JPET Fast Forward. Published on July 19, 2017 as DOI: 10.1124/jpet.117.241091
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Journal of Pharmacology and Experimental Therapeutics: Mini-Review

(Revised version: JPET/2017/241091)

Enhancing studies of pharmacodynamic mechanisms via measurements of metabolic flux: Fundamental concepts and guiding principles for using stable isotope tracers.

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Running title: Supporting pharmacodynamics studies via tracer kinetics.

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Document Statistics:

Text pages = 40

Tables = 0

Figures = 8

References = 83

Word count Abstract = 208

Word count Introduction = 705

Word count Discussion = 5403

Non-Standard Abbreviations:

Fractional Synthesis Rate = FSR

Fractional Clearance Rate = FCR

Abstract.

Drug discovery and development efforts are largely based around a common expectation, namely, direct or indirect action on a cellular process (e.g. statin-mediated enzyme inhibition or insulin-stimulated receptor activation) will have a beneficial impact on physiological homeostasis. To expand on this, one could argue that virtually all pharmacological interventions attempt to influence the flow of traffic in a biochemical network, irrespective of disease or modality. Since stable isotope tracer kinetic methods provide a measure of traffic flow (i.e. metabolic flux), their inclusion in study designs can yield novel information regarding pathway biology; the application of such methods requires the integration of knowledge in physiology, analytical chemistry and mathematical modeling. Herein, we review the fundamental concepts that surround the use of tracer kinetics, we define basic terms and outline guiding principles via theoretical and experimental problems. Specifically, one needs to (i) recognize the types of biochemical events that change isotopic enrichments, (ii) appreciate the distinction between fractional turnover and flux rate and (iii) be aware of the subtle differences between tracer kinetics and pharmacokinetics. Hopefully investigators can use the framework that is presented here to develop applications which address their specific questions surrounding biochemical flux and therein gain insight into the pathophysiology of disease states and examine pharmacodynamic mechanisms.

1. Why are measurements of metabolic flux important in drug discovery?

Many of the most challenging diseases are associated with metabolic dysregulation. Although genomic and proteomic analyses can suggest drug targets by contrasting healthy and disease states (Plenge, et al., 2013), these analyses describe isolated events and do not account for the translation of altered expression profiles into aberrant metabolic activities. Since biochemical pathways are controlled by compensatory and often redundant regulatory mechanisms, including feedback loops that maintain necessary functions under various insults, it is not surprising to encounter disconnects between expression profiles and biochemical flux. For example, although Ob/Ob mice have a downregulated expression of lipogenic genes in adipose tissue, tracer-based studies demonstrate increased triglyceride synthesis and *de novo* lipogenesis (Turner, et al., 2007). In other cases, one can observe changes in expression profiles which reflect the directional change of a metabolic flux, however, the magnitude of change that is observed at a given step in a pathway can lead to confusion when considering which reaction to target (Kasturi, et al., 2007). Although intuition might suggest that one should target genes and proteins which display the largest change in expression profile, sensitivity assessments suggest targeting expression signatures with the smallest change (Fell, 1997).

In contrast to the snapshots that are captured by expression or concentration profiles, isotope tracers allow one to quantify pathway flux, synthesis rate, and half-life. For example, Simvastatin does not immediately change the amount of cholesterol in isolated hepatocytes, however, flux measurements demonstrate an unambiguous effect on pathway biology, note the decrease in cholesterol synthesis (Figure 1). Similarly, the addition of fructose does not immediately alter cholesterol concentration, however, tracer data demonstrate an increase in cholesterol synthesis, which is inhibited by Simvastatin (Figure 1). Since metabolic flux can change independent of changes in an expression profile (Turner, et al., 2007) and since a flux rate must change before we can expect a concentration to change (Harding, et al., 2015) the ability to measure metabolic flux should enhance pharmacodynamic studies.

Because a lack of efficacy in patients remains a key reason why drug candidates fail (Plenge, et al., 2013;Cook, et al., 2014;Arrowsmith and Miller, 2013) it seems important to cross-reference a parts list (i.e. an expression profile) with an instruction manual (i.e. an integrative study of organ physiology and/or pathway biology). Presumably we all agree that roadmaps can help us find our way across a city, however, digital applications, which also report the traffic flow, are of the greatest utility. Since stable isotope tracer methods yield a measure of traffic flow and are well validated and safe for use in *in vitro* and *in vivo* systems (including humans), their application should enhance translational studies and yield mechanistic information through all phases of development (Turner and Hellerstein, 2005). In fact, "clinicaltrials.gov" contains many reports where tracer methods are being used to gain new knowledge into pathophysiological changes in disease states and to support drug discovery. Likewise, there are published examples where tracers informed on a pharmacodynamic mechanism of action (Stiede, et al., 2017;Reyes-Soffer, et al., 2017;Cascante, et al., 2002;Cuchel, et al., 1997;Hundal, et al., 2000;Petersen, et al., 2000), including fundamental studies that aim to explain the pathophysiology of disease states (Sunny, et al., 2011;Donnelly, et al., 2005;Cline, et al., 1999;Decaris, et al., 2017;Decaris, et al., 2015)

Although the ability to design and conduct tracer studies requires a general working knowledge of physiology, analytical chemistry and mathematical modeling (Wolfe and Chinkes, 2005;Waterlow, 2006), the general principles have been outlined in a number of excellent references (Zilversmit, 1960;ROBERTSON, 1957;LONDON, 1949;SOLOMON, 1949;REINER, 1953b;REINER, 1953a;Zierler, 1961;Heath and Barton, 1973). Unfortunately, one encounters discrepancies regarding "right" or "wrong" approaches (Previs and Kelley, 2015;Landau and Wahren, 1992;Landau, et al., 1998;Edland and Galasko, 2011;Katz, 1992). Our goal here is to review key issues that impact tracer-based studies and show that adherence to a few guidelines should help circumvent confusion regarding data interpretation and therein yield a clear understanding of pharmacodynamic mechanisms. Attention to

these seemingly subtle points should allow reliable applications and expand the utility of tracer methods in drug discovery studies. Rather than restate many of the mathematical expressions that can be found in the literature (Zilversmit, 1960;ROBERTSON, 1957;LONDON, 1949;SOLOMON, 1949;REINER, 1953b;REINER, 1953a;Zierler, 1961;Heath and Barton, 1973), where possible, we will use everyday examples to keep this review more conversational.

2. Basic definitions and principles surrounding tracer-based studies of metabolic flux.

A starting point centers on defining the term "**pool size**"; fundamentally, the pool size is a mass. For a circulating marker, the pool size represents the concentration multiplied by the distribution volume, e.g. mg analyte per ml x total ml = mg analyte. Obviously, one can measure the concentration of a circulating analyte (e.g. albumin) but it can be difficult to calculate the total mass of an analyte if one does not have knowledge of its distribution volume, e.g. albumin can be found in intravascular and extravascular spaces. Studies of lipoprotein flux often assume a distribution volume that represents ~ 4-5% of body mass (Lichtenstein, et al., 1990;Parhofer, et al., 1991). Although this assumption is reasonable, the volume can be ignored if it is proportional to body mass across groups, the analyte concentration then provides a marker of the relative pool size.

Second, we should differentiate between "**fractional turnover"** (i.e. fractional synthesis rate "FSR" and fractional clearance rate "FCR") and "**flux rate"**. FSR and FCR represent the movement of a proportion of a pool per unit of time (e.g. fraction of the analyte pool per min) whereas flux rate represents the movement of a mass per unit of time (e.g. mg of analyte per min) (Figure 2), these terms are described and related using the equations:

$$\textit{fractional turnover } (min^{-1}) = \textit{flux rate } (mg \times min^{-1}) / \textit{pool size } (mg) \quad \textit{(Eq 1)}$$

$$\textit{flux rate } (mg \times min^{-1}) = \textit{fractional turnover } (min^{-1}) \times \textit{pool size } (mg) \quad \textit{(Eq 2)}$$

As implied from Figure 2, the movement of molecules in a pool can be expressed in relative or absolute units, e.g. “per min” or “mg per min”, respectively. Note that flux rate is typically normalized against body weight, tissue mass, cell number, etc.

Studies often assume a metabolic steady-state, i.e. the endogenous pool size is not changing over the period of time when a tracer is administered, however, tracer levels may change over time (and may therefore be in an isotopic non-steady state). For example, if we wanted to measure the movement of glucose molecules in the plasma we could administer [U-$^{13}C_6$]glucose into the circulation and then plot a time course of its labeling; we could draw conclusions regarding the kinetics by examining the fractional turnover and/or the flux rate (Wang, et al., 2016;van Dijk, et al., 2013;Shipley and Clark, 1972). Investigators in the field of lipoprotein kinetics often use flux rate to describe production and FCR to describe removal (Millar, et al., 2015), readers should recognize that studies are generally run under conditions of a metabolic steady state where FSR equals FCR and production rate equals removal rate.

