Novel application of the “doubly labeled” water method: measuring CO₂ production and the tissue-specific dynamics of lipid and protein in vivo

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Bederman, Ilya R., Danielle A. Dufner, James C. Alexander, and Stephen F. Previs. Novel application of the “doubly labeled” water method: measuring CO₂ production and the tissue-specific dynamics of lipid and protein in vivo. Am J Physiol Endocrinol Metab 290: E1048–E1056, 2006. First published December 20, 2005; doi:10.1152/ajpendo.00340.2005.—The partitioning of whole body carbon flux between fat and lean compartments affects body composition. We hypothesized that it is possible to simultaneously determine whole body carbon (energy) balance and the dynamics of lipids and proteins in specific tissues in vivo. Growing C57BL/6J mice fed a high-fat low-carbohydrate diet were injected with a bolus of “doubly labeled” water (i.e., ²H₂O and H₂¹⁸O). The rate of CO₂ production was determined under conditions of non-steady-state precursor labeling, we hypothesized that it would be possible to measure lipid and protein dynamics after the administration of a bolus of ²H₂O. This modification offers a major advantage, since one can simultaneously administer a bolus of H₂¹⁸O and determine the rate of CO₂ production from the difference between the rates of elimination of ²H and ¹⁸O from body water. Because kinetic parameters can be determined during conditions of non-steady-state precursor labeling, we hypothesized that it would be possible to measure lipid and protein dynamics after the administration of a bolus of ²H₂O. This modification offers a major advantage, since one can simultaneously administer a bolus of H₂¹⁸O and determine the rate of CO₂ production from the difference between the rates of elimination of ²H and ¹⁸O from body water. We report here on a novel application of the “doubly labeled” water method. We demonstrate that by coupling the doubly labeled water method with measurements of ²H incorporation to specific end products, it is possible to 1) estimate whole body carbon balance by comparing dietary intake and CO₂ production and 2) determine the rates of biochemical reactions that influence the modeling of lipids and proteins in specific tissues in vivo.

MATERIALS AND METHODS

Chemicals and Supplies

Unless specified, all chemicals and reagents were purchased from Sigma-Aldrich. ²H₂O (99.9 atom percent excess) and H₂¹⁸O (10.4 atom percent excess) were purchased from Cambridge Isotopes (Andover, MA) and Isotec (Miamisburg, OH), respectively. Ion exchange resins were purchased from Bio-Rad (Hercules, CA). GC-MS supplies were purchased from Agilent Technologies (Wilmington, DE) and Alltech (Deerfield, IL). Enzymes were purchased from Roche (Indianapolis, IN). Diets were purchased from Research Diets (New Brunswick, NJ). Mice were purchased from Jackson Laboratories (Bar Harbor, ME).

Biological Experiments

On arrival, male C57BL/6J mice (5 wk old, n = 42) were fed a high-fat low-carbohydrate diet (no. D12451, kcal distribution equal to 45% fat, 35% carbohydrate, and 20% protein). After 5 days, mice were randomized into two groups, designated either “continuous” or “bolus,” and given an intraperitoneal injection of labeled water. Mice in the continuous group were injected with ²H₂O (20 μg/g body wt of 0.9 g NaCl in 1,000 ml 99% ²H₂O). After injection, mice were returned to their cages and maintained on 5% ²H₂O labeled drinking water for 5 days before being switched to regular drinking water.
water. This procedure maintains a steady-state $^2$H labeling of body water at $\sim-2.5\%$ (days 0-5), followed by an exponential elimination of the tracer from body water (after day 5), as previously described (3). Mice in the bolus group were injected with $^3$H- and $^{18}$O-labeled saline (40 $\mu$g/g body wt of 0.9 g NaCl in 500 ml 99% $^2$H$_2$O + 500 ml 10% $^3$H$_2^{18}$O). This will initially raise the body water enrichment to $\sim-2.5$ and $\sim-0.25\%$ of $^3$H and $^{18}$O, respectively. Mice in the bolus group were maintained on regular drinking water to allow for the exponential elimination of both $^3$H and $^{18}$O from body water. In all cases, to ensure the precision of the injection, mice were sedated by a brief exposure ($\sim15$ s) to Isoflurane (Baxter Pharmaceuticals, Deerfield, IL); mice regain consciousness within a minute with no apparent adverse side effects.

Mice in each group were fed ad libitum, and daily food consumption was measured. Mice were sedated on days 0, 2, 5, 7, 9, 12, and 15 postinjection ($n = 3$ per day per group). Blood and tissue samples were collected and quick-frozen in liquid nitrogen. Samples were stored at $\sim80^\circ$C until analyses.

