Use of stable isotopes to study carbohydrate and fat metabolism at the whole-body level

Andrew R. Coggan

Biomedical Mass Spectrometry Facility, University of Maryland School of Medicine, Department of Medicine, Division of Gerontology. VA Medical Center (BT18GR), 10 N Greene Street, Baltimore, MD 21201, USA

The present review discusses the advantages and limitations of using stable-isotope tracers to assess carbohydrate and fat metabolism at the whole-body level. One advantage of stable-(v. radioactive-) isotope tracers is the relative ease with which the location of a label within a molecule can be determined using selected-ion-monitoring GC–mass spectrometry (SIM–GC–MS). This technique minimizes potential problems due to label recycling, allows the use of multiple-labelled compounds simultaneously (e.g. to quantify glucose cycling), and perhaps most importantly, has led to the development of unique stable-isotope methods for, for example, quantifying gluconeogenesis. However, the limited sensitivity of SIM–GC–MS sometimes requires that relatively large amounts of a stable-isotope tracer be used, thus increasing cost and potentially altering metabolism. At least theoretically, stable- (or radioactive-) isotope tracers can also be used in conjunction with indirect calorimetry to estimate utilization of muscle glycogen or triacylglycerol stores, thus potentially circumventing the need to obtain muscle biopsies. These calculations, however, require certain critical assumptions, which if incorrect could lead to major errors in the values obtained. Despite such limitations, stable-isotope tracers provide a powerful and sometimes unique tool for investigating carbohydrate and fat metabolism at the whole-body level. With continuing advances in availability, instrumentation and methods, it is likely that stable-isotope tracers will become increasingly important in the immediate future.

Stable-isotope tracers: Exercise: Lipolysis

The use of stable-isotope tracers to assess in vivo metabolism has risen markedly during the last 20 years. This growth has been fuelled by a number of factors, including: (1) increased concern about the potential health and environmental consequences associated with the use of radioactive tracers; (2) increased commercial availability of highly-enriched stable-isotope-labelled compounds; (3) reduced cost and increased sensitivity of the instrumentation needed for their detection in biological samples; (4) increased recognition of the unique metabolic information that can be obtained by using stable-isotope tracers and selected-ion-monitoring (SIM) GC–mass spectrometry (MS). The purpose of the present review is to briefly discuss the use of stable-isotope tracer methods to study carbohydrate and fat metabolism at the whole-body level. Since space limitations preclude a comprehensive review of such a broad topic, the focus instead is on specific applications that illustrate some of the advantages and limitations of these methods.

Glucose kinetics

Stable-isotope tracers were first used to quantify glucose kinetics (i.e. rate of appearance (R_a), rate of disappearance (R_d), etc.) in human subjects in the late 1970s (Bier et al. 1977a,b; Kalhan et al. 1977). Since that time, they have been used to investigate glucose metabolism in a variety of physiological circumstances, e.g. pregnancy (Kalhan et al. 1979), burn injury (Burke et al. 1979), and exercise (Stanley et al. 1988; Coggan et al. 1990).

[6,6-D_2]glucose has generally been the tracer of choice in such studies, since there is almost no chance that both D will recycle back into the C-6 position of glucose following glycolysis and subsequent gluconeogenesis (about 80% of the label is lost during the formation of oxaloacetate from pyruvate, with further loss of label occurring as a result of the equilibration of oxaloacetate with malate and fumarate; Wajngot et al. 1989; Fig. 1). [6,6-D_2]glucose is therefore considered to be a non-recycling tracer and to give the best

Abbreviations: MIDA, mass isotopomer distribution analysis; MS, mass spectrometry; R_a, rate of appearance; R_d, rate of disappearance; SIM, selected-ion-monitoring.