Perhaps the following scenario may clarify the differences between fractional turnover and flux rate. Imagine a scenario where an adult is holding the hand of toddler while taking a walk. An observer could draw two different conclusions if (s)he were asked to explain “who is walking faster”. If the observer counts the number of steps the adult and toddler takes (s)he would likely conclude that the toddler is walking faster. Readers will recognize that the toddler’s little feet and shorter legs will require that they take more steps in the same amount of time (i.e. “walk faster”) as compared to the adult. However, the observer would also conclude that both the adult and the toddler covered the same total distance in the same amount of time (remember, they are holding hands so they start and end the walk together). Thus, one question could yield two apparent outcomes. Counting the number of steps per time is analogous to measuring a fractional turnover whereas counting the total distance is analogous to

measuring a flux rate; each provides a measure of activity but with very different meaning in regards to movement.

Fractional turnover is an interesting term that will be discussed later in the context of inferring mechanism of action, for now, readers should recognize the links to "**half-life"** and "**residence time"**. If studies have looked at a first-order process, then the fractional turnover is related to the half-life and residence time using the equations:

$$half\text{-}life = ln\ 2 \times fractional\ turnover^{-1} \qquad (Eq\ 3)$$

$$residence\ time = 1 \times fractional\ turnover^{-1} \qquad (Eq\ 4)$$

The **half-life** is the amount of time required for half of the pool to be replaced, while **residence time** is the average amount of time a molecule stays in the pool (Berman, et al., 1982). Accordingly, the time that is required to renew the pool is truly altered if the fractional turnover is changed, which is worth considering in the context of pharmacodynamic studies. For example, if we accept the hypothesis that oxidative stress can damage molecules within a given population, making them more likely to react with the body in a harmful way, then there is value in thinking about kinetics from the perspective of half-life and/or residence time. If a therapeutic could shorten the residence time of a harmful end-product one might be better positioned in terms of potential outcomes. Consider that each household will generate a certain amount of garbage every year, i.e. the absolute flux rate is fixed. Imagine a scenario in which we removed our trash every week vs every three months. The increased turnover (weekly vs quarterly garbage removal) has advantages even if we do not change the overall rate of garbage output. The concept of substrate cycling as a means of affecting metabolic control is based on the logic of having fast inter-conversions relative to net flux rates (Newsholme, 1978). Knowledge of half-life and/or residence time certainly provides a useful assessment of the physiological status.

An assumption is made when tracers are used to quantify fractional turnover and flux rate, i.e. there is no bias or discrimination between the absorption, distribution, metabolism or elimination of a tracer and a tracee. Readers may note that enzymologists exploit isotope effects in order to probe transition states (Cleland, 2005), however, physiological studies assume that there is no measureable difference between the fate of the tracer and the tracee (Wolfe and Chinkes, 2005;Waterlow, 2006), or, if there is a difference it is equal across groups. Because of this assumption extra consideration must be given to the events that affect isotopic "**enrichment**", this has special importance in pulse-chase experiments. Note that although many of the mathematical concepts used in tracer kinetics (Heath and Barton, 1973;LONDON, 1949;REINER, 1953b;REINER, 1953a;ROBERTSON, 1957;SOLOMON, 1949;Zierler, 1961;Zilversmit, 1960) are interchangeable with those used in pharmacokinetics (Rowland and Tozer, 2011;Gabrielsson and Weiner, 2000) there are a few key differences. For example, when a drug is administered via an infusion protocol, its concentration will increase. When the infusion is stopped, the concentration will decrease via the influence of a removal process, this is easy to visualize by plotting the concentration (y-axis) vs time (x-axis). A distinction must be appreciated when stable isotope tracers are used, the y-axis almost always reflects the "**enrichment**" of an analyte (a proportion of labeled-to-unlabeled molecules) which is in strong contrast to plots of drug concentration on the y-axis. Some readers will recognize that the literature surrounding stable isotope tracers contains terms including enrichment, mole fraction and tracer:tracee ratio (Wolfe and Chinkes, 2005;Ramakrishnan, 2006), although the equations and notation can impact certain experiments (Ramakrishnan, 2006) these terms are considered equivalent for our discussion.

Since we assume that labeled and unlabeled molecules are indiscriminately metabolized, it is not possible for clearance, degradation, elimination, or removal processes to affect the enrichment or change the proportion of labeled-to-unlabeled molecules (Previs, et al., 2004). Although one can cleverly

derive equations which imply that degradation affects isotope labeling (Holm, et al., 2013), tracer studies assume that removal processes do not change enrichment. Since our perspective is in contrast to the logic that is applied in some pulse-chase studies we will discuss how an appreciation of this point can simplify experimental designs and enhance data interpretations (Millar, et al., 2015;Bateman, et al., 2006;Mawuenyega, et al., 2010). Readers can test our assertion without any elaborate experimentation. Simply pour a cup of coffee and note the color, it will be black (this represents the endogenous "cold" tracee molecules). Adding a splash of milk (tracer) will make the color less black. This is analogous to what happens when one adds tracer to a system, the pool of endogenous molecules becomes enriched, the proportion of labeled-to-unlabeled molecules increases. When the infusion of the labeled precursor is discontinued one will observe a decrease in the enrichment of the product (e.g. protein) molecules, this decrease is thought to reflect clearance (Bateman, et al., 2006) which is not possible if labeled and unlabeled molecules undergo equal metabolism. If you sit back and take a drink you will recognize that there is now less coffee in your cup but you will note that the color has remained the same as it was before you started to drink. Clearance, removal, elimination or degradation processes will reduce the concentration of a product but they will not influence the proportion of labeled-to-unlabeled molecules, just as drinking from your cup will reduce the amount of coffee without influencing the proportion of milk-to-coffee. The color will only approach the original black color if one adds more coffee to a nearly empty cup, analogous to the synthesis of new unlabeled product molecules once the tracer (precursor) infusion is stopped.

The scenario that was just described draws out a distinction regarding "**metabolic steady-state**" and "**isotopic steady-state**" (Figure 2). Many biological problems are studied under conditions of a metabolic steady-state, i.e. the concentration of some end-product is not changing over the time. We observe a metabolic steady-state because production rates and removal rates are equal, however, we can also observe a change in isotopic enrichment over the same period. In the extreme setting, when we stop

the influx of new molecules and we only allow for the removal of existing molecules, we should observe a decrease in the amount of a target analyte but we should not observe a change in the enrichment. Indeed, we do observe changes in enrichment during a chase period, however, this is because new "cold" molecules are produced which therein replace the loss of a mixture of "cold" and "labeled" molecules; it is this production of "cold" molecules that subsequently maintains a metabolic steady-state.

A final definition concerns "**first-order reactions"** and **"zero-order reactions"** (Figure 3), i.e. when the reaction rate varies with the reactant concentration and when the reaction rate is independent of the reactant concentration, respectively. According to general principles, e.g. the Michaelis-Menton model of enzyme catalyzed reactions, numerous physiological events (including transport processes) have the potential to be first- and zero-order depending on the substrate concentration(s) and the Km. An example of this is seen in studies of triglyceride metabolism, circulating lipid levels can span a broad range and begin to saturate the removal process(es) at high, but physiologically relevant, concentrations (Grundy, et al., 1979). Mechanistic studies of lipoprotein lipase, which catalyzes the degradation of circulating triglycerides (Fielding, 1976), demonstrated that there is nearly a 10-fold difference between the Km in heart vs adipose tissue, implying that the heart will almost always be saturated with substrate (~ zero order kinetics) whereas the adipose tissue flux will respond to changes in circulating concentration (~ first-order kinetics). These types of tissue-specific differences may play an important role in maintaining normal lipid homeostasis (Previs, et al., 2014). A recent study concluded that most intracellular substrate concentrations exceed the Km implying that metabolic flux more closely approximates zero-order kinetics (Park, et al., 2016). This would imply that changes in substrate concentration will have little effect on pathway flux. A comparable cell-based study concluded that, in fact, reactions may not exceed the Km (Wahrheit, et al., 2014). Clearly, this matter of reaction order

should be considered on a case-by-case basis. A discussion of higher order reactions, which further complicate the modeling, is beyond the scope of our review.

3. Applying the definitions and concepts to a hypothetical metabolic problem.

Considering the terms that we have defined, one may ask, What are the most informative ways to characterize a kinetic phenotype? For example, investigators have tried to explain differences in pool sizes by comparing FSR to FCR (Bateman, et al., 2006) or by comparing production rate to FCR (Millar, et al., 2015). This section will consider a theoretical problem in order to look at whether these terms truly inform on biochemical traffic flow and allow one to draw conclusions regarding mechanisms that change pool size.