**Generation of Positional Isotopomers of Glycerol 3-Phosphate**

[$^3$H]glycerol 3-phosphate standards were generated by reducing 100 mg of glyceraldehyde 3-phosphate or 100 mg of dihydroxyacetone phosphate with 75 $\mu$L of NaBD$_4$ solution (0.44 g NaBD$_4$/ml 0.1 N NaOH). The reduction of glyceraldehyde 3-phosphate generated [$^3$H]glycerol 3-phosphate, and the reduction of dihydroxyacetone phosphate generated [$^2$H]glycerol 3-phosphate. The reaction was allowed to stand at room temperature for 1 h. Excess borodeuteride was destroyed by addition of 12 N HCl until the pH was acidic (pH $\sim1.0$). The sample was evaporated to dryness and then dissolved in 2.5 ml of 50% methanol-water (vol/vol). To remove methyl borate, the methanol-water was evaporated to dryness. The methanol-water treatment was repeated twice. The dry residues were dissolved in water and applied to AG 1-X8 ion exchange columns (formate form). The columns were first washed with 20 ml of water, and [$^3$H]glycerol 3-phosphates were then eluted in 20 ml of 4 N formic acid.

**Analytic Procedures**

$^3$H labeling of body water. The $^3$H labeling of body water was determined by exchange with acetone (37). Briefly, 40 $\mu$L of sample or standard were reacted with 2 $\mu$L of 10 N NaOH and 4 $\mu$L of a 5% (vol/vol) solution of acetone in acetonitrile for 24 h. Acetone was extracted by addition of 600 $\mu$L of chloroform followed by addition of $\sim0.5$ g Na$_2$SO$_4$. Samples were vigorously mixed, and a small aliquot of the chloroform was transferred to a GC-MS vial.

Acetone was analyzed using an Agilent 5973N-MSD equipped with an Agilent 6890 GC system, and a DB-17MS capillary column (30 m $\times$ 0.25 mm $\times$ 0.25 $\mu$m) was used in all analyses. The temperature program was as follows: 60°C initial, increase by 20°C/min to 100°C, increase by 50°C/min to 220°C, and hold for 1 min. The sample was injected at a split ratio of 40:1 with a helium flow 1 ml/min. G3P-TMS eluted at 2.4 min. The $^{18}$O enrichment was determined using the Agilent 5973N-MSD equipped with the Agilent 6890 GC system. A DB-17MS capillary column (30 m $\times$ 0.25 mm $\times$ 0.25 $\mu$m) was used in all analyses. The temperature program was 90°C initial, increase by 30°C/min to 240°C, and hold for 1 min. The split ratio was 20:1 with helium flow 1 ml/min. TMP eluted at $\sim2.4$ min. The $^3$H enrichment was determined using electron impact ionization (70 eV) and selected ion monitoring (10 ms dwell time) of m/z 140 to 142. The $^{18}$O enrichment was calculated from the signal ratio (142)/(142 + 140).

$^2$H labeling of triglyceride-bound glycerol. Total glycerides were extracted from frozen epididymal fat pads by first hydrolyzing both fat pads in 1 N KOH-ethanol (20:80, vol/vol) at 70°C. After 3 h, the hydrolysate was evaporated to dryness, and dry residue was dissolved in 3 ml of H$_2$O and acidified to $\sim$pH 1.0 by adding 6 N HCl.

Free fatty acids were extracted using diethyl ether (3 times with 4 ml). The solution of the aqueous solution was then adjusted to $\sim$7.0 (using 10 N NaOH). Free glycerol was converted to glycerol 3-phosphate by incubating $\sim$150 $\mu$g of glycerol in 1.5 ml of Tris-EDTA buffer (pH 7.4) containing 0.2 M ATP and 5 U of glycerokinase for 2 h at 37°C.

Glycerol 3-phosphate was purified by passing the reaction mixture over an AG 1-X8 ion exchange resin (formate form). The column was first washed with 20 ml of water, and glycerol 3-phosphate was then recovered by washing with 20 ml of 4 N formic acid. The eluent containing glycerol 3-phosphate was evaporated to dryness and converted to its tetratrimethylsilyl derivative (G3P-TMS). This was done by reacting the dry glycerol 3-phosphate residue with 100 $\mu$L of bis(trimethylsilyl) trifluoroacetamide + 10% trimethylchlorosilane (Regis, Morton Grove, IL) at 75°C for 30 min.

