BODY PROTEIN POOLS UNDERGO CONTINUAL RENEWAL through a constant cycle of synthesis and degradation. Alterations in the balance between synthetic and degradative rates produce changes in protein pool size that are fundamental for the ability of a biological system to adapt to intrinsic and environmental factors (26). Therefore, accurate methods to assess directly the protein synthetic and breakdown rates are necessary to investigate and understand the regulation of protein balance. The rate of protein synthesis can be measured from the rate of isotope-labeled amino acid incorporation into proteins (45, 47). With technical advances in mass spectrometers and the combination of techniques, the determination of the synthesis rate of small quantities of individual proteins is now possible (21, 24). The need for a comparable approach to measure protein-specific breakdown rates is also important for in vivo human research. However, the assessment of protein breakdown is more troublesome (18). Currently, the in vivo approach used routinely relies on isotopic dilution and arterial-venous catheterization (2, 6). More pools have been added to the method, improving validity (17), and the latest approach has been reworked and further refined by Zhang and colleagues (51–53). However, the same principle remains, i.e., to measure the dilution of free amino acid tracer enrichment at arterial, venous, and intracellular sites as a consequence of release of trace amino acids when proteins degrade. Although this technique has substantial utility, the protein origin of the amino acids remains unknown. Hence, it does not permit estimation of the breakdown rate of specific proteins as it does for the direct tracer incorporation approach for protein synthesis measurements. For this reason we developed a fractional breakdown rate (FBR) method in rats, allowing the measurements of FBR of individual proteins, and demonstrated the applicability of this approach in humans.

To meet the requirement of protein specificity, the analytical target in the protein breakdown approach must be the proteins themselves. If protein can be prelabeled with one or more labeled amino acids, then the rate of disappearance of labeled amino acids from protein can be measured to determine protein breakdown (14, 32, 34, 46, 49). Historically, this approach has been used in vitro on bacteria (14, 27, 36, 46), in cell cultures (41), and in chick skeletal muscle cells (49) as well as in human muscle cells (34). In all approaches, an isotope-labeled amino acid was introduced into proteins, and the average rate of protein breakdown was obtained by measuring the rate of release of labeled amino acids from the prelabeled protein structure. Although elegant approaches were developed (e.g., see Ref. 49), it became apparent that the reutilization of the recently released tracer by reuptake via new protein synthesis results in an underestimated breakdown rate, which seems hard to circumvent and avoid (1, 34). To avoid reincorporation of tracer amino acids, the tracer amino acids must be quickly removed via metabolism after release from protein breakdown or not reutilized at all. Molnar et al. (32) took advantage of the latter. They exposed growing rats to $^{18}$O$_2$ for 36 h in a closed environment, an approach developed some years earlier by Jackson and Heinenger (23), to label hydroxyproline in collagen proteins undergoing posttranslational hydroxylation of peptide-bound proline residues. Because hydroxyproline cannot be reutilized for protein synthesis once it is released from degraded proteins, the hydroxyproline tracer does not recycle. This approach is limited to proteins releasing specific posttranslationally modified amino acids that cannot recycle. Humphrey and Davies (20) tested the option to remove the label quickly from amino acids released from degraded proteins. They used tritiated water ($^{3}$H$_2$O) to transfer the $^3$H isotopes to...
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ring assumption also for the precursor-product approach to determine the FSR.

According to assumptions 1 and 2, the first equation can be written as
\[
\frac{dP}{dt} = r_S - k_B P = 0
\]
where \( r_S \) is the synthesis rate, \( k_B \) is the rate constant of the protein breakdown, and \( P \) is the size of the protein pool.

Equation 4 describes how the protein with labeled amino acids, \( P^* \), loses the label, depending on the protein breakdown rate, \( k_B \):

\[
\frac{dE}{dt} = k_B E = k_B E_{0e}
\]

where \( E \) is the enrichment at steady state. To measure the protein fractional synthesis rate (FSR), incorporation of tracer is measured typically by the change in enrichment (\( \Delta E \)) over a known period of time (\( \Delta t \)), where the precursor tracer enrichment is steady and equal to \( E_0 \) (enrichment at infinity). Circle shows tracer incorporated into the protein pool (\( P^* \)). C: time course of disappearance of tracer from the protein pool after input of tracer is stopped at various time points. The fractional breakdown rate (FBR; \( k_B \)) of a prelabeled protein pool can be calculated by measuring the rate of tracer disappearance from the protein pool (\( \Delta E \)) over \( \Delta t \). It is assumed that no released tracer is reincorporated into the protein.

Fig. 1. Model of protein kinetics. A: model of protein synthesis and breakdown. The mass of any protein pool (\( P \)) is defined by the rate of protein accretion by synthesis (\( S \)) and loss by breakdown processes (\( B \)). At tissue protein steady state, \( P \) is constant and \( S \) and \( B \) rates are equal. B: the exponential rise of tracer enrichment over time obtained by incorporation into a protein pool during constant infusion of tracer. The fractional synthesis rate (FSR; \( k_B \)) can be assessed by determining the initial rate of tracer enrichment increase in protein (\( \Delta E \)) during a short period of time (\( \Delta t \)), where the precursor tracer enrichment is steady and equal to \( E_0 \) (enrichment at infinity). Circle shows tracer incorporated into the protein pool (\( P^* \)). C: time course of disappearance of tracer from the protein pool after input of tracer is stopped at various time points. The fractional breakdown rate (FBR; \( k_B \)) of a prelabeled protein pool can be calculated by measuring the rate of tracer disappearance from the protein pool (\( \Delta E \)) over \( \Delta t \). It is assumed that no released tracer is reincorporated into the protein.

MATERIALS AND METHODS

Mathematical Derivation and Assumptions

The mathematical derivation and assumptions are outlined in Fig. 1. In a pool of proteins (\( P \)), continuous processes of synthesis (\( S \)) and breakdown (\( B \)) of individual proteins allow remodeling and renewal (Fig. 1A).

Assumption 1. Both the rate of incorporation of label into a protein and the disappearance of label from the protein follow first-order kinetics (as described in Fig. 1, B and C, respectively). For measures of synthesis (Fig. 1B), the appearance of label in a protein pool (given by the enrichment of tracer in the protein (\( E \)) will depend on the synthesis rate (\( k_S \)):

\[
E = E_0 (1 - e^{-k_S t})
\]

where \( E_0 \) is the enrichment at steady state. To measure the protein fractional synthesis rate (FSR), incorporation of tracer is measured typically by the change in enrichment (\( \Delta E \)) over a known period of time (\( \Delta t \)) (Fig. 1B).

