange reaction for pyruvate is much higher in intact cells than in a cell lysate (Fig. 1A), reflecting some limitation of the exchange rate by transporter activity, the apparent \( K_m \) (0.88 mM) determined assuming simple Michaelis-Menten kinetics (Equation 1) is much less than that determined by fitting the
exchange rate to Equation 3, which takes into account shared control of flux between the transporter and LDH. This gives a $K_m$ for pyruvate of 3.0 mM. If the transporter were entirely rate-limiting for isotope exchange flux ($V_{\text{MCT}} \approx 1.0; V_{\text{LDH}} \approx 0$) then the $K_m$ determined using simple Michaelis-Menten kinetics (Equation 1) would approach 3.0 mM. The higher $K_m$ determined by Harris et al. (13), which was obtained assuming simple Michaelis-Menten kinetics, implies that in breast cancer cells used in their study the membrane transporter may have a higher flux control coefficient for the exchange than the transporter in the EL-4 murine lymphoma cells used here. The individual isotope exchange velocities of LDH and the membrane transporter(s) ($V_{\text{LDH}}$ and $V_{\text{MCT}}$) determined using the cell lysis experiment and calculated using Equation 2 were 64 and 114 fmol min$^{-1}$ cell$^{-1}$, respectively, at a lactate concentration of 10 mM and pyruvate concentrations between 0.5 and 20 mM, whereas those determined in the intact cell by modulating LDH expression using a PI3K inhibitor were 72 and 196 fmol min$^{-1}$ cell$^{-1}$, respectively, at lactate and pyruvate concentrations of 40 mM. The LDH exchange velocity determined in the intact cell of 72 fmol min$^{-1}$ cell$^{-1}$ is appreciably lower than the experimentally determined value for $V_{\text{LDH}}$ in a cell lysate at 40 mM lactate and 20 mM pyruvate of 154 fmol/min/cell (the kinetic model predicts that increasing the pyruvate concentration to 40 mM will not increase the exchange velocity further). The discrepancy between these two values might indicate that the conditions used in the cell lysate, in terms of pH and NAD$^+$ concentration, do not accurately mimic the conditions in the cell. A lower than expected isotope exchange velocity has been observed previously for LDH in human erythrocytes (31). Regardless of the reason for this discrepancy, both experiments demonstrate that LDH and the membrane transporters share in the control of isotope flux. Inhibition of hyperpolarized $^{13}$C label exchange between pyruvate and lactate in tumor cells in vitro and tumors in vivo, through PI3K inhibitor-mediated reduction of LDH-A activity, has been reported recently (26), although in this previous study there was no analysis of the relative contributions of transporter and LDH-A activities to the observed isotope flux. Nevertheless, the preliminary tumor measurements in this earlier study demonstrate the feasibility of this approach for determining the flux control coefficients of LDH and the membrane transporters for isotope flux in vivo.

Although the isotope exchange data can be fit to the Michaelis-Menten equation (Equation 1), it is important to note that this is not appropriate. Fitting the calculated isotope exchange fluxes at low pyruvate concentrations (Fig. 5) to the Michaelis-Menten equation gives an apparent $K_m$ of 13 $\mu$M, whereas the true $K_m$ of LDH for pyruvate ($k_j/k_i$), determined using steady state enzyme kinetic measurements, is 125 $\mu$M (Table 1 and Fig. 2). This low apparent $K_m$ in the simulation of the isotope exchange experiment is due to a decrease in the equilibrium NADH concentration as the concentration of pyruvate is increased. If the NADH concentration in the model (Fig. 5) is fixed at 10 $\mu$M then the isotope exchange rate increases to much higher levels and the apparent $K_m$ is increased to 76 $\mu$M. The inappropriateness of analyzing the isotope exchange data using Michaelis-Menten-type kinetics is also demonstrated by examining the effects of increasing lactate concentration on the isotope exchange rate. Increasing the lactate concentration increases the isotope exchange rate (Fig. 3A), as has been observed previously (8), whereas it decreases the net flux between pyruvate and lactate determined using steady state kinetic measurements (Fig. 3C). These changes in flux are predicted by the rate equations for isotope exchange (Equations 8 and 9; see Fig. 3B) and for steady state flux between pyruvate and lactate (Equation 7; see Fig. 3D).

The steady state kinetic measurements (Figs. 2 and 3C) demonstrate that there is significant pyruvate inhibition of the enzyme, with a $K_i$ of 9.4 $\mu$M (the dissociation constant for pyruvate binding to the $E$-NAD$^+$ complex), which is similar to values reported previously (22). Pyruvate inhibition is complex because in addition to the rapid and reversible formation of the $E$-NAD$^+$-Pyr complex, pyruvate will, over a period of time, form a slowly reversible binary enzyme-inhibitor complex in which the inhibitor is the adduct of pyruvate and NAD$^+$ (t$_{1/2}$ for formation $>25$ s) (24). The theoretical curves show that we should also have expected to see pyruvate inhibition in the isotope exchange experiments (Figs. 1B and 3B), however, this was not observed experimentally (Figs. 1A and 3A). An important difference between the steady state kinetic and the isotope exchange experiments was the enzyme concentration. Based on fitting to the steady state kinetic data shown in Fig. 2, the enzyme concentration was $1.2 \times 10^{-9}$ M in the steady state kinetic experiments, $6 \times 10^{-7}$ M in the lysed cell preparations used for the isotope exchange experiments, and $1.1 \times 10^{-5}$ M in the intact cell (assuming a cell volume of $1.06 \times 10^{-15}$ ml/cell, based on a measured average cell diameter of 12.6 $\mu$m and assuming that the cell is a sphere). The concentration of LDH in steady state kinetic studies has been shown to affect pyruvate inhibition of the enzyme (32). Studies with LDH-5 (LDH-A), the predominant isofrom in skeletal muscle and in the EL-4 murine lymphoma cells used here, showed that the enzyme was inhibited significantly by pyruvate concentrations greater than 3.0 mM and at enzyme concentrations of $9 \times 10^{-9}$ M and lower. There was no pyruvate inhibition of the enzyme at concentrations of $1.8 \times 10^{-8}$ and $7.0 \times 10^{-6}$ M. This inhibition was more pronounced for the isofrom predominant in heart muscle (LDH-1 or LDH-B). Subsequent studies suggested that this relief of pyruvate inhibition at high LDH concentrations was due to the presence of other dehydrogenases in the tissue extract, which competed with LDH for NAD$^+$ (33).
Kinetic Studies with Hyperpolarized \([1-^{13}C]\)Pyruvate

In conclusion, we have shown how the relative importance of LDH and the membrane transporters in controlling exchange of \(^{13}\text{C}\) label between pyruvate and lactate can be determined experimentally and have demonstrated that both have significant control of isotope flux in the murine lymphoma cells used here and that this varies according to the lactate and pyruvate concentrations. This relative importance of LDH and membrane transporter activity is also likely to vary between different cell types. This experimental approach may be feasible clinically using drugs that are inhibitors of the PI3K pathway. The exchange kinetics are well described by a kinetic model based on rate constants determined in steady state kinetic experiments. This model should be useful in future clinical studies, for example, for determining the likely effects of endogenous lactate on the observed exchange flux and the effect of the lactate and pyruvate concentrations on the distribution of control between LDH and the transporters. Although the kinetic model predicted pyruvate inhibition of isotope exchange at high pyruvate concentrations this was not observed experimentally and is likely due to the high enzyme concentration in the cell and in the cell lysates used for the isotope exchange experiments.

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