Reply to letter to the editor: to D₂O or not to D₂O? What are the reasons we D₂O it at all?

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TO THE EDITOR: the estimation of protein synthesis has long been used as an outcome variable to better understand anabolic processes in skeletal muscle. Much of what we have learned about the regulation of muscle mass and protein metabolism has been a result of our ability to estimate synthesis rates under varying experimental conditions ranging from different types of mechanical loading to nutritional interventions.

A number of methodological approaches have been developed to assess protein synthesis that use a labeled precursor as a tracer whereby the rate of protein synthesis can be estimated by measuring the appearance of this tracer into muscle protein over time. To achieve this goal, there are a number of “assumptions” that must be considered while using these methodologies (4, 6). We will focus on two of these assumptions in response to the letter to the editor by Wilkinson et al. (19a). The first, and perhaps foremost, assumption is that the relationship of incorporated label is linear with rates of muscle protein synthesis. In other words, any change in the appearance of the label in skeletal muscle is indicative of a change in the rate of protein synthesis. A second assumption, often overlooked with recent methodological approaches, is that the contribution of amino acid “recycling” is minimal (or at least constant) at the time of the assessment.

First Assumption: The Relationship of Incorporated Label is Linear With Rates of Muscle Protein Synthesis

This assumption has received considerable attention from investigators and is considered a hallmark of a well-executed study. From the earliest work by Garlick and colleagues (4–6), later followed by Jefferson (12), it was understood that the concept of linearity between label incorporation and rates of synthesis could be interpretable only if the precursor pool was maintained in a steady state over the time of assessment. To accomplish this task, “flooding dose” methodologies were used to ensure rapid and equally distributed precursor pool labeling so that a constant ratio of the labeled to unlabeled tracer pool during the period of assessment was maintained to satisfy the assumption that alterations of label incorporation are indicative of altered rates of synthesis. Additionally, these measurements were conducted over relatively short periods of time to minimize any contributions of amino acid recycling (related to the second assumption described herein later), which could affect the ratio of the (fixed) labeled to unlabeled precursor pool by “diluting” the precursor pool and making estimations of protein synthesis difficult, if not impossible, unless closely monitored.

More recent iterations of methods designed to estimate synthesis rates have also paid considerable attention to the importance of the precursor pool. The “primed constant infusion” methodology, pioneered and employed largely by Paddon-Jones and colleagues (14, 15), Tipton and colleagues (18, 19), Wolfe and colleagues (22, 23), and others (1, 2, 16, 17), placed tremendous emphasis on the importance of the precursor pool. This methodology closely monitors the precursor pool in an effort to ensure stability (labeled/unlabeled ratio) leading up to the assessment of protein synthesis because it is understood that estimations of protein synthesis become problematic if the precursor pool is not in steady state during the period of measurement. Although the aforementioned method is the standard by which all estimations of protein synthesis are compared, many of the studies assessing protein synthesis under strict experimental conditions using this methodology do not necessarily extrapolate to a free-living state, which is due primarily to loss of control over the precursor pool outside of experimental settings. Typically, these studies are performed under fasted conditions whereby basal measures are taken when an individual is in bed (21).

Deuterium oxide in estimation of protein synthesis. To better understand rates of synthesis in free-living states, methodologies using deuterium oxide water have recently garnered some attention. Compared with methodologies using fixed label precursors, a distinct advantage of the deuterium labeling is that the tracer is manufactured by the cell and that the rate of manufacture of the label is consistent with the presence of deuterium in the total body water (3). Thus, amino acids that are subject to synthesis, transamination, etc., such as alanine, are readily labeled with deuterium while undergoing these processes, and the label remains fixed while they are incorporated into protein. Studies have concluded that a relationship persists between the amount of deuterium available and the proportion of enrichment of the amino acid in the fed state, even after feeding. This methodology may prove to be extremely useful when attempting to understand alterations of protein synthesis following a variety of treatments under conditions that more closely mimic free-living conditions over greater periods of time, such as hours, days, or perhaps even weeks. Although the approach may not be practical for assessing the acute impact of specific signaling molecules on protein synthesis, the method may prove beneficial in attempting to understand the role of exercise or chronic nutrient status on anabolic responses over time. Our group has considerable interest in the use of this methodology, and we have conducted studies directly comparing the use of this method against more traditional fixed label methodologies (11). Our findings have demonstrated that alterations of protein synthesis using the deuterium approach have been consistent with changes of protein synthesis using fixed label methods. However, although the approaches have proven to be qualitatively similar, our direct comparison efforts have shown that the estimation of
fractional synthesis rates is quantitatively higher using deuterium methodologies compared with fixed label approaches.

