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The rate of lactate production from glucose in hearts is not altered by per-deuteration of glucose

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Abstract

This study was designed to determine whether perdeuterated glucose experiences a kinetic isotope effect (KIE) as glucose passes through glycolysis and is further oxidized in the tricarboxylic acid (TCA) cycle. Metabolism of deuterated glucose was investigated in two groups of perfused rat hearts. The control group was supplied with a 1:1 mixture of [U-¹³C₆]glucose and [1,6-¹³C₂]glucose, while the experimental group received [U-¹³C₆,U-²H₇]glucose and [1,6-¹³C₂]glucose. Tissue extracts were analyzed by ¹H, ²H and proton-decoupled ¹³C NMR spectroscopy. Extensive ²H-¹³C scalar coupling plus chemical shift isotope effects were observed in the proton-decoupled ¹³C NMR spectra of lactate, alanine and glutamate. A small but measureable (~8%) difference in the rate of conversion of [U-¹³C₆]glucose vs. [1,6-¹³C₂]glucose to lactate, likely reflecting rates of C–C bond breakage in the aldolase reaction, but conversion of [U-¹³C₆]glucose versus [U-¹³C₆,U-²H₇]glucose to lactate did not differ. This shows that the presence of deuterium in glucose does not alter glycolytic flux. However, there were two distinct effects of deuteration on metabolism of glucose to alanine and oxidation of glucose in the TCA. First, alanine undergoes extensive exchange of methyl deuterons with solvent protons in the alanine amino transferase reaction. Second, there is a substantial kinetic isotope effect in metabolism of [U-¹³C₆,U-²H₇]glucose to alanine and glutamate. In the presence of [U-¹³C₆,U-²H₇]glucose, alanine and lactate are not in rapid exchange with the same pool of pyruvate. These studies indicate that the appearance of hyperpolarized ¹³C-lactate from hyperpolarized [U-¹³C₆,U-²H₇]glucose is not substantially influenced by a deuterium kinetic isotope effect.

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Graphical Abstract

Keywords

kinetic isotope effect; heart; perfusion; glucose metabolism; NMR; deuterium

INTRODUCTION

Stable isotopes are widely used to investigate metabolism. ^{13}C labelling of substrates and analysis of the products by NMR spectroscopy is valuable because of the information encoded in the chemical shift and ^{13}C - ^{13}C scalar coupling [1]. Since many applications are restricted by the poor sensitivity of ^{13}C and the low concentration of metabolic products, there is strong interest in hyperpolarization methods which temporarily increase ^{13}C polarization by 10,000-fold or more [2]. Since T_1 decay of ^{13}C polarization limits the duration of data acquisition, incorporation of ^2H prolongs the T_1 of ^{13}C in some molecules, enabling a longer period of observation [2]. Metabolism of hyperpolarized $[\text{U-}^2\text{H}_7, \text{U-}^{13}\text{C}_6]$ glucose to hyperpolarized $[\text{U-}^{13}\text{C}_3]$ lactate has been described in *Escherichia coli* [3], yeast [4], breast cancer cells [5] and lymphoma [6]. These findings are important because imaging of hyperpolarized lactate derived from glucose could provide a direct measure of glycolysis and thereby offer a fundamentally new method for investigating carbohydrate metabolism in vivo [6]. Conclusions about flux through the glycolytic pathway derived from these experiments are only valid if flux through the pathway is not influenced by ^2H in glucose. Some enzymes are known to be sensitive to isotopic ^1H to ^2H replacement in substrates and display a kinetic isotope effect (KIE). A primary isotope effect refers to an altered rate of reaction when a heavy isotope is in a chemical bond that is broken [3,7], while secondary isotope effects occur when no chemical bonds are broken but an altered rate is still observed [8–10]. The typical result is to slow the reaction kinetics, although exceptions have been reported [11]. The KIE in the individual reactions of glycolysis have been studied in isolated systems with the conclusion that a KIE during pyruvate enolization and in the triose phosphate isomerase reaction are substantial [12–15]. Little is known, however, about the effects of deuteration on overall flux of glucose through the entire pathway. Some models of complex metabolic systems assume that the KIE at each step are additive [16] implying that even small effects of ^2H at each reaction in glycolysis would jeopardize the assumption that propagation of hyperpolarized $[\text{U-}^2\text{H}_7, \text{U-}^{13}\text{C}_6]$ glucose through the system is proportional to glycolysis.