The $^3$H labeling of G3P-TMS was determined using the Agilent 5973N-MSD equipped with the Agilent 6890 GC system. A DB-17MS capillary column (30 m $\times$ 0.25 mm $\times$ 0.25 $\mu$m) was used in all analyses. The temperature program was 120°C initial, increase by 10°C/min to 200°C, increase by 50°C/min to 240°C, 2 min hold time. The split ratio was 40:1 with helium flow 1 ml/min. G3P-TMS eluted at $\sim$8.0 min. Electrospray ionization was used in all analyses. Selective ion monitoring of fragments m/z 357 and 358 (containing carbons 2 and 3 and the respective carbon-bound hydrogens) and 445 and 446 (containing carbons 1, 2, and 3 and the respective carbon-bound hydrogens) was performed using a dwell time of 10 ms per ion (8).

Note that glycerol has biological asymmetry, and glycerokinase selectively phosphorylates carbon 3 (5, 26, 32). In this study, we determined the labeling of $^3$H that is bound to carbon 1 of triglyceride-glycerol from the difference of the molar percent enrichments (MPEs) of (358)/(357) and (446)/(445) (i.e., the labeling of $^3$H bound to carbons 1 and 2) was subtracted from the labeling of $^3$H bound to carbons 1 and 2 (8). Calculations were based on the use of known standards of G3P-TMS.

Concentration of triglyceride-bound glycerol. The concentration of triglyceride-bound glycerol was determined by enzymatic/spectrophotometric assay. Briefly, epididymal fat pads were hydrolyzed, and free glycerol was obtained as described above. Glycerol was then digested in a known amount of Tris-EDTA buffer (pH 7.4) and its concentration determined using an enzymatic assay (Free Glycerol Reagent, Sigma).

$^2$H labeling of protein-bound alanine from heart muscle. Hearts were homogenized in 10% (wt/vol) trichloroacetic acid (5 ml acid/g tissue). The protein pellet was washed three times with 5% trichloroacetic acid and then dissolved in 6 N HCl (5 ml/0.5 g tissue). The sample was reacted at 100°C for 18 h and then diluted 10-fold.

Innovative Methodology
Innovative Methodology

E1050 PARTITIONING CARBON FLUX IN VIVO

Alanine was then recovered following ion exchange chromatography. Briefly, samples were loaded onto an AG 50W-X8 ion exchange resin (hydrogen form), and the column was washed with 20 ml of H2O. Next, alanine was eluted by washing with 20 ml of 4 N NH4OH, and the NH4OH fraction was evaporated to dryness.

The 2H labeling of alanine was determined on its methyl-8 derivative, formed by reaction with N,N-dimethylformamide dimethylacetal (Pierce, Rockford, IL) (33). Samples were analyzed under electron impact ionization, the Agilent 5973N-MSD equipped with the Agilent 6890 GC system. A DB-17MS capillary column (30 m x 0.25 mm x 0.25 μm) was used in all analyses. The temperature program was 90°C initial, hold for 5 min, increase by 5°C/min to 130°C, increase by 40°C/min to 240°C, and hold for 5 min. The split ratio was 10:1 with a helium flow of 1 ml/min. Alanine eluted at ~12 min. The mass spectrometer was operated in the electron impact mode. Selective ion monitoring of the molecular ion was performed, i.e., m/z 158 and 159 (total 2H labeling of alanine), using a dwell time of 10 ms per ion.

Mathematical Model for Describing Triglyceride and Protein Kinetics

Steady-state isotope precursor labeling protocol. Rates of synthesis and degradation of triglycerides and proteins were determined by fitting the concentration and 2H labeling data (3). In the case of the continuous group (the steady-state 2H labeling of body water), data were fitted as described (3). First, the concentration of triglyceride-bound glycerol or total protein is modeled. The incorporation of 2H from water into lipids or proteins is modeled using a single-compartment model, assuming that the labeling of plasma water reflects that of water in adipose tissue and muscle, respectively. The time-dependent labeling of 2H in water is c(t). 2H is eliminated from plasma at a rate c'. The parameters of basic interest, i.e., the rates of triglyceride or protein synthesis and degradation (S and D, respectively, expressed in units of mass per day), are estimated from the data using nonlinear least-squares fitting.