If we assume that biological processes follow some type of saturation model, e.g. Michaelis-Menton, an interesting relationship appears regarding fractional turnover and flux rate. In fact, fractional turnover (FSR or FCR) does not reflect metabolic flux as the reaction approaches zero-order (Figure 3). For example, as the concentration increases from 150 to 300 units the flux remains virtually constant (~ 18 and ~ 19 units x min^{-1}, respectively). However, the fractional turnover (i.e. the rate of product formation divided by the substrate concentration) substantially decreases over the same range (from 0.12 to 0.062, respectively). This example demonstrates that as the reaction approaches saturation wide shifts in the fractional turnover (nearly doubling here) reflect virtually no change in the flux rate (~ 6% difference here); i.e. 150 units x 0.12 min^{-1} = 18 units x min^{-1} vs 300 units x 0.062 min^{-1} = 18.6 units x min^{-1}

When the reaction approximates first-order kinetics (Figure 3) we see that a change in the concentration (from 20 to 60 units) is now associated with more dramatic changes in both the flux rate and the fractional turnover (from 10 to 15 units x min^{-1} and from 0.50 to 0.25, respectively). However, although

the magnitude of the change in fractional turnover now more closely reflects the change in flux the directionality is opposite; a marked increase in the fractional turnover represents a sizeable decrease in the flux; i.e. 20 units x 0.5 min^{-1} = 10 units x min^{-1} vs 60 units x 0.25 min^{-1} = 15 units x min^{-1}. According to this example, if one observes an increase in FCR it is not possible to conclude that this reflects an increase in the removal rate. Fractional turnover certainly provides a measure of relative metabolic activity but this should not be confused with a measure of biochemical flow.

This example demonstrates that fractional turnover and flux rate can both be used to characterize a metabolic phenotype however they may signify opposite effects. This will be discussed in more detail below since it is of central importance in various studies and since our view appears to go against commonly applied logic (Bateman, et al., 2006;Bateman, et al., 2007;Dobrowolska, et al., 2014;Elbert, et al., 2015;Millar, et al., 2015). Our perspective on this topic follows comments that were made by Steele regarding the meaning of "turnover constants" and "reaction rate constants", he also demonstrated that tracer decay curves can create an illusion of being first-order even if the reaction is not following such a scheme (Steele R, 1971). A deeper consideration of those matters is beyond the scope of this discussion.

4. Studying metabolic flux *in vivo*: Measurements of water turnover.

We now consider a simple metabolic problem to examine the concepts surrounding pool size, fractional turnover and flux rate using an *in vivo* model. Water kinetics were measured in C57Bl/6J mice (28.2 ± 1.3 g mean ± SEM, n = 3) and Sprague-Dawley rats (348 ± 10 g mean ± SEM, n = 3), animals were allowed free access to 5% [^{2}H]labeled drinking water for 7 days and then switched to regular drinking water for an additional 7 days. There were several reasons for contrasting water kinetics in mice vs rats. First, this protocol will generate data in which there is an exponential rise in plasma water labeling while animals are exposed to [^{2}H]labeled drinking water and a decay of enrichment when the animals are switched to

regular drinking water, i.e. this is a pulse-chase experiment under conditions of a metabolic steady-state (Bateman, et al., 2006). Second, we could draw out concepts regarding different pool sizes and link points regarding fractional turnover and flux rate. We could experimentally determine whether comparing FSR to FCR or production rate to FCR would yield insight regarding the differences in pool size. Finally, we could cross-validate tracer estimates of water kinetics with an orthogonal (non-tracer based) approach, i.e. measure the change in weight of the water bottles over 24-hour intervals; since some spillage can occur this approach may slightly overestimate the true water turnover but will still provide a reasonable surrogate assessment. These studies were approved by our Institutional Animal Care and Use Committee.

As expected, the water intake (determined by weighing the bottles) was different between mice and rats, 6.1 ± 0.5 and 26.2 ± 1.6 ml per 24 hour period, respectively (mean ± SEM, Figure 4). Since rodents obtain ~ 60-70% of their water by drinking, with the remainder coming from digestion and respiration (Lee, et al., 1994), the total water flux is ~ 9 and ~ 40 ml per day in mice and rats, respectively. Since these are lean animals, fed a standard low-fat rodent chow, the total body water pool (i.e. body weight x 0.70) is ~ 20 and ~ 244 ml in mice and rats. Therefore, the fractional turnover is ~ 9/20 or 0.45 and ~ 40/244 or 0.16 pools of water per day, in mice and rats, respectively.

The [^{2}H]labeling of plasma water followed the expected trends for a pulse-chase experiment (Figure 4), the ascending and descending enrichments were fit to single exponentials for individual animals using GraphPad Prism. Since the animals are in a metabolic steady-state one expects agreement between the increase and the decrease in enrichment values (assigned to the terms FSR and FCR, respectively), furthermore, since body weight was stable the water pool was therefore not changing with time and the input and output rates of water flux are equal. As compared to rats, mice display a greater fractional turnover, however, the water flux (Eq 2) was reduced in mice vs rats (Figure 5). The overall data set

demonstrates that tracer-based estimates of fractional turnover and absolute flux rate agree with direct measurements of water consumption.

Can we use these kinetic data to explain why the pool sizes are different? Although the decrease in water labeling (what some might call the "FCR") is ~ 3 times lower in rats vs mice (Figure 5, Panel B), the water flux is actually ~ 4 times greater in rats vs mice (Figure 5, Panel C). Therefore, fractional turnover does not really provide a useful index of mass-balance. Knowing that the "FCR" is lower in rats cannot explain the existence of a greater pool size of body water; water pool size and turnover are in a metabolic steady-state and not changing over the course of the study. This example demonstrates that knowledge of absolute flux inputs (i.e. water intake and production) and fractional turnover cannot allow one to make statements regarding why pool sizes are different or whether the pool size will change over time (Bateman, et al., 2006;Millar, et al., 2015;Bateman, et al., 2007;Dobrowolska, et al., 2014;Elbert, et al., 2015). Stated another way, if we are told that 20 people enter a restaurant every hour and 5% of the total people who are there leave over the same interval could we determine if the number of people inside the establishment were increasing, decreasing or remaining constant? We could only make statements regarding a change in the number of people inside the restaurant if we knew how many people were inside at the start or if we compared the number of people entering with the number of people leaving.

5. Using tracer studies to make a sound interpretation of physiological regulation.

To this point we have defined critical terms (Figure 2) and outlined their application in the context of theoretical (Figure 3) and *in vivo* studies (Figure 4 and 5). Hopefully, readers are becoming familiar with high-level concepts that are used in tracer studies, there are now two issues that should be considered in order to best utilize tracer studies. First, how does one approach experimental designs to determine

biochemical flux? Second, how can one ensure some confidence regarding the data interpretation? We will consider these questions by reviewing technical details that affect investigational plans.

5.a. How can we measure FSR and FCR?

If studies are conducted under a metabolic steady-state one can infer the fractional turnover by injecting a tracer into a pool and then measuring its dilution over time (Wolfe and Chinkes, 2005;Waterlow, 2006). This approach works reasonably well in cases where one is interested in the kinetics of small molecules (e.g. glucose, Figure 2) (Wang, et al., 2016;van Dijk, et al., 2013) however it is more complicated when macromolecules are considered, e.g. it is not practical to quantify proteome dynamics via the administration of a mixture of pre-labeled proteins.

To determine the kinetics of larger molecules one typically follows the movement of a labeled precursor. For example, pulse-chase methods have been proposed for quantifying the FSR and FCR of a protein (Bateman, et al., 2006). Briefly, the FSR is estimated during the infusion of [^{13}C]leucine via its incorporation into a protein of interest (i.e. pulse), the "FCR" is estimated when the tracer infusion is terminated via the decrease in [^{13}C]labeling of the respective protein(s) (i.e. chase). This approach suggested that healthy and disease subjects present with comparable FSRs but that a reduced FCR contributes to the accumulation of amyloid protein in human neurodegeneration (Mawuenyega, et al., 2010). When this logic was applied in a recent drug development study the data suggested that the drug delivery vehicle induced a favorable imbalance in amyloid homeostasis; the FCR was nearly twice that of the FSR (Figure 2 in ref (Dobrowolska, et al., 2014)). The implications of these observations are profound, if the pulse-chase method is correct (Bateman, et al., 2006) then one could consider the vehicle as a viable drug candidate (Dobrowolska, et al., 2014). It was suggested that the conclusions may need to be reexamined in the context of a new mathematical model (Dobrowolska, et al., 2014;Elbert, et al., 2015). While we recognize the need for rigor when modeling the pathways, especially in cases where

there are inaccessible pools (e.g. CSF serving as a surrogate for brain), it is possible that there are misunderstandings of the principles surrounding pulse-chase protocols. This would not impact on the true efficacy of the drug but it would raise questions regarding our understanding of the pathophysiology in the disease state (Mawuenyega, et al., 2010) and limit our ability to examine the mechanism of drug action (Dobrowolska, et al., 2014). Considering this scenario we thought that an explanation of the logic surrounding the measurements might be helpful.