Corresponding author: Dr Andrew R. Coggan, fax +1 410 605 7913, email coggan@grecc.umaryland.edu
estimate of ‘true’ endogenous glucose production (Bier et al. 1977b). However, [U-13 C]glucose or [1-13 C]glucose can also be used, provided that recycling of the 13 C label during gluconeogenesis is taken into account. With GC–MS analysis, this can be readily done by selectively monitoring or measuring specific ionized glucose molecules or molecular fragments to differentiate the infused tracer from labelled glucose derived from it as a result of in vivo metabolism. When quantifying [U-13 C]glucose enrichment, for example, one can monitor only ions with a mass : charge 6 atomic mass units greater than that of natural glucose (i.e. only ions at m+6), thus excluding from the measurement partially-labelled glucose molecules (i.e. at m+1 – m+5) formed as a result of glycolysis and subsequent gluconeogenesis (the probability of m+6 glucose being reformed in vivo is quite small; Tsering & Kalhan, 1983; Coggan et al. 1990). With [1-13 C]glucose, the data can be corrected for the effect of label recycling by measuring the increase in the m+1 abundance of an ion fragment that does not contain C-1. The [1-13 C]glucose enrichment can then be calculated by subtracting this non-specific increase, taking into account the probability of the label recycling into the various positions of glucose. Thus, when plasma glucose enrichment is measured by combusting the glucose to CO2 and then quantifying the 13 CO2 enrichment using isotope-ratio MS, [U-13 C]glucose and [1-13 C]glucose will function as recycling tracers and will tend to underestimate the total glucose Ra, just like [U-14 C]glucose and [1-14 C]glucose.

The earlier discussion illustrates one of the main advantages of using stable-isotope tracers and SIM-GC–MS, and that is the relative ease with which the position of a label within a molecule can be determined. In contrast, when using a radioactive tracer and liquid-scintillation counting (or when using combustion–isotope-ratio MS) it is usually difficult, if not impossible, to obtain such positional information. In addition, with stable-isotope tracers the variable of interest, i.e. tracer : tracee, is measured directly during the analysis, whereas with radioactive tracers tracer : tracee (i.e. the specific activity) is calculated from separate measurements of radioactivity and tracee concentration. This procedure may introduce additional variability into subsequent calculations. For these reasons, use of the appropriate stable-isotope tracer, in theory, may provide a more accurate and/or precise estimate of the true glucose Ra than use of the analogous radioactive tracer. In reality, however, values for glucose Ra obtained using comparable stable and radioactive tracers (e.g. [6,6-2 H]glucose v. [6-2 H]glucose) are equivalent (Haigh et al. 1982; Argoud et al. 1987), and thus in this regard the two methods can be
considered to be interchangeable. On the other hand, the high selectivity provided by SIM-GC–MS often makes possible the simultaneous use of multiple \(^{2}H\)- or \(^{13}C\)-substituted tracers (for example, see Shulman et al. 1985), something that usually cannot be done when using \(^{2}H\)- or \(^{13}C\)-labelled compounds. Alternatively and/or in addition, another tracer can be added as an internal standard during sample processing, such that the tracee concentration (needed, for example, to calculate \(R_a\) in non-steady-state) can be conveniently and precisely measured using SIM-GC–MS at the same time as the enrichment (for example, see Goromaru et al. 1994).

**Gluconeogenesis**

The estimation of gluconeogenesis has been another common application of stable-isotope tracers. Until recently, the approaches used have simply paralleled those previously developed for use with radioactive tracers. For example, Bier et al. (1977a) infused \([2,3-{^{13}}C]\)alanine into two subjects and measured the resultant increase in glucose enrichment to determine the fractional contribution of gluconeogenesis from alanine to the total glucose \(R_a\) (i.e. the precursor–product approach). Similarly, Wolfe et al. (1979) measured glucose \(R_a\) using both a non-recycling tracer (i.e. \([6,6-{^{2}}H\])glucose) and a recycling tracer (i.e. \([U-{^{13}}C]\)glucose, with enrichment measured using combustion–isotope-ratio MS), and used the difference between the two measurements as an index of gluconeogenesis (i.e. the C-recycling method). Other workers have used similar approaches to study glucose metabolism in type 2 diabetes (Zadwarzki et al. 1988), exercise (Stanley et al. 1988) etc. As when using radioactive tracers, however, it has long been recognized that such methods markedly underestimate the actual rate of gluconeogenesis. This underestimation is due to: (1) dilution of the \(^{13}C\) label in the intrahepatic pyruvate pool (the true precursor for the gluconeogenic pathway) by unlabelled pyruvate or lactate or alanine produced via gut or hepatic metabolism; (2) further dilution of the label in the oxaloacetate pool of liver mitochondria due to ‘crossover’ between the gluconeogenic and tricarboxylic pathways; (3) loss of the label as \(^{13}CO_2\) as a result of the phosphoenolpyruvate carboxykinase (EC 4.1.1.49) reaction; (4) failure to allow sufficient time to achieve steady-state labelling of the product (glucose) pool. In addition, neither approach accounts for the contribution of glyceral to glucose synthesis. While the latter is normally relatively minor, it can be important when the rate of lipolysis is greatly stimulated, for example, during prolonged fasting (Baba et al. 1995).

