Stable Isotopes in Human Nutrition
Laboratory Methods and Research Applications

Edited by

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The existence of stable, non-radioactive isotopes of the elements and the ability to measure these isotopes by mass spectrometry were first recognized in the early and mid-1920s (Aston, 1927). However, stable isotopes were not used for nutrition-related research until the 1930s, when Schoenheimer and Rittenberg (1935a,b) fed linseed oil partially hydrogenated with deuterium to mice and discovered that approximately one-third of the deuterium-labelled fatty acids were incorporated into the fat tissues of the mice. These ‘godfathers’ of stable isotope methodologies also laid the groundwork for the use of stable hydrogen isotopes to study fat and cholesterol metabolism (Rittenberg and Schoenheimer, 1937) as well as the use of stable nitrogen isotopes to study protein metabolism (Foster et al., 1938). Schoenheimer and Rittenberg were also the first investigators to use a tracer labelled with multiple isotopes ($^{2}$H,$^{15}$N-leucine) to investigate protein metabolism (Schoenheimer et al., 1939).

Another significant development in the use of stable isotopes in nutrition-related research was the discovery of the doubly labelled water ($^{2}$H$_{2}^{18}$O) method for the determination of daily energy expenditure by Lifson and McClintock (1966). The $^{2}$H$_{2}^{18}$O method quickly became the ‘gold standard’ for estimation of daily energy expenditure under free-living conditions. The most recent significant development in stable isotope methodologies is the $^{13}$CO$_{2}$ urea breath test (Klein et al., 1996, 1997). The $^{13}$CO$_{2}$ urea breath test represents the very first stable isotope method to receive approval from the Federal Drug Administration for the diagnosis of Helicobacter pylori infection and the first stable isotope method to become a commercialized medical diagnostic product.

For mineral isotopes, the initial human studies using mineral stable isotopes were reported for iron and calcium in the 1960s. McPherson (1965) reported on methods for using stable calcium isotopes in 1965. One of the key first practical studies was by Heaney and Skillman (1971), who reported a remarkable study using $^{48}$Ca administered intravenously to 15 women aged 15–28 years to measure calcium kinetics at different stages of pregnancy. These initial studies utilized neutron activation to determine the isotopic content of blood, urine and faecal samples.

However, neutron activation of isotope ratios is relatively cumbersome compared with mass spectrometric determinations. In 1972, Moore and Machlan (1972) reported a mass spectrometric technique for measuring calcium stable isotope enrichment from blood and urine samples using a specially constructed thermal ionization mass spectrometer.

A turning point in the use of these isotopes came with the possibility of performing analysis using more widely available commercial mass spectrometers. The use of a standard quadrupole thermal ionization mass spectrometer to measure calcium isotope ratios was reported by Yergey et al. (1980). These pioneering studies led to the much more widespread use of mineral isotopes in the late 1980s and early 1990s.

Although radioactive tracers have long been used extensively to provide quantitative information on rates of absorption, synthesis, degradation and turnover in biochemistry, physiology and nutrition research, stable isotopic tracers have gained tremendous popularity because of the ever-increasing restrictions and cost of disposal on the use of radioactive isotopic tracers. With the phenomenal advances in mass spectrometry, accurate and precise isotopic abundance measurements can now be made in permanent gases, fluids, products of combustion, individual organic molecular compounds, as well as in mineral elements. The various mass spectrometric techniques for stable isotope abundance measurements have been elegantly discussed by Hachey et al. (1987) and will not be repeated in this book.

The applications of stable isotopes in nutrition-related research are essentially limitless. The primary purpose of this book is to give readers a brief glimpse into the manufacturing processes of the various stable isotopic tracers as well as a basic understanding of the principles, study designs, sample requirements, sample preparation techniques, instrumentation requirements and methods of calculation for several stable isotope methods commonly used in nutrition-related research. The book is written with the intention of generating excitement among new users and serves as a basic reference guide to experienced users. Therefore, the book is appropriate for graduate-level curriculum in nutrition and for those at any level who wish to obtain an understanding of these methods.
References


Production of Stable Isotopes for Nutrition-related Research

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Isotope

An isotope is one of two or more species of atoms of a chemical element with the same atomic number and position in the periodic table and nearly identical chemical behaviour but with different atomic masses and physical properties.

Before the early 1900s it was generally assumed that the mass of a standard number of atoms of any given element was a basic characteristic of the element. It was also thought that all the atoms of an element were the same and, in particular, had the same mass. The first evidence that two substances with the same chemical properties did not have to be physically identical came from the study of the radioactivity of the heavy elements. Between 1906 and 1907 several investigators showed that ionium (a decay product of uranium) and radiothorium (a decay product of thorium), when mixed with thorium, could not be separated from it by any chemical means. The two substances had radioactive properties quite different from those of thorium and could be shown to have atomic masses differing by several units from that of thorium. The term isotope was introduced in 1913 by the English chemist Frederick Soddy to cover such situations. Not long after the acceptance of these ideas as applied to the heavy elements came indications that isotopes might exist in the main group of naturally occurring stable elements. In 1919 F.W. Aston of England showed conclusively that neon consisted chiefly of two atomic species. This success was followed by the discovery that chlorine had two isotopes. It soon became clear that most elements consisted of a mixture.
of isotopes, each with an atomic mass close to an integer on the atomic mass scale.

In most cases, only the stable isotopes of elements can be found in nature. The unstable, or radioactive, forms decay (break down) spontaneously into entirely different elements at characteristic rates because their ratio of neutrons to protons is either too low or too high for stability. Isotopes of all the elements heavier than bismuth are radioactive. Some of these, such as uranium, do occur naturally because their isotopes have long half-lives.

Production of Stable Isotopes

As one can imagine, the ability to extract and purify the small amount of the rare from the common isotope can be daunting. A few examples will be given here so that the readers can appreciate the complexity of the production processes.

Cryogenic separation of nitrogen

Dry air is composed of 78% nitrogen, 20% oxygen, 1% argon, and a remaining 1% that includes carbon dioxide and many trace gases. Air separation processes take raw air input and isolate one or more of these components to within 95–99.99+% purity. Usually, either pure nitrogen or oxygen is produced.

A useful method for isolating nitrogen has been cryogenic distillation. Additional information on cryogenic distillation can be found at: http://www.cheme.cmu.edu/course/06302/airsep2/intro/credits.htm

Cryogenic separation is a distillation process that occurs at temperatures close to −170°C. At this temperature, air starts to liquify. Before separation can occur, there are specific operation conditions that must be achieved. Distillation requires two phases, gas and liquid. Air must be very cold for this to happen. For this instance, at 1 atmosphere, nitrogen is a liquid at −196°C. A pressure of 8–10 times atmospheric pressure is required for the cryogenic separation. These conditions are achieved via compression and heat exchange; cold air exiting the column is used to cool air entering it. Nitrogen is more volatile than oxygen and comes off as the distillate product.

A cryogenic air separation plant is expensive and large; the distilla-
tion column is several storeys high and must be well insulated. Consequently, it only becomes economically feasible to separate air in this way when a large amount is needed.

**Production of deuterium**

The concentration of deuterium in hydrogen on earth is 0.015%. The commercial enrichment process starts with a cascade that exploits the shift of a chemical equilibrium with temperature. The reaction used in Canada (previously also used in the United States) is

$$^1\text{H}_2\text{HO} + ^1\text{H}_2\text{S} \rightarrow ^1\text{H}_2\text{O} + ^1\text{H}_2\text{HS}$$

There is a large temperature effect on the equilibrium constant for this reaction. This cascade enriches the D concentration in the water up to about 1%. After that, vacuum distillation cascades are used, followed by electrolysis to ‘polish’ the heavy water (very pure heavy water being needed for use in heavy-water moderated reactors, since even small amounts of hydrogen can absorb neutrons). It is also possible to separate deuterium by cryogenic distillation. The large isotope effect in electrolysis means that a plant that makes hydrogen from electrolysis of water could produce water enriched in deuterium as a by-product.

Alternatively, it is feasible to extract the $^2\text{H}_2\text{O}$ if the concentration is at least 135 ppm or 135 mg l$^{-1}$. The concentrations in Canada range from 140 to 155 mg l$^{-1}$ depending on rainfall temperature in the Great Lakes. The Canadian nuclear fission reactor system uses heavy water ($^2\text{H}_2\text{O}$) as its coolant/moderator and so Canada began production of $^2\text{H}_2\text{O}$ since no one else was extracting it (although the USA had stockpiles from the days when it operated the Savannah River plant). The process used is called Girdler-Sulfide process and was developed in the USA in the 1940s. Canadians revamped it and made significant improvements. There are at least 50 stages of enrichment used before the final product of 99.5% $^2\text{H}_2\text{O}$ is obtained via molecular distillation.

**Synthesis of labelled compounds**

Commercially available labelled products are either ‘specifically’ labelled or ‘U’ ‘uniformly/universally’ labelled. For example, d-glucose (1-$^{13}\text{C}$,
99%) where only the carbon atoms at the 1 position of the glucose molecule are labelled with $^{13}\text{C}$ and 99 out of every 100 carboxyl atoms of glucose are replaced with $^{13}\text{C}$.

**Uniformly labelled compounds**

In general, uniform labelling is derived from a biosynthetic growth. The organism, whether bacteria, algae or cell culture, is grown in an environment where the naturally occurring isotope of interest is replaced with stable isotopes or enriched isotopes (i.e. $^{13}\text{C}$, $^{15}\text{N}$, $^{2}\text{H}$). The bacteria will grow exponentially when put into a glucose/ammonium salts medium and aerated at 37$^\circ\text{C}$. Class I enzymes will degrade the carbon substrate, glucose, to other substances including CO$_2$ whose chemical energy can be used by the organism. The growth curve of an organism can be plotted as growth vs. time. If the nutrients are limited, growth eventually stops when the substrates are consumed. If the limiting nutrients are isotopically labelled then as the cells enter the stationary phase the cellular components should have incorporated the isotope into cellular components. It would be possible to keep a culture in a steady state of exponential growth by diluting it constantly and providing fresh medium.

Labelled products can then be removed from these complex mixes with a variety of biochemical strategies including centrifugation, high performance liquid chromatography and other techniques. These separations yield uniformly labelled carbohydrates, fatty acids, amino acids and other cellular components.

**Specific labelled compounds**

Specific labelling of a molecule is typically accomplished by traditional organic chemistry methods. The advantage of this ‘pot’ chemistry is the ability to scale the synthesis to control synthetic strategy and yields efficiency. Since many of these methods are proprietary, they will not be presented here.

**Production of isotopic metals**

Prior to World War II most of the separation methods focused on large-scale production for the war effort. A notable example was the heavy water plant in Norway, which focused on the separation of deuterium.
With respect to isotopic metals the development of a device named with the acronym ‘cal-u-tron’ was envisioned at the University of California by adapting the configuration of a cyclotron. This device specialized in the separation of elements from magnesium ($M_r 24.305$) to uranium ($M_r 236.2$). As part of the war effort, the predecessor to the Department of Energy (DOE), the then Atomic Energy Commission, established a full-scale production facility to be constructed in the rural farming community of Oak Ridge, Tennessee. At the height of construction over 100,000 people were based in this community, working towards the common goal of the separation of uranium. From a post war effort the calutrons were utilized as a research tool to provide science and emerging medical industries with a much-needed source of stable isotopes. During this same period, and for the same purposes, a similar facility was constructed in the town of Lesnoy, Russia. Using the same ‘calutron’ principle isotope separation occurred in this facility using the massive SU-20 and experimental E7 calutrons.

The calutron separation method essentially ‘rips’ apart the element magnetically and centrifugally into its isotopic constituents while in an excited state. The element is ionized and injected into a magnetic field where it is thereby deflected, or bent according to its mass. Heavier isotopic species are ‘bent’ the least, and the lightest are subject to a greater field effect and thus have a smaller arc. The trajectory of the masses can be accurately calculated to ascertain the best angle of deflection and thus collection. In the case of calcium, six different ‘streams’ or ‘beams’ are achieved, one beam for each isotope. The beams are then further separated by a cooled metal pocket, which solidifies the metal on contact and hence collects the isotopes in metal form. The collectors or ‘pockets’ are then extracted from the calutron, at which time the isotope target is removed and chemically purified in the lab. While considered a scientific dinosaur due to its sheer mass, electrical consumption, manpower requirements and low throughput, the calutron has an advantage over other more current production methods in that all the isotopes of an element can be separated simultaneously.

Today there are only three calutron facilities in existence, and only two of these are functioning. The calutron facility located in Oak Ridge, Tennessee, has been closed since 1998 and plans for decommissioning are underway. Prior to the shut down the DOE produced significant quantities that will supply the needs of research well into the future.

The two remaining calutrons are located in Russia, one in Lesnoy and one at the Kurchatov Institute outside Moscow. Russian calutron technology is quite advanced and high enrichments of most elements have been achieved.

Once the isotopes have been separated and chemically purified, they are assayed on site using standard ICP-MS techniques. The materials are then sent directly to distributors in sealed ampoules under strict shipping
guidelines and procedures. The materials are then checked and re-
processed at the distributor whereby further reductions or alterations are
completed. Calcium, for example, is received in the carbonate form and
is reduced to the oxide or converted into the salt CaCl$_2$ routinely. The dis-
tributor should determine the conversion efficiency or homogeneity with
repeated assays.
The Measurement of Protein Kinetics with Stable Isotope Tracers

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Historical Perspective of Tracer Techniques

Because protein underlies all structural and dynamic functions in living organisms research into protein metabolism has contributed immensely to our present understanding of how metabolic and physiological homeostasis is maintained in good health and altered by diseases. Protein turnover (or kinetics), i.e. the rate at which protein is synthesized and broken down, is a fundamental biological process, and at the whole body level it is defined as the sum of turnovers of all the individual proteins of the body.

In terms of scientific history, the measurement of protein kinetics in humans can be considered a relatively young field, which started with the pioneering work of Sprinson and Rittenberg (1949). Using a single dose of $[^{15}N]$glycine as tracer, they developed a method of measuring the rate of protein synthesis based on a two-pool model, a pool of body protein and a labile metabolic nitrogen pool comprising a mixture of dietary amino-nitrogen and amino-nitrogen released from the breakdown of proteins. The model assumed that the metabolic nitrogen pool is homogeneous, that the amino acids of the metabolic pool, including those of dietary origin, are either used to synthesize proteins or oxidized and the nitrogen excreted in the urine, that distribution of the tracer throughout the pool is completed in 3–6 h and that the tracer leaves the pool according to first-order kinetics. Later experiments, however, questioned the validity of the last two assumptions (Tschudy et al., 1959). These shortcomings were addressed by a technique devised by Picou and Taylor-Roberts (1969) in which the tracer was administered by a constant
infusion. This method was the forerunner of the well-established end-product method still widely used today.

The basis of most methods used to estimate protein turnover is to measure the whole body flux of an amino acid or of amino-nitrogen. Flux is the rate of flow of amino acids from protein breakdown and the diet through the body’s metabolic amino acid pool into protein and other metabolic pathways such as oxidation. Once flux is determined, because dietary intake and oxidation can be measured, protein breakdown and synthesis can be estimated.

**Measurement of Whole-body Protein Kinetics**

Most measurements of protein kinetics are at the level of the whole body. It is the sum of the turnovers of all individual proteins that are being broken down and renewed at different rates. Although it provides useful information about overall protein homeostasis and responses to disease and intervention, it is limited in the information available for specific organs and tissue beds, which are invariably different and respond differently to nutrition and different pathologies. For example, in severely stressed states, there is a decrease in muscle protein synthesis rate, but in the liver there is an increase in the rate of synthesis of secretory proteins (Jahoor et al., 1999a). Despite its limitation, this method enables identification of the role of changes in synthesis and breakdown rates in mediating the protein metabolic response to different physiological and pathological conditions, for example, whether the loss of protein in trauma is due to a stimulated breakdown rate relative to synthesis or to a suppression of synthesis relative to breakdown. It also gives information about how protein and amino acid homeostasis are regulated by nutrients, cytokines, hormones and pharmacological compounds. Hence, it provides useful information for direction of clinical intervention. The two most commonly used methods to measure whole-body protein kinetics are the precursor and end-product methods. Both methods utilize the same simple two-pool model, first proposed by Sprinson and Rittenberg (1949) and later modified by Picou and Taylor-Roberts (1969), and are based on a set of common assumptions.