5.a.1. Quantifying the FSR.

The FSR can be estimated by infusing a labeled precursor and then measuring its incorporation into a product, e.g. [^{13}C]leucine ⇢ [^{13}C]amyloid protein, however, there are caveats to consider. In short term protocols one can measure the pseudo-linear change in product labeling vs time and compare that to the precursor labeling using the equation (Foster, et al., 1993):

FSR = (change in protein labeling / change in time) / precursor labeling *(Eq 5)*

The "precursor labeling" can be (i) plasma or compartment (e.g. cerebrospinal fluid "CSF") [^{13}C]leucine, (ii) plasma or compartment [^{13}C]keto-isocaproate or (iii) the [^{13}C]leucine labeling in some rapidly turning over protein that is made in the same cells as the target protein of interest (Waterlow, 2006). If we consider i, ii and iii, there are 5 variants of Eq 5. Since one can observe sizeable differences between the [^{13}C]leucine labeling in plasma and CSF, this matter was carefully considered during the development of the protocol used in the amyloid studies, it was concluded that plasma [^{13}C]leucine represents the precursor labeling in Eq 5 for studies of amyloid kinetics (Cook, et al., 2010;Bateman, et al., 2006). Studies of apolipoprotein kinetics have also demonstrated that the labeling of plasma amino acids is considerably greater than that of intracellular amino acids (Lichtenstein, et al., 1990), however, those investigators typically avoid making the claim that the labeling of a plasma amino acid reflects the

precursor labeling, they rely on a surrogate measure of intracellular amino acid labeling as a proxy for the precursor labeling. A main take-home message is that one can obtain different estimates of FSR depending on what analyte is used to represent the precursor labeling, some experimentation may be required to build confidence in one's choice (Zhou, et al., 2015).

In longer term studies and/or in cases where a protein turns over more rapidly one can estimate the FSR from the temporal change in labeling as the protein approaches its asymptotic enrichment using the equation (Foster, et al., 1993):

$$protein\ labeling_{time\ z} = protein\ labeling_{asymptotic\ enrichment} \cdot (1 - e^{-FSR \cdot time\ z}) \qquad (Eq\ 6)$$

Assuming that samples are collected at appropriate times, for example, more rapidly turning over proteins will require earlier sampling, and assuming that there are no errors in the data acquisition then Eq 5 and Eq 6 will yield the same values. Since Eq 6 circumvents the need to consider a value for the precursor labeling the analyses are somewhat simplified, investigators estimate the time it takes for the protein to reach steady-state labeling (Foster, et al., 1993). However, the primary assumption surrounding Eq 6 is that the precursor labeling remains stable over time (note that adjustments can be made to correct for changes in precursor labeling (Bederman, et al., 2006;Ramakrishnan, 2006). In summary, there are at least 6 approaches for estimating the FSR during the infusion of the labeled precursor, i.e. 5 variants of Eq 5 and Eq 6. Each approach has assumptions, and therefore potential limitations, these should be recognized when choosing how to estimate the FSR.

5.a.2. Quantifying the FCR.

The term "FCR" often refers to the later part of a pulse-chase protocol, when the infusion of the precursor (e.g. [^{13}C]leucine) has been terminated and one measures the decrease in product labeling

(e.g. [^{13}C]amyloid) (Bateman, et al., 2006). Since tracer studies assume indiscriminate metabolism of tracer and tracee it is not possible for clearance, removal, elimination or degradation processes to affect the labeling; just as drinking coffee after we add a splash of milk will not reverse the color of the mixture in our cup. In fact, we can estimate the FSR during the "washout" phase (Previs, et al., 2004). Therefore, in addition to the six approaches for estimating FSR (noted earlier) a seventh approach for assessing FSR involves measuring the decrease in enrichment during a "chase" (Previs, et al., 2004). However, one should recognize a caveat regarding tracer recycling (Waterlow, 2006). For example, during the tracer infusion period the labeled amino acid precursor will be incorporated into many proteins, when the precursor infusion is terminated labeled amino acids will be released as labeled proteins are degraded, those labeled amino acids can be reincorporated into newly synthesized proteins (Poole, 1971;Muramatsu, et al., 1963). Tracer recycling can lead to a slower dilution of label from a protein of interest, i.e. a slower decrease in protein labeling can be observed during the washout phase (as compared against the increase in protein labeling that occurs during the infusion period). In cases where tracer recycling occurs, the "chase" phase will yield little insight into the true fractional turnover of a protein, the decrease in enrichment that is observed during the "chase" (FCR) can appear to be slower than the increase that is observed during the "pulse" (FSR) because of recycling (Mawuenyega, et al., 2010).

We recognize that intuition may lead one to doubt these comments so we remind readers of examples where tracer dilution is used, in combination with arterio-venous catheterization, to estimate organ balance (Ekberg, et al., 1999;Mittendorfer, et al., 1998). For example, a decrease in enrichment that is observed as the tracer moves from the arterial circulation across the organ (with subsequent sampling of the venous circulation) represents the production of cold molecules. We could use another example surrounding standard isotope dilution assays. Suppose that we had a test tube which contained a certain amount of plasma to which we spiked in a labeled standard, e.g. [^{13}C]leucine. If we mix the contents of

the stock tube and then make numerous smaller aliquots we will eventually deplete the main stock tube and yet every aliquot will contain the exact same isotopic enrichment as the initial stock. Clearly, the removal of material from the primary stock tube does not change its enrichment.

There are two questions that should now be addressed regarding FCR. First, can tracers yield a value for FCR? Yes, this is possible. Studies that use radiolabeled tracers offer some advantages since one can plot the radioactive counts (independent of the endogenous "cold" tracee) on the y-axis and therein estimate FCR. Because stable isotope protocols almost exclusively plot a measure of enrichment (Bateman, et al., 2006;Cook, et al., 2010;Mawuenyega, et al., 2010) one should convert the y-axis to a tracer mass.

This brings us to our second question regarding "FCR" which we should rephrase as "Does knowledge of the fractional turnover offer any advantage over knowledge of the flux rate?". Some may be thinking that if a study examines a system under in a metabolic steady-state, where FSR equals FCR (Parhofer, et al., 1991), it may seem pointless to consider this matter further. However, we believe that there is merit in expressing, or contrasting, fractional turnover against flux rate; readers should refer to Eq 3 and 4 where fractional turnover is linked to half-life and/or residence time, this matter is further considered in next section. We believe that the term "fractional turnover" is over utilized and that investigators should consider the use of half-life or residence time. Although experienced investigators know that this statement is more about semantics, it may be helpful to those who are new to the field.

5.b. Using tracers to predict or explain changes in pool size: Learning about mechanism of action.

If our goal is to predict whether a pool size is changing it can be misleading to compare the FSR with the FCR (Bateman, et al., 2006;Dobrowolska, et al., 2014;Mawuenyega, et al., 2010) especially as the reactions approach saturation and/or in cases of a metabolic steady-state. Likewise, it can be

challenging to accurately estimate these end-points given the assumptions regarding the measurements. However, can we gain insight regarding a mechanism of action by comparing the production rate with the FCR? (Millar, et al., 2015). For example, studies may randomize disease subjects to a placebo or a treatment group, the treatment may then modulate the concentration of an end-point, e.g. lower apoB lipoprotein. At some point after initiating the randomization and treatment an investigator may run a metabolic flux study in an attempt to explain the change in concentration of the end-point between groups (e.g. tracers are administered during Period 3, Figure 6). Conclusions are typically made in which input is described using an absolute value (production rate, e.g. μmol x kg^{-1} x hr^{-1}) and removal is described using a relative value (FCR, e.g. pool x hr^{-1}), the expectation is that one can explain how the treatment changed the end-point (Millar, et al., 2015).

There are a few matters to consider regarding the scenario above. Once a metabolic steady-state is reached (e.g. Figure 6, Period 1 or 3) the FSR equals the FCR and the production rate equals the removal rate. We can appreciate that one can find apparent differences in pathway activity between groups if inputs and outputs are expressed using different units however this can result from the dependency of the terms (Eq 1 and 2) and the fact that a pool size has changed, e.g. if production rate is the same across two groups but pool sizes are different we somewhat obligated to observe a change in FCR because the terms are all linked. Hopefully we can agree that comparing pathway activities which are expressed in different units (absolute flux compared to a fractional turnover) is confusing and potentially misleading in terms of explaining a mechanism.