As a result of these limitations, we (Coggan et al. 1995) and other workers (Tappy et al. 1992; Haesler et al. 1994) have used the rate of incorporation of \(H^{13}CO_3^-\) into glucose as an estimate of the rate of gluconeogenesis. \(HCO_3^-\) is not a gluconeogenic precursor in the net sense, but gluconeogenically-derived glucose becomes labelled as a result of \(CO_2\) fixation by pyruvate carboxylase (EC 6.4.1.1) and the redistribution of the label within oxaloacetate via its equilibration with malate and fumarate (Fig. 2). The major advantage of this approach is that labelled \(HCO_3^-\) rapidly diffuses across membranes, and thus equilibrates better across the arterial, portal venous and intrahepatic precursor pools than, for example, labelled lactate. This process minimizes the underestimation that results from the inability to measure the true precursor enrichment. In addition, it is much easier to maintain a constant precursor (i.e. \(^{13}CO_2\)) enrichment under different conditions, since changes in \(CO_2\) production can generally be anticipated and the rate of \(HCO_3^-\) infusion varied proportionately. Nonetheless, the \(HCO_3^-\)-incorporation technique still does not account for

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**Fig. 2.** The principle underlying the use of \(^{13}C\)-labelled bicarbonate to trace the gluconeogenic pathway. PEPCK, phosphoenolpyruvate carboxykinase (EC 4.1.1.49); PEP, phosphoenolpyruvate; pyruvate carboxylase, EC 6.4.1.1.
dilution of the labelled oxaloacetate produced during gluconeogenesis by unlabelled oxaloacetate produced via the tricarboxylic acid cycle, and is not sensitive to gluconeogenesis from glycerol. The method will also underestimate gluconeogenesis if equilibration of the $^{13}$C label between C-1 and C-4 of oxaloacetate is incomplete.

The limitations of previous tracer methods for quantifying gluconeogenesis has led to the recent development of several unique stable-isotope approaches to the problem. Perhaps the simplest of these approaches, at least conceptually, is that first introduced by Tayek & Katz (1996) and subsequently modified and used by Tayek & Katz (1997) and Katz & Tayek (1998). These investigators infused $[U-^{13}$C]glucose and measured the recycling of the label into glucose isotopomers containing less than six (in actuality, one to three) $^{13}$C (in this regard, the method can be viewed as an extension of the classical C-recycling technique). Dilution of labelled pyruvate by non-glucose-C (e.g. from glycogen or amino acids) was estimated by measuring the $^{13}$C enrichment of plasma lactate, assuming that this was the same as pyruvate or lactate enrichment within the liver. Dilution or loss of label in the conversion of pyruvate to phosphoenolpyruvate was estimated by comparing the ‘output’ of $^{13}$C-labelled glucose with the ‘input’ of $^{13}$C in the form of labelled pyruvate or lactate. Gluconeogenesis was then calculated by multiplying glucose $R_{p}$ by the percentage of glucose-C recycled and by the latter two correction factors. If accurate, this relatively simple method would be quite useful for investigators interested in glucose metabolism. However, as discussed by Landau et al. (1998) and Landau (1999), the equations proposed by Tayek & Katz (1996, 1997) fail to distinguish adequately between simple isotope exchange and actual dilution of the label by unlabelled C fluxing to glucose. Furthermore, even if correct equations are used, the method does not account for gluconeogenesis from glycerol or amino acids that bypass pyruvate (e.g. glutamine), and the assumption that arterial lactate enrichment is similar to intrahepatic pyruvate enrichment may not hold under all circumstances. Consequently, this method, like those previously discussed, underestimates the actual rate of gluconeogenesis.

