Mass spectrometry-based microassay of $^2$H and $^{13}$C plasma glucose labeling to quantify liver metabolic fluxes in vivo

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Hasenour CM, Wall ML, Ridley DE, Hughey CC, James FD, Wasserman DH, Young JD. Mass spectrometry-based microassay of $^2$H and $^{13}$C plasma glucose labeling to quantify liver metabolic fluxes in vivo. Am J Physiol Endocrinol Metab 309: E191–E203, 2015. First published May 19, 2015; doi:10.1152/ajpendo.00003.2015.—Mouse models designed to examine hepatic metabolism are critical to diabetes and obesity research. Thus, a microscale method to quantitatively assess hepatic glucose and intermediary metabolism in conscious, unrestrained mice was developed. $[1^{3}$C$]_2$propionate, $[2^{1}$H$]_2$water, and $[6,6-{2^{1}$H$}_2]$glucose isotopes were delivered intravenously in short-(9-h) and long-term-fasted (19-h) C57BL/6J mice. GC-MS and mass isotopomer distribution (MID) analysis were performed on three 40-μl arterial plasma glucose samples obtained during the euglycemic isotopic steady state. Model-based regression of hepatic glucose and citric acid cycle (CAC)-related fluxes was performed using a comprehensive isotopomer model to track carbon and hydrogen atom transitions through the network and thereby simulate the MIDs of measured fragment ions. Glucose-6-phosphate production from glycogen diminished, and endogenous glucose production was exclusively gluconeogenic with prolonged fasting. Gluconeogenic flux from phosphoenolpyruvate (PEP) remained stable, whereas that from glycerol modestly increased from short- to long-term fasting. CAC fluxes were measured using mass isotopomer distributions (MIDs) of six glucose fragment ions, mass isotopomer distributions (MIDs) of six glucose fragment ions were measured by gas chromatography-mass spectrometry (GC-MS) analysis of a single 40-μl plasma glucose sample. Chronic catheterization allowed for multiple plasma samples to be obtained over time from conscious, unrestrained short- (9-h) and long-term-fasted (19-h) mice. Serial sampling permits confirmation of isotopic steady state and maximizes the precision of glucose MID measurements. Unlike NMR, positional $^2$H and $^{13}$C enrichments of plasma glucose cannot be determined directly by GC-MS analysis. To circumvent this drawback, plasma glucose samples were converted into three separate derivatives to produce unique GC-MS fragment ions previously validated for accuracy and precision (4, 59). Analysis of these fragment ions provided 29 independent mass isotopomer abundances that were regressed using a comprehensive isotopomer model to simultaneously quantify in vivo fluxes through seven independent reactions involved in liver glucose and CAC metabolism. Relative and absolute fluxes obtained from fasted C57BL/6J mice in these studies align with those previously measured using NMR techniques (13, 51, 54). The large amount of mass isotopomer data obtained from each sample results in a least-squares solution that is highly overdetermined and can be statistically assessed to detect errors in measurements or model formulation. Furthermore, our computational approach can accommodate a broad range of modeling assumptions, isotopomer tracers, and measurement inputs without the need to introduce ad hoc mathematical approximations. As

MOUSE MODELS OF HUMAN DISEASE have become a sine qua non of biomedical research. This is evident in diabetes research, where specialized techniques have been adapted to the conscious mouse to dissect the influence of genes, hormones, pharmaceutical agents, and other factors in regulating in vivo glucose metabolism (5, 17, 26). Metabolic research has been further bolstered by the development of nuclear magnetic resonance (NMR)-based approaches for quantifying intermediary fluxes in a number of tissues in vivo. $^{13}$C-glucose, $[2^{1}$H$]_2$water, and $^{13}$C-propionate administration to humans (57) or rats (28, 30), followed by NMR analysis of isotopically labeled blood glucose, has been developed previously to assess in vivo glucose and citric acid cycle (CAC) fluxes in the liver. The application of these methods to the mouse in vivo has been used to investigate metabolic disease progression (51) as well as the effect of murine strain on the coupling of energy and glucose-producing pathways in the liver (13). Flux analysis applied to transgenic mice has the potential to transform paradigms of genetic control over hepatic metabolism in physiological and pathophysiological states in vivo. Streamlining NMR methods for application to the large number of mouse models of metabolic disease is constrained by equipment costs and lengthy sample analyses. Furthermore, the low sensitivity of NMR requires plasma volumes (~0.5 ml) from the mouse that obviate replicate steady-state sampling, induce stress, and render tissues unsuitable for subsequent analyses (13, 51).

The method presented herein eliminates many of the barriers that hinder the application of $^2$H/$^{13}$C flux analysis to the conscious mouse. After infusion of a combination of stable isotope tracers similar to that used by Satapati et al. (51) (i.e., $[6,6-{2^{1}$H$}_2]$glucose, $[2^{1}$H$]_2$water, and $[^{13}$C$]_2$propionate), mass isotopomer distributions (MIDs) of six glucose fragment ions were measured by gas chromatography-mass spectrometry (GC-MS) analysis of a single 40-μl plasma glucose sample. Chronic catheterization allowed for multiple plasma samples to be obtained over time from conscious, unrestrained short- (9-h) and long-term-fasted (19-h) mice. Serial sampling permits confirmation of isotopic steady state and maximizes the precision of glucose MID measurements. Unlike NMR, positional $^2$H and $^{13}$C enrichments of plasma glucose cannot be determined directly by GC-MS analysis. To circumvent this drawback, plasma glucose samples were converted into three separate derivatives to produce six unique GC-MS fragment ions previously validated for accuracy and precision (4, 59). Analysis of these fragment ions provided 29 independent mass isotopomer abundances that were regressed using a comprehensive isotopomer model to simultaneously quantify in vivo fluxes through seven independent reactions involved in liver glucose and CAC metabolism. Relative and absolute fluxes obtained from fasted C57BL/6J mice in these studies align with those previously measured using NMR techniques (13, 51, 54). The large amount of mass isotopomer data obtained from each sample results in a least-squares solution that is highly overdetermined and can be statistically assessed to detect errors in measurements or model formulation. Furthermore, our computational approach can accommodate a broad range of modeling assumptions, isotopomer tracers, and measurement inputs without the need to introduce ad hoc mathematical approximations. As
a result, the methodology can be readily adapted to study other nutritional and genetic states in the in vivo mouse.

