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A novel oral tracer procedure for measurement of habitual myofibrillar protein synthesis†

A. J. MacDonald[1], A. C. Small[3], C. A. Greig[1], H. Husi[2], J. A. Ross[2], N. A. Stephens[1], K. C. H. Fearon[1] and T. Preston[3]*

[1]Clinical and Surgical Sciences, University of Edinburgh, The Chancellor's Building, 49 Little France Crescent, Edinburgh EH16 4SB, UK
[2]Tissue Injury and Repair Group, University of Edinburgh, The Chancellor's Building, 49 Little France Crescent, Edinburgh EH16 4SB, UK
[3]Scottish Universities Environmental Research Centre, University of Glasgow, Rankine Avenue, Scottish Enterprise Technology Park, East Kilbride G75 0QF, UK

RATIONALE: Conventionally, myofibrillar protein synthesis is measured over time periods of hours. In clinical studies, interventions occur over weeks. Functional measures over such periods may be more representative. We aimed to develop a novel method to determine myofibrillar protein fractional synthetic rate (FSR) to estimate habitual rates, while avoiding intravenous tracer infusions.
METHODS: Four healthy males were given 100 g water enriched to 70 Atom % with ^2H_2O as a single oral bolus. Vastus-lateralis needle biopsies were performed and plasma samples collected, 3–13 days post-dose. ^2H enrichment in body water was measured in plasma using continuous flow isotope ratio mass spectrometry (IRMS). Myofibrillar protein was isolated from muscle biopsies and acid hydrolysed. ^2H enrichment of protein-bound and plasma-free alanine was measured by gas chromatography (GC)/pyrolysis/IRMS. Myofibrillar protein FSR was calculated (% day^{-1}).
RESULTS: The tracer bolus raised the initial enrichment of body water to 1514 ppm ^2H excess. Water elimination followed a simple exponential. The average elimination half-time was 8.3 days. Plasma alanine, labelled during *de novo* synthesis, followed the same elimination kinetics as water. The weighted average myofibrillar protein FSR from the four subjects was 1.38 % day^{-1} (range, 1.0–1.9 % day^{-1}).
CONCLUSIONS: Myofibrillar protein FSR was measured in free-living healthy individuals over 3–13 days. Using a single oral ^2H_2O bolus, endogenous labelling of alanine occurred in a predictable manner giving estimates of synthesis comparable with published values. Furthermore, the protocol does not compromise the ability to measure other important metabolic processes such as total energy expenditure. Copyright © 2013 John Wiley & Sons, Ltd.

Muscle wasting is common in a number of diseases such as cancer cachexia, chronic obstructive pulmonary disease, cardiac failure, HIV-AIDS, and renal failure.[1] It is believed that the body's normal mechanisms for muscle growth and repair are impaired in these conditions. It is therefore important to be able to assess skeletal muscle protein fractional synthetic rate (FSR) accurately in order to develop hypotheses for the pathophysiology of muscle wasting in these conditions and also to monitor response to proposed treatments in clinical trials. The measurement of muscle FSR is also of interest in other situations such as sarcopenia (the process of muscle loss seen in ageing), muscle atrophy seen during prolonged bed rest or spaceflight, and sports physiology, where there is increased muscle synthesis during athletic training.[2] Skeletal muscle FSR is measured by the introduction of a labelled tracer, the incorporation of which can be measured in skeletal muscle biopsies. The conventional methodology involves the administration of amino acids such as leucine or phenylalanine labelled with stable isotopes such as ^{13}C or ^2H, and less frequently ^{15}N. Many other stable isotope labelled amino acids have been applied for specific biological and analytical reasons (e.g., glycine, valine, proline, lysine). These protocols require the labelled amino acid to be administered intravenously either as a large bolus (flooding dose protocol) or as a primed constant infusion.[3,4] There are a number of disadvantages with these protocols: (a) administration of supplemental non-dispensable amino acids can stimulate protein synthesis, so may alter the variable of interest; (b) use of an infusion requires the protocol to be undertaken in a controlled study environment, places additional burden on the subject and is likely to restrict their usual levels of activity; (c) infusion protocols are performed over short time periods and are

* *Correspondence to:* T. Preston, Scottish Universities Environmental Research Centre, University of Glasgow, Rankine Avenue, Scottish Enterprise Technology Park, East Kilbride G75 0QF, UK.
E-mail: Tom.Preston@glasgow.ac.uk

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unable to measure the cumulative effects of diseases such as cancer cachexia, treatments or response to exercise training that usually occur over time periods of weeks; and (d) with i.v. infusions, technical difficulties while estimating precursor pool enrichment usually involve the assumption of isotopic equilibrium between a plasma proxy and *t*RNA bound amino acids within the cell. While the latter may be accepted with respect to the use of circulating α-ketoisocaproate for intramuscular 1-^{13}C-leucine, and hippuric acid for intrahepatic ^{15}N-glycine, it remains less certain for other tracer/tissue combinations.[4,5]