Because the mass of triglyceride (expressed in μmol) increased linearly with time (m, expressed in μmol per day), the total amount of labeled triglyceride at time t satisfies the differential equation

$$\frac{dh(t)}{dt} = Sc(t) - \frac{Dh(t)}{mt + b}$$

where h equals the 2H labeling of triglyceride (expressed in μmol 2H). The parameter c(t) is essentially constant while mice are maintained on 2H-labeled drinking water, yet it decays exponentially (at c') once mice are switched to regular water. The parameter c' is estimated from the data.

Differential Eq. 1 is then solved in closed form for the removal of 2H-labeled drinking water. That equation is

$$v(t) = \frac{sc_0}{b + mt} \left[ \left( \frac{b + mt}{S} \right)^{s} \left( \frac{b + mt}{b + mt} \right)^{s} \right]^{\frac{-1}{m}} + \frac{b + 5m}{S} \left( \frac{b + mt}{b + mt} \right)^{s} \left( \frac{b + mt}{mt} \right)^{0(t-5)} + \frac{sc_0}{b + mt} \left( \frac{a}{S} \right) e^{\left( \frac{b}{m} + T \right) \frac{m}{m}} \left( \frac{S}{m} \right) a \left( \frac{b + 5m}{mt} \right)^{0(t-5)}$$

where c0 = c(0) and v0 = v(0) represent the 2H labeling (expressed in % enrichment) of body water and triglyceride-glycerol, respectively, at the time when 2H2O is withdrawn, θ is the unit step function (0 for the negative argument and 1 for positive argument; this incorporates the change from enriched to unenriched water at day 5), a is the decay constant for labeled body water, and Γ(a,ζ) is the incomplete gamma function

$$\Gamma(a,\zeta) = \int_{\zeta}^{\infty} e^{-x}x^{a-1}dx.$$  

The model was solved by using a linear least square fit of the data regarding the mass of triglycerides in adipose tissue. The only remaining parameters that require estimation are the rate of synthesis (S) and the rate of degradation (D = S – m). A Levenberg-Marquardt nonlinear fit (equal weights on all data points) is used to compute those parameters.

A similar approach was used to quantify protein dynamics in heart muscle. However, because the mass of the tissue remained constant during the course of the experiment, the term m is reduced to 0. Therefore, a modified differential equation is used, namely

$$\frac{dv(t)}{dt} = Sc(t) - \frac{Sh(t)}{b}$$

The data are then fitted using differential Eq. 4 and solving. The equation is

$$v(t) = \frac{c_0}{m} \left[ 1 - e^{-\frac{t}{m}} + \left( 1 - e^{-\frac{t}{m}} \right) S + a(e^{-\frac{t}{m}} - 1)b(0(t - 5)) \right]$$

Non-steady-state isotope precursor labeling protocol. Rates of synthesis and degradation of triglycerides and proteins were calculated from labeling data of mice that received a single bolus of 2H2O (i.e., under the conditions of non-steady-state 2H labeling of body water). As described above, the pool size was first modeled, followed by a fit of the 2H labeling data. In the case of the bolus group, a new mathematical model was developed where S and D are estimated using the equation

$$v(t) = \frac{1}{m} \left( \frac{m}{a} \frac{c_0}{S} S \left( \frac{b}{m} + t \right) - \frac{S}{m} \Gamma \left( \frac{S}{m} \right) - \frac{S}{m} \Gamma \left( \frac{S}{m} \frac{a}{b} \right) \right.$$  

and for constant total mass

$$v(t) = \frac{c_0}{S} S \left( e^{-\frac{t}{m}} - e^{-\frac{t}{m}} \right)$$

The best-fit solutions were determined and are stated as the rate ± the 95% confidence interval observed between the measured data and the model predictions.

Calculations

The rate of CO2 production (mmol/day) was calculated using the equation

$$\text{Calculations}$$

$$\text{Calculations}$$
CO$_2$ production = \((0.481 \times N) \times [(1.01 \times k^{18}O) \\
- (1.04 \times k^2H)] - (0.0246 \times 1.05 \times N \times (k^{18}O - k^2H))\) (8)

where the fractional biological elimination constants (k$^{18}$O and k$^2$H, in days) are determined from the decrease in the labeling of body water and total body water (N, in mmol) is determined from the initial dilution of the tracers (i.e., the average pool size obtained from the dilution of each tracer) (28).

Unless noted, data are expressed as means ± SE.