A more promising method for quantifying gluconeogenesis is to infuse a $^{13}$C-labelled gluconeogenic precursor and to use the observed mass isotopomer distribution in glucose to derive the true precursor enrichment based on probabilities, an approach that has been termed mass isotopomer distribution analysis (MIDA). Initially applied to the study of the synthesis of larger polymeric molecules such as fatty acids (Hellerstein et al. 1991) or cholesterol (Neese et al. 1993), MIDA can in theory also be used to quantify gluconeogenesis, since glucose can be viewed as a dimer formed from two triose subunits (Neese et al. 1995). The potential advantage of this approach is that it would circumvent the problems previously discussed that result in the underestimation of gluconeogenesis by other tracer techniques. However, when Landau et al. (1995a) infused $[U-^{13}$C]glycerol into normal subjects who had fasted for 60 h and calculated the proportion of glucose derived from gluconeogenesis using MIDA, they found that only about half the glucose was seemingly derived via this pathway, as opposed to the approximately 100 % that would be expected. This underestimation apparently resulted because the precursor (i.e. triose phosphate) pool of the liver is not homogeneous in space and/or time (a required assumption of MIDA; Brunengraber et al. 1997). Based on studies of perfused rat livers and live rats and monkeys, this inhomogeneity seems to result from: (1) the rapid uptake of glycerol by periportal cells and thus a large concentration gradient for glycerol across the liver; (2) the release of unlabelled glycerol by the liver, presumably the result of the activity of yet-to-be identified lipases (Previs et al. 1995). Thus, in contrast to earlier reports (Neese et al. 1995), $^{13}$C-labelled glycerol does not appear to be a suitable tracer for quantifying gluconeogenesis using MIDA. However, when Previs et al. (1995) used $^{13}$C-labelled lactate, apparently valid estimates for the proportion of glucose derived from gluconeogenesis were obtained in vivo (but not in situ), presumably because of rapid equilibration of lactate and pyruvate enrichment via erythrocyte metabolism before and during passage through the liver. Thus, when using the appropriate tracer MIDA may yet still prove to be a accurate way of quantifying gluconeogenesis in human subjects.

Finally, gluconeogenesis can also be quantified by measuring the incorporation of $^3$H from ingested or infused $^2$H$_2$O into glucose (Landau et al., 1995b). This method is described in detail in the accompanying paper by Landau (1999). Briefly, however, the principle is as follows: interconversion of malate and fumarate in liver mitochondria results in $^3$H being bound to the C-3 of malate, which subsequently becomes the C-2 of phosphoenolpyruvate and eventually the C-5 of glucose (Fig. 3). Gluconeogenesis from glycerol also results in labelling of the C-5 of glucose, via incorporation of the label into the C-2 of glyceraldehyde-3-phosphate during its equilibration with dihydroxyacetone phosphate (Fig. 4). The C-2 position of glucose produced via gluconeogenesis or glycogenolysis becomes labelled as a result of cycling between glucose, glyceraldehyde-3-phosphate, fructose-6-phosphate, and fructose-l,6-bisphosphate. Thus, the value for the enrichment in C-5: enrichment in C-2 of glucose following $^3$H$_2$O administration is equal to the proportion of glucose derived from gluconeogenesis at the time that molecule of glucose was formed. This method has been used previously in animal studies (using $^3$H$_2$O), but until recently the amount of $^3$H$_2$O or even $^2$H$_2$O required would have been more than can be safely used in human subjects. However, by chemically isolating the C-2 or C-5 of glucose and their associated H in the form of formaldehyde and then converting six molecules formaldehyde to one molecule hexamethylenetetraamine, Landau et al. (1995b) were able to amplify the enrichment 6-fold before measurement, and thus were able to quantify gluconeogenesis using a dose of $^2$H$_2$O that raised body water enrichment to only approximately 0.5 %. This simple but elegant method has considerable appeal, as it requires minimal assumptions, and measures gluconeogenesis from glycerol as well as from pyruvate-level precursors. The procedures for preparing the hexamethylenetetraamine derivative are technically quite involved, however, and only time will tell whether the method will see widespread application.
Glycogen utilization