The purpose of this study was to compare the relative rates of conversion of [U- $^{13}\text{C}_6$]glucose and [U- $^{13}\text{C}_6$,U- $^2\text{H}_7$]glucose to alanine, lactate and acetyl-CoA in a highly oxidative tissue, the isolated rat heart. Metabolism of [1,6- $^{13}\text{C}_2$]glucose was used as an internal reference. The design takes advantage of the known effects of ^2H on the ^{13}C NMR spectrum [17]. Upon substitution of ^1H with ^2H , nearby ^{13}C signals are generally shifted upfield in proportion to bond distances and the number of substituted ^2H nuclei. Scalar coupling effects were also informative. Upon coupling of a ^{13}C nucleus to ^2H ($I = 1$), a 1:1:1 triplet is observed in the proton-decoupled ^{13}C NMR spectrum. Two ^2H nuclei bonded to a single ^{13}C produce a 1:2:3:2:1 quintet, and three covalently bonded ^2H nuclei generate a 1:3:6:7:6:3:1 septet in the ^{13}C NMR spectrum. Deconvolution of the ^2H - ^{13}C couplings and chemical shift effects allowed measurement of complex mixtures of ^2H and ^{13}C isotopomers. To assist with the characterization of the resulting mixtures, a reference compound with different degrees of deuteration in lactate and pyruvate was prepared. In spite of the prior evidence for a significant KIE in some reactions of glycolysis and the suggestion that these effects are additive in vivo, deuteration of glucose had no measureable impact on the rate of production of lactate in the functioning heart. These results suggest that rate of production of hyperpolarized [U- $^{13}\text{C}_3$]lactate from hyperpolarized [U- $^2\text{H}_7$, U- $^{13}\text{C}_6$]glucose is not influenced by deuteration. In addition to information about metabolism of glucose to lactate, the initial goal of the study, this experiment provided an unexpectedly rich source of information about metabolism of glucose to alanine and glutamate. Production of alanine and glutamate was quite sensitive to the presence of deuterium in upstream glucose. We also observed proton exchange in the methyl group of lactate and alanine, previously described in isolated enzyme preparations [18] and now demonstrated in mammalian tissue.

MATERIALS & METHODS

[1,6- $^{13}\text{C}_2$]glucose, [U- $^{13}\text{C}_6$]glucose, [3- $^{13}\text{C}_1$,3-3- $^2\text{H}_3$]sodium pyruvate and [U- $^2\text{H}_7$,U- $^{13}\text{C}_6$]glucose were purchased from Sigma-Aldrich Isotec (Miamisburg, OH) or Cambridge Isotope Laboratories (Tewksbury, MA) and used without further purification. The [3- $^{13}\text{C}_1$,3-3- $^2\text{H}_3$]sodium pyruvate was obtained as mixture of all ^2H isotopomers: [3- $^{13}\text{C}_1$,3-3- $^2\text{H}_3$],[3- $^{13}\text{C}_1$,3-3- $^2\text{H}_2$],[3- $^{13}\text{C}_1$,3- $^2\text{H}_1$] and [3- $^{13}\text{C}_1$]sodium pyruvate

Heart Perfusions

The protocol was approved by the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center. Sprague Dawley rats, 250–300 grams, were purchased from Charles River (Cambridge, MA). All animals were fed ad libitum. Isoflurane (1.5–2%) used for general anesthesia. Depth of anesthesia was assessed by observation of the respiratory rate, paw pinch reflex, and palpebral reflex of the animal. Under general anesthesia, hearts were rapidly excised and arrested in ice-cold perfusion medium. Hearts were perfused in the Langendorff mode using a non-recirculating medium at a constant perfusion pressure of 100 cm H_2O . The medium contained 25 mM NaHCO_3 , 118 mM NaCl , 4.7 mM KCl , 1.2 mM MgSO_4 , 1.2 mM KH_2PO_4 , and 1.25 mM CaCl_2 and was bubbled continuously with a 95/5 mixture of O_2/CO_2 gas to maintain a pH of 7.4. The perfusion medium was maintained at 37° C using a water bath. Two sets of perfusion conditions were used. Hearts in group 1 were supplied with 5 mM [1,6- $^{13}\text{C}_2$]glucose plus 5

mM [U- $^{13}\text{C}_6$]glucose (n=4) while hearts in group 2 were supplied with 5 mM [1,6- $^{13}\text{C}_2$]glucose plus 5 mM [U- $^{13}\text{C}_6$, U- $^2\text{H}_7$]glucose (n=5). In both groups, the perfusate was treated with insulin (1 microunit / ml). Metabolism of [1,6- $^{13}\text{C}_2$]glucose to [3- $^{13}\text{C}_1$]lactate was assumed constant between the two groups. Hearts were freeze-clamped after 30 min of perfusion. Frozen tissue was extracted using perchloric acid [19,20], then dissolved in 5% D_2O , 0.5 mM DSS- d_3 , 1mM EDTA in H_2O at pH 7 and analyzed by ^1H , ^2H and ^{13}C NMR spectroscopy.