One solution to these difficulties is to use 'heavy water' or deuterium oxide (^2H_2O) as tracer.[6] This may be administered orally where it mixes rapidly through the body. Amino acids become intrinsically labelled through the usual metabolic processes. A protocol of this kind avoids supplemental amino acid administration and potentially allows continuation of the procedure in the subject's home or workplace. As the labelling of free amino acids will be considerably faster than their rate of incorporation into muscle protein, either plasma ^2H_2O or plasma-free amino acids may be used as the precursor when calculating skeletal muscle protein FSR. Alanine, in particular, is efficiently labelled with four hydrogen atoms (C2 and C3) being derived from body water. Endogenous or intrinsic labelling by an oral ^2H_2O tracer has been used previously in studies of skeletal muscle synthesis in animal models and in studies of plasma and liver proteins.[8,9] ^2H_2O has also been used to study lipogenesis, cell proliferation and glucose and carbohydrate metabolism in humans and animals.[10] These studies have been able to achieve relatively high enrichments in protein-bound amino acids through measuring fast turnover proteins, giving doses orally or with intravenous or intraperitoneal ^2H_2O in animal models. Recently, its use has been extended to measuring muscle protein synthesis and cell proliferation in elderly subjects over a period of 6 weeks using daily top-up doses.[7]

We believe that an alternative approach is possible, being simple and effective while much less burdensome on subjects, whose frailty may be the focus of the study. Our aim has been to develop a method to measure habitual rates of protein synthesis over periods of 1 to 2 weeks. We have adopted a single bolus approach, where a modest oral dose of ^2H_2O is taken on the first day without subsequent top-up doses. No assumptions of elimination kinetics are necessary as body water is eliminated at a slow and steady rate, which is measured in every subject. Total Body Water (TBW) can be calculated from the intercept of the elimination curve, which is the most accurate method. Fat Free Mass (FFM) and body fat can be derived from TBW. Furthermore, TEE can be measured by simple addition of $H_2^{18}O$ to the bolus dose, should this be indicated. Macronutrient precursor molecules come into rapid equilibrium with body water during *de novo* synthesis. Their kinetics closely follows that of ^2H_2O (see below). A compromise of this approach is that ^2H enrichment in all precursor pools will decline slowly in parallel with body water. However, this is similar to the decline in tracer enrichment over the measurement period of the flooding dose protocol.[3] Precursor enrichment can be estimated precisely by calculating an area under the curve of the elimination kinetics (see below). Should Isotope Ratio Mass Spectrometry (IRMS) be used for all measurements, as reported here, larger doses are unnecessary. Sample size permitting, ^2H enrichments of 10–40 ppm ^2H excess in protein-bound amino acids are measureable with 1 ppm precision. Target body water enrichments of 1000–4000 ppm ^2H excess, a tenth of the target for GC/MS, are readily achieved. The suitability of IRMS for measuring higher enrichments as well as natural abundance is seldom appreciated. We therefore decided to apply a single tracer bolus with IRMS throughout. Unlike GC/MS, IRMS lacks the capability to characterise isotopomer distribution. However, by analysing both body water and free alanine, we could estimate the degree to which precursor enrichment had equilibrated with body water, while permitting comparison with published Mass Isotopomer Distribution Analysis (MIDA) values. In this study we examined the feasibility of a protocol to measure skeletal muscle fractional synthetic rate in free-living healthy volunteers by following the incorporation of ^2H-alanine into myofibrillar protein over a 2-week period using a single oral tracer dose of ^2H_2O combined with IRMS.

EXPERIMENTAL

Human protocol

Four healthy male volunteers (median age: 37 years; median body mass index (BMI): 24.9 kg/m^2) were recruited into the study, which was designed to measure habitual skeletal muscle fractional synthetic rate. Baseline measures of height and weight were taken along with an initial whole blood sample. A single oral dose of ^2H_2O was given with drinking water (100 g of 70 Atom % ^2H). No adverse effects were observed. One or more *quadriceps* biopsies were taken from the *vastus lateralis* under local anaesthetic in each individual between 3 and 13 days after dosing. Blood samples were taken at the same time. Different time points were chosen deliberately for each subject. With three biopsies and blood samples in each of four subjects, a pattern of daily samples over the 3–13-day period could be achieved. No other specific instructions were given to participants who were encouraged to continue their regular diet and activity pattern over the course of the 2-week protocol. Blood samples were collected into EDTA-treated collection tubes, centrifuged at 1,500 *g* for 15 min and the serum was stored at –80°C prior to analysis. Skeletal muscle biopsies were snap-frozen in liquid nitrogen and stored at –80°C until processed. Ethical approval for the study was granted by the South Eastern Scotland Research Ethics Committee.