**Glossary: Metabolites and Central Reactions in the Flux Model**

- AcCoA: Acetyl-CoA
- Akg: α-Ketoglutarate
- BPG: 1,3-Bisphosphoglycerate
- Cit: Citrate
- DHAP: Dihydroxyacetone phosphate
- F6P: Fructose-6-phosphate
- FBP: Fructose-1,6-bisphosphate
- G6P: Glucose-6-phosphate
- GA3P: Glyceraldehyde-3-phosphate
- Glc/Gluc: Glucose
- Lac: Lactate
- Oac: Oxaloacetate
- PEP: Phosphoenolpyruvate
- PropCoA: Propionyl-CoA
- Pyr: Pyruvate
- Succylin-CoA: Succinyl-CoA
- VCS: Flux from Oac and AcCoA to Cit
- VEndoRa: Endogenous glucose production
- VEnol: Flux from PEP to BPG
- VGK: Flux from glycerol to DHAP
- VInf: [6,6-2H2]glucose infusion
- VLDH: Non-PEP-derived, unlabeled sources of anaplerosis to Pyr
- VPC: Flux from Pyr to Oac
- VPCC: Flux from PropCoA to SuccCoA
- VPCK: Flux from Oac to PEP
- VPK + ME: Contribution of pyruvate kinase (PK) and malic enzyme (ME) to Pyr
- VPGYGL: Flux from glycogen to G6P
- VSDH: Flux from SuccCoA to Oac

**In Vivo Procedures in the Mouse**

All procedures were performed with approval from the Vanderbilt Animal Care and Use Committee. Eight-week-old male C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME) and studied 2–3 wk after arrival. Mice were kept on a 12:12-h light-dark cycle in a temperature- (23°C) and humidity-stable environment and maintained on a standard chow diet (5L0D LabDiet, St. Louis, MO) with free access to water. Mice were chronically catheterized ~1 wk prior to study, as described previously (5). Briefly, catheters were implanted in the left common carotid artery and jugular vein for sampling and infusing, respectively. Mice were individually housed for postsurgical recovery and monitored for signs of distress.

On the day of the study, mice were placed in bedded containers without food or water and connected to sampling and infusion lines. An 80-μl arterial blood sample was drawn to determine the natural isotopic abundance of circulating glucose after 5 or 15 h of fasting. Directly after the baseline sample, a bolus of [2H2]water (99.9%) was delivered over 25 min to enrich total body water to 4.5%. A [6,6-2H2]glucose prime (440 μmol/kg) was dissolved in the bolus. Following the prime, [6,6-2H2]glucose was administered as a continuous infusion (4.4 μmol·kg⁻¹·min⁻¹) for the duration of the study. Sodium [13C3]propionate (i.e., [13C3]propionate (99%)) was delivered as a primed, continuous infusion starting 120 min after the [2H2]water bolus (Fig. 1A). All infusates were prepared in a 4.5% [2H2]water-saline solution unless otherwise specified. To test the sensitivity limits of the present technique, a graded reduction in the [13C3]propionate bolus and infusion was administered in three separate groups of long-term-fasted mice (1.10 mmol/kg + 0.035 mmol·kg⁻¹·min⁻¹ (standard), 0.55 mmol/kg + 0.027 mmol·kg⁻¹·min⁻¹ (one-half), and 0.28 mmol/kg + 0.014 mmol·kg⁻¹·min⁻¹ (one-fourth)) over a 120-min time course. For a single long-term-fasted mouse, plasma samples were collected 30 min earlier (~18–18.5 h of fasting); this difference did not result in appreciable differences in flux estimates. Stable isotopes were obtained from Cambridge Isotope Laboratories (Tewksbury, MA). Blood glucose was monitored (AccuCheck; Roche Diagnostics, Indianapolis, IN) and donor erythrocytes were infused to maintain hematocrit throughout the study (Fig. 1, A and B). Three blood samples (80 μl each) were collected in a 30-min phase 90 min after the [13C3]propionate prime was initiated. Arterial blood samples were centrifuged in EDTA-coated tubes for plasma isolation, and the
three 40-μl samples were stored at −20°C prior to glucose derivatization and GC-MS analysis. The duration between the \( [\text{H}_2] \) water bolus ([6,6-\( \text{H}_2 \)]) glucose prime and steady-state isotopic sampling was 3.5 to 4 h. Mice were rapidly euthanized through cervical dislocation immediately after the final steady-state sample.

**Preparation of Glucose Derivatives**

Plasma samples were divided into three aliquots and derivatized separately to obtain di-O-isopropylidene propionate, aldonitrile pentapropionate, and methylxime pentapropionate derivatives of glucose. For di-O-isopropylidene propionate preparation, proteins were precipitated from 20 μl of plasma using 300 μl of cold acetone, and the protein-free supernatant was evaporated to dryness in screw-cap culture tubes. Derivatization proceeded as described previously (4) to produce glucose 1,2,5,6-di-isopropylidene propionate. For aldonitrile and methylxime derivatization, proteins were precipitated from 10 μl of plasma using 300 μl of cold acetone, and the protein-free supernatants were evaporated to dryness in microcentrifuge tubes. Derivatizations then proceeded as described previously (4) to produce all derivatives. All derivatizations were evaporated to dryness, dissolved in 100 μl of ethyl acetate, and transferred to GC injection vials with 250-μl glass inserts for GC-MS analysis.

**GC-MS Analysis**

GC-MS analysis was performed using an Agilent 7890A gas chromatography system with an HP-5 ms (30 m × 0.25 mm × 0.25 μm; Agilent J & W Scientific) capillary column interfaced with an Agilent 5975C mass spectrometer. For optimal signal to noise, injection volumes were varied between 1 and 5 μl with purge flow times between 30 and 60 s. Samples were injected into a 270°C injection port in splitless mode. Helium flow was maintained at 0.88 ml/min. For analysis of di-O-isopropylidene and aldonitrile derivatives, the column temperature was held at 80°C for 1 min, ramped to 200°C/min to 280°C, and held for 4 min and then ramped at 40°C/min to 325°C. For methylxime derivatives, the same oven program was used, except the ramp to 280°C was 10°C/min. After a 5-min solvent delay, the MS collected data in scan mode from m/z 300 to 320 for di-O-isopropylidene derivatives, m/z 100 to 500 for aldonitrile derivatives, and m/z 144 to 260 for methylxime derivatives. Each derivatization was integrated using a custom MATLAB function (2) to produce glucose 1,2,5,6-di-isopropylidene propionate. For aldonitrile and methylxime derivatizations, proteins were precipitated from 10 μl of plasma using 300 μl of cold acetone, and the protein-free supernatant was evaporated to dryness in microcentrifuge tubes. Derivatizations then proceeded as described previously (4) to produce glucose aldonitrile pentapropionate and glucose methylxime pentapropionate. All derivatizations were evaporated to dryness, dissolved in 100 μl of ethyl acetate, and transferred to GC injection vials with 250-μl glass inserts for GC-MS analysis.