^1H , ^2H and ^{13}C NMR Spectroscopy

The principles and terminology for analysis of the ^{13}C NMR spectra have been described previously [1,20–22]. All high resolution NMR spectra were obtained using an Agilent 600MHz VNMRs Direct Drive console using a 3mm broadband probe. We assumed that once ^2H was released into intracellular water, it was no longer involved in metabolism. ^{13}C NMR spectra of the extracts were acquired as described previously [18,22–25]. The multiplets in the ^{13}C NMR spectrum were fit using a mixed Gaussian/Lorentzian lineshape in the TOPSPIN 3.5 Software (Bruker, Germany). Relative peak areas of the resonances adjacent to ^{12}C versus the ^{13}C satellites were measured in the ^1H NMR spectrum to obtain ^{13}C enrichments in the methyl carbon. The ^{13}C NMR spectra of glutamate were analyzed as described previously [21]. Significant differences were evaluated using GraphPad Prism (La Jolla, CA) using multiple t-tests with a significance level of $P < 0.05$.

Synthesis and NMR analysis of [3- ^{13}C , 3,3,3- $^2\text{H}_3$] lactate

A mixture of [3- $^{13}\text{C}_1$, 3,3,3- $^2\text{H}_3$]-, [3- $^{13}\text{C}_1$, 3,3- $^2\text{H}_2$]-, [3- $^{13}\text{C}_1$, 3- $^2\text{H}_1$]-pyruvate and [3- $^{13}\text{C}_1$] pyruvate obtained as a mixture from Sigma Aldrich Isotec (Miamisburg, OH) was partially reduced by dissolving it in H_2O and palladium on carbon. The reaction vessel was attached to a Parr hydrogenator for 4 hours to produce a mixture of approximately 50% lactate and pyruvate. The mixture was filtered and freeze-dried. The resulting mixture was analyzed by ^1H , ^{13}C { ^1H } and ^2H NMR spectroscopy. In all cases, the shorthand notation ^{13}C NMR indicates $^{13}\text{C}\{^1\text{H}\}$ NMR spectroscopy.

^2H and ^{13}C NMR Isotopomer Analysis of Pyruvate and Downstream Metabolites

These experimental conditions generate five different isotopomers of pyruvate (Figure 1). Unlabeled pyruvate originates from glycogen (Figure 1, [A]). Isotopomer [B] arises from the internal standard [1,6- $^{13}\text{C}_2$]glucose, and [C] is derived from the [U- $^{13}\text{C}_6$]glucose in the control hearts or from metabolism of [U- $^{13}\text{C}_6$, U- $^2\text{H}_7$]glucose to pyruvate followed by replacement of the ^2H by ^1H . During glycolysis, the deuterated glucose is converted to fructose-1,6-bisphosphate, which is then split into dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (G-3-P). G-3-P is derived from the 4, 5, 6 carbons of glucose and, therefore, contains two ^2H nuclei at the C3 position, while DHAP is derived from the 1, 2, 3 carbons of glucose where only one ^2H is retained in the C3 position. DHAP rapidly isomerizes to G-3-P creating a 1:1 mixture between two different deuterated species in [U- $^{13}\text{C}_3$]pyruvate with one ^2H , isotopomer [D] and two ^2H , isotopomer [E]. Any deviation from this 1:1 ratio must be due to a ^2H to ^1H replacement reaction involving pyruvate. The ^{13}C resonances of pyruvate are not directly observable in tissue extracts but resonances of alanine and lactate were readily detected and therefore analyzed.

All five species could arise from glycolysis in hearts supplied with [1,6- $^{13}\text{C}_2$]glucose plus [U- $^{13}\text{C}_6$,U- $^2\text{H}_7$]glucose (Figure 1, [A]–[E]) while three different species could be generated from hearts supplied with [1,6- $^{13}\text{C}_2$]glucose plus [U- $^{13}\text{C}_6$]glucose (Figure 1, [A]–[C]). These different isotopomers of pyruvate will also generate five distinct ^2H and ^{13}C labeling patterns in acetyl-CoA and in the carbon 4 moiety of α -ketoglutarate (Table 1).

Under these conditions, 19 lines with significant overlap (the sum of 4 subspectra shown in Figure 1) will be generated in the methyl ^{13}C resonance of lactate at ~20.8 ppm; repeated curve fitting of these resonances using a mixed Gaussian-Lorentzian lineshape to analyze relative fluxes resulted in significant scatter due to the large number of resonances. Instead the relative concentration of all 5 isotopomers (Figure 1) were determined from measurements taken largely from the C2 resonance of lactate in the proton-decoupled ^{13}C NMR spectrum and the ^1H NMR spectrum. As will be illustrated, the C2 resonances of lactate or alanine are sensitive to a 2-bond chemical shift isotope effect but there is no significant scalar coupling of C2 to the ^2H nuclei at C3. Furthermore, each measurement in C2 is the average of four measurements due to the underlying doublet of doublets in the ^{13}C NMR spectrum due to J_{12} and J_{23} . The following applies to lactate but identical calculations can be performed for alanine and glutamate can be analyzed using the C4 and C3 resonances:

- [A]/[B] determined from the ^1H NMR spectrum, the fraction of the central doublet in the methyl resonance at 1.33 ppm compared to the total area of the methyl proton resonance
- [B]/[C] determined from the ^{13}C NMR spectrum of the lactate methyl group, the ratio of lactate C3 singlet at 20.8 ppm to C3 doublet without covalently-bonded ^2H
- [C]/[D] determined from the ^{13}C NMR spectrum of lactate C2 at 69.1 ppm, nondeuterated lactate relative to monodeuterated lactate ($^2\text{H}_0/^2\text{H}_1$ in the methyl group)
- [D]/[E] determined from the ^{13}C NMR spectrum of lactate C2, monodeuterated lactate relative to bisdeuterated lactate ($^2\text{H}_1/^2\text{H}_2$ in the methyl group)

The sum of the relative concentrations of the 5 isotopomers (Figure 1) was assumed equal to 1. A MatLab script showing the solution of the simultaneous equations is provided in the supplementary material. From this script, the relative concentrations of all lactate or alanine isotopomers shown in Figure 1 were calculated. The glutamate resonances C3 and C4 can be analyzed in a similar way using the ratio of [B]/[C] from C4 direct analysis and [C]/[D] and [D]/[E] from the chemical shift isotope effects in C3. Glutamate species [A] was also calculated using the C3 and C4 resonances using a non-steady state approach described previously [21]. The results are summarized in Table 1.

RESULTS

Chemical Shift Assignments of Methyl-deuterated Lactate

The ^{13}C NMR spectra of tissue extracts were quite complex due to extensive ^2H and ^{13}C coupling. Resonance assignments were first checked using a chemically-synthesized mixture containing protonated [3- $^{13}\text{C}_1$]lactate, [3- $^{13}\text{C}_1$,3,3,3- $^2\text{H}_3$]lactate, [3- $^{13}\text{C}_1$,3,3- $^2\text{H}_2$]lactate, and [3- $^{13}\text{C}_1$,3- $^2\text{H}_1$]lactate. In the proton-decoupled ^{13}C NMR spectrum of the C3 position of lactate (Figure 2), the observed coupling patterns confirmed the scheme in Figure 1 for the mono- and bis-deuterated lactate. A 1:3:6:7:3:1 septet originating from [3- $^{13}\text{C}_1$,3,3,3- $^2\text{H}_3$]lactate was also observed. The chemical shift isotope effect for a 1-bond

substitution from ^1H to ^2H in the ^{13}C NMR spectrum was ~ 0.28 ppm per ^2H nucleus, and the one-bond $^2\text{H} - ^{13}\text{C}$ scalar coupling constant was essentially identical across all three species, 20.01 Hz. The ^1H NMR spectrum showed the expected pyruvate and lactate C3 doublets at 2.25 and 1.33 ppm respectively with three observable species (no ^2H , mono- and bis-) affected by a secondary chemical shift effect of 0.02 ppm per ^2H nucleus. The ^2H spectrum showed the deuterated species including the presence of the perdeuterated lactate and pyruvate. The measured $J(^1\text{H}-^2\text{H})$ was 2.34 Hz.

Minimal kinetic isotope effects were detected in metabolism of ^{13}C - ^2H -enriched glucose to lactate

The lactate C3 (Figure 3) and lactate C2 (Figure 4, panel A and B) resonances are the positions of interest due to direct deuterium coupling in carbon C3 and an expected chemical shift isotope effect in both positions. The actual ratio of $[1,6-^{13}\text{C}]$ glucose relative to $[\text{U}-^{13}\text{C}]$ glucose in the perfusion medium, measured from the singlet/doublet ratio in glucose C1 in the ^{13}C NMR of the tissue extracts, was $1:1.00 \pm 0.05$. Consequently, in the lactate C3 position, the protonated species [B] (singlet) and [C] (doublet) should be present in a 1:1 ratio in control hearts. The observed ratio of these two species was $1:0.92 \pm 0.01$. This small deviation from 1 likely stems from a small kinetic isotope effect of $[\text{U}-^{13}\text{C}]$ glucose relative to $[1,6-^{13}\text{C}]$ glucose, likely at the level of aldolase[26].

In hearts supplied with $[1,6-^{13}\text{C}_2]$ glucose and $[\text{U}-^{13}\text{C}_6, \text{U}-^2\text{H}_7]$ glucose, isotopomer [C] is also present, demonstrating that ^2H displacement occurred since [C] cannot arise directly from metabolism of $[\text{U}-^{13}\text{C}_6, \text{U}-^2\text{H}_7]$ glucose. However, in lactate, this amount is rather small ($\sim 6\%$ of the total lactate pool, Table 1) and the majority of the lactate derived from $[\text{U}-^{13}\text{C}_6, \text{U}-^2\text{H}_7]$ was deuterated, species [D] and [E], as expected (Table 1). The ratio of the lactate species derived from $[1,6-^{13}\text{C}_2]$ glucose relative to $[\text{U}-^{13}\text{C}_6, \text{U}-^2\text{H}_7]$ glucose is similar to the control hearts with $1:0.95 \pm 0.02$ (Table 1).