RESULTS

$^2$H from $^2$H$_2$O can be incorporated into five carbon-bound hydrogen positions of triglyceride-glycerol. We previously reported (3) that the rates of triglyceride synthesis and breakdown can be determined by measuring the labeling of $^2$H that is bound to carbon 1 of triglyceride-glycerol divided by 2. In this study, we aimed to simplify our previous efforts for measuring the positional labeling of $^2$H bound to carbon 1 of triglyceride-glycerol. Figure 1 shows the mass spectra of G3P-TMS. The fragment ion at $M$ – 15 is typically observed with trimethylsilyl derivatives producing the molecular ion (m/z 460) minus a methyl group (m/z 445; Fig. 1A). The shift in m/z 445 to 446 is expected, since $^2$H was incorporated during the reduction of the respective compounds with sodium borodeuteride (Fig. 1, A vs. B or C). The differential effect in shift observed at m/z 357 (to m/z 358) is consistent with the observations reported by Cronholm and Curstedt (8); i.e., the fragment ion at m/z 357 contains carbons 2 + 3 and their respective carbon-bound hydrogens (Fig. 1B, m/z 357 is shifted to m/z 358; Fig. 1C, no shift in m/z 357). The labeling of $^2$H bound to carbon 1 of triglyceride-glycerol was determined by subtracting the labeling of $^2$H bound to carbons 2 + 3 (m/z 357) from the labeling of $^2$H bound to carbons 1 + 2 + 3 (m/z 445).

We were concerned that it would be difficult to obtain precise measurements of low $^3$H enrichments of triglyceride-glycerol ($\sim$0.4 to 2% excess $^3$H) since the background labeling in each fragment ion is relatively high; the chemical derivative contains silicon and carbon that have $M$ + 1 isotopes equal to $\sim$5 and $\sim$1% of the predominant natural species, respectively (Fig. 1). Therefore, we examined the reproducibility of measurements of the background labeling in each fragment ion by means of repeated GC-MS measurements performed over several days (Table 1). Measurements were reproducible on an individual day; however, the measurements were somewhat variable over different days (Table 1).

Figure 2 demonstrates that mice gained weight (Fig. 2A; $y = 0.295x + 18.9$, $r^2 = 0.99$) and accumulated triglycerides in epididymal fat pads (Fig. 2B; $y = 9.08x + 90.7$, $r^2 = 0.78$; dashed lines represent 95% confidence interval). These data are

![Table 1. Determination of intra- and interday reproducibility of measurements of tetra-trimethylsilyl glycerol 3-phosphate background labeling](data:image/png;base64,iVBORw0KGgoAAAANSUhEUgAAAgAAAAAbgAAABEjX87AAAABJRU5ErkJggg==)

Data are expressed as means ± SE. CV, coefficient of variation for 5 replication determinations. Samples were assayed over various days using 5 injections per day. Abundance of 4 ion signals was measured, m/z 445 and 446 (containing carbons 1, 2, and 3 and respective carbon-bound hydrogens) and m/z 357 and 358 (containing carbons 2 and 3 and respective carbon-bound hydrogens). Data were tabulated as the natural mole percent in $M$ + 1, i.e., the background labeling.
consistent with our previous observations (3). During the experimental period, there was no change in the wet weight of the hearts, 142 ± 9 mg on day 0 vs. 144 ± 11 mg on day 15 (consistent with unpublished observations).

Figure 3 shows the labeling profiles of body water, glycerol from total epididymal fat pad triglycerides, and alanine from total heart muscle proteins. While mice in the continuous group were maintained on $^2$H$_2$O (days 0–5), the $^2$H labeling of body water remained constant (2.59 ± 0.10% $^2$H) and equaled ~50% that of the drinking water, consistent with our previous observations. During the experimental period, there was no change in the wet weight of the hearts, 142 ± 9 mg on day 0 vs. 144 ± 11 mg on day 15 (consistent with unpublished observations).

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Fig. 3. Labeling of body water, glycerol from epididymal fat pad triglycerides, and alanine from heart muscle proteins. Mice were randomized to 1 of 2 isotope labeling protocols. A: “continuous” mice were maintained on $^2$H$_2$O for 5 days before being switched to regular drinking water. B: “bolus” mice were given a single injection containing a mixture of $^2$H$_2$O and H$_2^{18}$O. $^2$H enrichment of body water ($) and that bound to carbon 1 of triglyceride-glycerol ($) and protein-alanine ($) were determined (A and B). In addition, $^{18}$O labeling of body water ($) was determined (B). Data are expressed as total labeling measured in each species. Inset: labeling of $^2$H and $^{18}$O of body water (normalized against labeling on day 0 and expressed in natural log scale). Elimination rates of $^2$H and $^{18}$O were determined from the slopes of regression analyses ($^2$H elimination: $y = -0.248x$, $r^2 = 0.901$; $^{18}$O elimination: $y = -0.485x$, $r^2 = 0.941$).