**Metabolic Flux Analysis**

A model of hepatic glucose production (Fig. 2A and Table A1) was constructed using the INCA software package (65) (accessible at http://mfa.vueinnovations.com/mfa). The hydrogen and carbon transitions were defined for each reaction. The model is based on the reaction network of Jones and colleagues (31, 32) and assumes 1) full equilibration of four-carbon intermediates in the CAC, 2) complete equilibration between G6P and F6P by phosphoglucoisomerase, 3) complete equilibration between GA3P and DHAP by triose phosphate isomerase, 4) no entry of labeled carbon from acetyl-CoA, and 5) no re-entry of CO2 formed in the reaction network. The model does not assume isotopes are present at trace quantities but instead accounts for the production of all possible labeled species that can form within the reaction network of Table A1. The model consists of 19 biochemical reactions, 22 metabolite nodes, and 424 mass isotopomer balance equations. Because the model is based on steady-state isotopomer balances, it does not depend on metabolic pool sizes and cannot be used to assess intermediate concentrations. A full description of the reaction network, isotopomer modeling approach, and relative importance of individual MID measurements to the variance of each flux estimate is available in the APPENDIX and the Supplemental Material.
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Fig. 3. Average relative abundance of m/z 301 isotopomers at baseline (−125 min) and isotopic steady state (90, 100, and 110 min) for long-term-fasted mice in the standard (n = 7; A), one-half (n = 8; B), and one-fourth (n = 8; C) [13C3]propionate infusion groups. The y-axes show the relative uncorrected abundance of each isotopomer detailed in the legend from M + 0 (unlabeled, m/z 301) to M + 4 (m/z 305).

(Supplemental Material for this article can be found online at the AJP-Endocrinology and Metabolism website).

The MIDs obtained from integration of GC-MS ion traces were imported into INCA. Flux through each reaction (Fig. 2A) was estimated relative to VCS (fixed at 100) by minimizing the sum of squared residuals (SSR) between simulated and experimentally determined MIDs. Flux estimates were repeated 25 times from random initial values. Goodness of fit was assessed by a chi-square test, and 95% confidence intervals were computed by evaluating the sensitivity of the sum-of-squared residuals to variations in flux values (1). All fits were accepted based on a chi-square test (P = 0.05) with 22 degrees of freedom (i.e., the regressions were overdetermined by 22 measurements). Relative fluxes were converted to absolute values using the known [6,6-2H2]glucose infusion rate (Vinf; Fig. 2A) and mouse weights. Flux estimates for the steady-state samples were averaged to obtain a representative set of values for each mouse.

To test the fifth assumption (listed on the previous page), two additional reactions were added to the base model: 1) a CO2 source reaction [CO2 source (A) → CO2 (A)] and 2) a CO2 sink reaction [CO2 (A) → CO2 sink (A)]. The revised model includes CO2 as a balanced metabolite, and 13CO2 generated in the CAC or through pyruvate cycling can be fixed by propionyl-CoA carboxylase or pyruvate carboxylase. Fluxes were estimated, and fits were accepted as described above.

RESULTS

Determination of an Optimal [13C3]propionate Infusion Rate

Blood glucose concentrations were consistent in mice receiving the standard, one-half, or one-fourth [13C3]propionate infusion (Fig. 1B). The propionate prime induced a uniform, transient elevation (14–20%) in blood glucose that subsided within 25 min. Plasma glucose samples collected from individual mice at −125, 90, 100, and 110 min (Fig. 1A) were analyzed by GC-MS. Baseline samples (−125 min) produced MIDs that matched the theoretical distribution expected based on the natural abundance of stable isotopes. For example, the m/z 301 fragment, which retains all carbon and hydrogen atoms from the parent glucose molecule (Fig. 2B), had <0.5% root mean square error relative to its theoretical MID. The isotopic steady state of plasma glucose was achieved within 90 min following the [13C3]propionate bolus (Fig. 3, A–C).

All steady-state MIDs were reflective of [13C3]propionate dose-dependent trends in plasma glucose enrichment (Fig. 4, A–F). A graded decrease in unlabeled isotopomers (M + 0) resulted from the tiered increase in [13C3]propionate in all glucose fragments (Figs. 2B and 4, A–F). The [2H2]water bolus, as expected, led to similar relative abundances of M + 1 isotopomers that result from single 2H incorporation. However, the abundance of higher mass isotopomers (M + 2 to M + 4) trended with the [13C3]propionate dose. The relative abundance of M + 2 to M + 4 isotopomers was highest in the standard infusion group and lowest in the one-fourth infusion group (Fig. 4, A–F). The MIDs for all isotopic steady-state samples were imported into INCA, and fluxes were estimated in the isotopomer model (Fig. 2A and Table A1), as specified in Metabolic Flux Analysis.

[13C3]propionate flux into the CAC (VPCC) exhibited a significant, infusion rate-dependent trend (Fig. 5A) consistent with differences in the relative abundance of higher mass isotopomers in the glucose fragments (Fig. 4, A–F). Flux
through \( V_{\text{PCC}} \) was linearly correlated with the infusion rate \( (r^2 = 0.9981) \). Other fluxes in the CAC, i.e., \( V_{\text{CS}} \) and flux from succinyl-CoA to Oac \( (V_{\text{SDH}}) \), were also significantly correlated with the \( [13\text{C}] \)propionate infusion rate (Fig. 5A). Gluconeogenesis from PEP \( (V_{\text{Enol}}) \) and \( V_{\text{Enol}R} \), however, was identical in all groups (Fig. 5A); this result is consistent with comparable blood glucose levels observed during the isotopic steady state (Fig. 1B).