Correspondingly, a tracer will disappear from a protein pool following the equation

\[
E = E_{0e} e^{-k_B t}
\]

where \( k_B \) is the fractional protein breakdown rate and \( E_{0e} \) is the label enrichment in the protein when the precursor label is removed. Our measurement of protein FBR measures the rate of label removal from the protein that has been labeled previously. As indicated in Fig. 1C, proteins that turn over faster will become labeled to a greater degree than proteins that turn over slower during a period of labeling by a constant delivery of labeled amino acid precursor (greater \( E_0 \) values). The goal of our method is to then measure the rate of tracer disappearance from the protein (\( \Delta E \)) over a period of time (\( \Delta t \)) after removal of labeled precursor.

Assumption 2. The protein pool mass of interest, \( P \), does not change significantly during the period of measurement, which is an underly-
k_B (\text{free alanine pool over time, and the rate constant of disappearance is (k_{\text{Ala}}) determined:})

\[ \frac{dE}{dt} = -k_B E \]

Setting E = P*/P, we get

\[ \frac{dP}{P} = \frac{dE}{k_B E} \]

where E is the tracer/trace enrichment (TTR) enrichment ratio.

Thus, Eq. 5 shows that the disappearance of label from a protein follows a mono-exponential decay with the rate constant, k_B, as depicted in Fig. 1C.

Assumption 3. When a labeled amino acid tracer is released from a protein via protein breakdown, reutilization of newly released labeled amino acid can be ignored if the label in free alanine is quickly removed from that amino acid upon release. For the present FBR method to work, sufficient label has to remain in the protein at the time of measurement. On the other hand, the label to be removed from the free pool of amino acids. Hence, only proteins with a significantly slower turnover rate than the label can be measured using this approach. The fraction of \(^{3}\text{H}\) label remaining in the free pool after the administration of \(^2\text{H}_2\text{O}\) is stopped can be measured in a preliminary study before any actual measurements of protein breakdown are done. The exponential disappearance of tracer measured from the free alanine pool over time, and the rate constant of disappearance is (k_{\text{Ala}}) determined:

\[ E_{t}(\text{Ala}) = E_{0}(\text{Ala}) \times e^{-k_{\text{Ala}}t} \]  

where \(E_{0}(\text{Ala})\) is the free alanine \(^2\text{H}\) enrichment at time t and \(E_{t}(\text{Ala})\) is the fitted enrichment at time t = 0. Assumption 3 requires that enough time elapse after \(^2\text{H}_2\text{O}\) administration is stopped for the alanine \(^2\text{H}\) enrichment to decline to a minimal level in the free pool. The minimum time (t_{\text{min}}) for that decline to occur can be calculated by rearrangement of Eq. 6:

\[ t_{\text{min}} = -\frac{1}{k_{\text{Ala}}} \ln \left( \frac{E_{t}(\text{Ala})}{E_{0}(\text{Ala})} \right) \]

A maximum threshold is defined [\(E_{t}(\text{Ala})/E_{0}(\text{Ala})\)], and from that threshold, the time needed to wait after discontinuing \(^2\text{H}_2\text{O}\) is calculated (t_{\text{min}}). For example, if we defined a maximal residual enrichment in the free alanine pool to be 10% [i.e., \(E_{t}(\text{Ala})/E_{0}(\text{Ala}) = 0.1\)], then the minimum time to wait after discontinuing \(^2\text{H}_2\text{O}\) would be \(t_{\text{min}} = 303/k_{\text{Ala}}\).

Animals and Procedures

Male Sprague-Dawley rats (3 wk old; Charles River Laboratories, St. Constant, QC, Canada) were housed in pairs with a 12:12-h light-dark cycle [controlled temperature: 18–26°C (average: 20°C); humidity: 30–70% (average: 55%)] and positive pressure. Food (Prolab RMH 3000, Lab Diet; Purina Mills Laboratory, St. Louis, MO), water, and physical activity were accessible ad libitum. Rats were allowed 5 days of acclimatization before any intervention was initiated. At the time of intervention (time zero), all rats were 26 days old and had an average body weight of 76.7 ± 0.7 g (means ± SE). All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and followed protocols approved by the Institutional Animal Care and Use Committee of the University of Vermont.

The deuterium oxide was administered to the rats via their drinking water, which was mixed with \(^2\text{H}_2\text{O}\) as 8% of total volume. Prior to the

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### Table 1. Serum protein amino acid M + 1 enrichments

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>M + 1 Enrichment (Em + 1), %</th>
<th>No. of (^3\text{H}) in Amino Acid (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>20.63 ± 0.13</td>
<td>3.97 ± 0.13</td>
</tr>
<tr>
<td>Glycine</td>
<td>9.23 ± 0.10</td>
<td>1.78 ± 0.06</td>
</tr>
<tr>
<td>Valine</td>
<td>3.57 ± 0.05</td>
<td>0.69 ± 0.02</td>
</tr>
<tr>
<td>Leucine</td>
<td>4.80 ± 0.05</td>
<td>0.92 ± 0.03</td>
</tr>
<tr>
<td>Proline</td>
<td>6.21 ± 0.08</td>
<td>1.19 ± 0.04</td>
</tr>
<tr>
<td>Serine</td>
<td>11.50 ± 0.13</td>
<td>2.21 ± 0.08</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>2.26 ± 0.10</td>
<td>0.44 ± 0.02</td>
</tr>
</tbody>
</table>

Values are means ± SE. The M + 1 enrichments of selected serum protein amino acids were measured in samples obtained weekly from days 14 through 42. The number of deuteriums in each amino acid was calculated by dividing the average M + 1 enrichment shown in the table by the mean water enrichment at steady state (\(E_{0}(\text{H}_2\text{O}) = 5.20 ± 0.17\)%).
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ion into the slow turnover proteins. Finally, a control group of rats (n = 5) were grown for 56 days on plain tap water and then euthanized to determine the natural background isotope ratio for amino acids derived from the various protein fractions. The natural background isotope ratios were used to calculate the actual enrichments (see Amino acid enrichment TTR calculation).

Experiment 2. Using what was learned in experiment 1, we then administered $^{2}$H$_{2}$O to rats to label body proteins with deuterium-labeled amino acids, and then we measured the disappearance rate of the deuterium from body water, from free amino acids, and from serum protein-derived amino acids. Eight rats were grown for 42 days on 8% vol $^{2}$H$_{2}$O-enriched drinking water. Thereafter, the rats returned to plain, unlabeled tap water for ≤38 days. During the washout period, frequent blood samples (every 4th day in subgroups of 4 rats) were obtained to follow the disappearance of the deuterium from serum water, free amino acids in serum, and serum protein amino acids. In addition, the rats were weighed every week to determine when the weekly weight gain started to level off (to comply with the assumption of a steady protein mass). The rats were euthanized and tissues harvested after 35 (n = 4) and 38 days (n = 4) on tap water.