Innovative Methodology

E1052 PARTITIONING CARBON FLUX IN VIVO

AJP-Endocrinol Metab • VOL 290 • MAY 2006 • www.ajpendo.org
observations, $^2$H was eliminated from body water by switching mice from $^2$H-labeled drinking water to regular water (days 6–15). The $^2$H enrichment of body water decayed exponentially to $\sim 0.28\%$ on day 15. The $t_{1/2}$ of body water, calculated from the elimination rate of $^2$H, was $\sim 2.3$ days. Again, this is consistent with our previous observations. The elimination of $^2$H from body water of mice in the bolus group (Fig. 3B: $t_{1/2}$ of $^2$H $\sim 2.8$ days) was similar to that observed in mice in the continuous group. As expected, the elimination of $^{18}$O from body water was greater than that of $^2$H (Fig. 3B: $t_{1/2}$ of $^{18}$O $\sim 1.4$ days). The calculated rate of $^2$H production was $\sim 74$ mmol/day.

Figure 4 shows the best fits of the mathematical models that were used to describe the $^2$H labeling of triglyceride-glycerol and protein-alanine. The calculated rates of synthesis and breakdown of triglycerides were $12.6 \pm 1.4$ and $3.8 \pm 1.4$ vs. $12.9 \pm 2.8$ and $4.1 \pm 2.8 \mu$mol/day, using the steady-state vs. the non-steady-state labeling protocol (Fig. 4, A vs. B, respectively). The data obtained from mice in the continuous group (Fig. 4A) agree with previously published data (3). The good agreement between the two labeling protocols supports the use of the non-steady-state model (Fig. 4, A vs. B). Also, these data are consistent with the observation that high-fat feeding promotes lipid accretion. As there was no change in the wet weight of the hearts, modeling the $^2$H labeling of protein-bound alanine yields equal rates of synthesis and breakdown of total cardiac protein with respect to each protocol, i.e., $14.4 \pm 1.1$ vs. $14.1 \pm 1.0$ mg wet wt/day, using the steady-state vs. the non-steady-state labeling protocol (Fig. 4, C and D, respectively). Overall, there was good agreement between the two labeling protocols.

DISCUSSION

Isotope tracers can be used to quantify the turnover rates of various intermediates and yield information regarding metabolic regulation (18, 22, 36, 38). However, studies often consider either one nutritional state (e.g., fed or fasted) and/or examine one component of a problem (e.g., measure lipid or protein dynamics). In that regard, the doubly labeled water method is a unique tracer method because it yields an integrative measurement of total energy expenditure, including basal metabolism, thermic effect of eating, and physical activity (16, 17, 19). In this report, we demonstrate a novel application of the doubly labeled water method; i.e., after the administration of a bolus containing $^2$H$_2$O and H$_2$18O, it is possible to measure the $^2$H labeling of lipids and proteins and thereby determine their rates of synthesis and degradation in vivo. Implementation of this approach required the development of a mathematical model to determine kinetic parameters under conditions of “non-steady-state” isotope precursor labeling. To further enhance the utilization of our method, we developed a simple GC-MS method for measuring the positional $^2$H labeling of triglyceride-glycerol. In discussing our observations, we first comment on the analytic developments, and second we comment on the biological outcomes of the study.

**Improving the Analytical Method for Measuring the $^2$H Labeling of Triglyceride-Glycerol**

The rate of triglyceride turnover is determined by measuring the labeling of $^2$H bound to carbon 1 of triglyceride-glycerol divided by 2 (the number of exchangeable hydrogens) (3). Previously, those measurements were made using the “hexa-