Preparation of skeletal muscle biopsies

All reagents were supplied by Sigma Chemicals (Poole, UK), unless otherwise stated. Skeletal muscle biopsies (median weight: 35 mg) were processed to isolate the myofibrillar component from sarcoplasmic proteins. Briefly, biopsies were homogenised using a glass hand homogeniser in homogenisation buffer A (250 mM sucrose; 100 mM KCl; 20 mM imidazole; 5 mM EGTA; 2 mM $MgCl_2$; pH 7). An insoluble component was discarded while the resulting suspension was transferred to a spin tube and centrifuged at 1500 *g* for 15 min. The supernatant was drawn off. The remaining pellet was washed in solution B containing the surfactant Triton X-100 (60 mM KCl; 30 mM imidazole; 2 mM $MgCl_2$; 0.1% Triton-X-100; pH 6.8) three times before

two final washes in solution C (150 mM KCl; 20 mM imidazole; pH 6.8). The washed myofibrillar protein pellet was freeze-dried prior to acid hydrolysis. To avoid the need for additional biopsies to determine basal ^2H abundance in protein-bound alanine, albumin was extracted from each basal serum sample by differential solubilisation in ethanol.[11] Albumin and myofibrillar protein pellet samples were freeze-dried prior to acid hydrolysis. Samples of ~2 mg protein were aliquotted into 4-mL glass vials (Chromacol, Welwyn Garden City, UK) where, four at a time, they were placed in a PTFE cylinder. 0.5 mL 6 M HCl was added to the cylinder. Oxygen was excluded by gassing the container with nitrogen before it was sealed within a steel pressure vessel (Parr, Moline, IL, USA). Samples were hydrolysed by gas-phase acid hydrolysis at 150°C for 4 h.[12]

Nor-leucine internal standard (300 nmol) was added to each hydrolysis vial and the amino acids were dried in a vacuum desiccator over NaOH pellets. Amino acids were derivatised as ethoxy carbonyl ethyl esters.[13] Briefly, 0.4 mL of solvent (60:32:8, deionised water/ethanol/pyridine) was added and the sample was swirled to dissolve. Ethyl chloroformate (0.02 mL) was added. The tube was swirled to facilitate reaction and CO_2 release. Dichloromethane (3 mL) and sodium bicarbonate were added (0.75 mL, saturated). The sample tubes were shaken to allow the phases to separate. Once the phases had separated, the upper phase was discarded and the lower phase was dried by addition of granular anhydrous Na_2SO_4. The residual solvent was blown off and the derivatised amino acids were re-dissolved in 0.3 mL hexane. They were transferred to 1.5-mL 'wineglass' GC vials for analysis and freezer storage. No independent protein analysis was undertaken as sample amino acid concentration could be estimated by comparison with the internal standard. Following GC analysis (see below) some samples contained insufficient sample and were rejected from the analysis. This was probably due to a combination of low protein content in the original biopsy and processing yield.

Preparation of serum samples

Serum samples (2 mL) were thawed for analysis and 1 mL (3×0.3 mL replicates) was used for analysis of ^2H enrichment in body water by continuous flow IRMS following gaseous equilibration with 20% H_2 in He in the presence of a platinum catalyst (20-22, SerCon, Crewe, UK).[14] Each sample batch included working standards calibrated against international reference waters. Gravimetric dilutions of each subject's dose were analysed against the same standards to facilitate total body water estimation.[15] One mL of each serum sample was processed for the analysis of free amino acid enrichment by cation exchange. Briefly, nor-leucine (300 nmol) was added as the internal standard. The samples were ultrafiltered to remove proteins and diluted to 8 mL with deionised water. The samples were acidified to pH 2 and amino acids were loaded onto 2-mL bed volume columns of Dowex 50WX8-200 cation-exchange resin and eluted with 5 mL 2 M NH_4OH and 5 mL deionised water to purify. After vacuum drying into 4-mL vials, samples were divided into two equal aliquots and derivatised as ethoxy carbonyl ethyl esters, exactly as for the protein hydrolysates.

GC/pyrolysis/IRMS of amino acids

An Agilent 7890 gas chromatograph (Agilent, Wokingham, UK) with a heated split/splitless injector was used. A DB wax column (60 m × 32 mm × 0.5 μm film) with 5 m 0.32 mm deactivated silica retention gap was employed. The system was fitted with an Agilent 7890 16-place autosampler. Injection volumes of 5 μL were used with the option of making several injections in rapid succession, using the 'solvent effect' to focus relatively large injection volumes at the head of the column. An optimal injection delivered approximately 2–6 nmol of analyte. The oven temperature programme was: start at 50°C and hold for 1 min; ramp at 25°C/min to 150°C; ramp at 2.5°C/min to 200°C; ramp at 1°C/min to 220°C; ramp at 7.5°C/min to 250°C with a 1 min hold. A constant carrier flow of 1.6 mL/min helium was used with a pressure programme. The low flow rate was used to maximise sample-to-carrier ratio at the open/split into the ion source. The gas chromatograph was fitted with a capillary flow technology Deans switch (Agilent) which, under computerised pressure control, diverted the sample flow to a flame ionisation detector (FID) or to a 1350°C capillary pyrolysis furnace (Nu Instruments, Wrexham, UK) to convert analytes into H_2 gas prior to the open/split. During installation, a length of 200 μm i.d. deactivated silica tubing between the Deans switch and the FID was trimmed to match the carrier flow at the open/split. The Deans switch did not noticeably cause the peak resolution to deteriorate. It was activated after 26 min and again after 44 min to cause peaks eluting between these times to be diverted towards the pyrolysis furnace. The pyrolysis furnace consisted of a 320 mm length of 0.5 mm i.d. alumina tubing, filled loosely with alumina wool. It was conditioned with 1-μL injections of hexane each time it was replaced and at the start of each analytical 'campaign'. The pyrolysis tube connected directly to the open/spilt via a short length of 0.32 mm i.d. silica tubing.