Suppose that one monitored his/her bank account on a monthly basis. If you knew that you had a certain amount of savings one month and then the next month your savings was decreased by half you might be concerned. However, you will immediately realize that although we could monitor all transactions going forward we will never explain why our savings decreased in the previous month. To

determine why our balance changed would require that we examine those transactions that occurred during the period of time when the balance actually changed (e.g. Figure 6, Period 2).

Readers may be left wondering if we can explain a mechanism of action when we observe difference concentrations of a given end-point between two groups. Hopefully there is some general agreement that studies which are run under a metabolic steady-state cannot explain how the system arrives at a new set point (e.g. Figure 6, Period 3)(Millar, et al., 2015). Nevertheless, there is value in probing for kinetic differences between steady-state systems.

Perhaps readers may now begin to appreciate the merits of studies that examine non steady-state conditions where pool size changes over the period of time when tracers are administered (e.g. add tracers during Period 2 of Figure 6). For example, if we measure the change in pool size over time and determine the synthesis rate then we can calculate the degradation rate (Figure 6, Period 2) (Bederman, et al., 2009;Bederman, et al., 2006;Brunengraber, et al., 2003;Ratheiser, et al., 1999). Although those designs are not practical in many instances, the synthesis rate reflects the degradation rate for a given group if/when the pool size is stable even if different pool sizes are observed between groups (Figure 6, Period 3 and Figures 3 and 4). Likewise, investigators can consider the use of perturbation tests that acutely move systems from a metabolic steady-state (Vaitheesvaran, et al., 2010;McLaren, et al., 2016;Chavez-Jauregui, et al., 2010;Donnelly, et al., 2005). Those approaches can unmask hidden phenotypes and address questions regarding metabolic flexibility and physiological response capacity. Whereas a fractional turnover, measured in a metabolic steady-state, may suggest an altered half-life, acute stressors (e.g. a meal tolerance test) might allow one to examine relative V_{max}- and K_m-type parameters and therein suggest biochemical events that might be contributing to the phenotype.

5.c. Relating tracer dose, area under the curve (AUC), and metabolic activity: Quantifying the conversion of a precursor to a product.

Our final discussion centers on the relation between an AUC and a metabolic activity. Although this concept is immediately obvious in pharmacokinetic modeling, i.e. clearance = dose of drug / AUC, it has limited meaning in tracer studies unless one accounts for isotope dilutions and exchanges (Jin, et al., 2013;McLaren, et al., 2013). Just as knowledge of bioavailability and distribution are needed in order for AUC to accurately reflect the metabolic rate surrounding drug clearance (Rowland and Tozer, 2011;Gabrielsson and Weiner, 2000), similar caveats apply when tracers are used to infer metabolic activity. For example, we might give a known dose of a labeled precursor to control and drug-treated subjects and then observe differences in the movement of the tracer from a precursor pool to a product pool (e.g. the conversion of [$^{2}H_5$]glycerol⇢[$^{2}H_5$]triglyceride or [$^{13}C_3$]lactate⇢[^{13}C]glucose is reduced in drug-treated vs control subjects). In order to draw meaningful conclusions regarding pathway activity, we must account for the fact that the isotope labeling could (i) be "scrambled" as it undergoes conversion to the product and/or (ii) experience differential dilution caused by changes in the half-life and/or the amount of endogenous tracee observed in the groups (Jin, et al., 2013;McLaren, et al., 2013).

Figure 7 outlines an example regarding dilution effect(s). The metabolic scheme (Panel A) shows that a precursor can either be irreversibly lost or converted to a product, the precursor can also mix with a side compartment that "scrambles" the isotopic distribution pattern (see Figure 3, ref (Previs, et al., 2014)). Figure 7 demonstrates the temporal change in product labeling if the same amount of tracer is given to two subjects as a single bolus and the only difference between the subjects is the conversion of precursor→product (i.e. the difference in product labeling in Panel B reflects a difference in the parameter k_2, we expect a 50% reduction in one vs another). Panel C and D also demonstrate differences between the product labeling despite the fact that k_2 is the same in the respective subjects, these data reflect changes in the precursor system and have nothing to do with the conversion of

JPET Fast Forward. Published on July 19, 2017 as DOI: 10.1124/jpet.117.241091
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precursor→product; Panel C demonstrates what happens if the precursor pool size is increased by a factor of two whereas Panel D demonstrates what happens if the half-life of the precursor is reduced by a factor of two. It is necessary to recognize the impact of these events if tracers are used to study pharmacodynamics and/or quantify target engagement activity (Landry, et al., 2011).

Figure 8 expands on the problem by demonstrating the effect of isotopic scrambling. Note that although we administered [U-$^{13}C_3$]lactate (M+3) the dominant glucose species is M+2, this is consistent with known biochemical schemes (Krebs, et al., 1966;WEINMAN, et al., 1957) and experimental data (Katz, et al., 1993;Landau, et al., 1998). In addition to the [^{13}C] scrambling, if we only consider the abundance of [^{13}C]labeled glucose as a readout of pathway activity we would conclude that octanoate is an inhibitor of glucose production. This is not true, glucose production is equal in the groups (Figure 8, inset), the addition of octanoate actually adds more [^{12}C]substrate which therein dilutes the [^{13}C]lactate as it undergoes conversion to glucose (WEINMAN, et al., 1957). Since enzymes and pathways affect the simultaneous conversion of tracers and tracees it is critical to measure enrichment, one should generally avoid measuring the fate of the tracer alone (Landry, et al., 2011).

6. Summary and final conclusions.

Hopefully readers will recognize that tracer kinetics add a new dimension in studies of pathway biology, measurements of metabolic flux can yield novel insight regarding studies of pharmacodynamic mechanisms (Figure 1). We have reviewed examples that represent broad concepts in the field of tracer kinetics in order to outline first-principles (Figure 2 and 3), presumably we have made the subject matter more conversational by minimizing the discussion of equations and emphasizing parallel problems. We recognize that some aspects of our review differ markedly from the literature, we apologize to authors who may feel singled out in this report, there is no intention to discredit the efforts of any individual or group.

We believe that stable isotope tracer methods can yield novel insight into questions surrounding biochemical flux and inform on the pathophysiology in disease states. Our theoretical model (Figure 3) along with experimental measurements of water flux (Figures 4 and 5) can be used to represent a number of scenarios including “pulse-chase” designs and studies of different metabolic states. Of course, the scenario that we have used (e.g. a single compartment with a stable pool size) represents the simplest type of problem.

Since the concentration of an end-point reflects the difference between the rates of synthesis and degradation one can infer degradation rates by measuring the temporal change in pool size and the rate of synthesis (Figure 6, Period 2). Readers should appreciate the fact that tracers and tracees have the same fate. Since one can derive estimates of fractional turnover and flux rate from the labeling “pulse” phase of an experiment it may not be necessary to measure dilution during the “chase” phase, perhaps our discussion can help to simplify experimental designs. As well, the subtle disconnect between the mathematical modeling that is applied to pharmacokinetics and tracer kinetics requires attention (Figures 7 and 8). Metabolic flux can affect the downstream labeling in precursor:product relationships, therefore a careful consideration of analytical requirements is needed. Attention to these matters can allow investigators to reliably estimate flux even in cases when isotope exchange is present.

Author Contributions.

Participated in the research design: NAD, SPW, YC, HZ, DGM, TPR, DGJ, DM, MDE, DEK, SFP, TK

Conducted experiments: NAD, SPW, YC, HZ, DGM, DM, SFP

Contributed new reagents or analytical tools: not applicable

Performed data analyses: NAD, SPW, YC, HZ, DGM, TPR, DGJ, DM, SFP, TK

Wrote or contributed to the writing of the manuscript: NAD, SPW, YC, HZ, DGM, TPR, DGJ, DM, MDE, DEK, SFP, TK

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Figure legends.

Figure 1. Contrasting static and kinetic assessments of cholesterol homeostasis. Primary human hepatocytes were incubated in the presence of 10 mM glucose and 10% [^{2}H]water for 36 hours. Standard media was supplemented with an additional 10 mM fructose and/or 1 μM Simvastatin for the entire incubation period. Cholesterol content and [^{2}H]labeling were determined using gas chromatography-mass spectrometry following saponification, extraction and acetylation (Jensen, et al., 2012). The relative signal intensity (a surrogate of total content, shaded bars) is comparable in all conditions but there were marked differences in the contribution of newly made cholesterol (solid bars, * $p < 0.01$, n = 6 wells per condition, data shown as mean ± SEM).