Fig. 4. Best fit of $^2$H labeling of glycerol from epididymal fat pad triglycerides and alanine from heart muscle proteins. Mathematical models were used to fit $^2$H labeling of glycerol and alanine isolated from epididymal fat pad triglycerides and heart muscle proteins, respectively. A and C: best fit of data obtained from mice in continuous group. B and D: best fit of data obtained from mice in bolus group. Virtually identical rates of synthesis and degradation of triglycerides were obtained using either protocol: A 12.6 $\pm$ 1.4 and 3.8 $\pm$ 1.4 vs. B 12.9 $\pm$ 2.8 and 4.1 $\pm$ 2.8 $\mu$mol/day, using the steady-state vs. the non-steady-state labeling protocol, respectively. Also, modeling the $^2$H labeling of protein-bound alanine yielded equal rates of synthesis and degradation regardless of the protocol: C 14.4 $\pm$ 1.1 vs. D 14.1 $\pm$ 1.0 mg wet wt/day, using the steady-state vs. the non-steady-state labeling protocol. Note: scales used on the y-axis are different in Figs. 3 and 4. Data are modeled using a precursor-to-product labeling ratio, where the precursor is $^2$H labeling of body water and the product is labeling of $^2$H bound to carbon 1 of triglyceride-glycerol divided by 2 and labeling of $^2$H bound to total protein-bound alanine divided by 3.7 (no. of exchangeable hydrogens in the respective products); see MATERIALS AND METHODS.
methylenetetramine method” (6, 13). Although novel and effective, the hexamethylenetetramine method requires HPLC equipment and a manually operated distillation apparatus. The former makes the method costly, and the latter makes the method less than ideal when large numbers of samples are processed.

Because improving the analytic throughput should facilitate the use of $^2$H$_2$O for measuring triglyceride kinetics, we examined the applicability of a GC-MS method for measuring the positional labeling of triglyceride-glycerol (8). Our concern was to obtain reliable measurements of low $^3$H enrichment over a relatively high background; e.g., in the current study, biological samples were enriched $\sim$0.4–2.0% excess $^3$H over $\sim$20–26% background labeling (Fig. 3). The high background (Table 1) is a consequence of the fact that the trimethylsilyl derivative of glycerol 3-phosphate contains silicon and carbon, which have $M + 1$ isotopes of $\sim$5 and $\sim$1%, respectively, of the predominant natural species. We found that the overall GC-MS method has good precision; i.e., the average coefficient of variation for measuring the background labeling of carbon 1 is $\sim$1.3% (Table 1). Because the average background labeling of carbon 1 is $\sim$4.5%, one can expect $\sim$0.06% absolute variation in the measurements (i.e., $4.5 \times 0.013$).

It is clear from Table 1 that GC-MS analyses of glycerol 3-phosphate labeling have rather poor accuracy, the absolute interdaily variation in background labeling ranging over $\sim$0.2% (Table 1). Therefore, it is not possible to reliably determine the $^2$H labeling by use of a theoretical model, i.e., by correcting the natural background from experimental samples (36). Consequently, we generated $^2$H-labeled standards by reducing commercially available glyceraldehyde 3-phosphate with sodium borodeuteride (Fig. 1). These standards offer two advantages. First, the analyses of known standards account for daily variations in the performance of the GC-MS instrument. Second, it is possible to amplify the detection of $^2$H labeling by integrating the leading edge of a chromatographic peak; i.e., biasing the ion chromatogram integration routine enhances the detection of $^2$H labeling (14).

It should be noted that other investigators are using $^2$H$_2$O to study triglyceride turnover (7, 34). It appears that the primary difference between the views expressed herein and those of Hellerstein and colleagues centers on how one defines/determines the product labeling (7, 34). It seems that they believe that it is sufficient to use a statistical method to determine the equilibrium of label incorporation in a product molecule, whereas we have used analytical methods to measure the labeling of specific hydrogen atoms in a product molecule. It may be that in certain instances the same results (regarding biochemical flux) will be obtained regardless of how the product labeling is defined. Examining the latter point is an area of ongoing research by our laboratory, especially since using the total product labeling increases the sensitivity of the measurements (see below).

**Advantage of Non-Steady-State Isotope Precursor Labeling**

Approximately 50 years ago, Lifson and colleagues (16, 17) demonstrated the use of the doubly labeled water method for determining rates of CO$_2$ production in vivo. Since those pioneering studies, investigators have refined and extended the method to measure CO$_2$ production in various species, including humans (27, 36). We hypothesized that it would be possible to further extend the doubly labeled water method and determine the flux of metabolic pathways by measuring the $^3$H labeling of certain biochemical end products. Our hypothesis was formulated on the classical studies of Schoenheimer and Rittenberg performed $\sim$80 years ago (30). Validation of this non-steady-state approach was performed against tracer studies in which a “steady-state” precursor was maintained.