Due to the extensive overlap in the proton-decoupled ^{13}C NMR spectrum, the individual deuterated species, [D] and [E], are not easily discernable as illustrated in Figure 3, but were analyzed with the knowledge gained from the ^{13}C coupling constants and secondary shifts seen in the reference mixture, described above. The resulting full assignments are shown in Figure 3. The doublet of triplets ([D]) and the doublet of pentets ([E]) were easily identified and quantified. Two additional resonances were also detected at 20.7 ppm and 20.56 ppm that do not follow in either of the patterns of [D] or [E], but could occur due to ^2H exchange at C2, causing secondary chemical shift isotope effects. The ^1H and ^2H NMR spectra of the extracts confirmed the observations of the ^{13}C NMR spectra and can be found in the supporting information.

Significant Kinetic Isotope Effects and Proton Substitution in Alanine

The carbon resonances of alanine should mirror those of lactate in terms of coupling constants and ^2H -induced isotope shifts. In control hearts, the ratio [B]: [C] ($[3-^{13}\text{C}_1]$ alanine: $[\text{U}-^{13}\text{C}_3]$ alanine) was $1:0.89 \pm 0.01$, essentially identical to the observation in lactate, again close but not identical to the expected 1:1 ratio. Since alanine and lactate

both exchange rapidly with pyruvate, essentially identical enrichment patterns were anticipated and observed in the absence of glucose deuteration.

Unexpectedly in the presence of [U- $^{13}\text{C}_6$,U- $^2\text{H}_7$]glucose, the relative concentrations of ^2H and ^{13}C species of alanine differed substantially from lactate (Figure 4) in two important respects. First, species [C], [U- $^{13}\text{C}_3$]alanine, was much more abundant than the corresponding ^{13}C labeling in lactate, indicating more extensive exchange of ^2H for ^1H in the methyl hydrogens, compared to lactate (Table 1). Second, in the hearts exposed to the mixture of [1,6- $^{13}\text{C}_2$]glucose and [U- $^{13}\text{C}_6$,U- $^2\text{H}_7$]glucose, the fraction of ^{13}C -labeled glucose derived from [U- $^{13}\text{C}_6$,U- $^2\text{H}_7$]glucose was significantly reduced, $1:0.57 \pm 0.02$. Together, these data demonstrate that metabolism of non-deuterated glucose to alanine and to lactate yielded essentially identical ^{13}C labeling patterns, as expected, but the presence of ^2H in glucose markedly altered metabolism of glucose to alanine by deuterium exchange and by reducing the fraction of alanine derived from uniformly-enriched glucose.

Oxidation of [U- $^{13}\text{C}_6$,U- $^2\text{H}_7$]glucose in the Citric Acid Cycle

Proton-decoupled ^{13}C NMR spectra of glutamate C4 and C3 are shown in Figure 5. Very little deuterated glutamate was observed in either resonance, but small shifted resonances in both C3 and C4 due to a chemical shift isotope effect from ^2H were clearly present. The ratio [2- $^{13}\text{C}_1$]acetyl-CoA / [1,2- $^{13}\text{C}_2$]acetyl-CoA, determined by a “direct analysis” of the glutamate C4 resonance [(S+D34)/(D45+Q)] [21], was $1:0.89 \pm 0.04$ in hearts supplied with a 1:1 mixture of [1,6- $^{13}\text{C}_2$]glucose plus [U- $^{13}\text{C}_6$]glucose, similar to the ratios observed for alanine and lactate. However, the C4 spectrum from hearts supplied with a 1:1 mixture of [1,6- $^{13}\text{C}_2$]glucose plus [U- $^{13}\text{C}_6$, U- $^2\text{H}_7$]glucose was dominated by glutamate isotopomers derived from [1,6- $^{13}\text{C}_2$]glucose, the singlet and doublet due to $J_{3,4}$. In this group of hearts, the ratio [2- $^{13}\text{C}_1$]acetyl-CoA / [1,2- $^{13}\text{C}_2$]acetyl-CoA was $1:0.34 \pm 0.1$, indicating that the oxidation of [U- $^{13}\text{C}_6$,U- $^2\text{H}_7$]glucose in the TCA cycle was significantly inhibited compared to [U- $^{13}\text{C}_6$]glucose. The C3 resonance experienced similar chemical shift isotope effects as the lactate and alanine C2 resonances.

DISCUSSION

Isotope substitution experiments are widely used to probe essential biological processes. With replacement of ^{13}C for ^{12}C , the mass change is relatively small and a common implicit assumption is the absence of significant ^{13}C isotope effects on enzyme-catalyzed reactions [27]. In other studies, the ^{13}C isotope effect in carbon-carbon bond-breaking reactions was small, 1–3%, based on results from aldolase in rabbit muscle [26], malic enzyme in chicken liver [28], and 6-phosphogluconate dehydrogenase in bacteria [29–31]. Others, however, have concluded that corrections for ^{13}C isotope effects are necessary in specialized systems [32,33]. In contrast to the replacement of ^{13}C for ^{12}C , substitution of ^2H for ^1H essentially doubles the mass of the hydrogen. Since there is a large percentage change in mass, significant slowing of bond-breaking reactions is generally observed in individual reactions, and a significant reduction of flux through a multi-step reaction in vivo such as glycolysis might be anticipated. Since only a single carbon-carbon bond is broken in glycolysis, it was surprising to find a small but detectable KIE due to ^{13}C in the current study. Conversely,