Fast duration in this study was sufficient to deplete liver glycogen in the mouse (5). However, the one-half and one-fourth \( [13\text{C}] \)propionate infusion groups displayed dose-dependent trends in flux from glycogen to G6P \( (V_{\text{PGY}}) \) and glycerol to DHAP \( (V_{\text{GL}}) \) (Fig. 5A). Reductions in glucose enrichment with one-half and one-fourth \( [13\text{C}] \)propionate infusion rates appeared to induce a model artifact impairing the resolution of \( V_{\text{PGL}} \) and \( V_{\text{PGY}} \). Higher-mass (M + 3 and M + 4) isotopomers of glucose form in the condensation of a \( ^{13}\text{C} \)-labeled triose-phosphate intermediate (derived from CAC) with an unlabeled triose-phosphate (potentially derived from glycerol). For many fragments, M + 3 and M + 4 isotopomers were reduced in the one-half infusion group and completely absent in the one-fourth infusion group (Fig. 4). The low abundance of these high-mass isotopomers likely contributed to the model’s inability to accurately determine \( V_{\text{PGY}} \) in the one-half and one-fourth infusion groups. Simulations confirmed that \( V_{\text{PGL}} \) and \( V_{\text{PGY}} \) are inversely correlated in the model; fixing \( V_{\text{PGY}} \) artificially low resulted in a proportional increase in \( V_{\text{PGL}} \), which mimicked the experimental effect. A similar issue was observed by Antoniewicz et al. (1) in their retrospective analysis of two previous \( [\text{U}^{-13}\text{C}] \)glucose infusion studies in humans, where decreased abundance of high-mass isotopomers was implicated as the cause of poor identifiability for \( V_{\text{PGY}} \) and \( V_{\text{PGL}} \).

Intragroup flux estimates were highly reproducible. This precision is reflected in the standard errors shown in Fig. 5A. Sensitivity analysis of the best-fit model estimates indicated a gradual loss of flux identifiability with decreasing \( [13\text{C}] \)propionate infusion rates. The 95% flux confidence intervals for samples in the standard infusion group were parabolic and narrow with a well-defined optimal point. Parabolic confidence intervals in the one-half infusion group were wider than those in the standard infusion group with less defined optimal points. Furthermore, confidence intervals for samples in the one-fourth infusion group were very long and flat on one or both sides of the optimal point, which was poorly defined. Representative confidence intervals for \( V_{\text{LDH}} \) are provided for each group in Fig. 5, B–D. Reductions in glucose enrichment likely obfuscated flux identifiability in the one-fourth and, to a lesser degree, one-half infusion rate groups (Fig. 4).

Although the results at all infusion rates were highly precise, the standard infusion rate was chosen for future experimentation due to its improved flux identifiability. The following section evaluates glucose and CAC-related fluxes using the standard infusion rate in short- and long-term-fasted C57BL/6J mice.

**Effect of Fasting Period**

Food was removed for 5 or 15 h prior to baseline sample acquisition (−125 min; Fig. 1A). Three plasma sample replicates were drawn from arterial circulation during the stable isotopic steady state between 3.5 and 4 h following the baseline
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Fig. 6. A: average blood glucose measurements taken over the experimental time course for short- (○ and solid line; n = 5) and long-term-fasted (● and dotted line; n = 7) mice. Data are presented as means ± SE and compared using a t-test with equal variance; *P < 0.05. B: average mass isotopomer distributions for fragment m/z 301 from baseline (−125 min) and isotopic steady-state (90, 100, and 110 min) samples from short-term-fasted mice (n = 5). C: comparison of the average relative abundance of m/z 301 isotopomers between the short- (n = 5) and long-term-fasted (n = 7) mice at isotopic steady state; data are presented as means ± SE. The standard [13C3]propionate infusion was administered to mice in both groups. At 90–110 min, mice in the short- and long-term groups were fasted −9 and 19 h, respectively. For B and C, the relative uncorrected abundance is shown for each mass isotopomer from m/z 301 (M + 0) to m/z 305 (M + 4).

sample (90, 100, and 110 min; Fig. 1A). Blood glucose measurements were taken throughout the course of the experiment. As anticipated, short-term-fasted mice had significantly higher blood glucose than those on a long-term fast (Fig. 6A). The [13C3]propionate prime (0 min; Fig. 6A) induced a similar, small transient increase in blood glucose that receded shortly after administration. Average blood glucose levels from 100 to 120 min were not different between the groups (Fig. 6A).

Plasma glucose samples obtained throughout the study were derivatized and analyzed by GC-MS. The average MID for the m/z 301 fragment confirmed steady-state enrichment of plasma glucose in both groups between 90 and 110 min (Figs. 3A and 6B). As expected, short- and long-term-fasted mice exhibited clear differences in stable isotopic labeling (Fig. 6C). The deviation in MIDs between the short- and long-term-fasting groups translated into differences in flux estimates following MFA (Fig. 7).

Increased fast duration provoked expected trends in the source of glucose produced from the liver (Fig. 7, A–C). Glycogen-derived G6P (VPYGL) was significantly diminished after long-term fasting; as a result, glycerol (VGK) and PEP (VEndoRa) were used exclusively by the long-term-fasted mice for hepatic glucose production (Fig. 7, A and B). This shift is also evident when fluxes are reported relative to VEndoRa (Fig. 7C). Changes in flux rates were consistent with the differences in enrichment observed in Fig. 6C.

VCS was reduced (78 to 59 μmol·kg⁻¹·min⁻¹) and pyruvate cycling increased with long-term fasting (Fig. 7B). Increased anaplerosis (VPCC) was balanced by cataplerotic flux (VPCP) with the return of PEP to pyruvate through VPCK + ME (Fig. 7). Although absolute fluxes were similar, relative anaplerosis from unlabeled metabolites (VLDH) increased with fast duration (Fig. 7C and Table 1). The absolute flux of [13C3]propionate into the CAC (VCCC) was not significantly different between the short- and long-term-fasting groups (Fig. 7, A and B). Absolute changes in CAC and related fluxes translated to changes in flux ratios (Table 1).