**The two-pool model**

The model of protein metabolism most commonly used to estimate whole-body protein synthesis and breakdown rates (Fig. 3.1) has essen-
tially the same characteristics as the two-pool model described by Picou and Taylor-Roberts (1969). The model groups all the metabolically available free amino acids of the body into a single metabolic pool of amino acids (or amino-nitrogen) and all body proteins, regardless of their individual rates of turnover, into a single protein pool. Amino acids from food or total parenteral nutrition (TPN), ($I$) and from the breakdown of body proteins ($B$) enter the metabolic pool and are used either to synthesize proteins ($S$) or oxidized ($O$) to the excretory products CO$_2$, ammonia and urea. Other routes of disposal, e.g. to nucleotides, are considered negligible. The rules of the model are that: (i) the pools are in steady state during the course of a study, i.e. constant in size; (ii) material exchanged between the pools or introduced into them mixes completely and instantaneously with that already present; and (iii) a constant fraction of each pool is exchanged in unit time (exponential or first-order kinetics). Based on rule (i), at steady state amino acids (or amino-nitrogen) are entering ($I + B$) and leaving ($S + O$) the metabolic amino acid pool at the same rate and this in turn is equal to the flux ($Q$) or total turnover rate of the metabolic amino acid (or amino-nitrogen) pool, so that:

$$Q = I + B = S + O. \quad (1)$$

By estimating flux and oxidation with a stable isotope tracer, $B$ and $S$ can be easily calculated from:

$$B = Q - I \text{ and } S = Q - O \quad (2)$$
Estimating flux with isotopic tracers (the precursor method)

Based on the fact that a nutritionally essential amino acid cannot be synthesized de novo, its flux consists of only two sources, nutritional intake and protein breakdown. Hence, in the precursor method of measuring whole-body protein kinetics, the flux and oxidation of an essential amino acid are measured and the rate of the amino acid entering the metabolic pool from protein breakdown \((Q - I)\) and exiting the pool to synthesize protein \((Q - O)\) are calculated. These rates can be used directly as indices of protein breakdown and synthesis rates or converted to units of protein based on the mixed body protein content of the essential amino acid used as tracer. Using similar reasoning, that once an essential amino acid is oxidized it is no longer available for protein synthesis, the net balance of an essential amino acid can be calculated as the difference between nutritional intake and oxidation \((I - O)\) and used as an index of whole-body protein balance.

This approach was first applied in humans by Waterlow (1967), who infused the radioactive tracer \([\text{U-14C}]\)lysine for 30 h. At present a stable-isotope-labelled essential amino acid (\([\text{13C}]\)leucine is most widely used) is usually given by bolus injection or constant infusion and its enrichment in plasma or urine is measured. A stochastic method of analysis is employed to determine flux (Waterlow et al., 1978). This approach ignores all the countless pools and components of whole-body protein metabolism, it is not concerned with transfers between individual pools, and it focuses only on the overall end results. It is based on the following assumptions:

1. The metabolism of the tracer amino acid reflects that of the body’s pool (of endogenous and exogenous origin).
2. Protein synthesis occurs from a homogeneous precursor pool (the metabolic amino acid pool). Flux through this pool can be measured by sampling plasma or urine.
3. Recycling of isotope from labelled protein is negligible during the course of an experiment.
4. Synthesis rate can be calculated as Flux – Oxidation as other metabolic pathways of disposable are negligible.

Flux can be estimated either by administering the tracer by constant infusion or as a single dose. In the single-dose method, the dose \((d)\) of labelled amino acid is injected intravenously and blood sampled over a sufficient period of time to establish an enrichment–time decay curve. Based on the assumptions above, the net amount of tracer exiting the metabolic amino acid pool at any moment is \(Q\epsilon\), where \(Q\) is the rate at which the tracer amino acid leaves the pool for protein synthesis and oxidation, i.e. its flux, and \(\epsilon\) is its plasma enrich-
ment. After a sufficiently long time, all of $d$ will be cleared from the pool, so that:

$$d = Q \int \varepsilon_i \, dt,$$

from which flux ($Q$) = dose / $\int \varepsilon_i \, dt$

where $\int \varepsilon_i \, dt$ is the area under the enrichment–time curve.

In the precursor method the most widely used approach to determine the flux of an essential amino acid employs a constant infusion of the tracer. When the labelled amino acid is given by constant infusion, its isotopic enrichment in the metabolic amino acid pool will reach a constant value (plateau) with time. At that time the amount of tracer, $d$, entering the pool is equal to the amount leaving ($\text{flux} \times \varepsilon$). That is $d = Q \times \varepsilon$ at plateau, from which flux ($Q$) = $d / \varepsilon_{\text{plateau}}$, where $\varepsilon_{\text{plateau}}$ is the isotopic enrichment of the amino acid in plasma (or urine) at isotopic steady state. To shorten the time taken to reach an isotopic steady state in plasma, a priming dose of the tracer is usually given as a bolus injection prior to the constant infusion. For most labelled essential amino acids used as tracers, the priming dose given is equivalent to ~1 h of the constant infusion.

**Estimating oxidation**

Protein oxidation or catabolism is usually estimated from the rate of oxidation of the tracer. This can be determined very easily with the constant infusion approach by measuring the rate of excretion of $^{13}$CO$_2$ in breath, if the tracer is a $^{13}$C-labelled amino acid, or the rate of urinary excretion of $^{15}$N, if the tracer is an $^{15}$N-labelled amino acid. Assuming a physiological steady state, i.e. the size of the protein and metabolic amino acid pools are constant, then according to the rules of the two-pool model, the amount of unlabelled amino acids entering the metabolic pool is equal to the amount leaving the pool, and, hence, the percentage entering the pool that is oxidized will also be constant. Likewise during the constant infusion of a tracer, when the isotopic enrichment in the metabolic amino acid pool reaches a constant value (isotopic steady state), the amount of tracer entering and leaving the pool will be equal and the fraction of tracer entering the pool that is oxidized should be constant and the same value as that of the unlabelled amino acid being traced. That is:

$$\text{Fraction of tracer dose oxidized} = \frac{\text{production rate of excretory label}}{\text{tracer dose rate (d)}}$$

The absolute rate of oxidation of the amino acid being traced can be obtained by multiplying its rate of entry into the pool (i.e. its
flux) by the fraction oxidized:

\[
\text{Rate of oxidation (O)} = \frac{d}{\epsilon_{\text{plateau}}} \times \frac{\text{(production rate of excretory label/(d)),}}}{\epsilon_{\text{plateau}}}
\]

i.e. \( O = \) production rate of excretory label \( \epsilon_{\text{plateau}} \).

In the case of a \(^{13}\text{C}\)-labelled amino acid, the rate of production of \(^{13}\text{C}\)O\(_2\) is usually calculated from:

\[
\text{\(^{13}\text{CO}_2\) production rate} = \left(\frac{V_{\text{CO}_2}}{f}\right) \times \epsilon_{\text{CO}_2}
\]

where \( V_{\text{CO}_2} \) is the rate of excretion of carbon dioxide (which can be measured by indirect calorimetry), \( f \) corrects for the fraction of bicarbonate produced in the body that is not excreted during the course of the experiment (\( f = \sim 0.8 \) under most conditions), and \( \epsilon_{\text{CO}_2} \) is the increase in isotopic enrichment over baseline of CO\(_2\) in breath at isotopic steady state. In the case of an \(^{15}\text{N}\)-labelled amino acid, the production rate of excretory \(^{15}\text{N}\) can be calculated as the product of the rate of excretion of nitrogen in urine and the increase in isotopic enrichment over baseline of urinary nitrogen.

The \(^{15}\text{N}\) end-product method

In the end-product method, an \(^{15}\text{N}\)-labelled amino acid, usually \(^{[15}\text{N}\)glycine, is administered and the flux of nitrogen through the amino-nitrogen pool is determined, as described by Picou and Taylor-Roberts (1969). Either \(^{[15}\text{N}\)glycine or uniformly labelled \(^{[15}\text{N}\)protein is given intravenously by constant infusion or by intragastric constant infusion with collection of timed urine samples. Isotopic measurements are made on urinary urea or ammonia (or both), which is assumed to be representative of the isotopic enrichment of the metabolic amino-nitrogen pool. Protein oxidation is derived from total nitrogen excretion in the urine. The method also makes the following assumptions:

1. An excreted nitrogenous end-product, e.g. urea, is derived from the same amino-nitrogen pool from which protein is synthesized.
2. At steady state, the precursor amino-nitrogen and the newly formed end-product (ammonia or urea) will have the same enrichment. Then the proportion of tracer excreted in urea or ammonia is the same as the proportion of amino-nitrogen disposed of via urea or ammonia.

Based on assumption (2),

\[
e_{X}/d = E_{X}/Q, \text{ from which } Q = dE_{X}/e_{X}
\]

(3)
where $Q$ is nitrogen flux, $E_X$ is the urinary excretion of the end-product, either ammonia or urea, $d$ is the dose of isotopic nitrogen in the glycine tracer and $e_X$ is the rate of excretion of $[^{15}\text{N}]$ammonia or -urea in urine. In order to calculate nitrogen utilized for protein synthesis ($S$), total urinary nitrogen excretion ($\text{g N kg}^{-1} \text{day}^{-1}$), a measure of protein oxidation ($O$), is also determined by measuring urinary nitrogen concentration. From Equation (2) above, $S = Q - O$ and $B = Q - I$. All values are then multiplied by 6.25 to convert g of nitrogen to g of protein.

In the original method described by Picou and Taylor-Roberts (1969), either an unprimed constant infusion of $[^{15}\text{N}]$glycine (intravenous) or an $[^{15}\text{N}]$egg protein (intragastric) was used to measure nitrogen flux. A major disadvantage of this approach is the long time taken to reach an isotopic steady state in urinary urea, increasing the possibility of isotope recycling back into the metabolic pool from the protein pool and also making the method unsuitable for clinical application. These problems have now been overcome by administering a priming dose of the tracer before the constant or intermittent administration of the isotope to shorten the time taken to reach isotopic steady state (de Benoist et al., 1985; Jackson et al., 1987). With this approach, Jackson et al. (1987) demonstrated that with a correct priming dose, a plateau enrichment can be achieved in urinary ammonia after 1 h and in urinary urea after 6 h.

**Single-dose method**

Because the time required to perform the unprimed constant-infusion $[^{15}\text{N}]$glycine method was impractical in most clinical situations, it was largely replaced by a single-dose approach, which is completed in 9 h. In this method, a single dose of $[^{15}\text{N}]$glycine is administered orally or intravenously and urine is collected over a 9 h period (Grove and Jackson, 1995). A blood sample is taken before the isotope is given and at 9 h to determine changes in urea concentration and isotopic enrichment, a procedure necessary to calculate changes in the size of the body urea pool and also the amount of $[^{15}\text{N}]$urea retained in the body’s urea pool. Nitrogen flux can be calculated either from the urinary ammonia or urea isotopic enrichment data. This method is based on the principle that the cumulative excretion of isotope in an end-product, $X$, will tend towards a plateau when all of the isotope that will be excreted has been cleared from the metabolic and excretory pools. Then the proportion of the tracer dose ($d$) oxidized and excreted is equal to the proportion of flux ($Q$) that is oxidized and excreted. Hence, flux can be calculated using Equation (3) above. However, the units are all expressed per 9 h, i.e. $Q$ is nitrogen flux.
(g) $9h^{-1}$, $E_X$ is the urinary excretion of the end-product, either ammonia or urea (g N $9h^{-1}$), $d$ is the dose of isotopic nitrogen in the glycine tracer (g of $^{15}$N) and $e_X$ is the amount of $^{15}$N excreted in the urine as ammonia in $9$ h (g of $^{15}$NH$_3$ $9h^{-1}$) or in the case of urea, the sum of the amount excreted in $9$ h plus the amount retained in the body urea pool at the end of $9$ h (g of $[^{15}$N]urea $9h^{-1}$). The rate of ammonia excretion is usually taken to be the same as urinary excretion during the $9$ h period. Urea excretion rate, however, is the amount excreted in urine after adjusting for any change in the body pool. That is,

$$\text{Urea-nitrogen excreted} = \text{urinary urea-nitrogen} - \left[ (\text{plasma urea-nitrogen conc. at time 0 h} - \text{plasma urea-nitrogen conc. at time 9 h}) \times \text{body water} \right]/0.92,$$

where the factor 0.92 corrects for the fact that the water content of plasma is 92%. In order to calculate nitrogen utilized for protein synthesis ($S$), total urinary nitrogen excretion (g N $9h^{-1}$), a measure of protein oxidation ($O$), is also determined by measuring total urinary nitrogen concentration. From Equation (2), $S = Q - O$ and $B = Q - I$. All values are then multiplied by 6.25 to convert g of nitrogen to g of protein.

Grove and Jackson (1995) compared the ‘prime/intermittent-dose end-product’ approach over 18 h with the ‘single-dose end-product’ approach, with urine collected for periods of 9–48 h. Nitrogen flux was derived using oral $[^{15}$N]glycine and measurement of $^{15}$N enrichment in urinary NH$_3$ or urea. They found that the single-dose approach, with urine collection over 9 h, gave results that were consistently higher than the prime/intermittent-dose approach. The extent of the difference was influenced by the duration of time over which the cumulative excretion of isotope in urine was determined, and with NH$_3$ as the end-product, the most consistent estimates of nitrogen flux could be obtained with a urine collection of at least 12 h and no greater than 24 h. With urea as the end-product, correction for the label retained in the body pool at 9 h gave similar results for nitrogen flux to those derived from the total excretion of $^{15}$N in urea over 24 h. They concluded that measurements of protein turnover similar to those obtained with the prime/intermittent-dose approach can be obtained with the single-dose approach in the fed state either from the excretion of label in urinary NH$_3$ over a period of 12 h or with the excretion of $^{15}$N in urinary urea over a period of 24 h.
A Practical Example: the [1-13C]Leucine Method

Experimental protocol

The most widely used method of measuring whole-body protein kinetics employs a 3 or 4 h constant intravenous infusion of [1-13C]-leucine at a rate of ~ 5 µmol kg⁻¹ h⁻¹ with a priming dose equivalent to 60 min of the constant infusion given as a bolus injection at the beginning to prime the leucine pool. The bicarbonate pool is also primed with 3 µmol kg⁻¹ NaH¹³CO₂ to shorten the time taken to reach an isotopic steady state in expired CO₂. Five blood and expired air samples are collected before, and at 0.25 h intervals during the last hour of isotope infusion. CO₂ production is measured either by dilution of NaH¹³CO₂ or estimated from $V_{CO₂}$ measured with a metabolic cart. Since intracellular leucine is the metabolic pool sampled for both protein synthesis and oxidation, the desired isotopic enrichment for estimation of flux is the intracellular leucine enrichment. An advantage of this method is that when labelled leucine is used as a tracer, the enrichment of plasma alpha keto isocaproic acid ($\alpha$-KICA) can be used to estimate the leucine precursor pool enrichment because plasma $\alpha$-KICA is derived intracellularly from the transamination of leucine (Fig. 3.2), and hence its enrichment will be equal to that of intracellular leucine (Horber et al., 1989). Therefore, leucine flux ($Q$) = $d/\varepsilon_{\alpha$-KICA}$, where $d$ is the rate of infusion of labelled leucine and $\varepsilon_{\alpha$-KICA}$ is the steady state isotopic enrichment of plasma $\alpha$-KICA. In the post-absorptive state, since dietary intake = 0, leucine derived from protein breakdown is obtained as $B = Q - d$, and in the fed state, $B = Q - (I + d)$, where $I$ is dietary intake.

A common misconception is that dietary intake should be first corrected for splanchnic uptake because 'some of the dietary leucine will be
taken up and utilized by the splanchnic tissues prior to its labelling by the intravenous tracer’ (El-Khoury, 1999). This is an erroneous assumption because the intravenous tracer is presented to all organs and tissue beds perfused by arterial blood. Hence, the tracer will be diluted by unlabelled leucine entering the metabolic free pools of all organs of the body including those comprising the splanchnic bed. This will also include all dietary leucine flowing into gut mucosal cells. On the other hand, if a tracer is being administered enterally to measure flux, it is important to correct for the fraction of tracer taken up and utilized by the splanchnic bed during first pass because that fraction of tracer will not be available to be presented to and diluted by unlabelled leucine in non-splanchnic organs and tissues.

Leucine oxidation is determined by dividing the rate of production (Ra) of $^{13}$CO$_2$ by the enrichment of the precursor pool ($\varepsilon_{\alpha-KICA}$).

$$\text{Total leucine oxidation (O)} = \frac{Ra_{CO2} \times \varepsilon_{CO2}}{\varepsilon_{\alpha-KICA}}$$

where $Ra_{CO2}$ is the rate of production of carbon dioxide and $\varepsilon_{CO2}$ is the steady state carbon dioxide isotopic enrichment in breath. $Ra_{CO2}$ is calculated as $V_{CO2}/f$ where $f$ = ~0.8, the correction factor to account for the fraction of $^{13}$CO$_2$ derived from leucine that is retained in the body’s bicarbonate pool during the course of the infusion. Leucine used for protein synthesis can now be calculated as $Q - O$ and protein balance, an index of net synthesis or net catabolism, as $(I + d) - O$.

Units of $Q$, $B$ and $S$ are $\mu$mol kg$^{-1}$ body weight/time or $\mu$mol kg$^{-1}$ lean body mass/time. They can be converted to units of protein by dividing by the concentration of leucine (590 $\mu$mol g$^{-1}$ protein) in mixed body protein.

**Measurement of protein kinetics of individual organs and tissues**

*Based on rate of incorporation of tracer*

In recent years the method of choice for measurement of protein synthesis rate in individual organs and tissues has been the constant infusion of a labelled amino acid. This method is also based on the two-pool precursor-product model. The purpose of the constant infusion is to produce a constant isotopic enrichment in the precursor amino acid pool, i.e. when the isotopic enrichment of the tracer amino acid in the precursor pool is constant, the rate of incorporation of label into the protein product is a direct function of the fractional rate of synthesis, $K_s$, of the protein (Waterlow, 1967). $K_s$ can be determined from the relationship:
\[ \delta I.E_{\text{protein}} / \delta \text{time} = K_s \times I.E_{\text{plateau}} \]

where \( \delta I.E_{\text{protein}} \) is the increase in isotopic enrichment of the protein over a certain time interval, \( \delta \text{time} \), \( K_s \) is the fractional rate of synthesis of the protein and \( I.E_{\text{plateau}} \) is the plateau isotopic enrichment of the tracer in the precursor pool from which the protein is synthesized. The fractional rate of synthesis is then obtained as:

\[ K_s = (\delta I.E_{\text{protein}} / \delta \text{time}) / I.E_{\text{plateau}} \] (4)

The full derivation of this equation can be found in Chapter 10 of Waterlow et al. (1978).