Experiment 3. At this point, we had determined the amount of time necessary to label the slow turnover tissue proteins (experiment 1) and the time necessary to allow the tracer to disappear from the precursor pool (experiment 2). The purpose of the third experiment was to measure the disappearance rate of deuterium-labeled amino acid tracer from various tissue proteins and calculate the FBR of slow turnover structural proteins. Thirty-two young rats at the age of 26 days were primed with $^{2}$H$_{2}$O and grown on 8% volume deuterium oxide in their drinking water for 42 days. On day 42, the four subgroups of rats were returned drinking plain tap water for another 21 days (age: 89 days), at which point groups of eight rats were euthanized and tissues harvested on four different days (days 0, 1, 2, and 4). The protein-bound amino acid tracer enrichments in the various tissues at these time points were used to calculate the FBRs (see Calculation of rat FBR).

Rat Sample Preparation and Analysis

Serum water sample preparation. Serum was obtained from whole blood that was allowed to clot and subsequently spun (6 min at 3,200 g) in Vacutte tubes (Z serum sep clot activator; Greiner Bio-one, Kremsmünster, Germany). After testing several methods to obtain serum water, we ended up simply diluting aliquots of serum (~5 mg) 1:250 by weight with tap water to obtain a deuterium enrichment within the measurable range for isotope ratio mass spectrometer (IRMS) analysis.

Serum Water Deuterium Abundance Analysis By Gas Chromatograph-P-IRMS

Determination of $^{2}$H/$^{1}$H ratios in body water was performed using an Agilent 6890A gas chromatograph (GC) coupled with a PDZ Europa (Cheshire, UK) model 20/20 IRMS via a pyrolysis reactor and a PDZ Europa Orchid GC module. Conditions used were GC inlet temperature of 200°C, injection split 30:1 onto a 5-m deactivated silica column kept isothermal at 200°C, and a helium carrier gas flow of 2 ml/min, which served to isolate water vapor and carry it to the pyrolysis reactor (350-mm length, 0.8-mm ID aluminum oxide tube-packed 0.1-mm nickel wires held at 1,050°C). Prior to each day’s deuterium water analyses, the pyrolysis reactor was conditioned by injection of 10 μl of hexane to provide a carbon source for water pyrolysis to hydrogen gas and carbon monoxide. The pyrolysis gas product subsequently entered the Orchid module containing a Naion water trap (Permapure, Toms River, NJ) and a Poraplot Q column (Varian, Palo Alto, CA) that transferred the hydrogen on to the IRMS. The IRMS was calibrated for $^{2}$H enrichment measurement by injection of water standards of known $^{2}$H content before each day’s analyses. All samples were run seven times, and all enrichments (in ppm $^{2}$H) are the mean of the last four analyses.

Serum water enrichment TTR calculation. The deuterium enrichment as TTR of $^{3}$H-enriched water was calculated by first subtracting the abundance of deuterium in unlabeled water from that in the enriched sample and subsequently correcting the enrichment (in ppm) with a factor derived from the daily measured deuterium abundances in the standards and then multiplying by 250 (the dilution value of the serum). This $^{2}$H enrichment value (in ppm) was finally converted to TTR in percent.

Serum free amino acid preparation. A 40-μl aliquot of serum was added to 400 μl of ice cold acetone to precipitate serum proteins and then centrifuged. The supernatant was removed and evaporated at 70°C under a stream of nitrogen. The amino acids were then derivatized using MTBSTFA + 1% TBDMCS, and acetonitrile was mixed 1:1 and heated at 70°C for 30 min to allow maximal tert-butyldimethylsilyl (TBDMS) derivitization.

Serum protein-bound amino acid preparation. The acetone-precipitated serum proteins were transferred to glass vials that contained a 1-ml slurry of cation exchange resin and 1 ml of 0.1 M HCl. The vials were capped and left overnight (~15 h) at 110°C to hydrolyze proteins. The liberated free amino acids were purified over columns containing washed and acidified cation exchange resin and eluted with 2 × 1 ml of 4 M NaH$_{11}$O, which was subsequently evaporated under a stream of nitrogen at 70°C. Amino acids were derivatized as the TBDMS derivative, as described above.

Tissue protein-bound amino acid preparation. Frozen (~80°C) skeletal muscle, heart muscle, and liver specimens were thawed in 1 ml of ice-cold homogenization buffer (0.15 M NaCl, 0.25 M sucrose, 0.2 mM EGTA, 0.2 mM EDTA, 0.5% Triton X-100, and 0.02 M Tris, pH 7.4) and subsequently homogenized using a Polytron RT-2100 with a dispersing aggregate size 2EC (Kinematica, Luzern, Switzerland). The homogenized tissues were left at 4°C for ≥1 h. The muscle homogenates were spun (800 g, 4°C, 20 min) to pellet myofibrillar and collagen proteins. Added to the muscle pellets was 1 ml of a myofibrillar protein solubilization buffer (0.7 M KCl, 0.1 M tetrasodium pyrophosphate), which was left for 1 h at 4°C and spun (1,200 g, 4°C, 20 min) to pellet collagen proteins. The supernatant containing the myofibrillar proteins was removed, and 2.3 volumes of ethanol were added to precipitate proteins, and the samples were spun (1,600 g, 4°C, 20 min) to pellet proteins. The pelleted proteins from the liver specimens were precipitated after homogenization by centrifugation (4,000 g, 4°C, 20 min), and the supernatant was discarded. The tendon samples were thawed in 1 ml of collagen precipitation buffer (0.7 M KCl, 0.02 M Tris, pH 7.4), which was left at 4°C for 1 h and spun (3,200 g, 4°C, 20 min) to pellet collagen proteins. All protein isolates were washed once with an ice-cold 80% propanol solution, left for 30 min, and subsequently spun (1,600 g, 4°C, 20 min), and the supernatant was discarded before the samples were hydrolyzed overnight (~15 h) at 110°C in a 1-ml slurry of cation exchange resin and 1 ml of 0.1 M HCl. The free amino acids were purified and derivatized as the TBDMS derivative, as described above.

Amino acid deuterium abundance analysis by gas chromatography/ mass spectrometry. Rat serum-free and protein amino acids as well as tissue protein amino acid $^{2}$H enrichments were determined by gas chromatography/mass spectrometry (GC-MS) (Agilent 5973; Agilent Technologies, Palo Alto, CA). Samples were injected onto a capillary column (29 m × 0.25 mm, film thickness 0.5 μM DB-17, J & W Scientific; Agilent Technologies) that was programmed over a temperature range of 155 to 280°C. The mass spectrometer was operated in electron ionization mode and selected ion monitoring. For most amino acids, the [M-57] ion fragment and one mass higher was monitored, except for leucine, where the [M-159] fragment and one mass higher was used. The specific ions monitored were mass-charge ratios 260 and 261 for alanine, 246 and 247 for glycine, 288 and 289 for valine, 200 and 201 for leucine, 286 and 287 for proline, 390 and 391 for serine, and 336 and 337 for phenylalanine. All sample
isotope ratios reported are the mean of four runs with an overall 95% confidence interval within the range of 0.065–0.133%. Correspondingly, the pooled inter-rat variation was 0.71–1.92%.