Effect of CO2 Recycling

Previous in vivo research suggests that reincorporation of respired 13CO2 can be significant in the liver (9). This violates an assumption of our model and of previous NMR studies (31, 32). An advantage of the isotopomering approach presented here is that the base model can be readily modified to test the effects of varying assumptions. Thus, our base model (Table A1) was revised to allow intrahepatic 13CO2 recycling, as described in Metabolic Flux Analysis. The MIDs from each mouse were then refitted to the revised model. The average fit to the model improved within each group, as indicated by a decrease in SSR compared with the base model (Table 2). The improvement in fit was most significant (≥50% decrease in SSR) for long-term-fasted mice. Although the predicted CO2 pool enrichment was not significantly different between short- and long-term-fasted mice receiving the standard [13C3]propionate infusion (Table 2), CO2 fixation was greater in the long-term-fasted mice (Fig. 7), allowing for a more significant change in MIDs and minimized SSRs. The enrichment of the CO2 pool trended with the infusion rate (Table 2), since CO2 is produced during the conversion of [13C3]propionate to succinyl-CoA.

CAC and pyruvate cycle fluxes changed when CO2 recycling was included in the model. Differences in CAC-related fluxes among the three [13C3]propionate infusion groups became insignificant (Fig. 8A), suggesting that the infusion rate may not significantly alter intermediary metabolism. As expected, the VPCC estimates showed an infusion rate-dependent change (Fig. 8A). Discrepancies in VGC and VPYGL were still apparent and related to low enrichment and poor flux identifiability in the one-half and one-fourth infusion groups (Fig. 8A). However, the infusion rate-dependent trends in VCS and VSDH that were previously observed (Fig. 5) were no longer significant when 13CO2 reincorporation was introduced into the model. This finding suggests that the presence of internal CO2 recycling may produce a quantitative bias in certain CAC fluxes when estimated using the base model. Despite this result, the qualitative differences observed between the short- and long-term-fasted mice did not change when glucose labeling data were analyzed using the revised model (Fig. 8B).

DISCUSSION

Technology to assess liver metabolic flux in the conscious, unrestrained mouse has lagged behind the development of mouse models of metabolic diseases. This paucity in sophisti-
cated flux analyses methods has forced investigators to rely on crude in vivo assays and static tissue measurements to decipher complex oxidative and glucose phenotypes. Such studies often infer metabolic pathway alterations indirectly from changes in enzyme expression rather than from direct measurements of metabolic fluxes. This can be misleading, as metabolic fluxes are controlled by substrate availability as well as allosteric feedback and covalent modifications. Also, mRNA or protein abundances often do not correlate strongly with pathway feedback and covalent modifications. Also, mRNA or protein expression rather than from direct measurements of enzyme expression. This can be misleading, as metabolic fluxes can be inferred from substrate availability and allosteric feedback, and are not directly measured.

Table 1. Relative effect of fasting on hepatic CAC-related fluxes in C57BL/6J mice

<table>
<thead>
<tr>
<th>Relative CAC-Related Fluxes</th>
<th>9-h Fast</th>
<th>19-h Fast</th>
</tr>
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<tbody>
<tr>
<td>PEP → BPG (V_{Enol}/V_{CS})</td>
<td>1.06 ± 0.03</td>
<td>1.59 ± 0.09</td>
</tr>
<tr>
<td>PEP → Pyruvate (V_{PK + ME}/V_{CS})</td>
<td>0.47 ± 0.11</td>
<td>1.44 ± 0.08</td>
</tr>
<tr>
<td>Lactate → Pyruvate (V_{LDH}/V_{CS})</td>
<td>0.84 ± 0.03</td>
<td>1.23 ± 0.08</td>
</tr>
<tr>
<td>Pyruvate → Oac (V_{PCK}/V_{CS})</td>
<td>1.31 ± 0.10</td>
<td>2.67 ± 0.13</td>
</tr>
<tr>
<td>Oac → PEP (V_{PCK}/V_{CS})</td>
<td>1.53 ± 0.11</td>
<td>3.03 ± 0.14</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE (n = 5–7) and compared using a t-test with equal variance. CAC, citric acid cycle; PEP, phosphoenolpyruvate; BPG, 1,3-bisphosphoglycerate; Oac, oxaloacetate. Relative hepatic fluxes related to the CAC were determined by dividing $V_{Enol}$, $V_{PK + ME}$, $V_{LDH}$, $V_{PC}$, and $V_{PCK}$ by flux through citrate synthase ($V_{CS}$). Comparisons were made between short- (9 h) and long-term-fasted (19 h) mice administered the standard [13C3]propionate infusion. $V_{Enol}$ was maintained in triose units for these calculations. *P < 0.05 vs. 9-h fast.

Table 2. Effect of CO2 recycling on SSR and simulated CO2 enrichment

<table>
<thead>
<tr>
<th>Fasting Period</th>
<th>[13C3]Propionate Infusion Rate</th>
<th>SSR without CO2 Recycling</th>
<th>SSR with CO2 Recycling</th>
<th>Simulated CO2 Enrichment, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Short Standard</td>
<td>6.1 ± 0.8</td>
<td>4.5 ± 0.3</td>
<td>4.8 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>Long Standard</td>
<td>26.2 ± 1.5</td>
<td>5.2 ± 0.8</td>
<td>5.2 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>Long One-half</td>
<td>18.9 ± 4.7</td>
<td>9.6 ± 4.3</td>
<td>4.2 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>Long One-fourth</td>
<td>6.3 ± 0.5</td>
<td>3.4 ± 0.3</td>
<td>2.3 ± 0.2</td>
<td></td>
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</tbody>
</table>

Values are expressed as mean enrichment ± SE; short/standard, n = 5; long/standard, n = 7; long/one-half, n = 8; long/one-fourth, n = 8. SSR, sum of squared residuals. Simulated mass isotope distribution. The best-fit model was compared with the experimental MIDs, and the SSR was calculated. SSRs for the 3 isotopic steady-state samples were averaged to obtain a representative value for each mouse. Shown here is the mean of the representative SSRs ± SE for each group fit to the base model (SSR without CO2 recycling) or to the revised model that allowed internal CO2 recycling (SSR with CO2 recycling). Enrichment of the hepatic CO2 pool (i.e., the relative abundance of [13C2]CO2) was simulated through the best-fit model for each mouse.
Fig. 8. Comparison of model-estimated fluxes with CO₂ recycling from long-term-fasted mice in the standard (black bars; n = 7), one-half (striped bars; n = 8), and one-fourth (open bars; n = 8) [¹³C₃]propionate infusion groups (A) and from short- (open bars; n = 5) and long-term-fasted (black bars; n = 7) mice administered the standard [¹³C₃]propionate infusion (B). MFA results within each group were averaged to obtain a representative set of values for each mouse. Data are presented as means ± SE. V_EOK and V_Eonol are reported in hexose units. Infusion groups were compared by 1-way ANOVA and Tukey-Kramer pairwise comparisons; letters above the bars indicate statistically separated groups. Fasting groups were compared using a t-test with equal variance. *P < 0.05.