IRMS conditions

The mass spectrometer used was a Horizon (Nu Instruments) which was controlled by proprietary software. The ion source was operated at 950 μA trap current and the tuning parameters were optimised to provide flat-topped *m/z* 3/2 ratio traces with a low and stable H_3^+ contribution. The latter was typically 7 ppm/nA. All analyses were performed by comparison with a pure H_2 reference gas. In addition, the *m/z* 3/2 ratios of neutral amino acids in the region of the chromatogram selected for analysis by IRMS were compared with that of the internal standard, nor-leucine. The amino acids were alanine, valine, iso-leucine, leucine, glycine, nor-leucine and proline (Fig. 2). Peak areas for *m/z* 2 of <1 nA were rejected as being too low for accurate ratio analysis. iso-leucine peak areas were frequently below this threshold. Above 1 nA major peak area, the precision was routinely close to 1 ppm ^2H ($\delta^2H = 6$ ‰) in samples near natural abundance.

A number of precautions were taken to maximise sensitivity: the choices of the column bore and phase ratio were made in order to maximise loading without compromising resolution; the ion source was operated at high trap current and tuned according to the manufacturer's recommendations; the helium carrier gas

flow rate was minimised to increase hydrogen gas concentration while not compromising separation; the pyrolysis furnace was operated at high temperature to ensure high conversion yield and minimal fractionation; the sample loading was optimised to ensure intense peaks while not compromising separation due to column overload. These precautions were necessary as the ^{2}H natural abundance is low and analytical precision worsens with decreasing peak area. In contrast to ^{13}C analysis, compound-specific ^{2}H analysis requires more attention to be paid to these details as, in practice, the dynamic range for maximum precision is often less. Sample loading of the order of 1 nmol analyte injected produced peak areas above 1 nA. The peak shape and resolution deteriorated due to 'fronting' when more than 10 nmol of an analyte was injected. Increasing the injection volume improved the situation with low concentration samples (see above). Nevertheless, some samples, in particular hydrolysates of proteins isolated with low yield, failed to give sufficient peak area for accurate analysis.

As an independent test of the system's performance, a series of ^{2}H-alanine standards was created, encompassing the full range of enrichment from natural abundance, through low enrichment protein-bound amino acids to high enrichment free amino acids. This was constructed by gravimetric dilution of a commercial ^{2}H-alanine ($^{2}H_4$-L-alanine 99 Atom % ^{2}H, CDN Isotopes, Quebec, Canada) with natural abundance L-alanine (Sigma). The whole curve was constructed from 10 individual solutions ranging from natural abundance to 5400 ppm ^{2}H excess in H_2 as analysed, where 4/15 of the hydrogen atoms in the alanine derivative were labelled. The maximum enrichment that IRMS can measure is governed by sample size, the dynamic range of head amplifiers and the analyte. The head amplifier gain is often determined by ^{13}C analysis at natural abundance, with 100-fold difference between the major and minor beam feedback resistors. In practice with modern amplifiers operating at 50 V, analysis of a far greater range of enrichments is possible than is generally appreciated, without resorting to altering the amplifier gain. Indeed, for H_2 analysis the limitation is more likely to be imposed by the fact that H_2 analysis is conducted on dual collector instruments where, above 330 000 ppm excess ^{2}H when for a diatomic species major and minor beam intensity are equal, a triple collector capable of analysing all H_2 isotopomers would seem more suitable. However, the latter is not feasible when using slow-resolution continuous flow IRMS to measure H_2 with helium as carrier. As a maximum of 4/15 hydrogen atoms in the alanine derivative used are labelled, the maximum possible enrichment is 267 000 ppm ^{2}H excess. IRMS is thus capable of analysing the full range of conceivable enrichments. Additional limitations may be imposed if the mass spectrometer is run by a research group that usually measures stable isotope natural abundance, due to understandable but surmountable concern over cross-contamination.

As with unknown samples, the alanine gravimetric series included nor-leucine as internal standard. These standards were derivatised and analysed as ethoxy carbonyl ethyl esters in the same manner as the samples. Data was expressed as raw delta values (ratio) by the IRMS software by comparison with a reference gas. Once transferred to a spreadsheet, the results were normalised to the internal standard ratio and converted into ppm ^{2}H excess by subtraction of the basal value. The slope of the resulting curve of measured versus calculated enrichment was 0.94, the intercept was not different from zero and the Pearson correlation coefficient was 0.98. Thus, this exercise revealed that the combination of GC, pyrolysis and IRMS was capable of performing well over a range of ^{2}H enrichments that exceeded by 10-fold the expected maximum enrichment in this protocol. Insertion of enriched samples before natural abundance samples, to test for memory, revealed the system to be memory-free over this range. Furthermore, the problem of sensitivity alluded to above diminishes as the minor peak area increases with increasing enrichment.

In the absence of a source of independent reference materials for stable isotope enriched substrates, individual laboratories are left to construct their own gravimetric standard curves and maintain working calibration standards. When the aim is to determine accurate rates of biological processes using stable isotope tracers, accuracy can be assured by adopting a robust procedure. In the context of measuring protein FSR, if both precursor and product are measured by the same instrument over ranges verified by use of a common gravimetric standard series, as the method of calculating FSR includes measured terms in the numerator and denominator any measurement bias will largely be cancelled. Should independent methods be used to measure precursor and product, great care should be taken with their intercalibration.