**Amino acid enrichment TTR calculation.** The area intensity [M+1]/M isotope ratio was determined for each amino acid measured by GC-MS where M is the base fragment ion and M+1 is the fragment ion one mass heavier. The M+1 tracer/tracee enrichment ratio (E_{M+1}) of each sample was calculated by subtracting the measured natural background (BKGD) ratio from the labeled sample ratio (LAB):

\[
E_{M+1} = \frac{[M+1]}{M}_{LAB} - \frac{[M+1]}{M}_{BKGD}
\]

The number of sites exchanging deuterium atoms in vivo with \(^2\text{H}_2\text{O}\) on each individual amino acid (n_H) was calculated by dividing the M+1 enrichment by the water enrichment at equilibrium:

\[
n_H = \frac{E_{M+1}}{E_{H_2O}}
\]

The standard error of the number of exchanged hydrogens for each amino acid (SE_{n_H}) was approximated by the equation

\[
SE_{n_H} = \sqrt{\left(\frac{SE_{E_{M+1}}}{n_H} + \frac{SE_{E_{H_2O}}}{E_{H_2O}}\right)^2}
\]

where it is assumed that 1) the measurements of the number of exchanged hydrogens of each amino acid are independent and no correlation exists between them, and 2) an equal number (H) of measurements was performed for the enrichment of each amino acid M+1 and deuterated water (n = 5).

The actual \(^2\text{H}\) tracer/tracee enrichment ratio of each amino acid (E) was then calculated:

\[
E = \frac{E_{M+1}}{n_H}E
\]

**Calculation of rat FBR.** Using rats allowed us to collect tissue at only one time point per animal. Therefore, in experiment 3, we averaged the protein-bound alanine \(^2\text{H}\) enrichments from the unrelated subgroups (n = 8/group): days 0, 1, 2, and 4 for each protein type. To calculate the disappearance rate of \(^2\text{H}\) alanine from the tissue one mass heavier. The M/H11001 alanine enrichments would be negligible.

Seven healthy men (age: 31 ± 2 yr; height: 184 ± 2 cm; body mass index: 22.3 ± 0.7 kg/m\(^2\); lean body mass: 66.1 ± 2.3 kg) gave their written consent to participate in the study that was approved by the local ethics committee of Copenhagen municipal (H-1-2010-007). On day 0, all subjects drank a total of 233 ± 8 ml of \(^2\text{H}_2\text{O}\) (estimated to be ~0.5% of total body water (9, 42)) mixed 1:2 with tap water given as six divided hourly oral doses. All subjects had a blood sample taken prior to administration of the first bolus of \(^2\text{H}\) water. In four subjects, blood was drawn hourly until 1 h after the last bolus. Blood samples were obtained from all subjects on days 1, 2, 3, 7, 14, 17, 24, 41, 50, 59, 70, 80, and 85. Muscle specimens were collected from five subjects and skin biopsies from two subjects to determine the abundance of labeled alanine in different tissue proteins at various time points. All subjects had biopsies taken on day 42 to verify the proximity between the alanine \(^2\text{H}\) enrichment in proteins and precursor. Tissue biopsies were obtained again on days 80, 85, and 90. Tissue sample sites were prepared with local anesthesia (1% lidocaine). Skin was taken from the posterior hip with a sterile 4-mm disposable Biopsy Punch (Miltex, York, PA), and muscle was sampled from vastus lateralis with a 14-gauge disposable core tissue biopsy needle (MN1410) using a Biopsy Instrument (MGI1522, Bard Magnum; Bard, Covington, GA). All tissue samples were immediately frozen in liquid nitrogen and saved at ~80°C until later analysis.

**Human serum-free and protein-bound alanine preparation.** Aliquots (100 μl) of serum were added to 500 μl of 50% acetic acid left for 30 min and then poured over acidified AG 50W-X8 cation resin columns. The alanine acids were eluted by adding 2 × 1 ml of 4 M NH₄OH to the columns. The eluent was collected and evaporated under a stream of nitrogen at 70°C. TBDMS derivatization was performed as described previously.

**Serum-free deuterated alanine enrichment analysis by GC-MS.** The alanine abundances in human serum samples were determined by GC-MS (Finnigan Mat Automass II MS, Finnigan), as described for the rat samples with small changes that allowed for the use of larger samples.

**Protein-bound alanine preparation.** Amino acids from serum proteins were isolated in a similar manner as per the rat samples, with small changes that allowed for use of larger samples. Proteins were isolated from 400-μl aliquots of serum by precipitation by adding 1 ml of 50% acetic acid with subsequent centrifugation. Amino acids were isolated from proteins in frozen muscle and skin specimens after homogenization (FastPrep 120A-230; Thermo Savant, Holbrook, NY) in 1 ml of ice-cold homogenization buffer, as used with the rat samples. Homogenates were treated as per the rat samples to isolate myofibrillar proteins. These protein isolates and the serum protein isolates were hydrolyzed overnight at 110°C in 1 ml of 6 M HCl. The free amino acids were purified over cation resin columns and derivatized as the NAP derivative (11).

**Deuterium-labeled protein-bound alanine enrichment analysis by GC-P-IRMS.** The alanine abundances in human serum, myofibrillar, and skin collagen proteins were analyzed on a GC-P-IRMS system (GC Combustion III, Delta Plus XL; Thermo Finnigan). The NAP-amino acid samples were injected in the PTV mode with the GC inlet initially at 45°C for 1 min then elevated to 280°C with 20°C/min. The GC column (30 m × 0.25 mm film thickness, 1 μm DB-1701; J & W Scientific) was ramped from 45 to 280°C. All GC column effluent passed into a high-temperature reduction reactor (1,450°C) where the organic compounds were reduced to hydrogen gas and carbon monoxide before entering the IRMS.

**Calculation of human protein FBR.** For the human experiment, we obtained paired samples and were able to determine the FBR of each individual’s muscle protein fraction. By log transformation of the measured enrichment values, we determined the slope, k, of the ln(E) vs. time curve as an average across the time between the latter three biopsies (10 days) as well as the adjacent biopsies (5 days).
Statistics

Changes in body weights in deuterium-labeled and control rats were compared by a two-factor (water and time) ANOVA with repeated measures. The level of deuterium enrichments in serum water and in free and protein-bound alanine turned out not to be normally distributed and thus was tested using a nonparametric one-way ANOVA (Kruskal-Wallis) test followed by Dunn’s multiple comparison post hoc test. Finally, the alanine $^2$H enrichments in the various tissue protein fractions obtained in experiment 3 were compared using a one-way ANOVA. When a significant effect was seen, the Holm-Sidak post hoc test was applied to compare individual enrichments. For the human experiment 4, the differences in tissue protein-bound alanine were compared by a one-way ANOVA with repeated measures, applying a Holm-Sidak post hoc test. A value of $P < 0.05$ was defined as significant. Unless otherwise stated, all values are means ± SE. All statistical tests were performed in SigmaPlot 11 (Systat Software, San Jose, CA).