Innovative Methodology

The absolute and relative rate of the predominant anaplerotic flux in our model (V_PC and V_PCE/NCS, respectively) increased with fast duration and was balanced by cataplerotic flux to PEP (V_PCK). Pyruvate carboxylase is critically reliant on acetyl-CoA for its activity (35, 58). Hepatic acetyl-CoA supply increases with prolonged fasting (56), and its production from β-oxidation is a putative mechanism for the stimulation of gluconeogenesis by fatty acids (19, 50, 62). Perfusion of the rat liver with octanoate, which bypasses extramitochondrial transport and presumably enters the β-oxidation sequence directly, increases the rate of pyruvate carboxylation relative to pyruvate dehydrogenation and citric acid cycle flux (19). Octanoate-mediated changes in relative anaplerosis induce a small increase in gluconeogenesis (19). However, no change in V_Eonol was observed in the transition from the 9- to 19-h fast in the conscious mouse despite an increment in anaplerosis/cataplerosis. In fact, flux to PEP was balanced by the return of carbon to pyruvate through V_PK + ME. The absolute and relative rate of pyruvate cycling observed in the 19 h fasted cohort is within the range calculated from NMR in the prolonged fasted mouse (13, 51, 54). Although very small, flux from propionate trended toward an increase with prolonged fasting.

The increase in V_PK + ME observed in the transition from a 9- to 19-h fast was an intriguing result. A body of literature substantiates glucagon’s inhibitory effect on flux through pyruvate kinase (10, 23, 27, 49). We hypothesized that V_PK + ME might diminish due to a fast-induced increase in glucagon. The results confirm a role for pyruvate cycling in the prolonged fasted mouse. The metabolic rate of the mouse leads to rapid changes in liver and whole body physiology with progressive fasting (5, 45, 46); increases in plasma glucagon and reductions in liver glycogen have been observed after 6 h of food removal in the mouse (45). Thus, the change in V_PK + ME with fasting may be indicative of glucagon’s evanescent effect in the liver (20). However, this speculation is a rather basic explanation for a set of complexly regulated reactions (21). Moreover, V_PK + ME represents total recycling of cataplerotic carbon to pyruvate and is not exclusively a reflection of pyruvate kinase. The relationship between pyruvate cycling and total anaplerosis/cataplerosis (V_PK + ME/V_PCK) observed here (~0.3 to 0.5) is comparable with the ratio observed elsewhere in the short- and long-term-fasted mouse (~0.3 to 0.6) (13, 51, 54). Pyruvate cycling appears pliable to physiological, pharmacological, and pathological changes in hepatic metabolism in humans (43, 57) and rodents (29, 41, 47, 51).

Physiological conditions characterized by sustained gluconeogenesis profoundly alter hepatic energy state in rodents (8, 15, 18, 56). A decrease in liver energy state supports AMP-activated protein kinase activation (7, 8, 15, 25), which promotes the β-oxidation of long-chain fatty acids in the mitochondria. The rise in acetyl-CoA (18, 56) and ketogenesis (51, 60) observed in fasting and exercise supports this thesis. Furthermore, proxy measurements of the NADH/NAD⁺ ratio indicate a more reduced state in the cytoplasmic and mitochondrial compartments of the hepatocyte with prolonged fasting (53, 61). A modest reduction in citrate synthase flux (V_CS) was observed in the 19-h-fasted mouse. This result is consistent with a depression in citrate synthase flux in isolated livers from brief and prolonged fasted rats (41). A comparison of the results from two separate in vivo studies in Sprague-Dawley rats exhibits a similar trend, although there are clear differences in study design (28, 30). As detailed by Williamson and Cooper (63), multiple mechanisms govern citrate synthase flux and the rate of the CAC. Accordingly, two negative feedback modifiers, intramitochondrial NADH/NAD⁺ and ATP, increase with hepatocyte exposure to glucagon (55).

Glucose production derived from PEP (V_Eonol) was similar during the 9- and 19-h fasts, yet a relative increase in gluconeogenesis (V_Eonol/V_CS) was observed. The results measured here (V_Eonol/V_CS = ~1.6) through GC-MS and metabolic flux analysis fall within the range recently obtained using NMR in...
prolonged fasting mice (1.5–1.9) (51). Concern over whether measurements of intermediary flux are compatible with the energy requirements of gluconeogenesis was once the subject of considerable debate (34, 38, 40). Landau (38) deduced that the inclusion of reducing cofactors generated through the β-oxidation of fatty acids, in addition to the complete oxidation of acetyl-CoA in the CAC, would yield sufficient ATP to meet the liver’s energy requirements in 60-h-fasted humans. Nevertheless, rodents experience a reduction in hepatic energy state in response to prolonged nutrient deprivation and exhaustive exercise (see aforementioned citations). Placing glucose-producing and CAC-related fluxes in the context of cytosolic and mitochondrial energy state in the conscious mouse is largely conjecture. Accurate measurements of hepatic adenine nucleotides from rodents requires freeze-clamping methods that prevent the distinction of compartmental concentrations (16).

Considerations and Critique of Study Design

The scientific value of flux analysis in mouse models with genetic and/or dietary manipulation cannot be overstated. Thus, the studies herein employed methods that improve the efficiency and accessibility of measurements of hepatic intermediary flux in the mouse. Stable isotopes were elected over radioactive tracers because they provide a much richer set of isotopomer data (36) required for simultaneous determination of all fluxes in our model. Because only 40 μl of plasma is required for GC-MS analysis (4), steady-state isotopic labeling of circulating glucose was determinable from samples acquired in series from the carotid arteries of conscious, unrestrained mice. Derivatization procedures require moderate benchtop effort and GC-MS analysis time, which equates to an average analysis rate of 12 plasma samples/day. Although artery and venous catheterization are not routine surgical procedures in all laboratories, they are feasible and becoming increasingly more common in the mouse. The dual-catheter model permits the analyses of other plasma and tissue parameters from a single mouse without stress (5).