Calculations

The fractional synthetic rate (FSR; % day^{-1} or % h^{-1}) was calculated as:

$$\mathrm{FSR} = \frac{\text{Change of myofibrillar protein-bound alanine enrichment with time}}{\text{Average free alanine enrichment}}$$

Alternatively,

$$\mathrm{FSR} = \frac{\text{Change of myofibrillar protein-bound alanine enrichment with time}}{\text{Alanine enrichment estimated from average body water enrichment}}$$

The average free alanine or body water enrichment over each specific period was calculated from the area under the curve of the log-transformed elimination plot (Fig. 1).

Body composition

The ^{2}H dilution space was calculated from the intercept of the elimination plot divided by the dose, following the methodology used in the doubly labelled water protocol.[14,15] TBW was calculated from $^{2}H_2O$ space assuming a non-aqueous hydrogen exchange factor of 1.041. FFM was calculated from TBW by assuming that the FFM hydration was 73.2%.[16] Skeletal muscle mass (SMM; kg) was estimated from FFM using the MRI-validated equations of Wang and coworkers which describe the ratio of SMM/FFM with gender and age.[17] A

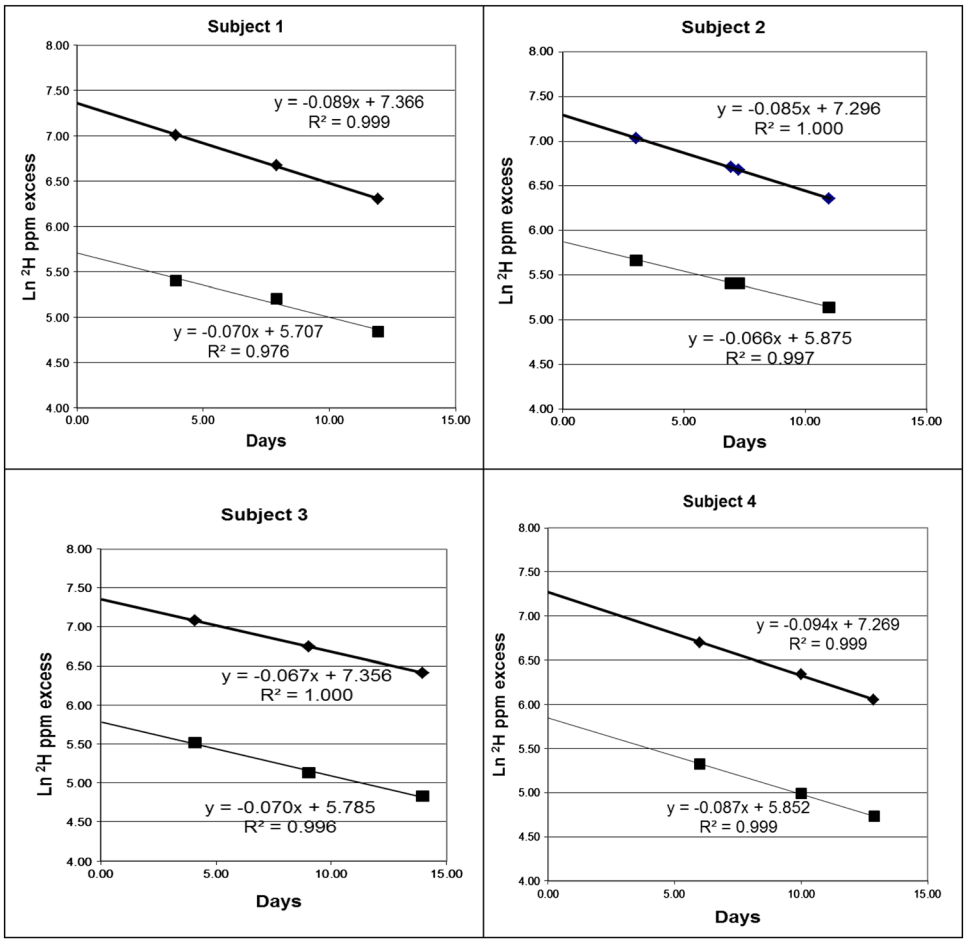

Figure 1. Body water and serum alanine enrichment (diamonds, body water; squares, serum alanine).

myofibrillar protein content of 12.4% SMM was assumed.[18] The absolute synthetic rate of myofibrillar protein (ASR; g day^{-1}) was calculated as:

$$ASR = FSR/100 \times SMM \times 1000 \times 12.4/100$$

Units

In this manuscript, the units Atom % ^2H have been used to describe ^2H abundance in the tracer stock as supplied. Due to low enrichment of the amino acids in this study, units of ppm ^2H excess (Atom % $^2H \times 10^4$ sample – Atom % ^2H basal $\times 10^4$) have been used.