RESULTS

Experiment 1

Rat growth while $^2$H$_2$O is consumed. Rats at the age of 26 days were randomized to receive deuterium-labeled water ($^2$H$_2$O; $n = 16$) or plain tap water (H$_2$O; $n = 5$). The starting body weights were similar ($^2$H$_2$O: 78.4 ± 1.5 g; H$_2$O: 73.4 ± 2.1 g; $P > 0.05$). During the following 56 days, rats fed $^2$H$_2$O were slightly, but statistically, heavier than the control rats after 7 days of being fed deuterium oxide ($P < 0.05$; Fig. 2A). However, the relative growth rate across 7 days was not statistically different between the groups (Fig. 2B). As expected, the rate of growth diminished as the rats got older.

Deuterium enrichment in the rats fed $^2$H$_2$O. The intraperitoneal priming dose, followed by administration of $^2$H$_2$O to the rats in their drinking water, elevated the rats’ body water deuterium enrichment (as measured from serum samples) to 3.88 ± 0.08% after 1 day and to 5.77 ± 0.05% after 7 days. Any fluctuations in the measured deuterium oxide enrichments after day 7 are ascribed to be a consequence of alterations in water and food intake. The mean body water enrichment during the deuterium labeling period from days 7–42 was 5.20 ± 0.17% (Fig. 3). Figure 3 also shows the M + 1 enrichments measured by GC-MS for free alanine in serum and alanine in serum protein from days 1–42. Both serum-free and serum protein alanine enrichments rose from days 0-14 (1-way ANOVA on ranks, Kruskal-Wallis, $P < 0.001$) and then reached a plateau.

The measurement of free alanine enrichment from serum was difficult because of the limited volume of serum available, hence resulting in incomplete data sets. However, a small serum sample contained more than adequate alanine in serum proteins for measurement. Therefore, after establishing that free alanine and serum protein alanine had equivalent deuterium enrichments at isotopic steady state, we used serum protein alanine enrichments as a measurement of free alanine enrichment in subsequent studies. Anticipating this to be true for other amino acids in the fast-turning over serum proteins, we also used serum protein as the proxy source for enrichment measurements for other amino acids.

The M + 1 enrichments measured by GC-MS of serum protein alanine, glycine, valine, leucine, proline, serine, and phenylalanine at isotopic steady state as well as the number of hydrogens that are exposed for exchange between water and each amino acid in vivo are shown in Table 1. Valine, leucine, proline, and phenylalanine had one hydrogen exposed for exchange, which would be the α-hydrogen that would exchange during transamination. Serine and glycine had two exchangeable hydrogens, and alanine exchanged four hydrogens, similar to the data of Previs et al. (38). As per the thinking of Previs et al. (38), we chose alanine as the tracer for determining the FBR of proteins because it has the highest number of exchangeable hydrogens and, therefore, produces the greatest M + 1 enrichment measurement by GCMS, giving the highest analytical sensitivity.

Deuterated alanine enrichment in rat proteins. The labeling of alanine with deuterium in tissue proteins was assessed by analyzing tissue samples from rats in the $^2$H$_2$O group euthanized on days 0, 7, 14, 21, 28, 35, 42, 49, and 56. The starting body weights in deuterium-labeled and control rats were compared by a two-factor (water and time) ANOVA with repeated measures. The level of deuterium enrichments in serum water and in free and protein-bound alanine turned out not to be normally distributed and thus was tested using a nonparametric one-way ANOVA (Kruskal-Wallis) test followed by Dunn’s multiple comparison post hoc test. Finally, the alanine $^2$H enrichments in the various tissue protein fractions obtained in experiment 3 were compared using a one-way ANOVA. When a significant effect was seen, the Holm-Sidak post hoc test was applied to compare individual enrichments. For the human experiment 4, the differences in tissue protein-bound alanine were compared by a one-way ANOVA with repeated measures, applying a Holm-Sidak post hoc test. A value of $P < 0.05$ was defined as significant. Unless otherwise stated, all values are means ± SE. All statistical tests were performed in SigmaPlot 11 (Systat Software, San Jose, CA).
Deuterium enrichments in serum-free alanine and protein-bound alanine (E[2H]) and in serum water (E[2H2O]). The symbols are M + 1 enrichments of free alanine in serum (○; n = 2–4/time point at left y-axis) and M + 1 enrichments of serum protein-bound alanine (●; n = 4–12/time point at left y-axis) and deuterium enrichment of serum water (●; n = 4–12/time point at right y-axis) from rats grown for 42 days on 8% volume deuterium-enriched drinking water. Values are means ± SE, although the error bars may be smaller than the symbols. A nonparametric 1-way ANOVA (Kruskal-Wallis) test was performed to define the time point when 2H enrichment was in isotopic steady state in each pool. Alanine deuterium enrichments were in isotopic steady state from days 14 to 42 in serum both free and protein bound, whereas the water enrichment fluctuated slightly more but had equilibrated in the body water pool by day 7. The mean deuterium enrichment for serum-free alanine at steady state is shown as a dashed line (20.66 ± 0.17%) and for body water as a solid line (5.20 ± 0.17%).

Experiment 2

Disappearance of deuterium from body water and serum proteins after removal of 2H2O drinking water. To estimate the minimum number of days that had to elapse after the 2H2O was removed before the FBR could be measured, eight rats were grown first for 42 days on 8% volume deuterium oxide drinking water and then placed on unlabeled drinking water for another 38 days. Figure 4 shows the disappearance of deuterium from serum water, free alanine in serum, and serum protein alanine of the rats (experimental days 42–80). The 2H enrichments decreased monoeXponentially as expected in body water, serum free alanine, and serum proteins (Fig. 4). Any remaining deuterium label in water and hence, free alanine after switching the rats from 2H2O to tap water can be re incor porated into the body proteins and thus underestimate the measured FBR. For this purpose, we needed to determine the maximal amount of residual free alanine 2H enrichment allowable based upon the exponential decay constant for serum free alanine 2H enrichment kAla. From this constant, we can calculate the minimum time (tmin) to get the enrichment down below a preset threshold. For example, based upon kAla = 17.9%/day (Fig. 4), we would have to wait 13 days before the residual free alanine 2H enrichment dropped to <10% of its initial value before the FBR could be measured. For convenience, we extended this period to 3 wk, or 21 days, of plain tap water. From Eq. 7 and the measured kAla, we estimate that the residual free alanine 2H enrichment was now only 2% of its initial value. We also note that this time occurs at a rat age where weekly growth was minimal (Fig. 2B).

Of the eight rats in experiment 2, four rats were euthanized at 35 days and four rats euthanized at 38 days after return to tap water to harvest tissues (experimental days 77 and 80). The alanine enrichments for nonsoluble mixed liver proteins and tendon collagen protein are shown in Fig. 4 and compared against the alanine enrichments in the same proteins in rats euthanized on the last day of 2H water administration (day 42).