Figure 2. Outline of metabolic flux terms and tracer logic. Three critical terms to consider are pool size, fractional turnover and flux rate. Fractional turnover and flux rate represent measures of pathway activity and they are related to each other through the pool size (Equations 1 and 2), different units can be used to describe production and removal of material from a pool (Panel A). Studies are most often run under conditions of a metabolic steady-state, where the amount of some endogenous tracee is not changing with time (Panel B, closed circles representing endogenous glucose). In those cases the FSR equals the FCR and the production rate equals the removal rate. Isotope tracers can be administered and measured under conditions of non-steady-state (Panel B, open circles representing [U-$^{13}C_6$]glucose). Note that (i) data should ideally be expressed as enrichment (i.e. labeled glucose / total glucose) and (ii) we expect equal loss of labeled and endogenous glucose from the pool, mass-balance is preserved since there is continuous production of unlabeled glucose.

Figure 3. Hypothetical model of reaction order in the context of an enzyme catalyzed reaction. According to the Michaelis-Menton model, the rate of product formation will reach a saturation point as the substrate concentration increases. Flux rate can be determined at each substrate concentration (solid symbols), i.e. $v = (V_{max} \times S)/(K_m + S)$. At lower concentrations there is a more direct relationship between the change in substrate concentration and the flux rate (e.g. first-order region), this relationship diminishes as the reaction approaches

saturation (e.g. zero-order region). If we assume a constant volume then we can calculate a fractional turnover at each substrate concentration (open symbols). Since we know the flux rate ("v" or product formation) and the pool size (substrate concentration x volume) we can see that the fractional turnover (flux rate / pool size, Eq 1) also changes over the course of the experiment, e.g. the fractional turnover decreases as the flux rate increases.

Figure 4. Water dynamics in rodents. The change in weight of the water bottles was determined over 24 hour intervals in mice (solid circles) and rats (open circles). Water intake was not effected during the period when animals were given [^{2}H]water vs regular tap water but there was a difference between mice and rats (Panel A, $p < 0.01$ using a 2-way t-test and assuming equal variance). The [^{2}H]labeling of plasma water (Shah, et al., 2010) increased over time when animals were given [^{2}H]water and there was an expected decrease in [^{2}H]labeling when animals were switched to regular tap water (Panel B). Data are shown as mean ± SEM, n = 3 per group per time point.

Figure 5. Tracer-based estimates of fractional turnover and flux rate of water. The water pool size was calculated by assuming that 70% of body weight is lean mass (Panel A). The enrichment data shown in Figure 4 were fit to single exponentials to determine the fractional turnover, the "FSR" was estimated from the ascending plots and the "FCR" was estimated from the descending plots (Panel B) (Eq 2). The water flux rate (Panel C) equals the product of the pool size (Panel A) and the fractional turnover (Panel B). There are no differences between the FSR and FCR data for a given group (paired t-test) but clear differences between pool size (Panel A), fractional turnover (Panel B) and flux rates (Panel C) between mice and rats in all cases ($p < 0.01$). Data are shown as mean ± SEM, n = 3 per group.

Figure 6. Product pool dynamics. Stable concentrations of a given product reflect a condition where synthesis (S) and degradation (D) are equal (Period 1 and 3), i.e. the system is in a metabolic steady-state. During

conditions where there is a change in steady-state concentration there will be an imbalance between S and D, e.g. S must be less than D when the concentration decreases (Period 2). When a new metabolic steady-state is reached S and D are again equal (Period 3).

F**igure 7. Precursor-product flux scenarios.** Panel A demonstrates that labeled precursors can either be irreversibly lost (k1) or converted to a product of interest (k2), some fraction can also equilibrate in a side compartment which may rearrange the isotopic labeling (designated "?"). In all cases we assumed that two subjects would exist, each would get the same dose of tracer. Panel B demonstrates the outcome that would be observed if the precursor→product conversion (k2) was reduced by 50% in one subject vs another; as expected, product labeling is reduced when the inhibitor is present. Panel C and D demonstrate comparable reductions in the product labeling even though k2 is equal in those cases. For example, in Panel C we let the precursor labeling be different by 50% in one subject vs another (e.g. suppose the same dose of tracer was given to each but the endogenous precursor pool was twice as large in the second subject) whereas in Panel D we let the precursor half-life change by a factor of two between the subjects. The solid line connecting the solid circles shows the different profiles of precursor labeling (Panel C and D) in the respective subjects, the dotted lines represent the corresponding product labeling.

Figure 8. Net flux vs isotopic dilution and exchange. The abbreviated scheme in Panel A demonstrates key steps in the gluconeogenic pathway that are relevant to the entry of various substrates. Previous experiments measured the isotopic distribution of glucose when rat livers with perfused in an open circuit with 0.1 mM glycerol and 1.0 mM [$^{13}C_3$]lactate ± octanoate (Previs, et al., 1995). The spectra contained in Panel B demonstrate marked "scrambling" of isotope labeling as the [U-$^{13}C_3$]lactate (M+3) precursor is converted to [^{13}C]glucose. The addition of octanoate leads to a reduction in the enrichment of glucose. If one considered the abundance of [^{13}C] to reflect the rate of glucose production one would erroneously conclude that octanoate acts as an inhibitor of the pathway flux (the inset demonstrates that there was no change in glucose

production). The differences in the [^{13}C]labeling reflect dilution of the tracer as the [$^{13}C_3$]lactate precursor is converted to [^{13}C]glucose. These data are reproduced from another report (Previs, et al., 1995).