RESULTS

^2H enrichment in body water and serum alanine

With a single oral dose, the body ^2H_2O enrichment increased to an average maximum of 1514 ppm ^2H excess (1435–1582 ppm; Fig. 1). These values are the enrichment at the time the dose was taken, calculated from the intercept of the elimination plot. The elimination half-time was 8.3 days (7–10). The average maximum measured enrichment in free alanine was 333 ppm ^2H excess (301–356 ppm).

Figure 2 shows an ion chromatogram (*m/z* 2 or major ion trace) representing myofibrillar protein-bound amino acids. Alanine, the principal analyte, elutes first and is clearly resolved from other neutral amino acids. nor-leucine was used as the internal standard, allowing concentration and isotopic calibration. All measured ^2H abundances were normalised to that of nor-leucine as this experienced the same derivatisation procedure, pyrolysis and flow conditions as all analytes.[19,20] Figure 3 shows all free pool data from each subject, indicating the enrichment of serum alanine relative to body water over time. This is presented as the ratio of free alanine enrichment to that of body water after correction of the free alanine term by the factor 15/4, as a theoretical maximum of 4 of the 15 hydrogen atoms in the analyte can come into equilibrium with body water. Should the theoretical number of hydrogen atoms become labelled, this plot should show a constant value of 4. There was no significant trend with time, indicating that alanine came rapidly into isotopic equilibrium with body water and remained in equilibrium over the period of study. The elimination rate of free ^2H-alanine mirrored that of body water (Figs. 1(a)–1(d)). The average number of hydrogen atoms in alanine that became labelled was 3.64, indicating that circulating free alanine reached 91% of the maximum theoretical enrichment.

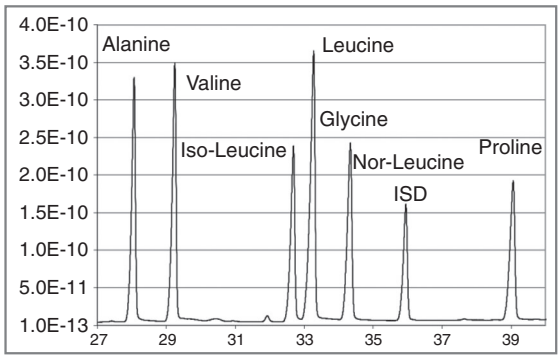

Figure 2. Ion chromatogram (*m/z* 2) of neutral amino acids.

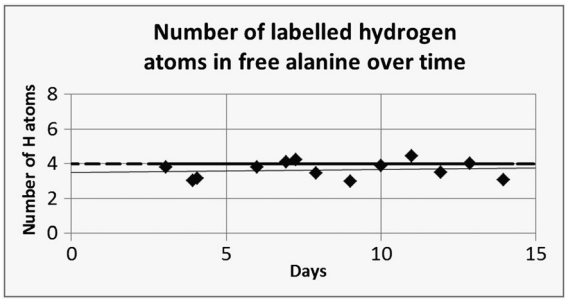

Figure 3. Estimated number of labelled hydrogen atoms in plasma-free alanine with time (see text for details).

The ^2H enrichment of other free neutral amino acids in the circulation was measured. Free glycine showed an average of 1.6 hydrogen atoms becoming labelled compared with a theoretical value of 2.[21] Valine showed a value of 1.07 compared with 1.0. Leucine showed a similar degree of labelling to valine. Free iso-leucine was of too low concentration for accurate analysis. In contrast, proline showed on average only 0.88 labelled atoms compared with a maximum theoretical value of 5. The hydrogen atom on the alpha carbon (C2) of the free branched chain and of other essential amino acids equilibrates rapidly with body water during transamination.[12] In contrast, it appears that proline acts as a non-dispensable amino acid, its free pool deriving from the diet and protein breakdown with little coming from *de novo* synthesis.[22,23]

Using circulating free alanine as the precursor, the habitual myofibrillar protein FSR ranged from 1.0 to 2.1% day^{-1} with an average of 1.43% day^{-1} (Table 1). The weighted average FSR per subject was 1.38% day, ranging from 1.0 to 1.9% day^{-1}. Using body water as the precursor with the assumption that 4 hydrogen atoms became labelled in free alanine, the habitual myofibrillar protein FSR was 1.16% day^{-1} and ranged from 0.9 to 1.5% day^{-1}. Using body water with the average number of labelled hydrogen atoms in circulating alanine as measured in this study (3.64), the average habitual myofibrillar protein FSR was 1.28% day^{-1} and ranged from 1.0 to 1.8% day^{-1}. Body composition analysis allowed estimates of absolute myofibrillar protein synthesis to be made. The habitual myofibrillar ASR ranged from 36.6 to 74.5 g day^{-1} with an average of 49.9 g day^{-1}. The variation in ASR was largely determined by the two-fold range in the FSR data (compare Table 1 with Table 2).