Hence, the minimum requirement for the calculation of the rate of protein synthesis with the precursor-product model is the measurement of the isotopic enrichment at two time points during the quasi-linear portion of the exponential rise in protein-bound amino acid labelling after the precursor pool enrichment, i.e. the isotopic enrichment of the tracer at the site of protein synthesis, has reached a plateau (Waterlow et al., 1978). This approach was used for the first time in humans by Halliday and McKeran (1975) to measure simultaneously the rates of synthesis of a hepatic-derived plasma protein, albumin, and muscle sarcoplasmic and myofibrillar proteins. They used an unprimed constant infusion of \([^{15}\text{N}]\)lysine for 21–30 h with timed intermittent blood sampling and two needle biopsies of the vastus lateralis muscle. Plasma lysine enrichment was used as a proxy of the true precursor pool isotopic enrichment in liver and muscle tissues.

A major disadvantage of this approach is that the very long time taken to reach an isotopic steady state in the precursor pool may lead to significant recycling of tracer back into the precursor pool. It may also be impossible to maintain a physiological steady state over such a long time. This problem has now been solved to some extent by administering a priming dose prior to the constant infusion to shorten the time taken to reach an isotopic steady state in the precursor pool. In addition, when the widely used primed-constant-infusion labelled leucine method is employed, the enrichment of plasma \( \alpha \)-KICA has been used by some investigators to estimate the precursor pool enrichment, on the assumption that plasma \( \alpha \)-KICA is derived intracellularly where it is in isotopic equilibrium with leucine (Horber et al., 1989). The advantage of this method is that plasma \( \alpha \)-KICA reaches plateau enrichment in a relatively short time after the start of a primed-constant infusion of labelled leucine (Horber et al., 1989). Another disadvantage of the constant-infusion tracer approach is the need to take multiple tissue samples at different times during the constant infusion of the tracer. In a study of lactase phlorizin hydrolase in piglets, we solved this problem by using staggered infusions of multiple isotopomers of the same tracer (labelled phenylalanine) for 6
h followed by a single biopsy of gut mucosa (Dudley et al., 1998). This approach enabled us to establish enrichment–time curves for both the precursor pool and the tissue protein from a single tissue sample.

Despite several improvements over the years, a major drawback of the constant-infusion approach remains the inability to measure the enrichment of the true precursor pool, i.e. the amino acyl-tRNA pool, because of the large tissue sample required and the tedious and elaborate procedure involved in its isolation and purification (Garlick et al., 1989). To circumvent this problem, in some studies (Bennet et al., 1989) the isotopic enrichment of the labelled amino acid in plasma (extracellular) and in the acid-soluble tissue extract (intracellular) have both been used as approximations of the true precursor pool, since animal studies have generally shown that the amino acyl-tRNA enrichment falls somewhere between the plasma and intracellular enrichments (Airhart et al., 1974; Khairallah and Mortimore, 1976; Schneible et al., 1981). Garlick et al. (1989) has rightfully argued, however, that the isotopic enrichment of the labelled amino acid in plasma or in the acid-soluble tissue extract still represents only an approximation of the true precursor pool enrichment. To overcome this problem of measuring the true precursor enrichment accurately in human studies, they adopted a flooding-dose technique, which is considerably shorter than the constant-infusion method (1.5 h versus 4–8 h) and permits an indirect estimate of the precursor pool enrichment by making measurements of the isotopic enrichment of the tracer amino acid in plasma (Garlick et al., 1989). This is possible because the isotope is given together with a large amount of the unlabelled amino acid, which causes rapid isotopic equilibration of the free extracellular and intracellular amino acid pools. Since the latter should also include the precursor pool from which amino acids are used to charge tRNA for protein synthesis, from a theoretical standpoint one can accurately estimate precursor pool enrichment by making measurements on plasma. The tracer is usually given intravenously together with a large bolus of the unlabelled amino acid followed by timed blood samples over a 1.5 h time period to define the enrichment–time curve and two tissue biopsies taken at the beginning and end of the sampling period. $K_s$ is calculated by dividing the increase in isotopic enrichment of the protein by the area under the curve for the precursor, i.e. the plasma enrichment–time curve from 0 to 90 min.

This approach was used for the first time in humans to measure the rate of synthesis of mixed muscle proteins (Garlick et al., 1989). In three different experimental protocols, a bolus intravenous injection of 0.05 g [1-13C]leucine kg$^{-1}$ was administered to healthy volunteers followed by timed blood and muscle tissue samples taken over intervals of 1.5, 2.0 or 3.0 h. The authors reported almost identical isotopic enrichments for leucine and $\alpha$-KICA in plasma and in the free pool of the muscle tissue just 20 min after injection of the bolus, suggesting that isotopic equilibra-
tion of the free extracellular and intracellular amino acid pools was achieved very quickly. They concluded that muscle protein synthesis can be measured conveniently in just 1.5 h with this approach. More recently they have confirmed that, after administration of a flooding dose of labelled phenylalanine in dogs, the isotopic enrichments of plasma-free phenylalanine and muscle phenylalanyl-tRNA were equalized between 20 and 45 min, although the enrichment of phenylalanyl-tRNA was lower at early time points (Caso et al., 2002). Based on this observation, they concluded that with a bolus injection of labelled phenylalanine, the enrichment of aminoacyl-tRNA, the true precursor pool for protein synthesis, can be assessed from more readily sampled plasma phenylalanine.

Because protein synthesis can be measured in a relatively short period of time with the flooding-dose method, it is convenient for measurements in hospitalized patients and to assess the acute effects of nutrients, cytokines, hormones and pharmacological compounds on the rate of synthesis of individual proteins. On the other hand, because the values for protein synthesis rate obtained with the flooding-dose technique are consistently higher by as much as 100% than the values obtained by the constant-infusion method (Pomposelli et al., 1985; Rennie et al., 1982), questions have been raised concerning the validity of the absolute values obtained with this method (Pomposelli et al., 1985; Watt et al., 1991). One possible explanation for this discrepancy is a direct stimulatory effect of the dose of amino acid (or that of the hormonal response it elicits) on protein synthesis rate (Garlick and Grant, 1988). There are excellent reviews (Garlick et al., 1994; Rennie et al., 1994) of the pros and cons of the constant-infusion and the flooding-dose methods in the literature.

Based on the dilution of tracer across organ/tissue bed

Another method used to measure protein synthesis in different organs and tissue beds is based on the dilution of a labelled amino acid across a particular organ or tissue bed. Net balance can also be obtained from the arteriovenous concentration difference (AV) multiplied by the blood flow rate through the organ or tissue bed. In humans, this method has mostly been used to estimate the rates of synthesis and breakdown of muscle protein in the leg or forearm (Cheng et al., 1987; Gore et al., 1991). The method is based on the assumption that the rate of appearance (Ra) of the amino acid (usually an essential amino acid such as phenylalanine, lysine or leucine) being traced in the organ or tissue bed reflects the breakdown of protein and the rate of its disappearance (Rd) mostly reflects its utilization for protein synthesis. If the amino acid being traced is also oxidized within the organ or tissue bed, then this can be determined by choosing a 13C-labelled tracer and subtracting its oxidation from the Rd to
get a more accurate estimate of protein synthesis. To use this method, the tracer amino acid is infused intravenously through a peripheral vein, and timed simultaneous arterial and venous blood samples are taken at isotopic steady state. Whereas the arterial blood can be taken from any artery on the assumption that the isotopic enrichment will be the same, the venous blood is taken from the dominant vein draining the organ or tissue bed being studied. Blood flow rate through the organ or tissue bed is measured either indirectly using the dilution or clearance of a dye, such as indocyanine green, or directly using real-time sensing devices that use electromagnetic, ultrasonic or Doppler shift technology.

**CALCULATION.** The total rate of appearance \( R_{at} \) of the amino acid being traced in an organ or tissue bed is the sum of arterial influx and that produced from within, i.e. endogenous \( R_a \). That is,

\[
R_{at} = R_a + \text{Influx}, \quad \text{from which, } R_a = R_{at} - \text{influx}
\]

where influx is equal to blood flow rate (\( F \)) multiplied by the arterial concentration (\( A \)) of the amino acid, and \( R_a \) can be calculated from the dilution of the tracer across the organ or tissue bed from \( R_a = \) amount of tracer entering/intracellular enrichment of amino acid being traced. Since venous enrichment is a good estimate of intracellular enrichment (Layman and Wolfe, 1987), then:

\[
R_a = \frac{FA \times A_e}{V_e}
\]

where \( A_e \) and \( V_e \) are the isotopic enrichments of the amino acid in arterial and venous blood. Substituting for \( R_{at} \) in Equation 5 above gives,

\[
R_a = \frac{FA \times A_e}{V_e} - FA, \quad \text{from which } R_a = FA\left(\frac{A_e}{V_e} - 1\right)
\]

where \( F \) is blood flow rate (ml min\(^{-1}\)), and \( A \) is the arterial concentration (\( \mu \text{mol ml}^{-1} \)) of the amino acid being traced. The units of \( R_a \) will be \( \mu \text{mol min}^{-1} \) or it can be standardized per kg body weight or per volume of the organ or tissue bed (e.g. per litre of leg) being studied.

Similarly, the rate of utilization of the amino acid, \( R_d \), for protein synthesis will be the difference between the total rate of appearance \( R_{at} \) of the amino acid being traced in an organ or tissue bed and the venous efflux (\( FV \)) plus oxidation (\( Ox \)) if the amino acid is oxidized within the organ, i.e.

\[
R_d = R_{at} - (FV + Ox)
\]

where \( Ox \) can be calculated from

\[
Ox = F(\nu CO_2 \times \nu CO_2e) - (\alpha CO_2 \times \alpha CO_2e)/V_e,
\]

where \( \nu CO_2 \) and \( \nu CO_2e \) are venous carbon dioxide concentra-
tration and isotopic enrichment, $aCO_2$ and $aCO_2e$ are arterial blood carbon dioxide concentration and $V_e$ is the isotopic enrichment of the amino acid in venous blood. Because lysine and phenylalanine are not known to be synthesized or catabolized in peripheral tissues (Goldberg and Chang, 1978; Barrett et al., 1987), these have been the tracers of choice to measure muscle protein breakdown and synthesis rates using this approach.

Measurement of plasma protein synthesis

As discussed above for organ and tissue proteins, a minimum requirement for the calculation of the rate of synthesis of a hepatic-derived plasma protein with the precursor-product model will be the measurement of the isotopic enrichment at two time points during the quasi-linear portion of the exponential rise in protein-bound amino acid labelling plus an estimate of the isotopic enrichment of the amino acid tracer at the site of protein incorporation, i.e. in the liver protein synthetic precursor pool (Waterlow et al., 1978). This is a major problem, because it is well established that a labelled amino acid taken up by the liver from the blood is substantially diluted in the hepatic-free pool by amino acids released from hepatic proteolysis. The ideal solution is to measure the steady-state labelling of the appropriate amino acyl t-RNA (Watt et al., 1991) in the liver, which severely limits its practicality in human studies.

An alternative is to use an indirect measurement of the intrahepatic protein synthetic precursor pool. Much of the early literature used urinary amino acid metabolites that were known to be synthesized from the tracer amino acid in the liver (Stein et al., 1978; Gersovitz et al., 1980). This approach has two disadvantages. First, it requires exceedingly long tracer infusions, because the fractional rates of turnover of the body pools of the metabolites are slow. Secondly, there is no assurance that the isotopic enrichment of a metabolite (e.g. hippuric acid) will be the same as the amino acid (e.g. glycine) being utilized for protein synthesis. More recent studies have used a prime-constant intravenous infusion of labelled leucine followed by measurements of the isotopic enrichment of plasma $\alpha$-KICA to estimate the intrahepatic leucine precursor pool (Horber et al., 1989). It has been argued, however, that plasma $\alpha$-KICA isotopic enrichment may not be an accurate marker of leucine isotopic enrichment in the hepatic protein synthetic precursor pool, because the major site of $\alpha$-KICA synthesis is the peripheral tissues (especially skeletal muscle). Data from our studies in pigs and humans support this argument. Significant differences between the steady state isotopic enrichments of circulating $\alpha$-KICA and very low density lipoprotein
apolipoprotein B-100 (VLDL-apoB-100)-bound leucine in both studies suggest that plasma α-KICA may not be an accurate marker of intrahepatic precursor pool leucine under all conditions (Reeds et al., 1992; Jahoor et al., 1994). For example, in a study in which labelled leucine was infused intravenously in adult human subjects in both the fed and fasted states, plasma VLDL-apoB-100-bound leucine was only ~ 70% as enriched as α-KICA in the fed state (Reeds et al., 1992).

Garlick and co-workers have also employed the flooding-dose technique to measure the rate of synthesis of hepatic-derived plasma proteins (Ballmer et al., 1990). This approach also has the technical advantage that the time required for appropriate labelling of the intracellular hepatic pool is considerably shorter than for the constant-infusion method, because the equilibration of the tracer into the hepatic-free pool is rapid (Ballmer et al., 1990). Unfortunately, the flooding-dose method may not be suitable for studies in infants, because multiple blood samples are necessary for the accurate estimation of the plasma-free amino acid labelling kinetics. In addition, some questions have been raised about the validity of this approach (Rennie et al., 1982; Watt et al., 1991; Smith et al., 1994). For example, it is still not known with certainty whether, in humans, the hepatic-free amino acid pool is completely equilibrated with the accessible plasma amino acid pool. Furthermore, the values for the rates of synthesis of albumin obtained simultaneously with the constant-infusion and flooding-dose technique are higher with the flooding-dose method, and there is evidence that the large dose of the tracer amino acid stimulates the rate of albumin synthesis (Smith et al., 1994). Although the absolute value obtained with the flooding-dose technique may not be totally accurate, the practical advantages of the method, such as a considerably shorter experimental period, make this method useful for testing the effects of a treatment in the same subject.

More recently, we (Jahoor et al., 1994, 1999a; Morlese et al., 1996, 1998) and others (Venkatesan et al., 1990) have used the steady-state enrichment of a labelled amino acid in a rapidly turning over hepatic-synthesized plasma protein, VLDL-apoB-100, as a marker of the intrahepatic enrichment of the tracer. This method is convenient because the VLDL-apoB-100-bound tracer amino acid reaches a plateau enrichment in 4–6 h and requires only ~ 0.5 ml of plasma for isolation of sufficient apoB-100 at several time points for gas chromatography–mass spectrometry (GC-MS) analysis. In a study in fasted pigs, we demonstrated that the rates of synthesis of albumin and fibrinogen were the same with three different tracers, labelled leucine, lysine and alanine (Jahoor et al., 1994). Although we did not simultaneously measure liver t-RNA enrichments in this study, we showed that, for the leucine and lysine tracers, the enrichment in apoB-100 was ~ 62% arterial plasma lysine (or leucine) enrichment and ~ 75% arterial plasma α-KICA. These results are almost identical to the ratios of the enrichments of hepatic t-RNA/arterial leucine (64%) and
t-RNA/arterial α-KICA (73%) reported by Bauman et al. (1994) in fasted pigs. The similarity of these findings suggests that the VLDL-apoB-100-bound tracer enrichment at plateau is the same as the hepatic t-RNA enrichments when the tracer is given by a prime-constant intravenous infusion. We have now adopted this approach in all of our human studies.

Another fortuitous outcome of one of our studies was the observation that, when labelled alanine is used as the tracer, the steady-state enrichment of plasma pyruvate agrees very closely with that of apoB-bound alanine in both the fed and fasted states (Reeds et al., 1992). On the basis of that observation, we infused pigs with uniformly labelled glucose (because of the unavailability of uniformly labelled alanine) to produce uniformly labelled alanine via pyruvate (Jahoor et al., 1994). We found that both plasma alanine and pyruvate were similarly enriched as apoB-bound alanine. Hence, plasma alanine or pyruvate may be used as surrogates to reflect accurately intrahepatic precursor-pool alanine enrichment (Schneible et al., 1981) The advantage of this approach is that plasma alanine reached isotopic equilibrium in a much shorter period of time compared with the time taken by VLDL-apoB-100 leucine and lysine (Jahoor et al., 1994). A disadvantage of this approach is that both uniformly labelled glucose and alanine are expensive.

A Practical Example: Prealbumin and VLDL-apoB-100 Synthesis

Because we use the steady-state isotopic enrichment of VLDL-apoB-100 to estimate the isotopic enrichment of the hepatic precursor pool in all of our studies, the fractional rate of synthesis of VLDL-apoB-100 can also be obtained in any study performed to measure the rate of synthesis of a particular hepatic-derived plasma protein. This is easily done by taking frequent additional blood samples to define the rise to plateau portion of the enrichment–time curve of VLDL-apoB-100. We will illustrate with a study designed to measure the rate of synthesis of prealbumin in adult human subjects using [2H3]leucine as the tracer.

Infusion protocol

Following a 10 h overnight fast, the subjects' weights and heights are measured and venous catheters inserted under local anaesthetic into each
forearm. One catheter is used for infusion of isotope and the other for blood sampling. A sterile solution of $[^2\text{H}_3]\text{leucine}$ is prepared in 4.5 g l$^{-1}$ saline and infused continuously for 8 h at 10 µmol kg$^{-1}$ h$^{-1}$ through the catheter in one forearm after a priming dose of 9 µmol kg$^{-1}$ is injected. A 4 ml blood sample is drawn before the start of the infusion, followed by additional blood samples at 1 h intervals for the rest of the infusion.