At this point, we had determined that 42 days of deuterium labeling followed by 21 days on unlabeled water was adequate first to label the proteins of interest and subsequently to reduce the deuterium enrichment of free alanine in serum and body water to negligible levels yet have sufficient deuterium retained in various slow turnover proteins. Because the rats were also weight stable on a weekly basis at this age, these conditions were selected for measuring protein breakdown rates in experiment 3.

Experiment 3

Measurement of the rat tissue protein FBRs. Rats (n = 32) were euthanized on days 0, 1, 2, and 4, corresponding to 21, 22, 23, and 25 days on unlabeled water, respectively, after 42 days of feeding with 2H water (Fig. 5). The mean body weights of the four subgroups of rats were not different at euthanization

Table 2. Deuterium enrichment in body water and in protein-bound alanine in serum and in various tissues

<table>
<thead>
<tr>
<th>Days on 8% Volume 2H2O</th>
<th>Body Water</th>
<th>Serum Protein</th>
<th>Liver</th>
<th>Soleus Mix</th>
<th>LV Myofilibrillar</th>
<th>LV Collagen</th>
<th>Tendon</th>
</tr>
</thead>
<tbody>
<tr>
<td>42#</td>
<td>4.69 ± 0.20</td>
<td>5.24 ± 0.11</td>
<td>5.22 ± 0.08</td>
<td>5.20 ± 0.07</td>
<td>4.93 ± 0.11</td>
<td>4.77 ± 0.06</td>
<td>4.29 ± 0.05</td>
</tr>
<tr>
<td>49#</td>
<td>4.86 ± 0.11</td>
<td>5.63 ± 0.08</td>
<td>5.43 ± 0.06</td>
<td>5.29 ± 0.09</td>
<td>5.06 ± 0.07</td>
<td>4.93 ± 0.06</td>
<td>4.52 ± 0.01</td>
</tr>
<tr>
<td>56</td>
<td>5.47 ± 0.06</td>
<td>6.01 ± 0.12</td>
<td>5.88 ± 0.14</td>
<td>5.80 ± 0.08</td>
<td>5.46 ± 0.11</td>
<td>5.25 ± 0.07</td>
<td>4.84 ± 0.09</td>
</tr>
<tr>
<td>63</td>
<td>5.60 ± 0.16</td>
<td>5.66 ± 0.04</td>
<td>5.56 ± 0.03</td>
<td>5.84 ± 0.03</td>
<td>5.24 ± 0.05</td>
<td>5.05 ± 0.02</td>
<td>4.78 ± 0.06</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 4/time point. LV, left ventricle. Alanine 2H enrichments (%) are E = E[2H] + r/nH, where nH = 4 for alanine. Protein fractions are serum protein (mainly albumin), liver mix (midlrobe nonsoluble liver proteins), LV myofilibrillar (heart left ventricle myofilibrillar proteins), LV collagen (heart left ventricle collagen proteins), and tendon (patella tendon collagen protein fraction). #Enrichments on day 42 were different from enrichments on days 56 and 63; ##enrichments on day 49 were different from enrichments on day 56.
A significant loss of protein-bound $^2$H alanine was found after 2 days in heart left ventricle collagen protein, after 3 days in heart left ventricle myofibrillar protein, EDL myofibrillar protein, soleus muscle myofibrillar and collagen proteins, and liver nonsoluble mixed proteins, and after 4 days in patella tendon collagen proteins. Thus, 2–4 days are needed between tissue collections to determine the FBR of slow turnover proteins in rats.

**Fig. 4.** Time course of deuterium enrichments in serum water, free alanine in serum, alanine in serum proteins, and alanine in tendon and liver proteins after removal of $^2$H water input. All rats consumed $^2$H$_2$O for the first 42 days and then plain tap water thereafter. The data start on day 42. • Serum water $^2$H enrichments ($n = 4$/time point); dotted line is the fitted exponential curve ($k = 18.03 \pm 0.22%/day$). $\triangle$, Serum-free alanine $^2$H enrichments ($n = 2–3$/time point); dashed line is the fitted exponential curve ($k = 17.94 \pm 0.82%/day$). Gray triangles, serum protein-bound alanine $^2$H enrichments ($n = 4$/time point); solid line is the fitted exponential curve ($k = 9.93 \pm 0.58%/day$). $\Box$, Tissue protein-bound alanine $^2$H enrichments for nonsoluble mixed liver proteins. $\bigcirc$, Tissue protein-bound alanine $^2$H enrichments for tendon collagen proteins. Values are means ± SE, although the error bars may be smaller than the symbols at some time points. The serum water and serum-free alanine enrichments overlap at several time points.

**Fig. 5.** Enrichment of $^2$H alanine in slow turnover tissue proteins. Points are the means ± SE of alanine $^2$H enrichments for 8 rats euthanized on each day. Dashed lines represent the exponential decay fitted to the 4 mean enrichments for each protein. The x-axis is the no. of days on plain tap water after 42 days on deuterated water. The experimental group abbreviations represent days of euthanization for FBR determination (days 0, 1, 2, and 4).
Table 3. Protein FBRs in rats

<table>
<thead>
<tr>
<th>Tissue/Protein</th>
<th>FBR, %/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skeletal muscle</td>
<td>1.50 ± 0.261%</td>
</tr>
<tr>
<td>Myofibrillar, soleus</td>
<td>1.50 ± 0.261%</td>
</tr>
<tr>
<td>Myofibrillar, EDL</td>
<td>1.66 ± 0.28%</td>
</tr>
<tr>
<td>Collagen, soleus</td>
<td>2.99 ± 0.66%</td>
</tr>
<tr>
<td>Heart muscle, left ventricle</td>
<td>5.30 ± 0.88%</td>
</tr>
<tr>
<td>Myofibrillar</td>
<td>0.66 ± 0.28%</td>
</tr>
<tr>
<td>Collagen</td>
<td>0.66 ± 0.28%</td>
</tr>
<tr>
<td>Tendon, collagen, patella</td>
<td>0.06 ± 0.50%</td>
</tr>
<tr>
<td>Liver, nonsoluble mix</td>
<td>5.00 ± 1.50%</td>
</tr>
</tbody>
</table>

Values are means ± SE. FBR, fractional breakdown rate; EDL, extensor digitorum longus. The FBR values are the slopes of the linear relation between the log-transformed 2H alanine enrichments determined on days 0, 1, 2, and 4. The soleus muscle in rats consists of 80% myosin heavy chain (MHC) I, and the remaining 20% is MHC IIa, whereas EDL consists of 10% MHC IIa and 90% MHC IIB’s fiber type II. The mixed liver proteins were the nonsoluble proteins pelleted by centrifugation after homogenization.