since multiple deuterium-carbon bonds are broken in glycolysis, a large ^2H kinetic isotope effect was anticipated yet little evidence of an effect of deuteration on conversion of glucose into lactate was found. These observations suggest that the appearance of hyperpolarized $[\text{U-}^{13}\text{C}]\text{lactate}$ from $[\text{U-}^{13}\text{C}_6, \text{U-}^2\text{H}_7]\text{glucose}$ is slightly inhibited by a small ^{13}C kinetic isotope effect but is essentially not impacted by the presence of deuterium.

Detection of Kinetic Isotope Effects by Analysis of Lactate

To analyze the potential KIE of perdeuterated glucose in glycolysis, it was necessary to consider all possible combinations of ^{13}C and ^2H that could occur in pyruvate derived from $[1,6\text{-}^{13}\text{C}_2]\text{glucose}$, $[\text{U-}^{13}\text{C}_6]\text{glucose}$, and $[\text{U-}^{13}\text{C}_6, \text{U-}^2\text{H}_7]\text{glucose}$. These possibilities are shown in Figure 1 and were measured by analysis of both alanine and lactate. Due to the complexity of the multiplets in C3 of both lactate and alanine, it was more accurate to analyze the signals in the C2 resonance shifted by chemical shift isotope effects (Figure 4). Using the ratios obtained for the shifted resonances (see Figures 3 and 4), it is straightforward to estimate the relative concentrations of the three uniformly ^{13}C labeled species (species [C], [D], and [E]). In lactate, the ratio of the protonated species to the mono- ^2H to the bis- ^2H species ([C]:[D]:[E]) was 0.20:0.25:0.55. The predominant species is [E], while there is an almost equivalent distribution of [C] and [D]. In principle, the production of [D] and [E] should be 1:1 because the C6 carbon of glucose carries two ^2H while the C1 carbon of glucose has only one ^2H . The relative concentration 1:1 ratio of [D] over [E] should be reflected in both lactate and alanine. However, the presence of species [C] confirmed a process whereby one or more deuterium atoms exchange with solvent protons. From the relative concentrations of lactate species, it appears that the species [D] is selectively targeted for this ^2H displacement reaction, as the concentration of [D] is much smaller than that of [E]. This is further confirmed by the relative concentration both species [C] and [D] being about the same as the total of species [E].

In the presence of perdeuterated glucose, the ratio between lactate species [B] relative to [C] + [D] + [E] should be sensitive to any KIE in glycolysis because [B] is derived solely from $[1,6\text{-}^{13}\text{C}_2]\text{glucose}$ while [C], [D] and [E] are derived exclusively from $[\text{U-}^{13}\text{C}_6, \text{U-}^2\text{H}_7]\text{glucose}$. Here, the ratio was 1:0.95, which indicates that deuteration does not have a substantial impact on the overall glycolytic flux to lactate. Presumably selective ^2H displacement occurs in the alanine transaminase reaction, followed by conversion of pyruvate to lactate. ^2H displacement has been observed previously in alanine transaminase (ALT) [34].

Proton Exchange and Kinetic Isotope Effects in Alanine Aminotransferase

Since the consequences of ^2H - ^{13}C coupling and chemical shift effects are essentially identical in alanine and lactate, a similar analysis was applied to alanine. A striking difference in the distribution of ^2H and ^{13}C isotopomers in alanine, compared to lactate, was apparent in two respects. First, the ratio of alanine derived from the internal standard $[1,6\text{-}^{13}\text{C}_2]\text{glucose}$ ([B]) relative to alanine derived from the $[\text{U-}^{13}\text{C}_6, \text{U-}^2\text{H}_7]\text{glucose}$ ([C],[D] and [E]) was 1:0.57, much lower than the 1:0.95 calculated for lactate (Table 1). This observation, based exclusively on measurements of ^{13}C isotopomers, indicates a marked effect of ^2H on alanine transaminase. Earlier solution studies [25] of the reaction mechanism

of ALT have suggested formation of an enamine derivative that requires breaking of a ^{13}C - ^2H bond in pyruvate. Based on typical effects of deuteration in biological reactions, a substantial KIE is expected. A second and related phenomenon was the marked difference in the relative concentration of deuterated species in alanine compared to lactate, apparent when comparing the C2 resonance in the ^{13}C NMR spectrum (Figure 4, [C] and [D]). In alanine, the ratio of the three species [C]:[D]:[E] was 0.68:0.24:0.08. In comparison to lactate C2, the bis-deuterated species [E] was markedly reduced while the protonated species [B] is the majority. This indicates that removal of one or more deuterium atoms occurs during conversion of pyruvate to alanine via ALT.