**Sample analyses**

Blood is drawn in pre-chilled tubes containing 10 µl of a solution consisting of 10% Na$_2$EDTA, 2% sodium azide, 1% merthiolate and 2% soybean trypsin inhibitor, immediately centrifuged at 2000 $\times$ g for 15 min at 4°C. The plasma is removed and stored at –70°C for later analysis.

Prealbumin is isolated from plasma by immunoprecipitation with anti-human prealbumin (Behring, Somerville, New Jersey) and VLDL-apoB-100 is separated by ultracentrifugation followed by isopropanol precipitation, as previously described (Jahoor et al., 1994, 1999; Morlese et al., 1996, 1998).

Although the precipitation of VLDL-apoB-100 by isopropanol is a simple and convenient method, there is concern that if the subjects are in the fed state there will be significant contamination of the isopropanol-precipitated VLDL-apoB-100 with gut-derived VLDL-apoB-48, a protein that can also be precipitated by isopropanol. In any fed study, however, pure apoB-100 can be immunoprecipitated from the VLDL isolate obtained by ultracentrifugation. The VLDL isolate is mixed with 100 µl of a solution of 66 mg antihuman apoB-100 (Calbiochem, La Jolla, California) ml$^{-1}$ and the solution made to 0.7 ml with 0.15 M NaCl containing 0.02% merthiolate. The mixture is incubated at 25°C for 1 h and then at 4°C for 24 h. To precipitate the protein–antibody complex, the tubes are centrifuged (5000 $\times$ g) at 4°C for 20 min, and the supernatant removed. The protein–antibody precipitates are delipidated by adding 1 ml of a 4:1 hexane/isopropanol solution, the mixture vortexed, centrifuged for 15 min at 5000 $\times$ g and the supernatant carefully removed. The precipitate is washed three times with 0.15 M NaCl and the precipitates treated with sample buffer for SDS–PAGE. The samples are loaded into 22.5 $\times$ 20 cm slabs of 3.5% stacking and 5% resolving polyacrylamide gels. The gels are electrophoresed in 25 mM Tris buffer (pH 8.3) containing SDS and glycine for about 7.5 h at 80 mA current. The gels are stained and destained as described before and the protein bands cut out and transferred into screw-top tubes.

The dried protein precipitates and gel bands are hydrolysed in 6 mol HCl l$^{-1}$ at 110°C for 12 h. The amino acids released from the protein are purified by cation-exchange chromatography and the tracer : tracee ratio
of the protein-derived leucine determined by negative chemical ionization GC-MS on a Hewlett-Packard 5988A GC-MS (Palo Alto, California). The amino acid is converted to the n-propyl ester, heptafluorobutyramide derivative, and leucine isotope ratio is determined by monitoring ions at m/z 349–352 as previously described (Jahoor et al., 1994, 1999; Morlese et al., 1996, 1998). Each sample is run on the GC-MS in triplicate and the average value used in the calculation.

**Calculation**

The fractional synthesis rate (FSR) of prealbumin is calculated using Equation (4) rewritten as:

$$\text{FSR} (\% \text{ day}^{-1}) = \left[ \frac{\text{IE}_{pr}.t_2 - \text{IE}_{pr}.t_1}{\text{IE}_{apoB-100\_pl}} \right] \times \frac{2400}{t_2 - t_1}$$

where $\text{IE}_{pr}.t_2 - \text{IE}_{pr}.t_1$ is the increase in isotopic enrichment of prealbumin-bound leucine over the period $(t_2 - t_1)$ h of the infusion, and $\text{IE}_{apoB-100\_pl}$ is the plateau isotopic enrichment of VLDL-apoB-100-bound leucine. In the case of VLDL-apoB-100, $(\text{IE}_{pr}.t_2 - \text{IE}_{pr}.t_1)$ is the increase in isotopic enrichment of VLDL-apoB-100-bound leucine during the time period $(t_2 - t_1)$ before a plateau is reached.

As shown in Fig. 3.3, VLDL-apoB-100-bound leucine reached a

![Graph](image-url)

**Fig. 3.3.** The tracer : tracee molar ratio of leucine incorporated into plasma VLDL-apoB-100, and into plasma prealbumin during an 8 h infusion of $[^2\text{H}_3]$leucine in an adult human subject.
plateau in isotopic enrichment after 5 h of the isotope infusion and there was a linear increase in the isotopic content of prealbumin during this period of time. Based on these isotopic enrichment–time curves it can be calculated that the FSR of VLDL-apoB-100 is 21% h⁻¹ and that of prealbumin is 68% day⁻¹.

**Conclusion**

Historically, use of the stable isotope tracer approach was severely limited because of lack of access to mass spectrometer technology and the availability of only a few labelled amino acids. This was due to both the high cost of the instruments and the need for a highly trained technical staff to prepare samples and to run and maintain the instruments. Furthermore, gas chromatograph mass spectrometers were unable to measure low isotope ratios accurately and gas isotope-ratio mass spectrometry (GIRMS) required a relatively large blood sample in order to isolate an adequate amount of the amino acid or protein to provide a sufficient quantity of N or CO₂ gas from the tracer amino acid for analysis. In the case of GIRMS analysis, a large amount of the tracer amino acid had to be isolated in a pure form for combustion, a requirement that involved laborious ion-exchange chromatography, which severely limited the use of this approach. Another major drawback was the requirement for long infusions to achieve an isotopic steady state. Since the early 1990s, however, a number of developments have made the use of stable isotope tracer techniques almost routine and hence more widespread. Both stable isotope-labelled amino acids and mass spectrometer technology has become more widely available at a relatively reasonable cost. In addition, improvements in GC-MS instruments, especially in computer-aided control and data acquisition, coupled to the high sensitivity afforded by negative chemical ionization, now enable accurate measurements of low isotope ratios in very small samples. These factors, plus the availability of amino acids labelled with multiple ²H- or ¹³C-atoms, mean that it is now possible to measure the low isotopic enrichment of a protein-derived amino acid tracer by GC-MS. In the case of the slower-turn-over-proteins, the amount of tracer incorporated into the protein may still be too low for measurement by GC-MS, making it necessary to use GIRMS analysis. Although the latter is more complicated as an analytical approach, with the advent of the gas chromatograph–combustion–isotope-ratio mass spectrometer (GC-C-IRMS), the difficulties associated with the GIRMS analysis are obviated. This instrument separates individual amino acids by GC and has a typical requirement of only ~ 20 nmol of CO₂ for adequate analysis.
In this chapter, different stable isotope tracer methods have been described that can be used to measure the kinetics of whole-body protein, the protein of specific organs and tissue beds and of individual plasma proteins. With the superior instruments available at present and the simpler sample processing required, these methods can be used conveniently in most metabolic units and in most clinical settings.

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Overview

Importance of minerals in human nutrition

Although considerable attention is devoted by the public to nutritional problems related to macronutrient intakes of protein, carbohydrates and fat, it is well recognized that important nutritional deficits, and occasionally excesses, occur for micronutrients, especially minerals. Among the most important of these are zinc, iron, calcium and magnesium. Fortunately, each of these has stable isotopes available that can be used in assessing nutrient requirements. In this chapter, we will consider the methods and some results relating to research studies using stable isotopes of calcium, magnesium and iron. Zinc will be considered in a separate chapter.

We will especially focus on techniques and findings related to research studies in children. Interest is increasing in the role of mineral nutrition in the health care of children. Mineral nutrition not only meets children’s growth and developmental needs, but also may limit or prevent disease processes (e.g. diarrhoea) and protect against future diseases (e.g. adult osteoporosis). Children are a particularly challenging group on which to perform nutritional research, not only because of their rapid growth, but because of the difficulties inherent in dietary regulation and sample collection in children (Abrams, 1999; Griffin and Abrams, 2001). Stable isotopes offer a unique opportunity to meet the need for evi-
Disease-based dietary guidelines. They also provide physiological information regarding nutrient metabolism that is otherwise unobtainable in children. Whereas radioactive isotopes may in some cases be used in adults, generally only stable isotopes are used at this time for nutritional studies of minerals in healthy children.

Diseases associated with low levels of calcium, magnesium and iron

Nutritional inadequacies during this critical period can have obvious and immediate effects. However, there are other, more insidious, long-term effects that need to be considered. Even marginal nutritional deficiencies during critical periods can have profound effects on later health and well-being. Adolescence, for example, is a period of unparalleled calcium accretion. Small differences in calcium balance during early puberty can have a significant effect on peak bone mineral mass and greatly increase the risk of later mortality and morbidity from osteoporosis (Heaney et al., 2000).

In children, calcium deficiency is primarily clinically related to the development of rickets (Committee on Nutrition, 1999). This is a severe disorder of inadequate bone mineralization. Classically, nutritional rickets is associated with vitamin D insufficiency and inadequate calcium absorption due to the lack of activated 1,25-dihydroxyvitamin D. However, in some cases, including premature infants and populations with very low calcium intakes, a deficiency of calcium can be causal or contributory to the development of childhood rickets (Thacher et al., 1999, 2000).

Of more widespread concern has been the need to obtain adequate bone minerals – calcium, phosphorus and magnesium – so as to achieve maximal bone mineralization ('peak bone mass') during adolescence. Achievement of optimal bone mass may be related to a lowered long-term risk of osteoporosis. There is some evidence that increased bone mass in childhood and adolescence is associated with fewer fractures during this period, but this relationship is less well developed (Institute of Medicine, 1997; Heaney et al., 2000).

In adults, calcium deficiency is firmly related to increasing severity of osteoporosis. It is well established that supplemental calcium can improve bone mineral density, although calcium alone is inadequate to prevent or treat osteoporosis. None the less, identifying optimal sources of dietary calcium and the consequences of therapies on calcium metabolism is important in understanding the development and treatment of osteoporosis in the elderly.

It is more difficult to identify directly the consequences of nutritional
deficits of magnesium. Magnesium is important in bone and muscle function. Deficiency conditions have been associated with a wide range of disorders including bone loss, diabetes and neurological impairment (Rude, 1998). A full understanding of the spectrum of magnesium deficiency conditions has partly been impaired by the difficulties associated with radioactive and stable magnesium studies (Avioli and Berman, 1966).

Iron deficiency is commonly associated with the development of anaemia, especially in high-risk populations including small children, pregnant women and athletes. Severe iron deficiency anaemia is particularly associated with increased morbidity and mortality in infancy and during pregnancy in developing countries where the incidence of iron deficiency anaemia is up to one-third of the at-risk population. Understanding dietary iron absorption is a critical part of nutritional intervention strategies throughout the world (Oski, 1993).

Approaches to setting dietary mineral requirements

The traditional method for determining mineral requirements is the mass balance. In this technique, net retention is determined from simultaneous measurements of intake and excretion. The effects of different levels of intake on net retention are calculated and an attempt is made to determine optimal intake levels based on these data (Matkovic and Heaney, 1992). Mass balances, however, have numerous limitations, especially when applied to paediatric populations. These include potential errors in accurately identifying both intake and total excretion of minerals, and the high cost and substantial difficulty of conducting long-term nutrition balance studies. It is especially difficult to maintain long-term dietary regulation and to perform accurate and complete urine and faecal collections in children. Moreover, mass balances do not provide direct information regarding mineral kinetics, or readily allow for determination of nutrient interactions (Abrams, 1999).

Historical Overview and Background

Isotope-based mineral studies

Studies using radioactive isotopes of calcium, magnesium and iron have been widely utilized during the past 60 years (Balfour et al., 1942; Aikawa
et al., 1960; Abrams, 1999). Although the radiation exposure from these tracers is relatively small, their use is not considered appropriate in healthy children or during pregnancy and lactation. In addition, a decrease in the use of radioactive mineral tracers in healthy adults may be occurring because of the radiation exposure and the cost of sample disposal.

The first publication describing the use of a stable isotope tracer in a human metabolic study was in 1963. An enriched stable isotope of iron, $^{58}$Fe, was injected into men in order to determine the plasma clearance of the stable isotope compared with the radioactive iron tracer, $^{59}$Fe. The initial human studies using mineral stable isotopes were reported in the 1960s. McPherson reported on methods for using stable calcium isotopes analysed by neutron activation analysis in 1965 (McPherson, 1965; Turnland, 1989). During the next 20 years, however, relatively few calcium stable isotope studies were performed, due to the lack of readily available methods for performing sample analysis (Yergey et al., 1980; Moore et al., 1985).

**Safety and comfort issues in the use of stable isotopes**

Stable isotopes of calcium, magnesium and iron have been used in thousands of clinical studies of all age groups with no reported complications related to their use. Because of their widespread use in clinical metabolic studies, we have extensively considered how to ensure the maximum safety in our clinical protocols, especially those involving children. Children clearly have a limited scope to consent for potentially uncomfortable procedures. Prior to the initiation of the study, children are interviewed and a screening blood sample taken. This provides an opportunity to assess whether the individual subjects are sufficiently comfortable with the process of venipuncture to allow kinetic studies to be carried out. We may choose not to enrol a subject who wishes to consent for the study because of possible difficulties with repeated phlebotomy. However, such subjects will often tolerate calcium kinetic studies as they require a single venipuncture to place an intravenous catheter, from which all subsequent blood samples can be taken.

If subjects express interest in the study, they and their parents are given full and complete details of the study, and independently sign consent forms. It is emphasized that enrolment in the study is optional and that subjects can either refuse to participate or can leave the study after enrolment. Clearly, for babies and toddlers no direct consent can be given from the actual study subject. This requires careful explanation of the risks and benefits of the study to the parents who must choose.
whether to enrol their small child. It is vitally important, however, that ethically conducted research be performed on infants and toddlers. It is not the case that they are ‘small adults’ who absorb and utilize minerals in the same way as adolescents and adults.

In practice, feedback from subjects has been overwhelmingly positive. We strive to make their ‘research’ experience an enjoyable one. In many cases, children have used the research experience as an informative way of learning more about nutrition and science. Indeed, most tears during the study period occur at discharge! Clearly, young children tolerate blood draws less well than adults. We always use a local anaesthetic cream on the skin. This has been very effective in reducing the discomfort associated with blood draws or intravenous catheter placement, and has made studies much more acceptable to children, as well as easier for investigators (Abrams, 1999; Griffin and Abrams, 2001).

Isotope Production, Supplies and Human Use
Preparation

Calutrons: history and methodology

Essentially all calcium and magnesium stable isotopes used in human studies are produced via calutrons. Calutrons were first operated at Oak Ridge, Tennessee, in 1944 for the production of enriched uranium for use in nuclear weapon development. They are essentially huge mass spectrometers capable of large-sized electromagnetic separation of isotopes. They were very expensive and difficult to use and were rapidly replaced in the weapons programme with other techniques for uranium separation. However, they remain the method of choice for the production of many mineral stable isotopes. A magnificent history of the calutron can be found in Yergey and Yergey (1997) and information regarding the production of the ‘atom bomb’ at: http://imglibe.lbl.gov/LBNL_Res_Rev/RR_online/81F/81fepi2.html (also at http://www.lbl.gov/Science-Articles/Research-Review/Magazine/1981/index.html).

Isotope supplies

Oak Ridge National Laboratories

Oak Ridge National Laboratories (ORNL) has, for many years, been the primary supplier of mineral stable isotopes for most of the nutritional
research world. However, production of isotopes at ORNL was stopped in the early 1990s and then restarted briefly in the mid-1990s (Abrams et al., 1992b). At present, the calutrons at ORNL are not operating and it appears extremely unlikely that they will ever be operated again, although a relatively large supply of highly enriched isotopes produced primarily prior to 1990 can still be purchased from ORNL.

However, the price of ORNL material is relatively high compared with other sources (see below), and there is generally no specific benefit to the nutrition researcher to using this material. Furthermore, since the calutrons at ORNL are unlikely to be operated again in the future, it is unnecessary to ‘support’ ongoing calutron operation by purchasing isotopes from them.

Russia

The unfortunate situation regarding ORNL has forced researchers to rely on alternative sources of mineral stable isotopes. Some isotopes, most notably zinc, can be produced by centrifuge techniques, which are available, for example, in The Netherlands (http://www.urenco.com/group.htm). However, there are currently no practical and cost-effective methods available for calcium, magnesium and iron isotope production other than via calutrons.

The only readily available alternative to ORNL for most researchers is to obtain isotopes produced in the former Soviet Union. These isotopes are produced primarily at a calutron facility located in Sverdlorsk, Russia, called ElectroKhimPribor (http://www.isotopetrace.com/ekp.htm). Although in the past, the supply, cost and importation of isotopes from Russia made their use difficult, this situation has been considerably improved in recent years. Currently, importation of Russian-produced calcium, magnesium and iron stable isotopes may be reliably arranged by several agents.

Isotope preparation for human use

Stable isotopes are usually obtained as the metal, or in the oxide, carbonate or chloride forms. The isotope needs to be converted into a soluble form prior to administration, and this is usually the soluble chloride or sulphate salt. This is done by dissolving the mineral in a small volume of concentrated acid, either hydrochloric or sulphuric acid. It is important to mention here that all acids used in isotope preparation and sample preparation need to be of ultra-high purity to avoid any trace contaminants.
The soluble isotope is diluted to the desired concentration, or further preparation is conducted if necessary, depending on the mineral and the form in which it is to be administered. If the isotope is to be administered intravenously, the solutions need to be sterile-filtered, packaged into vials and tested for sterility and pyrogenicity. This is usually carried out by a hospital pharmacy experienced in these procedures.