Experiment 4: Human Experiment

Human free and protein-bound alanine enrichments. All subjects experienced a mild vertigo starting ~30 min after intake of the first bolus of deuterated water and continuing until 2–3 h after the last bolus. Otherwise, no other side effects were experienced. One hour after drinking the first bolus of deuterated water, a marked elevation in deuterated alanine was present in the serum, and it continued to rise \( (n = 4; \text{Fig. 6A}) \). One hour after intake of the last \( ^2 \text{H}_2 \text{O} \) bolus, the alanine \( ^2 \text{H} \) enrichment reached a plateau and remained unchanged through day 2 \( (\text{Fig. 6A}) \). The alanine \( ^2 \text{H} \) enrichment in serum began to decline by day 3. The alanine \( ^2 \text{H} \) enrichment rose in serum protein exponentially at a slower rate over the 3-day measurement period \( (\text{Fig. 6A}) \). During the subsequent washout period, the serum-free alanine \( ^2 \text{H} \) enrichment declined exponentially \( (\text{Fig. 6B}) \) and returned to background levels after ~80 days. As expected, alanine \( ^2 \text{H} \) enrichments in serum protein rose slower and to a lesser peak enrichment than in the serum-free pool \( (\text{Fig. 6B}) \).

At 42 days after the initial administration of the deuterated water, we obtained vastus lateralis muscle biopsies in four subjects and skin biopsies in two subjects. We found that the alanine \( ^2 \text{H} \) enrichment in myofibrillar protein was \( 0.0449 ± 0.0054% \) \( (\text{Fig. 7}) \), which was similar to the enrichment in serum-free alanine \( (0.0486 ± 0.0389%; \text{Fig. 6B}) \). At the same time, the skin-derived collagen proteins had an enrichment of \( 0.0019 ± 0.0009% \) \( (\text{Fig. 7}) \).

On days 80, 85, and 90, we obtained muscle vastus lateralis biopsies from five subjects and skin biopsies from two subjects. The alanine \( ^2 \text{H} \) enrichment declined over time in the myofibrillar protein \( (\text{Fig. 7}) \). However, the alanine \( ^2 \text{H} \) enrichment in skin collagen protein had diminished to background levels \( (\text{data not shown}) \) by day 80. Thus, there was significant \( ^2 \text{H} \) enrichment remaining in the myofibrillar proteins to determine FBR but insignificant \( ^2 \text{H} \) enrichment in either serum protein or skin collagen protein alanine at this point in time. We fit the alanine \( ^2 \text{H} \) enrichment in the myofibrillar proteins to a monoexponential decay and calculated the FBRs of the myofibrillar proteins over 5-day periods: 80–85 days, \( 1.68 ± 0.348%/\text{day} \); 85–90 days: \( 1.76 ± 0.261%/\text{day} \); and over the 10-day period \( (80–90 \text{ days}) \): \( 1.73 ± 0.185%/\text{day} \).
acids exchanging the highest number of deuteriums, giving the highest analytical sensitivity. Previously, Previs et al. (38) and Rachdaoui et al. (39) determined in both humans and rodents, respectively, that alanine exchanges essentially four carbon-bound hydrogens in vivo. We also note almost complete exchange (3.97 ± 0.13) of these four hydrogens in alanine. Furthermore, the exchange of deuterium between water and alanine via the transamination reaction is very fast both during administration of $^2$H water (Fig. 3) and when rats were returned to unlabeled drinking water (Fig. 4), which is in accord with previous reports (5, 13).

An important point for measuring the rate of fractional protein breakdown by our method compared with measuring fractional synthetic rates of proteins is that the FBR measurement does not depend upon knowing the deuterium labeling of a precursor species. The FBR measurement requires only measurement of the rate of loss of deuterated amino acid enrichment from a protein. Alanine was chosen for use here because 1) alanine is relatively abundant in proteins, 2) alanine is readily measured by both GC-MS and GC-P-IRMS, 3) alanine hydrogens exchange rapidly with water hydrogens, resulting in quick deuterium equilibration with water, and 4) alanine has the highest number of exchangeable hydrogens of the amino acids measured and, therefore, provides the greatest analytical sensitivity.

We expected that one hydrogen would exchange for the essential amino acids that undergo transamination in vivo, i.e., leucine, isoleucine (not measured), valine, and phenylalanine. The exchange for leucine and valine was ~0.9 and ~0.7 hydrogens, respectively (Table 1) but only ~0.4 hydrogens for phenylalanine. This pattern of exchange in our rats is similar but higher than the pattern of exchange reported by Rachdaoui et al. (39) in mice.

As would be expected, glycine and serine each exchanged about two hydrogens. These results are consistent with what Rachdaoui et al. (39) found in mouse liver for glycine, but they found less than one hydrogen exchanged for serine. We did not measure glutamate or glutamine in protein because hydrolysis combines the two. But Rachdaoui et al. (39) measured free glutamate and glutamine in mouse liver and found the exchange of hydrogen to be ~3.5 and 3.0, respectively. Although proline is synthesized from glutamate, we found that the number of hydrogens exchanged in proline to be only 1.2. Rachdaoui et al. (39) found 2.2 protons exchanged in mouse liver-free proline, still lower than expected if it was synthesized solely from glutamate, suggesting that de novo proline synthesis from glutamate is a minor component of proline turnover via protein synthesis and breakdown. All in all, our conclusion from the results reported here and those reported previously (39) is that alanine is the only amino acid to exchange consistently the majority of its exposed hydrogens. The reason that this result is possible is that reversible transamination is a critical part of alanine metabolism, providing high throughput in both directions, which is in agreement with previous studies in humans using different labeled alanines (48).

Water Enrichment Determines the Tracer Enrichment

A crucial assumption in the model is that the amount of tracer amino acid in the free pool is negligible during the period of the breakdown measurement and, therefore, cannot return tracer to the protein. For this reason, we completed experiment 2 to determine when the $^2$H enrichment in body water and intracellular free alanine [measured from serum protein (albumin)-bound alanine] had diminished to inconsequential levels. At the same time, we wanted to keep the maximum amount of $^2$H labeling in alanine in the proteins for which we were trying to determine FBR to obtain the best analytical sensitivity. The optimal point in time to measure the protein FBR was, therefore, a compromise. In the present rat experiments, we defined that time to be about 21 days after removal of the $^2$H water intake. At this point, both body water and free alanine $^2$H enrichments had decreased to negligible levels (~0.1%) compared with the $^2$H enrichments of the slow turnover proteins of 1–4% (Fig. 5). This scheme works only for slow turnover proteins. Proteins that turnover quickly will lose $^2$H labeling, similar to what is seen for serum proteins (Fig. 4), and cannot have their FBR measured by this method.

An important advantage of using $^2$H water to label alanine is that labeling occurs quickly and that when $^2$H water is removed, the alanine labeling quickly disappears through exchange with unlabeled water. Thus, recycling and reincorporation of alanine $^2$H labeling during measurement of FBR will be negligible once the water labeling is relatively absent.