In the presence of $[\text{U-}^{13}\text{C}_6, \text{U-}^2\text{H}_7]\text{glucose}$, the ^{13}C and ^2H isotopomers of lactate and alanine differ substantially. It is generally assumed, because of the high activity of alanine aminotransferase and lactate dehydrogenase relative to flux through pyruvate, that tracer labeling in pyruvate is reflected equivalently in both alanine and lactate. However some earlier studies indicate that pyruvate is compartmented in the heart and that the tracer labeling of alanine and lactate are not equivalent [35–38]. Due to the additional information provided by the ^2H labels, it is possible to investigate which of the two pools reflect pyruvate that enters the mitochondria. Previous reports have shown that the labeling patterns in alanine but not lactate reflect the labeling pattern of pyruvate entering mitochondria and participating in the generation of acetyl-CoA [35,39,40].

Metabolism of Pyruvate to Acetyl-CoA and Glutamate

The deuterium label can only be retained in the C4 position of glutamate in all turns of the TCA cycle (Figure S3 in the Supporting Information). However, a 2-bond isotope chemical shift should be visible in the C3 position, similar to C2 of lactate and alanine. The C3 and C4 resonances of glutamate are highlighted in Figure 5. While deuterium coupling is observable in the C4 position, its analysis and the differentiation between the species is made difficult by the limited signal to noise ratio and complex nature of the C4 multiplet. However, the C3 position shows similar behavior to the C2 position of lactate and alanine. By applying the 3-bond chemical shift isotope effect information (0.02 ppm per ^2H) that was obtained previously, it is possible to characterize all resonances and distinguish between the [D] and the [E] species. It also allowed a determination of the relative concentrations of all species. The C3 resonance multiplet consists of a singlet derived from $[3\text{-}^{13}\text{C}_1]\text{pyruvate}$, a doublet due to ^{13}C coupling in C3 and C4 and a triplet due to coupling to both C2 and C4. However, the complexity of the multiplet still limits the analysis to the triplets of the fully labelled glutamate. The measured ratio of [C]:[D]:[E] was 0.74:0.019:0.07. Deuteration can also be observed for the other species (doublets), but cannot be analyzed quantitatively. Most of the glutamate detected by NMR was protonated (74%), only a small part of the glutamate derived from $[\text{U-}^{13}\text{C}_6, \text{U-}^2\text{H}_7]\text{glucose}$ was the deuterated species [D] or [E] (Table 1).

A direct ^{13}C isotopomer analysis of the glutamate C4 resonance showed that hearts presented with non-deuterated glucose derived an equivalent amount of acetyl-CoA from $[\text{U-}^{13}\text{C}_6]\text{glucose}$ and $[1,6\text{-}^{13}\text{C}_2]\text{glucose}$ as expected (Table 1). However in hearts presented with $[\text{U-}^{13}\text{C}_6, \text{U-}^2\text{H}_7]\text{glucose}$ plus $[1,6\text{-}^{13}\text{C}_2]\text{glucose}$, it was quite evident that less acetyl-CoA was derived from $[\text{U-}^{13}\text{C}_6, \text{U-}^2\text{H}_7]\text{glucose}$, consistent with a large KIE in the TCA

cycle. The difference can be directly observed in the C4 spectrum in Figure 4, and was quantified to be 1:0.34. In addition, the distribution of species of glutamate is similar to alanine with a high amount of protonated species [C] and a very low amount of the bis-deuterated species [E]. These observations are consistent with earlier reports that alanine but not lactate exchanges with the pyruvate pool undergoing decarboxylation by pyruvate dehydrogenase [35,40,41].

CONCLUSIONS

The effects of perdeuteration in glucose on metabolism was investigated using perfused hearts. The tissue extracts provided information by ^1H , ^{13}C and ^2H NMR spectroscopy. There was a minor effect of $[\text{U-}^{13}\text{C}_6]\text{glucose}$ vs. $[\text{1,6-}^{13}\text{C}_2]\text{glucose}$ attributed to a ^{13}C KIE. Even though multiple carbon-deuterium bonds are broken as glucose passes through glycolysis, there was no significant additional KIE of glucose to lactate, based on analysis of the lactate C3 and C2 resonances in the ^{13}C NMR spectrum. This result is, of course, important for interpretation of studies using hyperpolarized $[\text{U-}^{13}\text{C}_6, \text{U-}^2\text{H}_7]\text{glucose}$ and suggests that the appearance of hyperpolarized ^{13}C -enriched lactate accurately reflects glycolytic flux. However, significant displacement of ^2H occurred in ALT and was confirmed in intact tissue for the first time, accompanied by an apparent slowing of flux into alanine and glutamate produced from $[\text{U-}^2\text{H}_7, \text{U-}^{13}\text{C}_6]\text{glucose}$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- New isotopomers methods developed to investigate deuterium kinetic isotope effects using ^{13}C NMR
- Mixture of glucoses used in perfused rat hearts allows analysis of relative fluxes
- Kinetic Isotope effect observed in alanine and glutamate

Figure 1.