Part of the rise in the use of stable-isotope tracers, at least in exercise studies, stems from the desire for a less-invasive alternative to the muscle-biopsy technique for determining glycogen utilization. That is, by quantifying glucose $R_d$ with a tracer and total carbohydrate oxidation via indirect calorimetry, it is possible to estimate whole-body glycogen use from the difference between the two measurements (for example, see Romjin et al. 1993; Phillips et al. 1996; Coyle et al. 1997). This calculation must underestimate the true rate of muscle glycogen utilization, since: (1) it assumes that all glucose $R_d$ is oxidized; (2) C originating in muscle glycogen that pass through the plasma glucose pool on their way to CO$_2$ are counted as coming from plasma glucose, not muscle glycogen; (3) it neglects lactate accumulation. However, during low-to-moderate intensity exercise (1) the percentage of glucose $R_d$ oxidized is quite high (Coggan et al. 1990, 1992; Jeukendrup et al. 1999), (2) the rate of gluconeogenesis is low relative to the overall rate of carbohydrate oxidation (Coggan et al. 1995; Jeukendrup et al. 1999), (3) lactate accumulation is minimal. Thus, it seems likely that under such conditions this indirect method provides a reasonable approximation of the rate of glycogen use. It should be noted, however, that this approach has not been validated against other techniques, e.g. muscle biopsy or magnetic resonance spectroscopy. Indeed, it is not necessarily even possible to do so, since it is difficult to equate glycogen use measured in one or more muscles using such techniques with the overall whole-body rate of glycogen utilization as calculated using the combined tracer–indirect calorimetry approach.

Fatty acid and glycerol kinetics

Stable-isotope tracers have also been used to study fatty acid or glycerol kinetics for almost 20 years (for example, see Galster et al. 1981; Bougneres et al. 1982), but only in the last decade has this method become more commonplace. Using, for example, $[^1-^{13}C]$palmitate to trace free fatty acid $R_a$ and $R_d$ has the obvious benefit of not exposing the subject to radiation, but otherwise the method is really not much different from using a radioactive fatty acid tracer, and the two approaches have been shown to yield comparable results (Wolfe et al. 1980; Guo et al. 1997). There is perhaps more of an advantage to using a stable-isotope tracer when measuring glycerol kinetics, since it can be difficult to completely separate $^3$H- or $^{14}$C-labelled glycerol from radioactive glucose (produced in vivo via gluconeogenesis) before liquid-scintillation counting. Provided that this problem is overcome, however, equivalent values for glycerol $R_a$ and $R_d$ have the obvious benefit of not exposing the subject to radiation, but otherwise the method is really not much different from using a radioactive fatty acid tracer, and the two approaches have been shown to yield comparable results (Wolfe et al. 1980; Guo et al. 1997).
Using a stable- (or radioactive-) isotope tracer to quantify free fatty acid or glycerol kinetics has the advantage of being somewhat less invasive than, for example, arterio–venous sampling or microdialysis, and of providing an integrated measure of whole-body lipid metabolism. However, when both fatty acid and glycerol tracers are used together, along with indirect calorimetry, potentially even more information can be obtained. That is, assuming that the R\text{a}\text{ of glycerol is a direct measure of the rate of lipolysis, the overall rate of fatty acid–triacylglycerol cycling can in theory be calculated from the difference between the rate of fatty acid oxidation and three times the glycerol R\text{a} (since three fatty acids are released for every glycerol). Furthermore, provided that all lipolysis is occurring in adipose tissue, the difference between free fatty acid R\text{a} and three times the glycerol R\text{a} would be equal to the rate of intracellular recycling, with the difference between this value and the total rate of cycling equalling the rate of ‘extracellular’ recycling (extracellular in the sense that the free fatty acids have traversed the extracellular space before their re-esterification). Alternatively, when total fat oxidation is greater than free fatty acid R\text{a} and R\text{d} would be equal to the rate of intracellular recycling, with the difference between this value and the total rate of cycling equaling the rate of ‘extracellular’ recycling (extracellular in the sense that the free fatty acids have traversed the extracellular space before their re-esterification). Alternatively, when total fat oxidation is greater than free fatty acid R\text{a} and R\text{d} is well established, and there is essentially no question about the soundness of this approach for determining the flux of free fatty acids through the plasma (see Miles \& Jensen, 1991). The assumption that 100 % of the free fatty acid R\text{d} is oxidized is more tenuous, since a number of studies have shown that even during exercise this percentage is only 50–85 (Martin \textit{et al.} 1993; Kanaley \textit{et al.} 1995; Coyle \textit{et al.} 1997; Sidossis \& Coggan, 1998). As a result, studies using the combined tracer–indirect calorimetry approach (for example, see Romijn \textit{et al.} 1993) have tended to overestimate the contribution of plasma-borne free fatty acids and underestimate the contribution of other fatty acid sources (i.e. intramuscular and circulating triacylglycerols) to the overall rate of fatty acid oxidation. In theory, this problem can be overcome by directly measuring plasma free fatty acid oxidation using a 13C- (or 14C-) labelled tracer (for example, see Coyle \textit{et al.} 1997), although this does require making additional measurements and entails other uncertainties (see Sidossis \textit{et al.} 1995;
van Hall, 1999). However, in light of other considerations, determining the exact percentage of the free fatty acid $R_d$ that is oxidized may not be all that important.