The tracer cocktail administered in these studies capitalizes on previous work utilizing labeled propionate (31) and deuterated water (13, 32), which emerged following the foundational research of Landau and colleagues (38, 39) and Magnusson et al. (43). Our study inherits many of the strengths and weaknesses of previous isotopic flux methods (6, 9, 13, 14, 34, 38, 52). As described elsewhere (13, 31, 38), [13C3]propionate was selected for its avid hepatic extraction/utilization and afforded a reasonable basis of comparison with results obtained in the mouse using NMR. Nonetheless, the quantity of stable isotope required for sufficient tracee enrichment often results in a calculable effect on intermediary metabolism. For example, ingestion of 1.5 g of sodium [13C3]propionate is estimated to contribute between 6 and 12% of total anaplerotic flux in humans (76 kg) (33). The ratio of anaplerosis from propionate to total anaplerosis (Vpcc/Vpck) here is an average of 0.14 and 0.12 for 9- and 19-h-fasted mice, respectively. Halving the [13C3]propionate bolus and infusion led to a dose-dependent reduction in Vpcc and Vcs fluxes, the latter of which was abolished when CO2 recycling was admitted to the model. Despite the dose-dependent effects on anaplerosis from propionate, the infusion had no measurable effect on gluconeogenic flux from PEP (Vengol) or endogenous glucose production (VendoRa); to this end, circulating glucose was relatively stable throughout the study period. It should also be noted that the interpretation of the dose-dependent effects was complicated by low flux identifiability in the one-half and one-fourth infusion groups. Although concern is still warranted as to whether the quantity of labeled infusate masks subtle phenotypes in CAC-related fluxes, we found that fasting-mediated differences in intermediary fluxes are clearly distinguishable through the current technique.

\[ V_{PK} + ME \] flux encompasses the total return of carbon to pyruvate and does not solely reflect pyruvate kinase flux. Formation of labeled pyruvate may occur through several mechanisms, including sources that emanate from extrahepatic metabolism (e.g., Cori and alanine cycles). \( V_{LDH} \) requires similar attention. As presented here, flux through \( V_{LDH} \) represents the movement of unlabeled three-carbon metabolites to liver pyruvate. Furthermore, gluconeogenic cells other than liver parenchyma may contribute to the observed results. Elucidating limitations of the current approach provides avenues for further methodological optimization. For example, modi-
fying the base isotopomer model to allow internal recycling of labeled CO₂ resulted in better model fits and improved consistency in flux estimates across the three different infusion groups. Although the comparison between short- and long-term-fasted animals remained qualitatively similar, there were some quantitative differences between the fluxes produced by the two different models. Future work, including empirical measurement of liver CO₂ enrichment (perhaps through the analysis of urea labeling), will be necessary to unequivocally discriminate between models with/without CO₂ recycling.

Liver transaldolase activity has also been shown to have a limited effect on some prior measurements of glycogenolysis/gluconeogenesis obtained by NMR in moderately fasted subjects. Specifically, in studies contingent on the deuterated water method, an inability to account for the transaldolase reaction results in an overestimation of gluconeogenesis in the presence of glycogenolysis, which is demonstrated to be modest and correctable (11). The technique presented herein, however, is not solely reliant on the enrichment at the H2 and H5 positions for gluconeogenic and glycolytic fluxes. A heat map has been generated to display the relative importance of each mass isotopomer to flux estimates (Fig. A1). Several different MS fragment ions contribute to estimates of gluconeogenic (\( V_{FGK} \)) and glycolytic fluxes (\( V_{PITGL} \)) by the model. This redundancy is expected to impart increased robustness to the flux estimates because they depend on multiple independent measurements. As a result, the inclusion of transaldolase exchange in the model had no significant effect on flux estimates in either short- or long-term-fasted mice (not shown), justifying the omission of this reaction.

Flux estimation determined through a model-based regression of isotope-labeling measurements has formed the basis of many prior publications (22, 37, 42, 44). One disadvantage of a regression-based approach is that it does not enable the derivation of closed-form, analytical equations that relate the mass isotopomer measurements to estimated fluxes. On the other hand, this approach can accommodate a much broader range of modeling assumptions, isotopomer tracers, and measurement inputs without the need to introduce ad hoc mathematical approximations. In particular, our model can account for all possible labeled species produced within the network of Table A1 and simulate the full MIDs of all measurable glucose fragment ions. The effects of adding/removing reactions from the model or changing model assumptions can also be assessed without manually reformulating equations, since the INCA software package automates the steps of constructing and solving isotopomer balance equations for a given reaction network. As described in the preceding paragraphs, this allowed a direct assessment of the potential effects of CO₂ recycling and transaldolase exchange on flux estimates.

Methods to determine the enrichment of glucose from microvolumes of plasma (4) have broad applicability for assessing in vivo hepatic pathway fluxes. Unlike the approach presented by Antoniewicz et al. (4), the technique offered herein does not solve explicitly for the positional \( ^2H \) or \( ^13C \) enrichments based on MID measurements. Instead, a comprehensive isotopomer modeling approach has been developed to describe the movement of hydrogen and carbon atoms throughout the reaction network. All fluxes in the network are simultaneously estimated by regressing the model to the full set of MID measurements. The model generated for these studies is well suited to examine the effectiveness of other isotope tracers for estimating glucose and CAC fluxes in the liver. For example, tracer simulations\(^1\) utilizing \( ^13C_3 \)lactate yield relative fluxes similar to those obtained with \( ^13C_3 \)propionate, assuming the lactate pool in the liver is at least 50% enriched. The substitution of \( [6,6-^2H_2] - \) with \( [3,4-^{13}C_2] \)glucose should work equally well in estimating \( V_{EndoRa} \). However, simulations with \( [6,6-^2H_2] \)glucose and \( [^2H_2] \)water tracers alone cannot resolve fluxes below the PEP node in Fig. 2A. Determining the MIDs of other metabolites related to the model would 1) provide additional information to reduce the uncertainty of flux measurements and/or 2) allow for a model with greater complexity. Nonetheless, estimates from the current investigation have a high degree of confidence. Measurement of liver intermediary fluxes in varying genetic and nutritional states in the mouse is an attractive application for the current method.