Labelling patterns for metabolites in heart tissue extracts using a 1:1 mixture of [1,6- $^{13}\text{C}_2$]glucose and either [U- $^{13}\text{C}_6$]glucose (Species [A] to [C]) in the control hearts or [U- $^{13}\text{C}_6$,U- $^2\text{H}_7$]glucose (Species [A] to [E]) with position numbering below. A black sphere marks a labelled position. The ^{13}C coupling pattern of the C3 position of pyruvate is shown on the right.

Figure 2.

Proton decoupled ^{13}C NMR spectrum showing the C3 of ^{13}C and ^2H enriched lactate in H_2O . Four different deuterium isotopomers were present: $[3\text{-}^{13}\text{C}_1]\text{lactate}$, $[3\text{-}^{13}\text{C}_1, 3\text{-}^2\text{H}_1]\text{lactate}$, $[3\text{-}^{13}\text{C}_1, 3,3\text{-}^2\text{H}_2]\text{lactate}$, and $[3\text{-}^{13}\text{C}_1; 3,3,3\text{-}^2\text{H}_3]\text{lactate}$. The multiplets due to $^2\text{H}\text{-}^{13}\text{C}$ scalar coupling are shown.

Figure 3.

Proton decoupled ^{13}C NMR spectra showing the C3 signal from lactate at ~20.9 ppm. The upper panel is from a heart supplied with $[1,6-^{13}\text{C}_2]\text{glucose}$ plus $[\text{U-}^{13}\text{C}_6, \text{U-}^2\text{H}_7]\text{glucose}$. All assignments arising due to $^2\text{H-}^{13}\text{C}$ scalar coupling are shown, with labeling taken from Figure 1. The lower panel shows the ^{13}C NMR spectrum of lactate methyl carbon from a heart supplied with equimolar $[1,6-^{13}\text{C}_2]\text{glucose}$ plus $[\text{U-}^{13}\text{C}_6]\text{glucose}$. The * indicates resonances not part of species [A] to [E]

Figure 4.

Proton decoupled ^{13}C NMR spectrum of the C2 position of lactate and alanine. Panel (a) is from a heart supplied with $[1,6\text{-}^{13}\text{C}_2]\text{glucose}$ plus $[\text{U-}^{13}\text{C}_6]\text{glucose}$. As expected, the doublet of doublets due to $[\text{U-}^{13}\text{C}_3]\text{lactate}$ arising from $[\text{U-}^{13}\text{C}_6]\text{glucose}$ was easily observed. Panel (b) is from a heart supplied with $[1,6\text{-}^{13}\text{C}_2]\text{glucose}$ plus $[\text{U-}^{13}\text{C}_6, \text{U-}^2\text{H}_7]\text{glucose}$. Each doublet of doublets (or quartet) is sensitive to ^2H in the methyl position. Three quartets were observed, reflecting 0, 1 or 2 deuteriums in the methyl group. Panels (c) and (d) show the corresponding spectra of C2 of alanine. The dashed lines are a visual aid to identify the $[\text{C}]$ resonances.

Figure 5.

Proton decoupled ^{13}C NMR spectra showing the carbon 3 (27.7 ppm) and carbon 4 (34.2 ppm) regions of glutamate. Typical 9-line spectra in C4 reflect a singlet (S) due to $[4-^{13}\text{C}]$ glutamate, a doublet (D34) due to $[3,4-^{13}\text{C}]$ glutamate, a doublet (D45) due to $[4,5-^{13}\text{C}]$ glutamate, and a doublet of doublets or quartet (Q) due to $[3,4,5-^{13}\text{C}]$ glutamate. Similar assignments in the C3 are singlet (S), a doublet (D) due to the sum of $[3,4-^{13}\text{C}]$ glutamate plus $[2,3-^{13}\text{C}]$ glutamate, and a triplet (T) due to $[2,3,4-^{13}\text{C}]$ glutamate.

Overview of the relative concentrations of species [A] through [E] in both lactate and alanine as analyzed by the ¹H and ¹³C NMR spectrum calculated from the ratios from the ¹³C NMR spectrum C2 position [C–E], the ¹H NMR spectrum [A] and the C3 position [B]. The errors for species [A] are less than 30%, while for [B] through [E] the ratio they are contained within 10%. Expect the values marked with a *, where the error is 30%.

Table 1

Relative Concentrations						
Glucose + [1,6- ¹³ C ₂]	Metabolite	[A]	[B]	[C]	[D]	[E]
[U- ¹³ C ₆]	Lactate	0.448	0.287	0.264	x	x
	Alanine	0.427	0.304	0.270	x	x
	Glutamate	0.583	0.221	0.196	x	x
[U- ¹³ C ₆ , U- ² H ₇]	Lactate	0.376	0.323	0.060	0.076	0.168
	Alanine	0.300	0.446	0.168	0.065	0.023
	Glutamate	0.595	0.292	0.083	0.021	0.08
						1:0.33*