A greater limitation to the combined tracer–indirect calorimetry approach pertains to the use of glycerol $R_a$ as a direct quantitative measure of the rate of lipolysis, either specifically in adipose tissue (i.e. at rest; for example, see Wolfe & Peters, 1987; Klein et al. 1989; Vallerand et al. 1999), or in muscle and adipose tissue combined (i.e. during exercise; for example, see Romijn et al, 1993; Phillips et al. 1996). Interpretation of the data in this manner is based on the following assumptions: (1) all lipolysis is complete (i.e. each triacylglycerol molecule is completely hydrolysed to three fatty acids and one glycerol); (2) lipidic tissues lack the capacity to reutilize glycerol; (3) glycerol is not produced via any reactions other than triacylglycerol hydrolysis; (4) there is minimal lipolysis in hepatic or mesenteric tissues (the glycerol from which would be largely metabolized by the liver without entering the peripheral circulation). The last two requirements are probably met, or at least sufficiently so that no major error is introduced by assuming they are true (Landau et al. 1995a; Previs et al. 1995; see also Brunengraber et al. 1997). The first two assumptions, however, are somewhat more problematic. They have been justified on the basis that (1) only a very small percentage (0–1–0.5) of the esterified fatty acids in adipose tissue are present as mono- or diacylglycerols (Arner & Ostman, 1974), (2) the activity of glycerol kinase ($EC$ 2.7.1.30; necessary for glycerol utilization) is minimal or even absent in adipose tissue and muscle (see Lin, 1977). As discussed by Kurpad et al. (1994), however, what matters is not the amount of mono- or diacylglycerol relative to the amount of triacylglycerol, but the rate of mono- or diacylglycerol formation relative to the overall rate of fatty acid release. Theoretical calculations indicate that this factor can be quite significant when lipolysis is stimulated (Kurpad et al. 1994). Similarly, both older (Hagenfeldt & Wahren, 1968) and more recent (Elia et al. 1993; Coppack et al. 1999) $in vivo$ studies indicate that muscle at least has the capacity to reutilize glycerol, at least under certain circumstances. In light of such observations, to equate glycerol $R_a$ as being synonymous with whole-body lipolysis and to use it as a ‘gold standard’ on which to base further calculations may be questionable. At the same time, however, based on the changes in glycerol $R_a$ observed in response to a wide variety of factors (for example, see Wolfe & Peters, 1987; Klein et al. 1989; Romijn et al. 1993), it appears to at least provide a reasonable index of the overall lipolytic rate.

Even if glycerol $R_a$ were a direct measure of lipolysis, calculations of intracellular $v$. extracellular re-esterification or intramuscular lipolysis from such data may not be correct, because they assume that hydrolysis of circulating triacylglycerols is minimal. While the arterio–venous balance of triacylglycerols across, for example, adipose tissue is quite small relative to the arterial concentration, the release of glycerol via the action of lipoprotein lipase ($EC$ 3.1.1.34) appears to contribute significantly to whole-body glycerol $R_a$, especially when glucose and insulin are elevated (Coppack et al. 1999). By itself, however, this fact does not invalidate the use of glycerol $R_a$ as an index or even a direct measure of the overall rate of triacylglycerol lipolysis.

**Summary**

As discussed in the present review, stable-isotope tracers provide a powerful and sometimes unique tool for investigating carbohydrate and fat metabolism at the whole-body level. With continuing advances in availability, instrumentation and methods, it is likely that they will become increasingly important in the immediate future.

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**References**


Tracers in metabolic and nutrition research


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