Although isotopes of calcium and magnesium are very safe for intravenous administration, it is important to ensure that they are provided and delivered in a safe, sterile fashion. Therefore, we usually choose to have them dispensed as individual subject doses by a pharmacist in the same fashion as any other hospital or out-patient medication. Handling and disposal of vials and syringes is carried out as for any medication given intravenously.

Special consideration needs to be given to the administration of iron stable isotopes intravenously. There are rare reports of adverse reactions related to intravenous iron infusion (Fishbane and Kowalski, 2000), although no such reports have been identified from stable isotope research studies. None the less, we have chosen only to administer iron isotopes intravenously in a setting in which vital signs can be closely monitored during the initial infusion and where provisions are available for intervention if an adverse effect occurs. Generally, this would indicate a hospital or fully staffed clinic setting.

**Isotope Methodology: Calcium Isotope Studies**

**Calcium stable isotopes**

There are six naturally occurring stable isotopes of calcium. Their natural abundance and typical doses for their use in paediatric and clinical studies is shown in Table 4.1. Choice of isotopes and dosing levels depends greatly on the type of study being conducted (i.e. absorption or kinetic), the likely level of mineral absorption and the type and precision of analytical technique being used (O’Brien and Abrams, 1994). It is necessary for the study planner to discuss these issues specifically with the analytical team prior to dosing.

The greatest precision in measurement is usually obtained from analysis via thermal ionization mass spectrometry using a multi-collector system and with magnetic ionic separation. In this case, it is possible to measure accurately the rarest calcium isotope, $^{46}$Ca, and this isotope may therefore be administered so as to keep down the total calcium dose given. In most other circumstances, $^{46}$Ca is not administered and a combination of $^{42}$Ca, $^{43}$Ca and $^{44}$Ca are given. Depending on the set-up of the
mass spectrometer, it may be practical to give $^{48}\text{Ca}$ in addition as a third isotope and analyse it too.

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**Table 4.1.** Mineral stable isotopes frequently used in paediatric nutritional research.

<table>
<thead>
<tr>
<th>Isotope</th>
<th>Natural abundance (%)</th>
<th>Typical dose for mineral absorption studies$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{42}\text{Ca}$</td>
<td>0.65</td>
<td>1–2 mg IV</td>
</tr>
<tr>
<td>$^{44}\text{Ca}$</td>
<td>2.08</td>
<td>3–16 mg IV (10–15 mg p.o.)</td>
</tr>
<tr>
<td>$^{46}\text{Ca}$</td>
<td>0.0032</td>
<td>15–20 µg p.o.</td>
</tr>
<tr>
<td>$^{57}\text{Fe}^b$</td>
<td>2.14</td>
<td>5–15 mg p.o.</td>
</tr>
<tr>
<td>$^{58}\text{Fe}^b$</td>
<td>0.29</td>
<td>1–3 mg p.o. (0.2–0.4 mg IV)</td>
</tr>
<tr>
<td>$^{25}\text{Mg}$</td>
<td>10.0</td>
<td>0.3–0.5 mg kg$^{-1}$ IV</td>
</tr>
<tr>
<td>$^{26}\text{Mg}$</td>
<td>11.0</td>
<td>0.2–0.4 mg kg$^{-1}$ IV</td>
</tr>
</tbody>
</table>

$^a$IV, intravenously, p.o., orally. Typical doses are based on dual tracer studies involving adults and children over 1 year of age.  
$^b$In studies of infants less than 1 year of age, $^{57}\text{Fe}$ doses of 2–4 mg and $^{58}\text{Fe}$ doses of 0.2–0.5 mg are given orally.

Clinical protocols for absorption studies

In a mass balance study, the net absorption of a nutrient is calculated by measuring the difference between mineral input from the diet and total faecal mineral output (Matkovic and Heaney, 1992). The faecal output of minerals includes both unabsorbed dietary minerals and minerals that have been secreted into the intestine and not reabsorbed (usually referred to as endogenous faecal excretion). These two sources of mineral appearing in the faeces cannot be distinguished by a classical balance study.

Using isotopic methods, however, faecal calcium from these two sources may be measured and the ‘true’ dietary absorption fraction of minerals measured. Either a single or a dual-tracer technique may be utilized for this measurement (Heaney and Recker, 1994).

In the single-isotope technique, an isotope of the mineral is given by mouth. A complete faecal collection is carried out until virtually all the unabsorbed oral tracer has been recovered. The fraction of administered tracer that has been absorbed is calculated from the difference between the amount of the oral dose and the faecal isotope recovery.

This technique has the benefit that the calculated absorption represents only the dietary component of the element that is absorbed and does not include endogenous secretory losses. It also does not require the
use of an intravenous infusion. A disadvantage of the use of this oral tracer approach is that extended faecal collections are required (Abrams, 1999) and the accuracy of the results remains dependent on the completeness of the faecal collection.

We prefer to utilize a dual-tracer technique to measure calcium absorption. In this technique, one low-abundance calcium isotope is given orally and a different isotope is given intravenously (Bronner, 1962; Degrazia et al., 1965). Early in the morning of the study, subjects are instructed to empty their bladders and are given breakfast. Toward the end of the meal, the subjects are given an isotope of calcium that has been premixed (and allowed to equilibrate in the refrigerator for 12–24 h) with milk or juice. After breakfast, a different calcium isotope is administered intravenously over 2–3 min (Abrams et al., 1999).

The orally administered isotopic tracer is absorbed into a central body pool, which for calcium is believed to represent serum, extracellular fluid and some metabolically active bone (Abrams et al., 1992a). The oral tracer mixes with the intravenous (IV) tracer, which serves to ‘normalize’ for variations in calcium distribution pool mass among subjects (Abrams, 1998).

After administration of the tracers, a complete 24 h urine collection is carried out. The relative fraction of the oral vs. the IV tracer dose in this 24 h urine pool is determined and represents the fraction of the oral tracer dose that was absorbed. Spot determinations of urine or serum isotope levels of the tracers may also be used. However, for calcium, this method may not be as accurate as that determined from complete 24 h collections (Yergey et al., 1994). Because absorption is calculated from total urinary isotope recovery, it is not necessary to sequence exactly the time of administration of the oral and IV isotopes, as would be necessary if a single peak serum value were used.

In some research circumstances, it may be advantageous to compare the absorption of two types or sources of calcium. In theory this can be done by administering two oral isotopes and a third isotope intravenously. For example, $^{46}$Ca and $^{48}$Ca can be administered orally and $^{42}$Ca or $^{44}$Ca administered intravenously. The significant drawback to this approach is that it is only accurate if the two oral isotopes are given at relatively close times (probably within a few hours). This is feasible if the two sources are designed for use at the same meal, but problematic for studies of two types of dietary calcium supplements where co-administration might confound the data interpretation.

In general, we rarely use triple isotope studies for these evaluations. Rather we would choose to adapt a subject completely to one calcium source, complete a full evaluation of that source and then, after an appropriate washout and readaptation period, evaluate the subject again with a new dual-tracer study on the second calcium source. We believe this approach to be more reliable as a tool for comparing calcium bioavailability between dietary sources.
Measurement of bone calcium deposition and pool masses (kinetics)

Although isotopic methodologies are important as a means of simplifying the measurement of mineral absorption, a second important benefit of their use is that they allow kinetic models to be developed to understand the distribution and turnover of minerals after absorption.

To perform these kinetic studies, the calcium absorption protocol must be expanded to collect sequential serum and urine samples after isotope administration. Serum samples (0.5 ml) are obtained for isotope enrichment determination at 6, 12, 20, 30, 45, 60, 120, 180, 240 and 480 min after completion of the intravenous infusion of the calcium isotope. Studies in premature infants have collected fewer serum samples (Abrams et al., 1995). Additionally, at least two spot urine samples are collected daily for a total of 5–10 additional days (Abrams, 1999; Abrams et al., 1997, 1999) after isotope administration. Calculation of the kinetic pool masses is described in the section on Calculation of kinetic parameters, this chapter.

Isotope Methodology: Magnesium Isotope Studies

Clinical protocols for absorption studies

The clinical protocol for magnesium studies is similar in design to those for calcium with a few important distinctions.

We have shown that magnesium absorption, unlike calcium absorption, does not plateau after 24 h of urine collection post-dosing (Abrams et al., 1997). Instead, we found that the magnesium absorption fraction reached a plateau of > 95% of the final value at 60 h after the final oral dose, but was only an average of 80% of the final value at 24 h afterward. Therefore, we collect complete 72 h urine samples after dosing for magnesium stable isotope studies rather than the 24 h used for calcium studies.

Also, because there are only two isotopes of magnesium available for administration, it is not possible to perform a triple isotope study as described above for calcium.

Clinical protocols for kinetic studies

There are also two important differences in magnesium isotope kinetic studies compared with calcium studies. First, because the total amount of
Magnesium administered is relatively large, it must usually be given over 10–20 min to ensure that the patient tolerates it well. Secondly, we do not find close concordance between urine and serum enrichment levels in the first 5 days after tracer administration. Therefore, unlike calcium studies in which spot urine samples can be collected to reflect serum enrichment, it is necessary to continue to obtain daily serum samples throughout the magnesium kinetic study (Abrams and Ellis, 1998; Abrams and Wen, 1999).

**Isotope Methodology: Iron Stable Isotopes**

There are four naturally occurring iron stable isotopes. Of these, the lowest-abundance isotopes, $^{58}\text{Fe}$ and $^{57}\text{Fe}$, are most commonly used in human nutrition research. Iron stable isotopes are usually provided as iron metal or iron oxide powder and are converted to ferrous sulphate prior to oral administration. Both the supply and cost of these isotopes have remained relatively constant, or decreased, in recent years (Table 4.1). Because dosing of iron stable isotopes is dependent on enriching the circulating body iron pool (see below), the dose administered is usually dependent on the subject’s weight, and increases in proportion to weight and haemoglobin concentration (Abrams et al., 1994).

**Protocol for assessing iron red blood cell incorporation**

Isotope studies using iron stable isotopes take advantage of their localization into red blood cells to estimate directly the fraction of isotope that is incorporated into red blood cells ($\text{RBC}_{\text{inc}}$) from an orally administered dose. A sample of blood is obtained at least 14 days after dosing and the enrichment of the administered isotopes is calculated (Abrams et al., 1994).

**Analytical Techniques**

**Thermal ionization mass spectrometry**

Thermal ionization mass spectrometry (TIMS) is widely used for geological research throughout the world. Ion separation using both a quadrupole and magnetic sector are feasible, and equipment for both of these
have been commercially produced. However, quadrupole TIMS is not widely utilized due to its lower measurement precision. A major disadvantage of TIMS analysis is sample throughput; it is virtually impossible to analyse more than 20 samples per day using available equipment. TIMS instrumentation is relatively expensive and usually requires a dedicated operator. None the less, TIMS will continue to play a major role in stable isotope studies of mineral nutrition and metabolism due to its extremely high accuracy and precision (Woodhouse and Abrams, 2000).

**Analytical methods – calcium**

Calcium can be isolated from most serum and urine samples by precipitation using ammonium oxalate (Yergey et al., 1994). Adequate amounts of calcium can usually be recovered from 3 ml of urine or 0.5 ml of serum. Faecal samples require acid digestion and sometimes ion-exchange chemistry prior to precipitation and analysis. Extracted calcium samples are resuspended in 100 µl of 2–3% high purity nitric acid and 5 µl of each sample is loaded on to a degassed evaporation filament. For thermal ionization mass spectrometers, calcium samples are analysed indirectly using a ‘dual-filament’ technique (O’Brien and Abrams, 1994).

**Isotopic ratios**

The following is a description of the measurement technique using a Finnigan (Thermoquest, Bremen, Germany) magnetic section TIMS. Rhenium ionization and evaporation filaments are degassed under vacuum at 4.2 amperes (A) for 15 min prior to use. Filaments are heated at 1.5 A until a white precipitate is observed and then increased slowly to 2.0 A until a yellow precipitate is obtained. Ionization and sample-containing evaporation filaments are loaded on to a 13-position magazine and inserted into the mass spectrometer. Sample analysis starts after a vacuum of at least 2 × 10^-6 torr is achieved. Data are collected from each position by first increasing the ionization filament current to approximately 3 A and optimizing the signal intensity by adjusting the lens potentials and magazine position. The current on the evaporation filament is then slowly increased, and the magazine position and lens potentials are again optimized until a stable signal at m/z 44 is observed. Data are collected from each isotope of interest using multiple Faraday cup collectors. With the latest generation of this equipment, the mass spectrometer sequence can proceed automatically under computer control, although most available equipment still requires manual processing.

Ratios are made for each measured isotope against a naturally occur-
ring non-administered isotope such as $^{43}$Ca. A total of ten scans are made per block and replicate blocks are run until the relative accuracy for each measured isotope is 0.1% or lower. Each ratio is normalized for fractionation using a reference ratio of non-administered isotopes (e.g. $^{44}$Ca/$^{43}$Ca). Accuracy of this method is 0.1% for natural abundance samples compared with standard data, and relative standard deviations are 0.1–0.2% or better. With the most up-to-date equipment, precision of 0.01–0.02% is likely to be achieved.

Analytical methods – magnesium

Serum samples for TIMS analysis can be prepared using a relatively simple precipitation method (Vieira et al., 1994). In this method, concentrated NH$_4$OH is added to 1 ml aliquots of serum until a pH of 10.0 is achieved. Subsequently, 3.7 M NH$_4$Cl is added to give a concentration of 200 µg NH$_4$Cl µg$^{-1}$Mg. Five per cent 8-OH quinoline is then added to give a concentration of 20 µg 8-OH quinoline µg$^{-1}$Mg. Samples are centrifuged at 2000 r.p.m. for 10 min and the supernatant discarded. Precipitates are dried at 100°C for 30 min and then at 450°C for 3 h. The residues are resuspended in 3% HNO$_3$ prior to mass spectrometric analysis.

For urine and faecal samples, it is necessary to perform ion-exchange chromatography (Abrams and Wen, 1999). In this case, 2–3 ml of urine or acid-digested faeces is dried in a beaker on a hot plate. Then 5–10 ml of concentrated nitric acid is added to digest the sample completely, which is dried again. Subsequently, it is redissolved in 6 M HCl and dried on a hot plate. This material is reconstituted in 4 ml of 0.5 M HCl. A disposable polyethylene transfer pipette is used as the ion-exchange column. A frit is fitted into the column, which is then filled to the top of the narrow tube with cation exchange resin (AG 50W-X8, 100–200 mesh) in H$_2$O. The resin is washed with 4 ml of 6 M HCl, rinsed with 4 ml of H$_2$O, and then reconditioned with 2 ml of 0.5 M HCl. The sample solution is loaded on to the column and washed twice with 4 ml each of 0.5 M HCl. Magnesium is then eluted with 4 ml of 1 M HCl. The solution is taken to dryness and resuspended in 3% HNO$_3$, ready to be loaded on to the filament and analysed in the mass spectrometer.

Samples may be analysed using either the dual-filament approach used for calcium or the single-filament approach described below for iron. Because two isotopes are usually administered and there is only one non-administered isotope, it is not possible to adjust directly for fractionation in the thermal source. To minimize fractionation, all samples are analysed for the $^{26}$Mg/$^{24}$Mg and $^{25}$Mg/$^{24}$Mg ratios at a fixed temperature. We have found that, using this approach, our measurement precision and accuracy (for non-enriched samples) was 0.2% or better for all measured ratios.
Analytical methods – iron

For iron, blood samples of 0.5 ml are digested in 2–10 ml of concentrated HNO\textsubscript{3} in a titration flask on a hot plate at a sub-boiling temperature for 24 h. Samples are then dried and redissolved in 1–2 ml of 6 M HCl and loaded on a polyethylene column filled with anion exchange resin (AG-1 ×8, 100–200 mesh). After the sample solution has passed through the column, it is washed with 6 ml of 6 M HCl followed by 0.5 ml of 0.5 M HCl before the iron is extracted from the column by 1 ml of 0.5 M HCl. The extracted iron is dried, resuspended in 30–50 µl of 3% HNO\textsubscript{3} and loaded on to a filament for mass spectrometric analysis (Abrams et al., 1994).

Analysis using TIMS is similar to that for calcium and magnesium, except that a single-filament technique is used. In this case, a filament is preloaded with silica gel to enhance ionization. The purified iron sample is then loaded and a dilute mixture of phosphoric acid added to enhance ionization further. Extreme care is needed throughout the procedure to avoid contamination with nickel, which can confuse the data results. Other techniques using negative ion mode may increase the precision of measurements.