DISCUSSION

We have developed a new protocol that allows the habitual rates of myofibrillar protein synthesis to be measured with minimal disturbance to the subject. Our approach uses a single oral bolus to minimise subject burden while allowing habitual rates of myofibrillar protein synthesis to be measured over periods of approximately 2 weeks. Pioneering studies of the ^2H_2O-labelling approach in human subjects have aimed at elevating the ^2H enrichment in body water to a constant value over periods of weeks or months.[7–9,21,24] This has been accomplished by use of a bolus or priming dose with daily top-up doses. One reason for such long study periods being adopted is that, to avoid occasional side effects of larger doses, body water has been raised slowly to a particular target level. To calculate the optimal theoretical doses, we need to consider the pool size (TBW) and its clearance or elimination rate.[25] In the study reported here, the average elimination rate was –0.0834 day^{-1}, which is typical of adults undertaking moderate physical activity in a temperate climate. The average body water elimination half-time was 8.3 days (Ln2/0.0834). For a single pool compartment, the theoretical prime to constant infusion ratio is the inverse of the elimination rate,[8] or 11.9 in this example.

Table 1. Habitual myofibrillar protein fractional synthetic rates in four healthy adult males. Data were calculated using free alanine as precursor. Two subjects had repeat biopsies on different occasions

Subject	Interval (days)	Average precursor ppm ^2H excess	Myofibrillar protein ppm ^2H excess	Myofibrillar protein FSR % day^{-1}
1	4	262.1	22.2	2.1
1	12	197.8	40.4	1.7
2	3	321.8	12.8	1.3
2	7	282.8	19.7	1.0
3	9	237.8	31.7	1.5
4	13	198.7	25.3	1.0

Table 2. Habitual myofibrillar protein absolute synthetic rates in four healthy adult males

Subject	FFM (kg)	SMM (kg)	Myofibrillar protein (g)	Myofibrillar protein ASR g day^{-1}
1	53.4	27.9	3465	74.5
1	53.4	27.9	3465	59.2
2	56.9	29.5	3660	47.5
2	56.9	29.5	3660	36.6
3	53.8	28.5	3530	52.2
4	58.8	30.0	3717	36.7

To achieve a plateau in body water enrichment as rapidly as possible and to maintain this, we should give each subject 11.9 + 1 units of ^2H_2O on the first day, with daily top-ups totalling 1 unit of tracer. Few have applied these principles, instead using sub-optimal priming doses which have often resulted in steadily increasing enrichment with time over long labelling periods. However, McDevitt and co-workers used a prime-to-infusion ratio of approximately 12 in their studies, using ^2H_2O to measure *de novo* lipogenesis.[26]

Mass isotopomer distribution analysis (MIDA) facilitates the measurement of protein FSR while avoiding analysis of the free amino acid precursor pool.[27] Here, GC/MS (or LC/MS) is used to analyse isotopomer distribution. Although GC/MS facilitates MIDA, it has a minimum working enrichment of approximately 5000 ppm excess ^2H.[19] Some improvement in the GC/MS detection limit may be gained by specialised modes of analysis such as the 'fractionation method' as applied by Previs and co-workers, but this is orders of magnitude poorer than the detection limit of IRMS as used in the current study.[8,28]

The reason for targeting such high enrichments was that GC/MS was the chosen analytical technique. Despite long labelling times, the low turnover of skeletal muscle protein (FSR 1–2% per day) results in very low rates of ^2H incorporation. To overcome this, highly sensitive GC/pyrolysis/IRMS can be applied as first used to study the FSR of other macromolecules and as has been proposed by Gasier and co-workers for protein FSR.[24,29,30] One of the key features of IRMS is that simple gases are analysed, in this case H_2. This greatly improves the signal-to-noise ratio when analysing stable isotopes at low enrichment. Finally, even if the tracer prime/infusion ratio theory were followed strictly, it would remove the ability to measure Total Energy Expenditure (TEE) simultaneously by the doubly labelled water method and might compromise the ability to determine TBW, while increasing subject burden.[14,15]

Rates of protein synthesis in the present study compare with those in the literature.[31] An average myofibrillar protein FSR of 1.38% day^{-1} (0.058 % h^{-1}) compares with the mean value of 0.044% h^{-1} for the eight studies in the post-absorptive state as discussed by Smith and co-workers.[31] Despite the low FSR, the convention has been to quote hourly rates, perhaps because of the normally short duration of the measurement period. Smith and co-workers noted the wide variation of reported values in resting myofibrillar protein FSR, quoting a range of 0.031–0.061 % h^{-1}, and discussed a number of factors that might influence the results, including protocol duration and the uncertainty of the precursor enrichment, in addition to their being considerable inter-subject variability.[31] Direct comparison of habitual rates with literature values from acute studies is difficult and was not the aim of this paper. This would require adjustment of literature FSR data to approximate to the fasting and feeding periods of the day with an estimate of the persistence of anabolic stimuli. The range of FSR data observed in our pilot study led to a similar range in ASR data. The ^2H_2O protocol described here allows much longer study periods than conventional infusion methods, thus overcoming some of the issues relating to short study periods. It also simplifies the precursor analysis as alanine was shown to reach 91% of the theoretical enrichment, rapidly. Its accessible free pool appears to describe the intracellular precursor pool. No obvious trend was seen with measurement period over 3–13 days. The two subjects with repeat biopsies showed FSR within 13% of the mean but, as reported by Smith and co-workers, the range of FSR showed considerable inter-subject variability, even in our small pilot study.[31]