Comparing the FBRs With Existing FSRs

To validate the measured FBR values in the rats, we compared them with existing FSR values in comparable animals. For example, the FSR of fast fiber type muscles in adult Lister rats of similar size as our rats was 3.5 ± 0.4%/day, using a continuous infusion of radiolabeled tyrosine (3), which was comparable with our FBR on EDL muscle (Table 3). Furthermore, for mixed muscle protein in meal-fed rats, the FSR is reported to be in the range of 4.6–8.9%/day (3, 15, 22). Using deuterated water as label donor to amino acids but still measuring mixed muscle protein synthesis over short (4- to 6-h) intervals, Gasier et al. (16) and Yuan et al. (50) reported conflicting values ranging from 8 to 19%/day. Thus, even in the literature, the muscle FSR values of comparable animals seem to span a range where our FBR values fit in the lower end. This may be related to inherent differences between
methods and the fact that prolonged measurements of FSR seem to reduce the values (16). Muscle-derived collagen turnover rates in smaller rodents are more sparsely investigated than for contractile proteins. Laurent (28) as well as McAnulty and Laurent (29) have reported synthesis rates for mature collagen from rabbits and rats, respectively, in heart and skeletal muscle (5.2 ± 0.7 and 2.2 ± 0.3%/day, respectively) that are similar to our breakdown rates (Table 3).

By the use of the flooding dose tracer method, heart myofibrillar proteins have been shown to have a slightly higher protein synthesis rate (17.5 ± 0.6%/day) compared with skeletal muscle myofibrillar proteins (plantaris myofibrillar of 14.3 ± 1.1%/day) (37). We verified this difference between heart and skeletal muscle protein turnover rates, albeit with markedly lower values (Table 3). However, note that the reported skeletal muscle myofibrillar FSR is markedly higher in that reference than in some of the previous studies discussed above, emphasizing that it may be hard to compare the numerical values from study to study. Similarly, from liver we isolated a nonsoluble structural protein fraction containing slow turnover proteins and measured a much lower breakdown rate than reported for synthesis of mixed hepatic proteins, ranging from 23%/day in young, growing Holtzman albino rats (22) to 20–50%/day in male Wistar rats (15) and ≤90%/day in other rat strains (8, 30). Such differences between the turnover rates between studies are probably due to the abundance of different proteins in the tissue homogenates used. The FSR measurement relies on measuring incorporation of tracer into proteins and will include the weighted average of all the different proteins in that pool. If the pool of proteins includes some fast turnover proteins, then the FSR measured will be higher than if specific proteins of slower rate were measured alone, which the results from Gasier et al. (16) support. However, in the FBR measurement, faster turnover proteins that lose their alanine 2H label by the beginning of the sample collection window will not contribute to disappearance of [2H]alanine during the sample collection window and will not contribute to the FBR rate. Thus, the FBR measurement using 2H2O will measure slow turnover protein rates even with some contamination of faster turnover proteins in the sample. This divergence between FSR and FBR measurements can be avoided only by isolating single proteins and measuring their turnover rate, similar to work by Jaleel and colleagues (24, 25).

**Human Data**

We administered an average of 233 ml of deuterated water per adult subject to add about 0.5% deuterium to the total body water pool. The maximal measured free alanine 2H enrichment was 0.37 ± 0.02% 1 day after the administration (Fig. 6), corresponding to ~70–75% of the administered 2H dose remaining in the free pool. A similar dilution of the administered water enrichment was seen in the rat experiments, where 8% deuterium oxide in the drinking water elevated body water enrichment to 5.2%, i.e., ~70–75% labeling. This dilution is due to metabolic processes that constantly remove hydrogen from water and release hydrogen into body water from compounds not yet labeled (4).

In humans, free alanine became labeled with 2H, quickly approaching plateau enrichment shortly after intake of deuterated water began (Fig. 6A). During the subsequent 80-day washout period, no restrictions were given to the subjects, who varied in daily physical activity level. Three performed endurance-like training three to five times a week, one did resistance training, and the remaining subjects were not involved in organized exercise. These differences presumably resulted in some interindividual temporal variation in the turnover rate of body water (42) and hence, in free alanine 2H enrichment. However, there were measurable amounts of 2H remaining in the alanine in muscle contractile proteins at 80 days. We cannot translate these results to all populations, especially those that may be critically ill, very old, or especially sedentary. For these groups, pilot measurements should be performed to determine the adequate amount of deuterated water necessary to label the proteins of interest as well as the time adequate to allow the free label in alanine and/or water to disappear.

After ~40 days, we found the alanine 2H enrichment in plasma and in myofibrillar protein to be about 0.045%/day. Whereas the 2H enrichment in the free alanine pool was descending, the appearance of labeled alanine in the slow turnover myofibrillar proteins had accumulated up to this point in time. Hereafter, a net loss of label from these proteins starts, although some reincorporation will take place as long as the precursor contains label. However, only a very low amount of 2H had accumulated in skin-derived collagen protein-bound alanine (0.002% at 42 days). This low labeling limited our ability to detect 2H and, therefore, measure FBR in these very slow turnover proteins. For these proteins, a longer labeling period with 2H2O water with larger, repeated doses of deuterated water is required.

The FBR of vastus lateralis myofibrillar protein determined as an average over 10 days was 0.072 ± 0.008%/h. This value corresponds to the rate of myofibrillar protein synthesis that our group reported earlier using the direct incorporation precursor-product approach (12, 19, 31) and is similar in magnitude to the 8-wk synthesis rates reported by Robinson et al. (40) in humans.

**Practicalities**

Our approach described here is suitable only for proteins that have a markedly slower turnover rate than body water, because the 2H label has to be removed from and equilibrate with the free pool of tracer amino acid to prevent reincorporation into protein at the time when FBR is measured. Furthermore, the loading period of 2H alanine incorporation into proteins should be tailored to suit both the species and the turnover rates of the proteins to be measured. Because we are trying to measure the FBR while the protein pool is constant in mass, we want to minimize the sample collection window, that is, the time between the two biopsies obtained to determine protein alanine 2H enrichment needed to calculate FBR. On the other hand, a longer sample collection window is required to allow the detection of the loss of tracer enrichment in the very slow turnover proteins. Thus, the length of the sample window can be optimized to fit the turnover rate of the protein of interest. If done appropriately, the protein mass will undergo minimal change because the change in the mass of a protein is dependent on the protein’s turnover rate. Therefore, planning experiments require first estimating the turnover rates of the proteins measured. From this information and the sensitivity of the
mass spectrometry method that is used for sample analysis, the loading period to enrich the protein to be measured, the time for the intrinsic enrichment of the free (precursor) $^2$H alanine to diminish after stopping administration of the $^2$H$_2$O, and the sample collection window can all be calculated.

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DISCLOSURES

The authors declare no conflicts of interest, and there are no financial interests to disclose.

AUTHOR CONTRIBUTIONS


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