Inductively coupled plasma mass spectrometry (ICP-MS)

ICP-MS, the most recent of the MS techniques, is the most widely used metal isotope analysis technique and is an instrument specifically designed for trace element quantitation (Woodhouse and Abrams, 2000). Samples are introduced through a nebulizer into a high-temperature argon plasma produced by electrical discharge, where the solids are volatilized and ionized. The plasma is sampled at atmospheric pressure through a differentially pumped interface, and ions are usually separated by mass with a quadrupole mass spectrometer. In the mid-1990s, commercially produced ICP-MS instruments that separate ions using a magnetic field (high-resolution magnetic sector ICP-MS) became available through several manufacturers. These machines feature very high resolution with claims of analytical precision close to that achieved using TIMS, as well as very rapid analysis times (about 5 min per sample). Depending on the mineral, sample preparation is important for ICP-MS analysis as there are interferences, and some elements cannot be analysed easily due to these interferences at specific masses. The high-resolution magnetic sector instruments have less interference due to the high mass resolution. The high-resolution ICP-MS instruments spectrally separate interfering masses by coupling the Ar ICP source to a high-resolution mass spectrometer. For example, the high resolution magnetic sector instrument can resolve the mass signal of $^{56}$Fe from ArO$^+$, which cannot be done with quadrupole mass analysers and is the main reason why
iron isotopes are difficult to analyse with quadrupole ICP-MS. Furthermore, only limited sample preparation may be necessary using these instruments.

At present, ICP-MS is frequently used for magnesium and iron analysis (Coudray et al., 1997). However, only limited use has been made for calcium analysis. This situation may change in the near future as more experience is gained with the newer magnetic section machines. In particular the recent introduction of multi-collector magnetic sector ICP instruments may lead to much greater precision of measurements than was previously available. This would be especially important in studies of infants, where small amounts of iron are often used, and in adolescents and adults, where the relatively greater body mass requires a larger and more expensive dose than is optimal.

Neutron activation analysis (NAA)

NAA is primarily used for total element analysis, but can be used to determine isotopic enrichment. NAA for determination of stable isotope enrichment is based on the interaction of thermal neutrons from a nuclear reactor with the nuclei of the stable isotopes. Some isotopes do not result in the production of the radioactive nuclide and cannot be measured with NAA. Many of the original calcium stable studies were analysed using neutron activation analysis after administration of $^{46}\text{Ca}$ or $^{48}\text{Ca}$ (McPherson, 1965; Heaney and Skillman, 1971). Currently there is relatively limited use of this technique (Abrams, 1999).

Fast-atom bombardment mass spectrometry (FAB-MS)

FAB-MS is well known for the analysis of large, labile polar molecules, but has also been used for analysis of stable mineral isotopes, including calcium. In FAB-MS, samples are bombarded with argon or xenon atoms, and the charged species are then separated. Analysis time is about 30 min per sample, with precision of approximately 1% for calcium isotopes (Smith, 1983; Woodhouse and Abrams, 2000).

Data Interpretation

Calcium and magnesium studies

For calcium, the low natural abundance fraction of the principal tracers utilized allows direct calculation of a delta percentage excess ratio (meas-
ured as the ratio of the administered isotope to a non-administered isotope) using the following equation:

\[
\% \text{Excess} = \frac{\text{observed ratio} - \text{natural abundance ratio}}{\text{natural abundance ratio}} \times 100
\] (1)

The reference natural abundance values (expressed as a percentage) used in the calculations are: $^{42}\text{Ca}$, 0.647; $^{43}\text{Ca}$, 0.135; $^{44}\text{Ca}$, 2.086; $^{46}\text{Ca}$, 0.0032; and $^{48}\text{Ca}$, 0.187.

For magnesium, the relatively greater natural occurring fractions of the minor isotopes leads to a need to adjust for the mass of these isotopes. The optimal method for this is to calculate the tracer : tracee ratio, as described by Wastney et al. (1996). A somewhat simpler approach that may have practical utility has been described by Liu et al. (1989).

**Calculation of fractional absorption**

The mathematical methods for the calculation of $V_a$ (true calcium or magnesium absorption) are described below. The dynamics of the system are depicted in Fig. 4.1. The true fractional absorption of calcium or magnesium (alpha) is calculated as the ratio of the accumulated oral vs. intravenous tracer in urine during the 24 h (72 h for magnesium) after tracer administration:

\[
\alpha = \frac{\int_0^t \text{oral dose in urine}}{\int_0^t \text{IV dose in urine}}
\] (2)

**Fig. 4.1.** Calcium kinetic model. $V_a$, Total calcium absorbed; $V_{o+}$, bone calcium deposition rate; $V_{o-}$, bone calcium resorption rate; $V_i$, calcium intake; $V_{endo}$, endogenous faecal calcium excretion; $V_u$, urinary calcium excretion.
True calcium or magnesium absorption, $V_a$, is then calculated from alpha and $V_i$ (calcium or magnesium intake) as:

$$V_a = \alpha \times V_i \quad (3)$$

$V_f$ (endogenous faecal calcium or magnesium excretion) is measured after bolus administration of an intravenous tracer. In this technique, $V_f$ and $V_u$ are assumed to occur from a single central pool. This pool is believed to consist of plasma and some components of extracellular fluid, tissues and surface bone (Abrams et al., 1992a, 1995). Calcium or magnesium flow to stable bone (also muscle in the case of magnesium) from the central pool may involve intermediate, kinetically determined bone pools; however, these do not affect the calculation of $V_f$ or $V_u$. With this assumption, the ratio of $V_f/V_u$ is equal to the ratio of the accumulated tracer recovered in the stool compared with the tracer recovered in the urine. This can be expressed as follows:

$$V_f = \frac{\int_0^t IV \text{ dose in stool}}{\int_0^t IV \text{ dose in urine}} \times V_u \quad (4)$$

Net calcium retention, $V_{bal}$, is the difference between absorption and the sum of urinary excretion ($V_u$) and $V_f$:

$$V_{bal} = V_a - (V_u + V_f) \quad (5)$$

**Calculation of kinetic parameters**

Although isotopic tracers may simplify and enhance the assessment of mineral absorption and excretion compared with mass balance studies, perhaps the most significant benefit of their use in mineral metabolism studies is that they allow kinetic models to be developed for the metabolism of the minerals.

Compartmental models (Fig. 4.1) are useful for studying biological systems because compartments are often used to represent material within a component such as an organelle or tissue. They have been used to study metabolism from the cellular to the population level. Calcium has been modelled using three or four compartments to represent exchange with calcium in blood (Neer et al., 1967; Abrams, 1999; Abrams et al., 1992a, 1999).

We use a three-compartment model, where the compartments are
considered to represent calcium in serum and some extravascular fluid, interstitial fluid and exchangeable calcium on bone. There is loss from the third compartment that represents deposition of calcium into bone. Other loss pathways are into urine and into the gastrointestinal tract. Absorption occurs by a single pathway into the sampled compartment. Entry of natural calcium occurs into the gastrointestinal tract via the diet and into serum via bone resorption.

Compartmental modelling of calcium metabolism in children and adults leads to estimation of the rate at which these minerals are deposited in bone (often referred to as $V_{o+}$) and the size of the exchangeable bone calcium pool (Abrams et al., 2000). It is of significant interest to determine the rate at which calcium is re-absorbed from bone. This is somewhat more difficult to assess from most kinetic studies. It may be determined indirectly from these studies by assuming that distribution from the central compartment (Fig. 4.1) into bone (as part of growth in children) and excretion in the urine and stool equals input to this compartment from the diet (the measured absorption) and bone resorption (which can then be calculated). In children (especially infants), this kinetic analysis may need to account for body growth and possible pool size growth during the course of the study.

Magnesium kinetics is similarly determined using a three-compartment model, as described by Avioli and Berman (1966) and Sojka et al. (1997). In this case, magnesium is deposited from the third exchangeable compartment (pool) into a very slowly exchanging pool consisting primarily of tissue magnesium, including muscle and bone tissues. The third pool for magnesium is referred to as the exchangeable pool, $M_{comp}$ and efflux of magnesium from this pool as $V_{o+Mg}$. Although the third pool does not comprise the entire exchangeable pool, it is by far the largest pool and may be used to represent the exchangeable pool that is the source of magnesium deposited into long-term storage pools (Abrams and Ellis, 1998).

Iron studies

*Calculation of RBC iron incorporation and iron absorption*

The ratio of the administered isotope ($^{57}$Fe or $^{58}$Fe) is determined relative to $^{56}$Fe in the sample of blood, and the quantity of administered isotope incorporated into erythrocytes ($Fe_{inc}$) is determined from enriched (enr) and baseline (base) isotope ratios as (for $^{57}$Fe):
where \( \text{NA}_{57} \) is the natural fraction (by weight) of \(^{57}\text{Fe} \) (0.0214). Identical calculations are done for \(^{58}\text{Fe}_{\text{inc}} \), except that 0.00287 is used as the natural fraction of \(^{58}\text{Fe} \) (Kastenmayer et al., 1994).

\( \text{Fe}_{\text{circ}} \) is the child’s total circulating iron, calculated as the product of the child’s blood volume, the measured haemoglobin concentration and the concentration of iron in haemoglobin of 3.47 (mg g\(^{-1}\)). To calculate the red blood cell iron incorporation (RBC\( _{\text{inc}} \)), the total isotope incorporated is divided by the dose.

An important issue in this calculation is the value used for the child’s blood volume. It is not practical to measure this directly for each subject. Rather, an estimate is usually used. Although estimates of 65 ml kg\(^{-1}\) are generally used in adults, blood volume may be greater in small children, especially premature infants. In premature or small infants, most calculations use a volume of 80 ml kg\(^{-1}\). It should be recognized that the final calculated RBC\( _{\text{inc}} \) may have some imprecision. However, in most iron isotope studies comparison is made for values within individual subjects on two different diets. This small uncertainty in the actual RBC\( _{\text{inc}} \) therefore may not have an important effect on the final study findings.

We usually prefer to report the value for the RBC\( _{\text{inc}} \) as the end point for iron bioavailability. If the actual dietary iron absorbed by the body is the end point of interest, several methods may be utilized for its determination. The most common method is to assume that 80–90% of all the iron absorbed from the diet is incorporated into RBCs (Abrams et al., 1994; McDonald et al., 1998), and to divide the RBC\( _{\text{inc}} \) by 0.8 or 0.9 to calculate the fractional absorption.

### Clinical Examples

**Determining the effect of varying calcium intake on calcium and iron absorption in small children (Ames et al., 1999)**

**Overview**

This clinical study was conducted to evaluate the relative absorption of calcium from low- and high-calcium diets and also the effects of high-calcium diets on iron absorption in small children. It demonstrated the
combined use of both calcium and iron studies in each subject and the use of crossover design in these studies.

Methods

Eleven subjects (six boys and five girls), aged 3–5 years, were randomized to begin either a high (1180 ± 117 mg) or low (502 ± 99 mg) calcium intake diet. Subjects continued on this diet at home for 5 weeks to allow equilibration of the subjects on the diet.

At the end of this 5 week period, subjects were admitted to an in-patient metabolic ward. Baseline blood samples were obtained for measurement of haemoglobin concentration and serum ferritin. Ten micrograms of ⁴⁶Ca was then infused intravenously using a butterfly infusion set over 1 min. Subsequent to intravenous infusion of ⁴⁶Ca, study subjects were given breakfast, lunch and dinner similar to meals received at home. Added to each of these three meals was one roll into which ⁵⁸Fe (0.5 mg per roll) had been baked. In addition, subjects were given milk that contained ⁴⁴Ca (total dose 12 mg ⁴⁴Ca divided between three meals). Calcium isotopes were pre-equilibrated in milk for 12–24 h prior to administration. For studies utilizing high-calcium intakes, 120 ml of milk was used; for the low-calcium intakes, 60 ml of milk was used. A complete urine collection was started with the infusion of the ⁴⁶Ca. All subjects returned on the 15th day after receiving the isotopes at which time a blood sample was obtained for isotope ratio measurements. At that time, subjects were switched to the other type of diet (high or low calcium) for 5 weeks and the entire study repeated. Immediately prior to the second in-patient study, a baseline blood sample was obtained for iron isotope ratios and a spot urine sample collected for baseline calcium isotope ratios.

Results

Fractional absorption of calcium was significantly lower during the high-calcium diet compared with the low-calcium diet (24.4 ± 8.4% vs. 36.2 ± 7.1%; P < 0.001). Total calcium absorption (181 ± 50 mg day⁻¹ vs. 277 ± 91 mg day⁻¹; P = 0.002) was greater in children on a higher calcium intake. RBC incorporation of ⁵⁸Fe in the high- and low-calcium diets was not different (7.9 ± 5.5% vs. 6.9 ± 4.2%, respectively; P = 0.67). The geometric mean RBC incorporation values were 6.4% and 5.6% for high- and low-calcium intake levels, respectively.
Conclusions

We found that increasing dietary calcium in pre-school children to approximately 1200 mg day\(^{-1}\), compared with 500 mg day\(^{-1}\), resulted in a significant increase in total dietary calcium absorption. We found no evidence that increasing dietary calcium intake over this range impaired iron incorporation into RBCs. Our findings indicate that small children may benefit from calcium intakes similar to those recommended for older children without adverse effects on dietary iron utilization. Concerns expressed in this regard (Hallberg, 1998) may reflect absorption inhibition from single meals rather than a long-term failure to adapt to higher calcium intake.

Determining the effects of iron supplementation on iron absorption in pregnant women (O’Brien et al., 1999)

Overview

This study was conducted to examine the influence of iron status on iron absorption during pregnancy. In this study, the absorption of supplemental iron, RBC iron incorporation and iron status in 45 pregnant Peruvian women (33 ± 1 week gestation) was measured. This study demonstrated the use of tracers to evaluate the effects of iron supplementation on total absorbed iron in an at-risk group. In this case, the stable iron isotope was given intravenously as well as orally.

Methods

This study comprised two groups of women who consumed daily prenatal supplements containing 60 mg of iron (as ferrous sulphate) and 250 µg of folate (Group A) or the same supplement also containing 15 mg of zinc (as zinc sulphate) (Group B).

The third group of women (Group C) was recruited from the same community but did not receive prenatal supplementation because they did not seek medical attention until the third trimester of pregnancy.

This study was conducted in Villa El Salvador, a lower-income, peri-urban area of Lima, Peru. After a baseline blood sample (10 ml) had been collected, 0.6 mg of \(^{58}\)Fe was infused over a 10 min interval. Each woman consumed 10 mg of \(^{57}\)Fe tracer in 60–90 ml of a non-ascorbic-acid-containing flavoured drink made from a powdered mix using deionized water. Women in Group A and Group B also consumed a prenatal sup-
The prenatal supplement was identical to the supplement normally consumed except that the total iron content was reduced by 10 mg to keep the total iron dose of supplement and tracer constant at 60 mg. The women remained fasted for 1.5 h following dosing. To measure iron absorption, women returned to the clinic 2 weeks post-dosing and a 5 ml blood sample was obtained for analysis of iron isotopes.

**Results**

Percentage iron absorption did not significantly differ between treatment groups and was not significantly influenced by total doses of non-haem iron of 10 or 60 mg. Because of the larger supplemental iron intake, net iron absorption was significantly higher in women who consumed prenatal iron supplements compared with those who did not ($P < 0.0001$).

The only significant predictor of iron absorption was maternal ferritin status, with this being inversely related to percentage iron absorption. Women with the highest ferritin levels (> 30 µg l$^{-1}$; $n = 5$) absorbed an average of 6.8 ± 4.4% of the supplemental iron, whereas women whose serum ferritin levels were ≤ 30 µg l$^{-1}$ absorbed an average of 12.2 ± 5.3% ($n = 39$).

The fraction of the intravenous tracer incorporated into RBCs was significantly influenced by iron supplementation. Women who did not receive prenatal iron supplements incorporated a significantly larger proportion of the intravenous tracer into RBCs in comparison with women in Group A + B (91.5 ± 28% vs. 76.4 ± 13.1%; $P < 0.02$).

RBC iron incorporation decreases during pregnancy due to increases in the size of the intravenous distribution pool and other physiological changes of pregnancy (Balfour et al., 1942). In this study, a significantly higher RBC iron incorporation (92%) was found in women who did not consume prenatal iron supplements, possibly related to the more limited iron reserves in this group.

**References**


Evaluation of Trace Mineral Status and Bioavailability Using Stable Isotopes (Zinc, Copper, Selenium, Molybdenum)

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Introduction

The essentiality of many of the trace minerals has been known for decades. Although required in the diet in very small amounts (less than 100 mg day⁻¹), a dietary deficiency can have serious consequences. Trace minerals play a crucial role in many body functions, serving as cofactors for enzymes, components of hormones and participants in oxidation–reduction reactions. They have both regulatory and structural functions, and those for copper, selenium and zinc are summarized in Table 5.1.

Table 5.1. A summary of the biochemical functions of zinc, copper and selenium.

<table>
<thead>
<tr>
<th>Trace element</th>
<th>Biochemical functions</th>
</tr>
</thead>
</table>
| Zinc          | Constituent of numerous metalloenzymes involved in protein synthesis, DNR/RNA synthesis, bone metabolism, dark adaptation, oxygen transport, protection against free radical damage  
|               | Gene expression (zinc fingers)  
|               | Structural role: hair, bone |
| Copper        | Constituent of metalloenzymes involved in synthesis of neurotransmitters, synthesis of connective tissue proteins, redox reactions, free radical scavenging  
|               | Constituent of caeruloplasmin, required for the transport of iron an acute phase protein |
| Selenium      | Antioxidant defence (glutathione peroxidase)  
|               | Thyroid hormone function; formation of active T3 |

The determination of trace mineral requirements depends on a number of assumptions regarding the bioavailability of the nutrient, the amount absorbed and the mineral status of the individual or population. All these factors are interrelated and are difficult to quantify using traditional mass balance techniques. In recent years, stable isotope methodologies have proved to be an invaluable tool in the quantification of these parameters.

The purpose of this chapter is to review the stable isotope methodologies that have been applied to the determination of trace mineral absorption, bioavailability and status. To do this we will focus on the trace minerals that have been most extensively studied using stable isotopes, namely zinc (Zn), copper (Cu) and selenium (Se).