Wilkinson et al. (20) recently submitted a letter to the editor due to concerns about fractional synthesis rates presented in the work done by Lambert et al. (13). They highlight that the reported fractional synthesis rate (FSR) values from our work are considerably greater than “expected” values compared with the more traditional primed constant infusion methodologies. Their point of contention is that the FSRs from their work, also using deuterium methodologies, are almost perfectly aligned with FSRs reported using traditional methodologies and call into question the precision of our measurement. Aside from the fact that the values from their FSR data closely mimic primed constant infusion methodologies, it appears that they have focused exclusively on the quantitative differences between the papers and have ignored the physiology leading to those numbers.

Although we can only speculate as to why FSR is higher using deuterium oxide (at least in our hands), it likely has something to do with a “fixed” vs. “cell-labeling” precursor pool. The strength of cell-labeling approaches is that the precursor pool is rapidly equilibrated in the total body water (3), and thus, any aqueous cell capable of transamination/synthesis will have the capacity to label the precursor pool. A striking difference between fixed label and cell-labeling methodologies is that any amino acid undergoing this process has an opportunity to be labeled in a manner consistent with the amount of deuterium available. It must be noted that the calculation of protein synthesis is based on the percentage of labeled amino acids in bound protein relative to unlabeled amino acids in the same sample. Therefore, the deuterium oxide methodology allows us to label amino acids from any source, including those that have been recycled, which may greatly enhance the capacity to detect label compared with fixed-label methodologies employed over time.

Second Assumption: The Contribution of Amino Acid Recycling is Minimal (Or At Least Constant) At the Time of the Assessment

The second assumption listed above focuses specifically on this turnover issue. By definition, turnover is related to rates of synthesis and degradation of specific proteins or pools. If protein degradation was nonexistent, then the use of fixed label methodologies over time would not be an issue, as the rate of incorporation of label would be precisely linear with the rate of synthesis. However, protein turnover exists, and the capacity to estimate protein synthesis is diminished, particularly with extended periods of time, because one cannot predict how many times the label may have been incorporated into bound pools. The inability to systematically detect how many times a label has been incorporated coupled with the extended period of time will undoubtedly lead to an underestimated rate of protein synthesis. It should be noted that, under most conditions, rates of protein degradation, and thus contributions of recycled amino acids, may be relatively stable; however, many of the experiments using these methodologies are now routinely employed to assess FSR following activities/treatments with the expressed purpose of altering muscle protein metabolism, which includes not only FSRs, but also fractional breakdown rates. Under those conditions, the contribution of recycled amino acids may not be predictable/stable and may prove to be potentially problematic for estimations of protein synthesis. By nature, any change in protein turnover pool could potentially impact not only actual protein synthesis but also the estimation of protein synthesis.

Early work in this area complied with this assumption by limiting the studies to minutes (4, 6, 12), as opposed to hours or days. Our group has begun utilizing deuterium oxide methodologies (7–11, 13) to expand our capacity to label amino acids that have been derived from protein degradation prior to being reincorporated into new proteins. That said, there are limits to this capacity, as repeated turnover of a specific protein pool could become saturated with label, which would make it virtually impossible to detect any changes in FSR over continued periods of assessment. As one approaches saturation over time, the diminished ability to alter the labeled/unlabeled ratio of the tracer, coupled with extended period of time without the ability to detect changes in FSR, would ultimately give the appearance that FSR is being reduced over time. In our work, we assessed protein synthesis within a 24-h period to minimize the possibility of saturating any given protein pool with labeled alanine over time. We recognize that it is unlikely that a myofibrillar fraction could become saturated over the 8-day window that was used by Wilkinson et al. (20), but it certainly would be more saturated than the 24-h window from our work.

In their letter to the editor, Wilkinson et al. (19a) focused on instrumentation and sensitivity differences between the two papers and indicated that these differences were why their FSR values fall perfectly in line with prior work assessing FSR in humans. It must be noted, however, that there were other important differences between these two papers. For example, Wilkinson et al. (20) used a single bolus of deuterium oxide to assess FSR over an 8-day period, whereas we used multiple boluses to assess FSR within a 24-h period. Although we commend their transition to deuterium methodologies, it should be noted a single bolus of D2O assessed over multiple days may lead to an underestimation of protein synthesis as time continues. We acknowledge that their numbers are similar to that of previously published work using fixed-label methodologies but also find it somewhat surprising since most of the studies using fixed-label methodologies were conducted in a fasted state of net-negative nitrogen balance (and frequently during bedrest conditions for several hours prior to assessment) and thus not under free-living conditions.