APPENDIX

The reaction network used for in vivo metabolic flux analysis, shown schematically in Fig. 2A, is fully detailed in Table A1. The INCA software suite (65) automates the derivation of a mathematically exact system of isotopomer balance equations that defines the relationship between fluxes and measurable MIDs. A decomposition method is used to break isotopomers into elementary metabolite units (EMUs) to reduce computational burden (3). Each EMU includes a subset of metabolite atoms that are directly involved in precursor-product relationships, leading to measurable GC-MS fragment ions. EMUs are organized into size blocks, where size refers to the number of atoms included in the EMU. INCA employs further decoupling of large blocks based on a Dulmage-Mendelsohn matrix decomposition to increase computational efficiency (64). The full network of isotopomer balances breaks down into 108 EMUs organized into 13 EMU blocks. The EMU balance equation for each block can be expressed in matrix form: \( Ax + By = 0 \), where \( A \) and \( B \) are functions of fluxes and \( x \) and \( y \) are functions of EMU MIDs. The full set of EMU balance equations are depicted in Supplemental Table S1. In these equations, the fluxes in matrices \( A \) and \( B \) are defined in Table A1. Each EMU that comprises the \( x \) and \( y \) matrices is defined by the metabolite name and the subset of atoms it includes. EMU atom numbering is based on the atom transitions shown in Table A1. For example, carbon and hydrogen atoms in Gluc.ext are defined as AaBbCcDdEeFfg in the \( V_{EndoRa} \) model reaction. Atoms are numbered sequentially for EMU definitions, where \( A = 1, a = 2, B = 3, b = 4, \) etc. There are six Gluc.ext EMUs (Table A2) that correspond to the six measured glucose fragment ions shown in Fig. 2B. These measured EMUs are highlighted in bold in the EMU balance equations (Supplemental Table S1). The majority of pathway fluxes were informed by multiple MID measurements, as shown in Fig. A1.

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GRANTS

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\(^1\) In silico simulations indicate that \( [^13C_3] \)lactate and \( [3,4-^{13}C_2] \)glucose are sufficient to estimate fluxes in the current model with equivalent precision (as replacements for \( [^{13}C_3] \)propionate and \( [6,6-^2H_2] \)glucose, respectively) in combination with \( [^2H_2] \)water (M. L. Wall).
Table A1. Reaction network for metabolic flux analysis

<table>
<thead>
<tr>
<th>Flux</th>
<th>Reaction Network</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{EndoRa}$</td>
<td>$\text{G6P (AabBcDdEeFfg)} \rightarrow \text{Gluc.ext (AabBcDdEeFfg)}$</td>
</tr>
<tr>
<td>$V_{FGCL}$</td>
<td>$\text{Glycogen (AabBcDdEeFfg) + H (h)} \rightarrow \text{G6P (AabBcDdEeFfg) + H (h)}$</td>
</tr>
<tr>
<td>$V_{GPI}$</td>
<td>$\text{F6P (AabBcDdEeFfg) + H (h)} \rightarrow \text{G6P (AabBcDdEeFfg) + H (a)}$</td>
</tr>
<tr>
<td>$V_{ALD}^{-}$</td>
<td>$\text{DHAP (ChbAab) + GAP (DdEeFfg)} \rightarrow \text{G6P (AabBcDdEeFfg) + H (h)}$</td>
</tr>
<tr>
<td>$V_{ALDH}^{-}$</td>
<td>$\text{BPG (AabeCcd) + H (e) + H (f)} \rightarrow \text{GAP (AabeCcd) + 0.5*GAP (AabeCcd) + H (a) + H (b)}$</td>
</tr>
<tr>
<td>$V_{CK}$</td>
<td>$\text{Glycerol (AabBcCd) + H (f)} \rightarrow \text{GAP (AabeCcd) + 0.5*GAP (AabeCcd) + H (a) + H (b)}$</td>
</tr>
<tr>
<td>$V_{GAPDH}$</td>
<td>$\text{PEP (ABCbcd) + H (b)} \rightarrow \text{BPG (ABbcdC)}$</td>
</tr>
<tr>
<td>$V_{PEP}$</td>
<td>$\text{PEP (ABCab) + H (c)} \rightarrow \text{Pyr (ABCbcdC)}$</td>
</tr>
<tr>
<td>$V_{PC}$</td>
<td>$\text{Pyr (ABCbcdC) + CO2 (D) + H (f) + H (g)} \rightarrow \text{0.5*Oac (ABCfgD) + H (c) + H (d) + H (e)}$</td>
</tr>
<tr>
<td>$V_{PCK}$</td>
<td>$\text{Oac (ABCbcdC)} \rightarrow \text{PEP (ABcAb)} + \text{CO2 (D)}$</td>
</tr>
<tr>
<td>$V_{LDH}$</td>
<td>$\text{Lac (ABCbcCd)} \rightarrow \text{Pyr (ABCbcdC) + H (b)}$</td>
</tr>
</tbody>
</table>

CAC Reactions

- $V_{CE}$: $\text{Oac (AabeCDdC) + AcCoA (EFgh)} \rightarrow \text{Cit (DBcdEFghA) + H (h)}$
- $V_{DH}$: $\text{Cit (ABabCDeD) + H (e)} \rightarrow \text{Akg (ABCabdCde)} + \text{CO2 (F)}$
- $V_{OCDH}$: $\text{Akg (ABCabdCde)} \rightarrow \text{SucCoA (BCabdCde) + CO2 (A)}$
- $V_{SDH}$: $\text{SucCoA (ABCabcCde) + H (e) + H (f)} \rightarrow \text{0.5*Oac (ABCfd) + 0.5*Oac (DBcfa) + H (a) + H (b) + H (c) + H (d)}$

Infusates

- $V_{Inf}$: $\text{Gluc.inf (AabBcDdEeFfg)} \rightarrow \text{Gluc.ext (AabBcDdEeFfg)}$
- $V_{Inf}$: $\text{H.inf (a)} \rightarrow \text{H (a)}$
- $V_{Inf}$: $\text{H.inf (a)} \rightarrow \text{H (a)}$
- $V_{Inf}$: $\text{H.inf (a)} \rightarrow \text{H (a)}$
- $V_{Inf}$: $\text{H.inf (a)} \rightarrow \text{H (a)}$

Metabolites with Equivalent Hydrogen Atoms

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Carbon Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>AcCoA</td>
<td>2</td>
</tr>
<tr>
<td>Lactate</td>
<td>3</td>
</tr>
<tr>
<td>PropCoA</td>
<td>3</td>
</tr>
<tr>
<td>Pyr</td>
<td>3</td>
</tr>
</tbody>
</table>

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Innovative Methodology


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