**Importance of Trace Minerals in Human Nutrition and Evaluation of Status**

**Zinc**

The clinical consequences of severe zinc deficiency are easily identified and include bullous-pustular dermatitis, diarrhoea, alopecia, mental disturbances and impaired immune function (Prasad, 1991). Such symptoms are clearly demonstrated in patients with acrodematitis enteropathica, an autosomal recessive disorder affecting early infancy. The primary cause of the pathology of this condition is known to be severe zinc deficiency, resulting from a defect in the mechanism of zinc absorption. The condition is fatal if not treated (Moynahan, 1974).

Overt zinc deficiency resulting from nutritional inadequacies is rarely seen in developed countries; however, it is likely that subclinical zinc deficiency is more prevalent (Hambidge, 1989). A nationwide food consumption survey showed that approximately half of the adults in the USA consume less than two-thirds of the recommended zinc intake at that time (Murphy et al., 1992). The actual prevalence of zinc deficiency amongst populations cannot be assessed, however, because of a lack of definitive criteria for human zinc requirements. The determination of requirements is difficult, in part because the fraction of dietary zinc absorbed can vary considerably depending upon dietary zinc intake. A recent study of zinc homeostasis in a group of young men demonstrated that fractional zinc absorption increased from 0.26 to values approaching unity when dietary zinc levels were decreased from 12.2 mg day$^{-1}$ to 0.23 mg day$^{-1}$ (King et al., 2001).

Plasma or serum zinc concentration is the most widely used measure...
of zinc status, but the reliability of this index has been criticized (King, 1990). Some zinc-dependent enzymes, such as alkaline phosphatase, respond to a decrease in dietary zinc intake (Weisman and Høyer, 1985), and retinol-binding protein activity has been shown to respond to large changes in zinc status (Solomons, 1979). Although these can be useful as markers of severe deficiency, subclinical, marginal deficiency is more difficult to diagnose. Hair zinc concentration has been proposed as a useful index of zinc status in children (Hambidge et al., 1972). A correlation between hair and circulating zinc levels was reported following a study in Panamanian children (Klevay, 1970), although it can be argued that since hair growth slows down during zinc deficiency, hair at best can only give a historical reflection of status over a long period of time and does not necessarily reflect the current situation.

Unlike iron, zinc has no storage site in the human body. There are approximately 2.5–3 g of zinc in the human body, 57% of which is present in skeletal muscle (Jackson, 1988), but this zinc is not readily released in conditions of deficiency. It is hypothesized that there is a functional pool of zinc within the body that represents less than 10% of total body zinc. The zinc in this pool has a relatively rapid turnover rate, and once it becomes depleted, the biochemical consequences observed in zinc deficiency ensue (King, 1990). Stable isotope techniques, coupled with mathematical modelling, provide a means of quantifying this exchangeable pool of zinc, and are discussed in the section on ‘Isotope methodology – zinc’, this chapter.

Copper

The human body contains around 110 mg of copper (Linder, 1991) and its biochemical roles are summarized in Table 5.1. One of its most important functions is as a cofactor for lysyl oxidase, which is responsible for the formation of collagen cross-links in connective tissue and bone.

Primary copper deficiency is rare. Secondary copper deficiency may occur as a result of a number of factors including:

- Genetic factors (e.g. Menkes syndrome);
- Malabsorption syndromes (e.g. coeliac disease);
- Nutrient interactions (zinc/iron/ascorbic acid);
- Drugs and other chemicals that may alter copper metabolism (e.g. alcohol, diuretics) or chelate copper (e.g. D-penicillamine).

Clinical manifestations of copper deficiency include anaemia, neutropenia and bone abnormalities (Heller et al., 1978; Williams, 1983). Severe copper deficiency symptoms are observed in patients with Menkes
disease, an X-linked autosomal disorder of copper metabolism. A defect in the transport and intracellular metabolism of copper results in cellular copper deficiency despite a systemic excess of copper. Symptoms are well documented and include a characteristic alteration in the structure of hair, termed ‘steely hair’. Infants born with this condition may also display a failure to thrive, progressive cerebral degeneration, loss of skin and hair pigmentation, thrombosis and arterial rupture, and hypothermia. Sufferers seldom live beyond 4 years of age (Mercer, 1998).

The dietary recommendations for copper intake for adults range from 1.1 to 3 mg Cu day\(^{-1}\). A survey of diets from North America and Europe indicated that more than 30% of the diets provided less than 1 mg Cu day\(^{-1}\). Marginal copper deficiency is therefore likely to be widespread, particularly among the elderly (Thomas et al., 1988). The most commonly used index of copper status is plasma copper concentration. However, human copper deficiency studies indicate that this is not a reliable index of marginal copper deficiency (Milne, 1998). Copper in the plasma is bound primarily to caeruloplasmin, which is an acute-phase protein, and therefore the plasma copper concentration increases during the acute-phase response to stress. Plasma copper is also elevated in late pregnancy and in women taking oestrogen-based oral contraceptives and hormone-replacement therapy. Recent studies indicate that the activity of copper metalloenzymes present in blood cells, such as erythrocyte superoxide dismutase and platelet cytochrome c oxidase, may provide more useful indices of copper status (Milne, 1998).

Stable isotope studies of copper metabolism have been limited due to the existence of only two stable isotopes of copper, neither of which has a low natural abundance. However they have been used to provide information about copper absorption and bioavailability, including interactions with dietary components (see section on ‘Isotope methodology – copper’, this chapter).

**Selenium**

Selenium was recognized as an essential nutrient in animals in the late 1950s. Since then, the vital roles of selenium in human health have been identified, most notably in the activation of thyroid hormones and as a cofactor of glutathione peroxidase (Table 5.1). A large proportion of dietary selenium comes from plant foods. The levels of selenium in plants depends on selenium levels in the soil. Poor selenium status is endemic in areas of the world where soil levels are low, such as China, New Zealand and Finland (Combs, 2001). Primary selenium deficiency, known as Keshan disease, is characterized by a cardiomyopathy that primarily affects children and women of child-bearing age. The condition improves
markedly following selenium supplementation. However, the occurrence of the disease demonstrates a seasonal variation that cannot be explained by selenium status alone. It has been suggested that there may also be an infectious component to the disease (Levander and Burke, 1990).

In the UK, concern has been expressed at the fall in blood selenium levels in the population since the early 1990s. This fall coincides with a change in the major source of grain from the USA, where selenium levels in the soil are high, to Europe, where the soil levels are lower (Raymon, 1997). The clinical consequences of this fall in blood selenium levels are yet to be fully evaluated. There are two pools of selenium within the body. The selenocysteine pool, which is metabolically active, and the selenomethionine pool, which is considered to be a storage pool. The selenocysteine pool is tightly regulated and responds to changes in status, whereas the selenomethionine pool does not. The two pools are, however, connected such that when dietary selenium is restricted, the selenomethionine pool can slowly provide selenium to the selenocysteine pool for biochemical functions.

In addition to blood selenium levels, other biochemical indices of selenium status include glutathione peroxidase (GSHpx) activity and selenoprotein P concentration. GSHpx has been shown to be useful for the diagnosis of selenium deficiency, but the selenium requirement to achieve full expression of GSHpx activity is satisfied at intakes that are lower than those experienced by many population groups. An alternative approach to the determination of selenium status is to estimate the size of the exchangeable selenium pool using stable isotopes. This is discussed in the section on ‘Isotope methodology – selenium’, this chapter.

**Isotope Preparation for Human Use**

**Preparation as salts**

Isotopes can be purchased in the elemental, oxide, carbonate or chloride forms (Woodhouse and Abrams, 2000). The isotopes need to be converted into a soluble form for administration. Standard methods of achieving this have been described (Ducros et al., 1994; Lowe et al., 1997).

**Stability and sterility testing**

Stable isotope preparation for intravenous and oral use should, ideally, be performed by a licensed drug manufacturing unit with the facilities for
aseptic preparation. Preliminary work to establish the stability of the dose is required in order to assign an appropriate shelf-life for the product. Isotopes for intravenous (IV) use should then be tested for pyrogenicity in a clinical laboratory.

**Doses**

The dose of the isotope to be used depends on a number of factors including:

- The natural abundances of the enriched and reference isotopes.
- The mineral content expected in the samples to be analysed. For example, very little zinc is excreted in the urine, whereas urinary selenium levels are high.
- The fraction of the isotope dose expected in the samples. For faecal enrichment following an oral dose, knowledge of the expected absorption of the mineral is helpful, although for zinc and copper this depends on their dietary levels. For selenium, the fraction of an oral dose absorbed can be very high.
- The length of time detectable enrichment is required.
- The precision of the ratio measurement. This will depend on the instrument used for analysis (discussed in the section on Sample preparation and analysis, this chapter). It is common to set the detection limit for isotope ratio enrichment at three times the standard deviation of the baseline ratio (Sandstrom et al., 1993).

To prevent perturbation of normal tracee kinetics, the size of an intravenous enriched isotope dose should not exceed 10% of the total amount of tracee present in the plasma pool. Oral doses should not exceed the recommended daily intake of that mineral.

**Isotope Methodology – Zinc**

**Zinc stable isotopes**

There are five stable isotopes of zinc. These are given with their relative abundances in Table 5.2. The isotopes can be purchased at varying
levels of enrichment and, consequently, cost. The enrichment is an important factor when calculating tracer:tracee ratio (TTR) (see section on ‘Data interpretation’, this chapter), since the lower the enrichment of the isotope, the greater the contribution of the other isotopes to the dose. It is important that information on the presence of other isotopes should be obtained from the isotope supplier.

Table 5.2. The stable isotopes of zinc, copper and selenium.

<table>
<thead>
<tr>
<th>Isotope mass</th>
<th>Relative abundance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zinc</td>
<td></td>
</tr>
<tr>
<td>64</td>
<td>48.6</td>
</tr>
<tr>
<td>66</td>
<td>27.9</td>
</tr>
<tr>
<td>67</td>
<td>4.1</td>
</tr>
<tr>
<td>68</td>
<td>18.8</td>
</tr>
<tr>
<td>70</td>
<td>0.6</td>
</tr>
<tr>
<td>Copper</td>
<td></td>
</tr>
<tr>
<td>63</td>
<td>69.2</td>
</tr>
<tr>
<td>65</td>
<td>30.8</td>
</tr>
<tr>
<td>Selenium</td>
<td></td>
</tr>
<tr>
<td>74</td>
<td>0.9</td>
</tr>
<tr>
<td>76</td>
<td>9.0</td>
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<td>77</td>
<td>7.6</td>
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<td>78</td>
<td>23.6</td>
</tr>
<tr>
<td>80</td>
<td>49.7</td>
</tr>
<tr>
<td>82</td>
<td>9.2</td>
</tr>
</tbody>
</table>

Clinical protocols for absorption studies

There are a number of published methods for the determination of zinc absorption using one or two stable isotopes. Such isotope methods have an advantage over traditional mass balance methods as they enable some correction for absorbed, endogenously excreted zinc, which is not possible using simple balance.

Single-tracer techniques

Faecal monitoring is one of the most widely used techniques for the estimation of zinc absorption. It requires the oral administration of a single zinc isotope dose followed by complete stool collections for 10–12 days.
The isotope enrichment of the stools is used to calculate the fraction of the oral dose absorbed. A correction must be made, however, for the portion of the isotope dose that is absorbed and resecreted endogenously into the stools, which contributes to the stool enrichment. This can be done using a simple plot of the percentage of oral dose in the faeces with time (English et al., 1989), or using a technique involving the intravenous administration of a second stable zinc isotope (Rauscher and Fairweather-Tait, 1997).

A new single isotope tracer technique has recently been proposed (Yeung et al., 2000). This method requires oral administration of a stable zinc isotope and measurement of urinary isotope enrichment.

**Dual-tracer techniques**

There are two methods that require the simultaneous administration of two different stable isotopes of zinc. One isotope is administered intravenously and the other orally. Plasma isotope enrichment can then be analysed using deconvolution analysis (Yergey, 1994). This technique requires frequent sampling over a period of several days. An alternative method, the so-called ‘double isotopic tracer technique’ (DITR) is a less invasive technique (Lowe et al., 2000). Fractional zinc absorption can be determined from the isotope enrichment of a single plasma or urine sample taken 3 or more days after isotope administration (Friel et al., 1992).

**Clinical protocols for kinetic studies**

Data derived from kinetic studies of zinc metabolism are usually analysed using some kind of mathematical modelling technique (see section on ‘Data interpretation’, this chapter). The complexity of the intended analytical tool will, to a large extent, determine the complexity of the clinical protocol.

In order to design a protocol for the study of zinc kinetics, it is important to understand some of the basics of zinc metabolism. Following a bolus intravenous isotope infusion, zinc is rapidly cleared by the liver (Fig. 5.1a). Therefore, to define accurately the early tracer kinetics, sampling needs to be frequent during the first 30 min post-infusion. The main excretory route of zinc is via the endogenous secretions into the gastrointestinal (GI) tract, very little zinc being excreted in the urine. Figure 5.1a–c gives examples of plasma and faecal enrichment curves following oral (⁶⁷Zn) and intravenous (⁷⁰Zn) administration of stable isotope tracers.
Fig. 5.1. (a) Typical response curve of tracer : tracee ratio measured in the plasma following an intravenous dose of 0.4 mg $^{70}\text{Zn}$. Inset is to show the early time points (0–8 h) in more detail. (b) Typical response curve of tracer : tracee ratio measured in the plasma following an oral dose of 1.3 mg $^{67}\text{Zn}$. (c) Typical cumulative faecal excretion pattern of an orally administered dose of 1.3 mg $^{67}\text{Zn}$, expressed as a percentage of the dose.
Isotope Methodology – Copper

Clinical protocols for kinetic studies of copper

Since neither of the two stable isotopes of copper have a low natural abundance, relatively high doses are required in order to achieve enrichment over background (Table 5.2). The primary excretory route for copper is into the GI tract via the bile. Newly absorbed or intravenously infused copper is rapidly cleared by the liver and incorporated into caeruloplasmin before re-entering the blood plasma pool. This explains the reappearance of an intravenously administered isotope dose at 24–48 h post-administration shown in Fig. 5.2a. Figure 5.2b shows a typical plasma enrichment response to an oral dose of $^{65}$Cu.

Clinical protocols for copper absorption studies

Because of the existence of only one minor copper isotope, a simultaneous dual-tracer method is not possible. Therefore, the only tracer method currently available for measuring copper absorption is faecal monitoring after an oral dose. An example of a cumulative faecal excretion pattern of orally administered $^{65}$Cu is shown in Fig. 5.2c. Following an oral dose of $^{65}$Cu, complete stool collections are made for 5–10 days. In an attempt to shorten the faecal collection time, the use of rare-earth elements such as dysprosium as faecal markers has been suggested (Schuette et al., 1993). These elements are not absorbed and therefore excretion should equal 100%. Rare-earth elements are administered simultaneously with the oral isotope dose. The rationale is that a shortened collection time (3 days) can be used, by measuring the amount of rare earth element retrieved. The percentage retrieved should be equal to the percentage of the total amount of isotope that would be retrieved if collection were continued to completeness. This approach appears to be successful for some elements including iron, zinc and magnesium (Fairweather-Tait et al., 1997). This technique relies on parallel excretion kinetics of the rare-earth element and the mineral of interest, which does not appear to be the case for dysprosium and copper (Schuette et al., 1993); however, alternative rare-earth elements, such as holmium, may follow the transit of copper through the gut more accurately.
Fig 5.2. (a) Typical response curve of tracer: tracee ratio measured in the plasma following an intravenous dose of 0.8 mg $^{65}$Cu. Inset is to show the early time points (0–6 h) in more detail. (b) Typical response curve of tracer: tracee ratio measured in the plasma following an oral dose of 2.7 mg $^{65}$Cu. (c) Typical cumulative faecal excretion pattern of an orally administered dose of 2.7 mg $^{65}$Cu, expressed as a percentage of the dose.
Isotope Methodology – Selenium

Clinical protocols for kinetic studies

There are six naturally occurring stable isotopes of selenium, five of which have a relatively low natural abundance (Table 5.2). Although selenium status can be determined using straightforward non-isotopic techniques, stable isotopes of selenium have been used to estimate the size of the functional selenium pool as an index of functional body selenium status. Two techniques have been used: an in vivo isotope dilution technique (Janghorbani et al., 1991) and a simplified two-compartment model (Ducros et al., 1997). Janghorbani et al. (1991) described an isotope dilution method to estimate the size of the functional selenium pool, which they defined as the selenite-exchangeable metabolic pool (Se-EMP). This pool does not include selenomethionine-bound selenium, which is considered to be a storage pool and with which the isotope does not exchange over the time period studied. The protocol involved administration of an intravenous dose of 82 µg of $^{74}$Se in 500 ml of 0.9% saline as a constant infusion over a 4 h period. Twenty-four-hour urine collections were made for the following 14 days. Measurement of the urinary enrichment of the stable isotope coupled with a knowledge of the isotope retention (determined from the cumulative urinary excretion) at a given time post-isotope dose can be used to calculate the size of the Se-EMP. The mean size of this pool in a group of four subjects, determined 13 days after isotope administration, was 6.32 mg (± 0.20). The study demonstrated that the size of this pool responded to changes in dietary selenium intake, falling in response to dietary selenium restriction. An alternative technique of measuring the functional selenium pool was proposed by Ducros et al. (1994). The protocol involved administration of a bolus intravenous injection of 100 µg $^{74}$Se. Plasma isotope enrichment was measured at specific time points up to 6 months after isotope dosing. The plasma isotope disappearance curve could be described mathematically using an equation composed of two exponential terms, and was therefore analysed using a simple two-compartment model. The first compartment was 0.84 mg with a half-life of 1 day; the second was 2.47 mg with a half-life of 30 days. They demonstrated a significant correlation between plasma selenium level and the sum of the masses of selenium in the two pools ($r = 0.66$, $P < 0.01$). An example of a plasma response to an intravenous dose of $^{74}$Se is shown in Fig. 5.3.