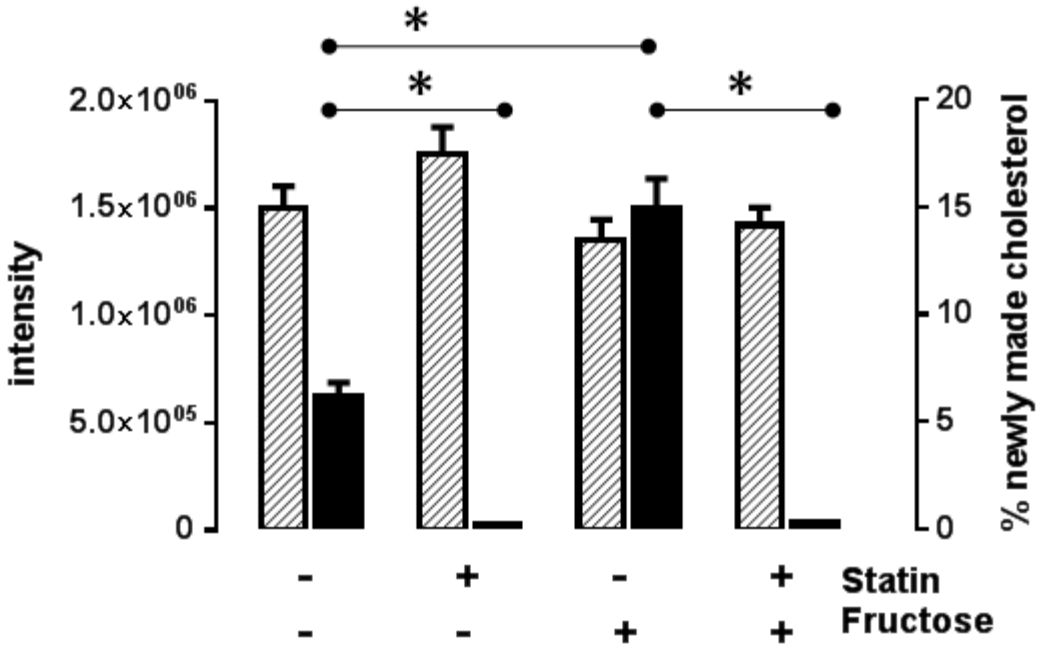


Figure 1

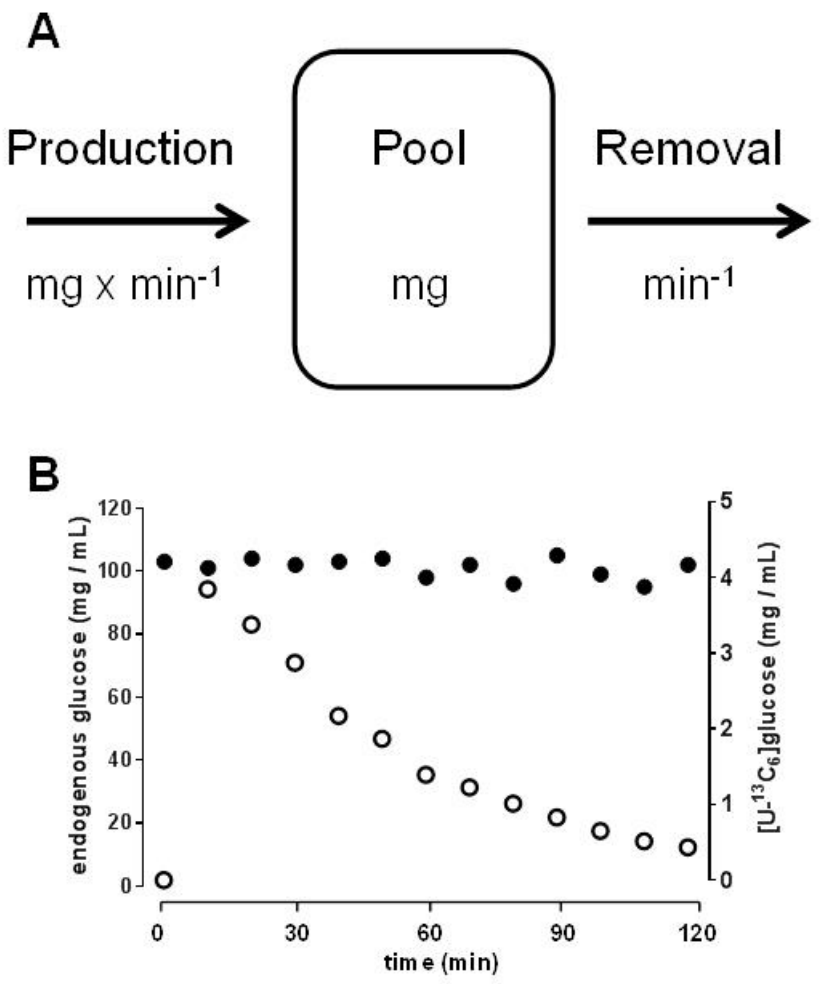
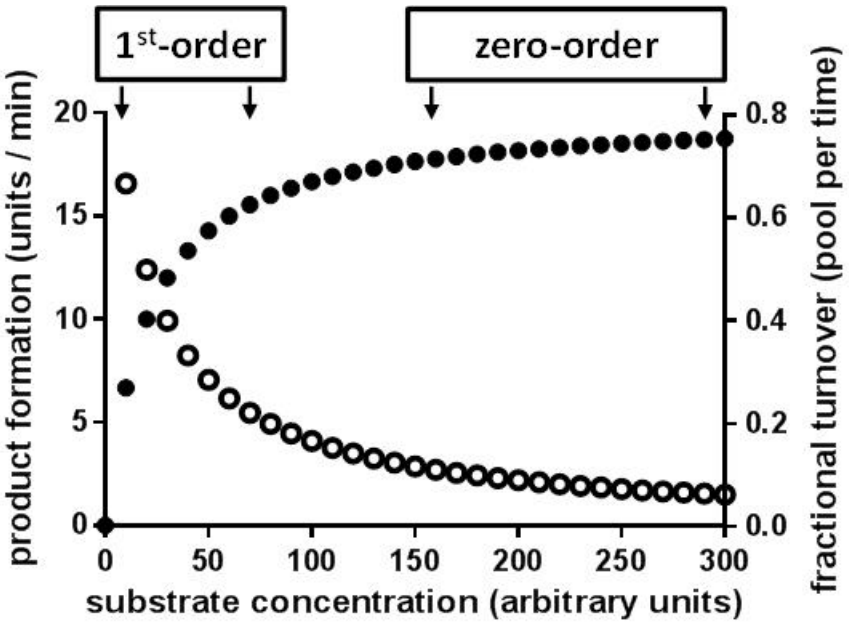


Figure 2



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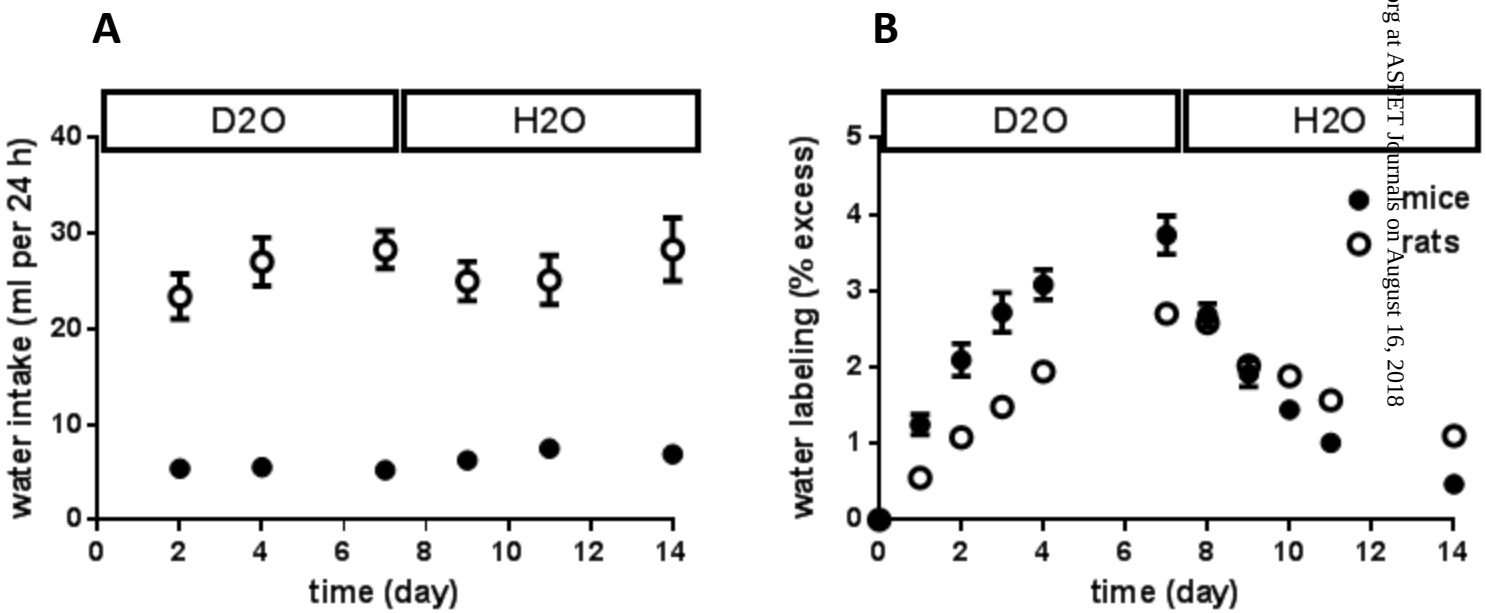
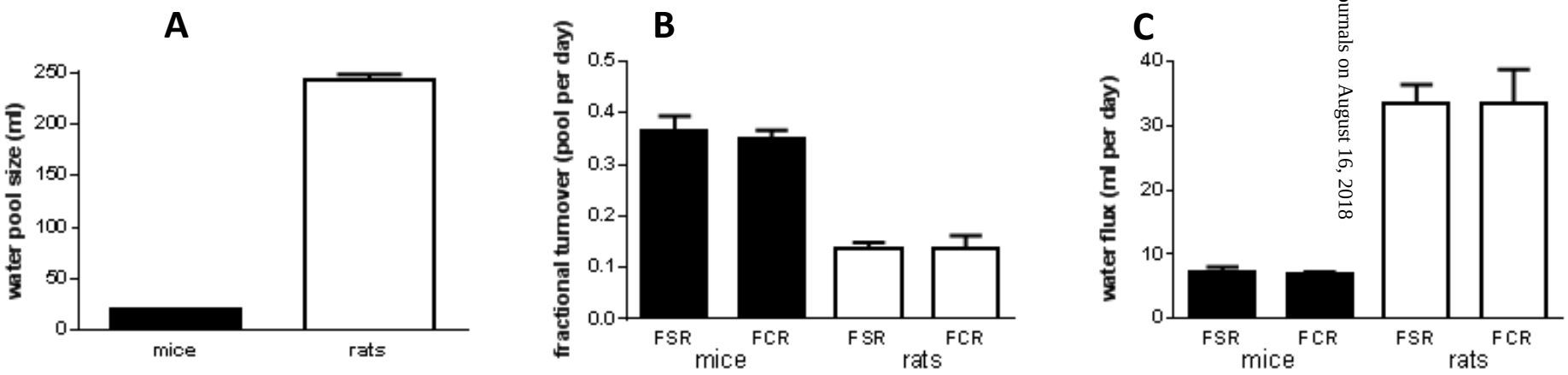