Rates of CO$_2$ production were calculated from the difference between the elimination rates of $^2$H and $^{13}$O from the body water (Fig. 3B, inset). We determined that mice produce $\sim$74 mmol of CO$_2$ per day. On comparing the rate of CO$_2$ production against the food intake ($\sim$93 mmol carbon per day), we found that mice retained $\sim$20% of the carbon intake. Although it is beyond the scope of this investigation to calculate the exact carbon balance (e.g., metabolic cages are needed to collect nonabsorbed food and/or solid waste), our findings are in strong agreement with the original studies of McClintock and Lifson (19). For example, in our study, mice were fed a high-fat diet and gained $\sim$295 mg body wt per day (Fig. 2). This is comparable to the gains reported by McClintock and Lifson; i.e., control mice retained $\sim$9% of total caloric intake and gained $\sim$200 mg body wt per day, and obese hyperphagic mice retained $\sim$20% of total caloric intake and gained $\sim$429 mg body wt per day (19).

Because C57BL/6J mice are sensitive to diet-induced obesity via high-fat feeding (23), we expected to observe lipid accretion (Fig. 2B). Regardless of the labeling protocol, we observed similar and substantial rates of incorporation of $^3$H into triglyceride-glycerol (Fig. 3, A and B). Despite the fact that the $^3$H labeling of triglyceride-glycerol is about three- to fivefold lower in the bolus group compared with the continuous group (Fig. 3), we observed strong agreement between the rates of triglyceride synthesis and degradation in both protocols (Fig. 4). This demonstrates the robust nature of these protocols and the reproducibility of the analyses.

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The observation of a substantial rate of triglyceride breakdown (equivalent to $\sim$25% the rate of triglyceride synthesis) during lipid accretion is especially intriguing. This clearly demonstrates that adipose tissue is a highly active storage site and not simply an inert depot of triglycerides. Our observation is consistent with the conclusions drawn by Schoenheimer and Rittenberg (29) and substantiates the recent hypothesis proposed by Frayn et al. (11). Finally, it should be noted that, by measuring the $^3$H labeling of triglyceride-bound fatty acids, it is possible to determine the contribution of de novo lipogenesis to the source of fatty acids used in triglyceride synthesis (not shown) (1, 3, 9).

Considering the current problem of obesity (20, 25), and since cardiac failure has been observed in cases of rapid weight loss (2, 35), we anticipate that future studies may need to examine whether therapeutic interventions (e.g., dietary, pharmacological, or surgical) that are designed to affect lipid accretion also affect protein turnover, especially in the heart. Consequently, we determined whether it would be possible to measure the rates of protein turnover while measuring lipid flux. By mathematically modeling the $^2$H labeling of protein-bound alanine, we were able to derive rates of protein synthesis and protein breakdown in the heart muscle (Fig. 3). We found that a best fit of the data yielded virtually identical rates of
protein synthesis and breakdown (Fig. 4). This should be expected, as the mass of the heart muscle did not change over the study period.

It should be noted that, although we determined protein turnover only in heart muscle, $^2$H$_2$O can be used to study the turnover of proteins in different tissues, the requirement being that one needs to isolate the tissues and/or proteins of interest (10, 24). Also, we (31) recently demonstrated that it is possible to measure the turnover of specific proteins without extensive purification by coupling the administration of $^2$H$_2$O with proteomic-based assays. Thus, in theory, one can readily quantify the synthesis and degradation of individual components of the proteome.

Summary and Conclusions

In summary, $^2$H- and $^{18}$O-labeled water can be used to quantify the contribution of anabolic and catabolic mechanisms that influence the dynamics of lipids and proteins. $^2$H$_2$O appears to be a particularly good tracer to use under conditions of non-steady-state isotope precursor labeling. First, because $^2$H rapidly distributes and equilibrates in body water, compartmentalization is not a major concern. Second, because body water has a relatively long half-life, there is a substantial amount of time for incorporation of $^2$H into slow-turning pools of macromolecules (e.g., triglycerides and proteins). Third, the elimination of $^2$H from body water follows a single exponential decay and occurs at a relatively slow rate, which facilitates measurements of $^2$H labeling.

We conclude that it is possible to extend the application of the doubly labeled water method and obtain measurements of CO$_2$ production and the tissue-specific dynamics of lipids and proteins in vivo. As the methods presented here can be applied in any animal resource center/laboratory, our approach should complement the existing tools for studying metabolic regulation, e.g., the hyperinsulinemic euglycemic clamp (12, 15). In particular, virtually no surgical expertise is required to administer doubly labeled water and collect tissue samples. Also, the analytical methods require a minimum of relatively common, high-throughput, gas chromatography-electron impact ionization-mass spectrometry equipment. Finally, although we have measured the incorporation of $^2$H into two classes of macromolecules (lipids and proteins), presumably one could study the dynamics of other important end products, e.g., DNA (21).

GRANTS

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REFERENCES