Our finding that, on average, 3.64 hydrogen atoms in free alanine come from body water compares with the MIDA analysis of plasma proteins in adult rats after long term labelling, where 3.6–3.7 alanine hydrogen atoms were shown to come from body water.[9] This may reflect incomplete labelling in the β hydrogen atoms (i.e., at C3), due to entry of unlabelled alanine, rather than at the α position (C2), as was observed through isotopomer analysis using GC/MS.[8] It is tempting to believe that these observations can be generalised, allowing prediction of precursor enrichment from body water. However, we prefer to measure free alanine to reduce assumptions of the degree of labelling from body water and to facilitate greater measurement accuracy through using the same instrument for the analysis of both precursor and product (as described under IRMS conditions above). Future extension of this approach to larger surgical biopsies of abdominal wall muscle will confirm this point, as larger samples of intracellular fluid should be accessible allowing the ^2H enrichment in serum alanine to be compared with that of intramuscular free alanine. This would add confidence to the belief that the ^2H enrichment of free alanine tracks the intracellular free pool as the latter is in dynamic equilibrium with its keto acid pyruvate, which influences its high rate of *de novo* synthesis and rapid equilibration with body water. The effects of dilution by protein breakdown and feeding appear to be modest, reducing the enrichment of free alanine to 91% of the theoretical value. The lack of an obvious trend when the data in Fig. 3 are plotted against time of day suggests that habitual periods of feeding do not greatly disturb the observed relationship.

The new protocol permits use of a single biopsy, as serum albumin was used to give basal protein-bound ^2H-alanine abundance. In future, urine samples may be substituted for blood samples as these have sufficient alanine and glycine for analysis, potentially reducing the required serum samples for this procedure to a single sample for the analysis of basal ^2H abundance in albumin-bound alanine. Urine has also proven a most suitable matrix in which to analyse ^2H_2O abundance for body composition and energy expenditure studies.[14,15] An endpoint within an elective surgical procedure would further reduce the impact of the protocol on frail subjects as it might then simply require a basal blood sample, a single oral tracer dose with a series of urine samples, ending when a biopsy is taken during surgery.

Simultaneous measurement of body composition allowed skeletal muscle mass and thus the absolute rate of myofibrillar protein synthesis to be estimated. The average absolute synthesis rate of myofibrillar protein of 49.4 g day^{-1} in our study emphasises the importance of contractile protein synthesis in terms of whole body protein turnover, in contributing some 25% of the daily total. With sarcoplasmic and connective tissue protein included, mixed skeletal muscle protein may contribute

30–50% of daily protein synthesis.[32] When developing this new protocol, maintaining compatibility with body composition measurement was regarded as an essential criterion as this allows future studies to relate findings to the subject's adiposity. The bolus protocol also ensures the ability to implement the doubly labelled water methodology should total energy expenditure be selected as an important outcome.

A single oral bolus of approximately 1 g ^2H_2O/kg body weight was used in this study. In contrast, the recent study of Robinson and co-workers used 3.5 kg ^2H_2O per subject, given as multiple daily boluses over 6 weeks.[7] In our study of healthy adult males, a dose of 100 g 70 Atom % ^2H_2O raised body water to an average of 1,514 ppm ^2H excess, falling exponentially with time as water was eliminated with a half-time of 8.3 days. A future increase in dose to achieve a target of 2000 ppm ^2H excess in body water could be met with very modest cost increase and an improvement in measurement sensitivity, should a protocol duration of 1 week be desirable. To achieve results close to target enrichment in all subjects, this might require preparation of separate doses for male and female subjects. Should future study designs call for a continuous labelling pattern to facilitate serial biopsies in an intervention study, there are two options. First, the procedure described here could be adapted to produce a constant 'infusion'. The same initial bolus (approximately 1 g ^2H_2O/kg) with modest top-up doses totalling some 0.083 g ^2H_2O/kg daily would suffice. The second option to facilitate a longitudinal study design would be to introduce a second and possibly larger bolus, some weeks after the first. It would also be advantageous in future studies to measure smaller protein samples as the smallest samples in this study were insufficient. Improvements in processing yield, injection efficiency and mass spectrometer sensitivity would permit analysis of smaller analyte quantities from human biopsies and, potentially, for analysis of the FSR of minor proteins. This being said, the GC/pyrolysis/IRMS system as used proved entirely suited to the new protocol. Indeed, modern continuous flow IRMS systems can accommodate more than one inlet. A gas inlet for body composition, energy expenditure and other analyses can be installed in parallel with an inlet for compound-specific isotope analysis.

There are many benefits of an extended tracer protocol: habitual rates describe the true integrated response of skeletal muscle protein to anabolic stimuli such as food, activity and hormones while the negative effects of fasting, inactivity, inflammatory mediators and other disease-related factors are also integrated; short-term variability such as diurnal cycles are damped by longer measurement periods; and reduced impact of precursor contamination on the accurate estimation of protein-bound tracers is a technical benefit of prolonged measurement periods. Questions relating to acute effects on skeletal muscle protein synthesis may still be addressed within the framework of this protocol as it does not compromise the ability to conduct a short-term infusion of, say, ^{13}C-leucine to examine specific stimuli, such as a single meal. In conclusion, the application of GC/pyrolysis/IRMS facilitated the implementation of a new protocol to measure skeletal muscle protein synthesis with minimal burden on the subjects while maintaining the ability to measure total energy expenditure.

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