An impetus for the use of deuterium methodologies was our recognition that experimentally controlled precursor pools may negatively impact the estimation of FSR (quantitatively), particularly when most of us accepted that food consumption and composition, blood flow, and activity could dramatically and acutely affect FSR. Again, given that during the 8-day protocol subjects were under free-living conditions where mechanical load, fluid shifts, and nutrition were given (20), it appears as though their FSR values are somewhat low given the experimental conditions. Methodological differences between the Wilkinson et al. (20) and Lambert et al. (13) papers make it extraordinarily difficult to directly compare the two studies for a number of reasons. First, it should be noted that a single bolus will diminish over time, and although this can be modeled by periodically assessing total body water, consideration must be given to previous work demonstrating that body water is

AJP-Endocrinol Metab • doi:10.1152/ajpendo.00136.2015 • www.ajpendo.org
rapidly equilibrated (3). That said, it stands to reason that this equilibration is equally rapid when the total body water is diluted with nonlabeled water. Although we acknowledge that this pool can be monitored, periodic assessments may not be able to capture the acute effect of hydration on the rapidly changed status of the precursor pool, particularly when only a single bolus is applied. So, depending on when hydration occurred vs. when the deuterium pool was assessed, it likely would serve to underestimate FSR given the rapidness of equilibration, especially over an 8-day window. Linear applications assessing the decline of deuterium over 8 days dismiss the immediate impact of dilution on total body water. Our rodent work overcame this issue not only by reducing the period of time for assessment but also by maintaining the deuterium precursor pool via enriching drinking water with deuterium oxide. Our human work bolstered the subjects with aliquots of deuterium oxide over time to maintain the enrichment throughout the 24-h assessment period.

Coupled to the consideration of actual deuterium availability, careful consideration must be given to the impact this availability has on the cell-labeling capacity of the cell. Each time an amino acid is liberated from the protein, its gain or loss of deuterated residues is dependent largely on the availability of deuterium for these transamination processes. As mentioned above, work from our predecessors has shown that the equilibration of deuterium in total body water is rapid (3, 7), which makes the use of deuterium to model precursor pools very attractive. Thus, although labeling may persist throughout the period, as the precursor pool diminishes, it should result not only in a reduced estimation but also an actual loss of label if previously labeled amino acids undergo transamination during that time. In Fig. 3 in the paper by Wilkinson et al. (20), the authors demonstrate that FSR is temporally diminished in the presence of exercise over 4 to 8 days of exercise compared with the first 4 days of exercise. Interestingly, using this methodology, it appears that even control legs are also declining as the experiment progresses. Furthermore, given the similarities of FSR when comparing 0–2, 0–4, and 0–8 days, compared with the “temporal” responses, it may be indicated that the robust response early in the experimental period (when the precursor pool was better controlled) may have allowed for the appearance of maintained FSR across the entire span of the experiment. One may be tempted to speculate that FSR, particularly with exercise, is most robust at the onset of the training but that within days the repeated acute effect of exercise dissipates. Because that speculation is not consistent with expectations of resistance exercise on muscle growth, it may also be indicated that the single bolus methodology over prolonged periods of time is not sufficient for the estimation of protein synthesis, even when the numbers are almost perfectly aligned with more traditional methodologies. Importantly, our FSR findings 24 h following acute exercise in the untrained and trained states (to some degree) were consistent with the physiological outcomes over time with regard to gains in muscle mass.

A final consideration is that prior research indicates that peak anabolic responses occur within the first 24 h and are relatively back to normal when assessed at the 48-h time point. Thus, the impact of an acute bout of exercise on FSR is likely more robust over the first 24 h of the experiment compared with the FSR response from 24 to 48 h. If so, the assessment of protein synthesis over the entire 48-h period after resistance exercise on skeletal muscle would serve to underestimate the impact of the acute anabolic response of the exercise, even if the deuterium enrichment of total body water was constant.

That said, it should not be surprising that estimates of FSR from Lambert et al. (13) are higher, as careful attention was paid to the maintenance of the precursor pool over the time of assessment, and the estimates were made during a period of time when the anabolic response of the muscle to exercise was optimal. However, it is gratifying that the work from Wilkinson et al. (20) maintains a heightened anabolic response over the 48-h period (at least over the first 4 days), which is ultimately the rationale for why our laboratory started using the methodology some years back.

In closing, although it is yet unclear why FSR values are not completely consistent quantitatively with differing methodological approaches, it is rather clear that there is a degree of qualitatively similar findings across studies using deuterium-based cell labeling compared with fixed-label approaches. When used appropriately, the use of deuterium methodologies will greatly expand our capacity to assess muscle protein synthesis over time and under free-living conditions, which should greatly enhance our understanding of anabolic processes.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

REFERENCES