Figure 4



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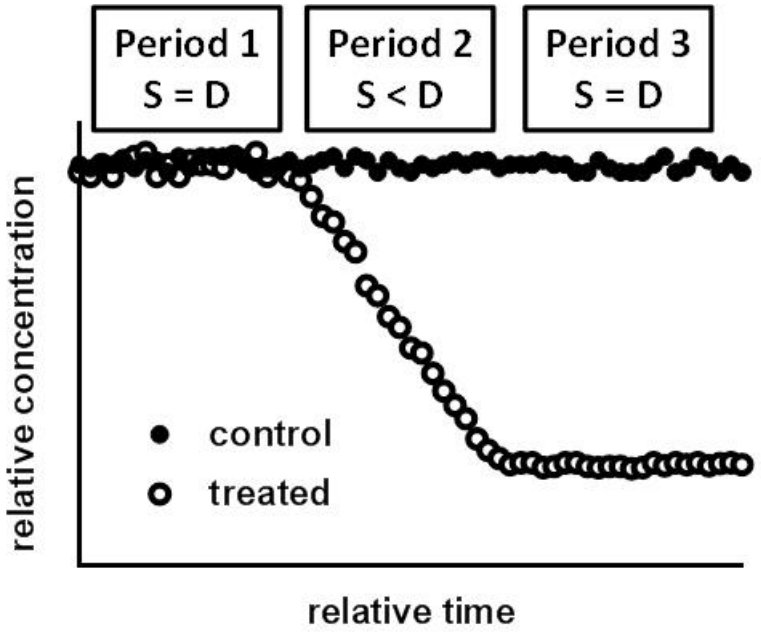

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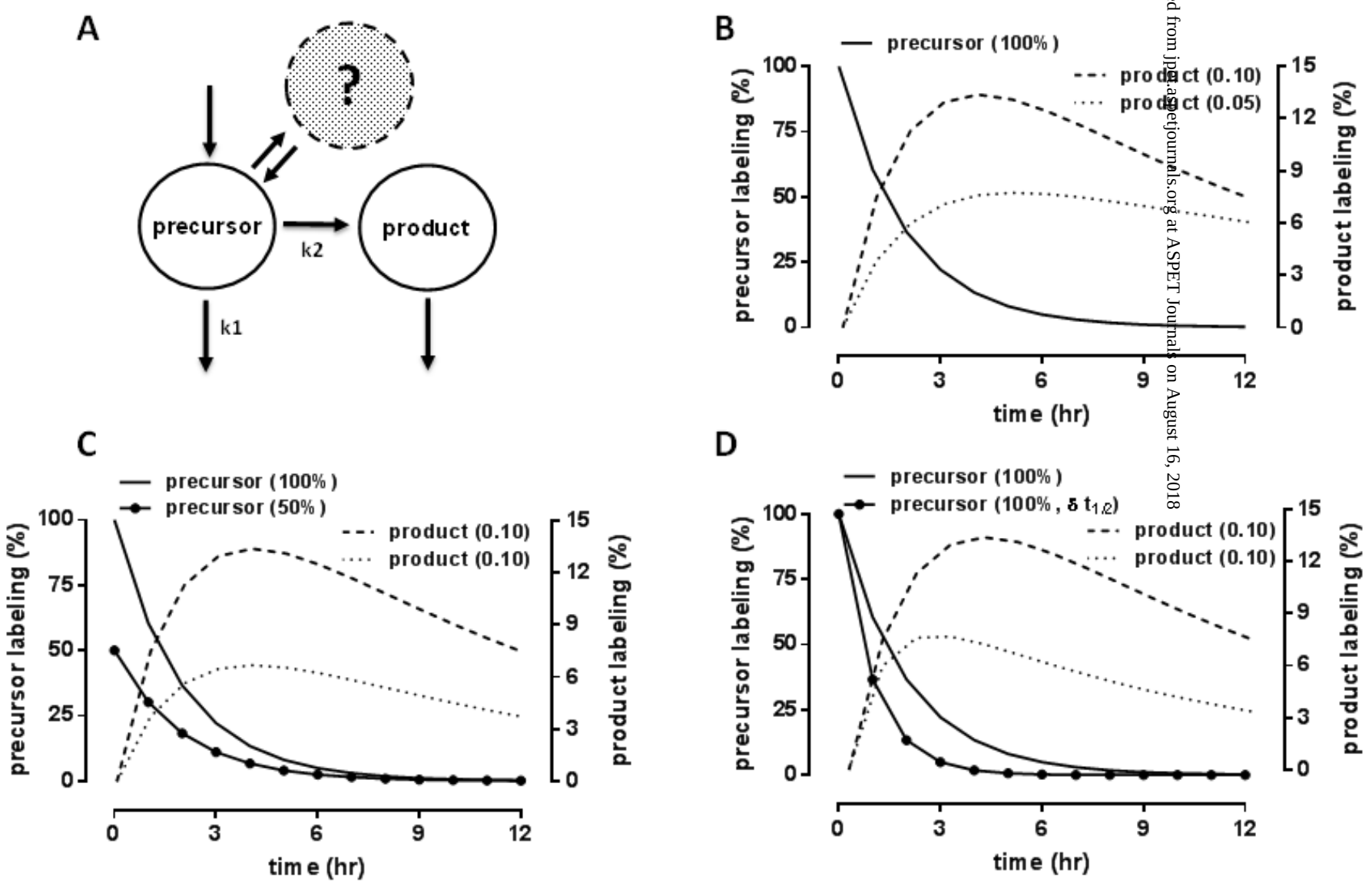

Figure 7

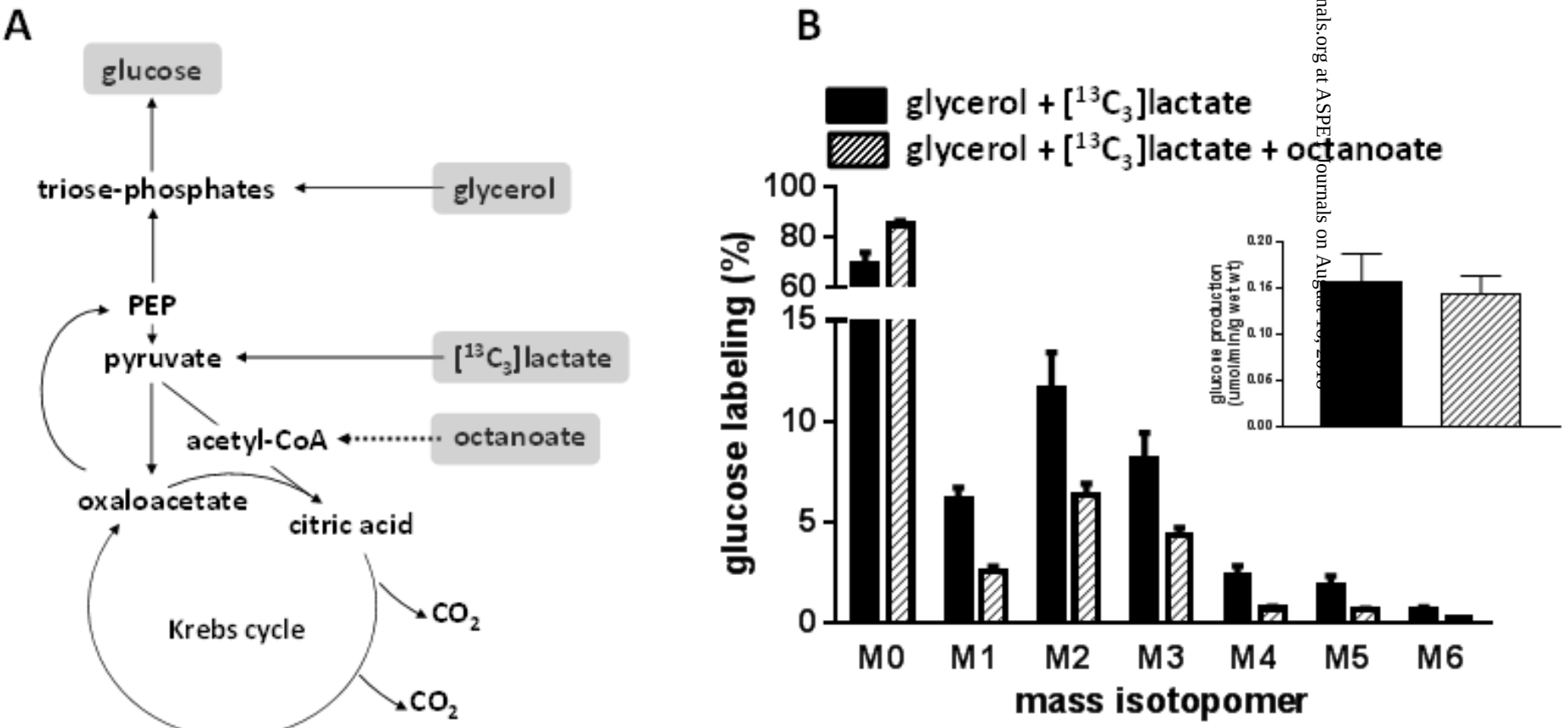

Figure 8