Clinical protocols for selenium absorption studies

Stable isotopes have been used extensively to examine the bioavailability of selenium from foods, by faecal monitoring or by dual-isotope ratio
measurements in urine (Fairweather-Tait et al., 2000). There are various chemical forms of selenium in food that affect its bioavailability. The two major organic forms, selenomethionine and selenocysteine, are the most abundant forms of selenium in food. These are absorbed via active transport mechanisms (Young et al., 1982). The inorganic forms, selenate (SeO$_4^{2-}$) and selenite (SeO$_3^{2-}$), are absorbed by sodium-mediated carrier transport and simple diffusion, respectively (Vendeland et al., 1992). The chemical form of the stable-isotope tracer used in a metabolic study will therefore be representative only of that dietary form of selenium (Fairweather-Tait et al., 2000), although intrinsically labelled foods have provided valuable information on selenium absorption from dietary sources (Sirichakwal et al., 1985; Finly, 1999).

Faecal monitoring can be used to estimate selenium absorption. As very little selenium is secreted into the GI tract, there is no need to correct the isotope excretion for endogenous losses. Oral doses of 100 µg of enriched selenium isotope have been used successfully in studies examining the absorption of selenate and selenite in adults (Van Dael et al., 2001). Collection of faecal samples should continue for at least 7 days post-dose, and a non-absorbable rare-earth element can
be used as a marker for complete stool recovery (Fairweather-Tait et al., 2000).

**Sample Preparation and Analysis**

Recent advances in inductively coupled plasma mass spectroscopy (ICP-MS) technology have improved the sensitivity and precision of this technique. The recently released ‘third generation’ ICP-MS instruments have overcome some of the problems of their predecessors, such as instability, intolerance to total dissolved solids and poor reliability. This, coupled with the rapid sample throughput and improved software interfaces, has meant that ICP-MS has become a relatively widely available and popular analytical tool for the measurement of stable isotope ratios in nutrition research. Samples are introduced through a nebulizer into a high-temperature argon plasma where solids are volatized and ionized. The ions are separated by mass using a quadrupole mass spectrometer. Isotope ratio precision can be further improved by the separation of ions using a magnetic field, as implemented by the latest magnetic sector ICP-MS. These instruments are reported to have higher accuracy and precision for stable isotope analysis than quadrupole, comparable to that of thermal ionization mass spectroscopy (TIMS) but with a much faster rate of sample throughput (Barnes, 1998; Woodhouse and Abrams, 2000).

The user-friendly software interface brings instrument operation and data acquisition within the realms of the non-analytical chemist. While this has obvious advantages, a note of caution is required for the interpretation of the data generated. Knowledge of the likely sources of interference from the sample matrix is essential. The greatest source of potential error in quadrupole ICP-MS is the occurrence of polyatomic species that are isobaric with the isotopes of the element of interest. Polyatomic ions arising from biological materials often include combinations of calcium, cadmium, chlorine, sulphur, oxygen, phosphorus, potassium and sodium (Barnes, 1998). Spectral overlaps by polyatomic species from the sample matrix and argon plasma background are particularly problematic for certain isotopes of copper and selenium. In the case of copper, the formation of the sodium–argon dimer at mass 63 is a major problem. This can be overcome by introducing a desalting step into the sample preparation procedure. This can be done by size-exclusion chromatography (Lyon et al., 1996).

The quadrupole instrument unit mass resolution is unable to resolve the most abundant isotope of selenium at mass 80 because of the formation of argon dimers resulting in isobaric interference. Where isotope
ratio is the desired endpoint, choosing alternative isotopes for the reference is a simple way around this.

Complex sample preparation procedures have been developed for the purification of individual elements for stable isotope ratio analysis. For plasma and faecal zinc and copper stable isotope enrichment determination, the organic matrix should be removed. Microwave digestion using ultrapure concentrated nitric acid in sealed teflon vessels ensures minimal contamination from exogenous sources, although alternative techniques include the use of a low-temperature asher (Turnlund and Keyes, 1990) or muffle furnace (Sian et al., 1996). Zinc and copper can be individually isolated from the digest by ion-exchange chromatography (Turnlund and Keyes, 1990). Inorganic salts present in urine samples can be removed using a chelating resin and the zinc and copper isolated from the sample by ion-exchange chromatography (Lowe et al., 2000).

Selenium stable isotope enrichment determination in plasma and urine can be carried out using a simple method that does not require any labour-intensive digestion of the sample matrix. This method adopts the Evans and Ebdon (1989) and Delves and Sieniawska (1997) approach of simply diluting the sample in the presence of a simple organic molecule. A simple 1:20 dilution of 200 µl volumes of serum or urine with a diluent containing 1% nitric acid, 0.5% butan-1-ol and 0.1% Triton X-100 enables accurate and precise measurements, as well as allowing long analytical runs without blockage of the torch. The butan-1-ol is present to reduce interferences from argon-adduct ions on selenium isotopes $^{77}$Se and $^{82}$Se, and the Triton X-100 helps to prevent blockage of the system and improve the nebulizer efficiency.

**Data Interpretation**

**Tracer : tracee ratio**

The mathematical treatment of isotope ratio data to obtain information such as fractional absorption was originally formulated using radioisotope data. The fundamental difference between radioisotope ratios and stable isotope ratios is that the radioisotope dose given is a true tracer in that there is normally no naturally occurring radioisotope in the system until the dose is administered, and therefore the isotope ratio measured is a true representation of the ratio of tracer to tracee present in the sample. In contrast, stable isotope ratios determined by mass spectrometry represent the ratio of total amounts of the ‘tracer’ isotope to the total amount of the reference isotopes. The measured tracer also contains
a contribution of 'cold' isotope, i.e. isotope that was already present in the sample according to its natural abundance. In addition, there will be a small contribution to the reference isotope from the dose administered. For example, an isotope that is highly enriched in $^{70}$Zn, for example to 90%, still contains 10% that is composed of the other isotopes. The relative abundances of these should be supplied with the isotope when purchased. Therefore, the measured isotope ratios need to be corrected for the presence of these in order to determine the true tracer:tracee ratio. Mathematical equations to make this correction have been published elsewhere (Buckley et al., 1985; Toffolo et al., 2000).

**Measurement error**

The estimation of model parameter values from data requires the assignment of statistical weights in the fitting process. The assigned weights should reflect the statistical uncertainty of the data. Although a number of factors contribute to data uncertainty, sample measurement error is relatively easily quantified (Janghorbani and Ting, 1990). When mixed methodologies are employed in the same modelling investigation, the relative size of measurement errors among the methods becomes important. For example, when measurements of tracee mass are used together with tracer data in model fitting, measurement errors together with replicate sample numbers permit the appropriate assignment of statistical weights to the two types of measurements (Ramberg et al., 1992).

**Compartmental models**

At its most basic level, a compartment model is a mathematical description of the relationship between two or more parameters. In the context of mineral metabolism, the design of the model is based on both experimental data and knowledge of the physiology of the system, and represents a working hypothesis of how the system behaves under the conditions in which the experiment was conducted. The mathematics enabling the calculation of the model parameters (mineral flux, turnover rate, compartment mass) is complex (Shipley and Clark, 1972), but a number of computer software programs have been developed to aid in the construction of compartmental models. One of the most widely used is SAAM/CONSAM (Foster and Boston, 1983), which has been updated to a more user-friendly program, SAAM II (SAAM Institute, Seattle,
Washington, USA). Multi compartmental models of zinc, copper and selenium have been developed using stable isotopes (Patterson et al., 1989; Scott and Turnlund, 1994a; Lowe et al., 1997). These models have provided valuable insights into trace mineral metabolism and homeostasis and have been used to investigate metabolic responses to changes in dietary intake (Scott and Turnlund, 1994b; King et al., 2001). In addition, a multi-compartment model of zinc metabolism has been used to compare methods for the determination of fractional zinc absorption (FZA) (Lowe et al., 2000) and to simulate data for the mathematical analysis of more simple techniques for the estimation of FZA (Shames et al., 2001).

In order to obtain sufficient data to develop a multi-compartment model, the clinical protocol may involve frequent sampling, complete urine and faecal collections and the use of more than one isotope tracer. Such a protocol is labour-intensive and is reliant on subject compliance; it can also be costly as it may require the subjects to be confined to a metabolic unit for a substantial period of time.

**Use of simplified models**

Simplified models require less data and the clinical protocols are less invasive. Such models can be useful tools in the determination of trace mineral status, for example the simple two-compartment model of selenium discussed above (Ducros et al., 1997). Another example is the technique described by Miller et al. (1994) to measure the exchangeable zinc pool. This approach lumps together the three most rapidly exchanging zinc pools defined by the more complex compartmental models. The size and turnover rate of this combined pool, termed the exchangeable zinc pool (EZP), can be estimated from the linear regression of a semi-log plot of plasma or urine isotope enrichment measured 3–9 days following an intravenous stable isotope dose. Although this simple model overestimates the true size of the combined pools, the study demonstrated that the estimated EZP mass responded to changes in dietary zinc intake and therefore has potential as an indicator of zinc status.

**Other trace minerals**

While this and the previous chapter have focused on the minerals most frequently studied by stable isotope techniques, it is worth mentioning
here that stable isotopes of molybdenum have also been used as tools for the investigation of molybdenum bioavailability and metabolism. Molybdenum is an essential trace mineral, acting as a cofactor for a number of enzymes including xanthine oxidase and aldehyde oxidase. There are seven stable isotopes of molybdenum: $^{92}$Mo (natural abundance 14.8%), $^{94}$Mo (9.3%), $^{95}$Mo (15.9%), $^{96}$Mo (16.7%), $^{97}$Mo (9.6%), $^{98}$Mo (24.1%), and $^{100}$Mo (9.6%). Using isotopically labelled foodstuffs (wheat, kale and soybeans) and measurements of isotope excretion, Turnlund et al. (1999) determined molybdenum absorption in a group of young women to be between 60 and 90%. Cantone et al. (1997) used plasma isotope enrichment, analysed by a simple two-compartment model, to calculate molybdenum absorption following an oral dose of either $^{95}$Mo or $^{96}$Mo. The 1 mg isotope doses were given on an empty stomach, and the calculated value of fractional molybdenum absorption from two volunteers was 0.53 and 0.57 (Cantone et al., 1993). A more complex compartmental model of molybdenum metabolism has been developed from both intravenously and orally administered isotopes in young men (Thompson and Turnlund, 1996).

**Clinical example**

One of the most interesting and clinically relevant ways in which stable isotopes have been used is in the differential diagnosis of Wilson’s disease (Lyon et al., 1995). Wilson’s disease is an inherited disorder characterized by a toxic accumulation of copper in the liver, brain and kidney. The condition results from the mutation of a gene that encodes a copper-transporting ATPase, which may cause a reduction in copper excretion via the bile and incorporation of copper into caeruloplasmin. The condition is fatal unless treated, therefore accurate and early diagnosis is vital. Lyon et al. (1995) described a technique using the stable isotope $^{65}$Cu to identify abnormalities of copper metabolism characteristic of Wilson’s disease. After an overnight fast, patients were given an oral dose of 3 mg $^{65}$Cu (2 mg for children). Blood samples were taken at specific time intervals for 72 h and the serum isotope enrichment determined by ICP-MS. Analysis of the $^{65}$Cu enrichment–time curve revealed that in patients with Wilson’s disease, the secondary rise in isotope enrichment seen in healthy subjects was absent, indicating a failure to incorporate $^{65}$Cu into caeruloplasmin. Interestingly, heterozygotes for the condition fell into three categories: those with a definite secondary $^{65}$Cu rise at 72 h, those with an impaired incorporation, and one patient with a complete failure to incorporate $^{65}$Cu. In addition, the heterozygotes demonstrated a significantly increased biological half-time of 43 days for the
clearance of $^{65}$Cu from the plasma pool compared with 18.5 days for control subjects.

## Concluding Remarks

Since stable isotopes were first used as probes for studies of mineral metabolism in 1963 (Lowman and Krivit, 1963), their significant contribution to the quantification of trace mineral absorption and to the understanding of the trace mineral metabolism has been unequivocal. Most work has been done on mineral absorption. However, with the introduction of user-friendly software to aid data analysis and computer modelling, advances have been made in the determination of mineral status by measuring exchangeable pools and compartmental modelling.

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Energy Utilization with Doubly Labelled Water ($\text{H}_2\text{O}^{18}$)

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Introduction

Energy is required for muscular activity, growth, reproduction and synthesis of metabolites such as proteins, fatty acids, nucleic acids and steroids, which are essential to maintain basal metabolic functions as well as optimal growth and development. Numerous methods, such as the food record, $[^{13}\text{C}]$bicarbonate infusion and indirect calorimetry, have been used to estimate energy expenditure (EE) in humans. The food record seldom reflects the true caloric content of ethnic foods and this procedure does not work well with children. It is also well documented that overweight individuals often under-report their food intake (Schoeller et al., 1990). The $[^{13}\text{C}]$bicarbonate infusion method is invasive and of short duration (< 24 h). Activity of the subject is restricted during the infusion. Therefore, EE measured by this method is not representative of the true daily EE of the free-living subject. Whole-room calorimetry is considered the gold standard for measuring EE in humans. Although the subject is free to move around in the calorimetric chamber, spontaneous physical activity is greatly reduced. Furthermore, the measurement is carried out under strictly controlled, artificial environmental conditions and often is of short duration (< 24 h). However, indirect calorimetry does provide important information about the basal metabolic rate, respiratory quotient, sedentary EE and sleeping metabolic rate.

The doubly labelled water ($\text{H}_2\text{O}^{18}$) method yields an average EE for a period of 5–14 days. The procedure is non-invasive, non-restrictive and reflective of actual EE under free-living conditions.

The purpose of this chapter is to describe the theory of the $^{2}\text{H}_2^{18}\text{O}$ method for the estimation of daily EE under free-living conditions, its assumptions, the analytical procedures and the equations used in its calculation.

**What is $^{2}\text{H}_2^{18}\text{O}$?**

The $^2\text{H}$ and $^{18}\text{O}$ are non-radioactive isotopes of hydrogen and oxygen. Hydrogen has two stable isotopes, $^1\text{H}$ and $^{2}\text{H}$. Oxygen has three stable isotopes, $^{16}\text{O}$, $^{17}\text{O}$ and $^{18}\text{O}$. The natural abundances of these stable hydrogen and oxygen isotopes are summarized in Table 6.1.

<table>
<thead>
<tr>
<th>Element</th>
<th>Isotope</th>
<th>Abundance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrogen</td>
<td>$^1\text{H}$</td>
<td>99.9844</td>
</tr>
<tr>
<td></td>
<td>$^2\text{H}$</td>
<td>0.0156</td>
</tr>
<tr>
<td>Oxygen</td>
<td>$^{16}\text{O}$</td>
<td>99.763</td>
</tr>
<tr>
<td></td>
<td>$^{17}\text{O}$</td>
<td>0.375</td>
</tr>
<tr>
<td></td>
<td>$^{18}\text{O}$</td>
<td>0.1995</td>
</tr>
</tbody>
</table>

The heavier stable isotopes of both hydrogen and oxygen are found in the human body and in the food and water we consume every day. The average daily intake and the amounts of these isotopes in a 50 kg adult are shown in Table 6.2. Therefore, the ingestion of $^{2}\text{H}_2^{18}\text{O}$ in the dosages routinely used in the estimation of daily EE is considered safe. In fact, the $^{2}\text{H}_2^{18}\text{O}$ method has been used extensively in studies with premature infants, newborns, children, adolescents, adults and pregnant women.

**Theory of the $^{2}\text{H}_2^{18}\text{O}$ Method**

The possibility of using the $^{2}\text{H}_2^{18}\text{O}$ method to estimate daily EE was first recognized by Lifson *et al.* (1949) and subsequently demonstrated and validated in small animal studies (Lifson and McClintock, 1966). Following oral ingestion of $^{2}\text{H}_2^{18}\text{O}$, the isotopes are rapidly distributed in body water (Fig. 6.1). With carbonic anhydrase, $^{18}\text{O}$ in body water also
rapidly reaches isotopic equilibrium with the bicarbonate or carbon dioxide (CO₂) in the body. As shown in Fig. 6.2, the rate of disappearance of deuterium (²H) from the body therefore reflects water turnover, whereas the rate of disappearance of ¹⁸O represents water turnover as well as CO₂ production (rCO₂).

Mathematically, water turnover (rH₂O) and rCO₂ can be presented as follows:

\[ r_{H_2O} = N \times k_H \]  
\[ r_{H_2O} + (2 \times r_{CO2}) = N \times k_O \]

where \( N \) is total body water and \( k_H \) and \( k_O \) are the fractional turnover rates of ²H and ¹⁸O as measured in the body fluid, respectively. Substituting \( r_{H_2O} \) from Equation (1) into Equation (2) and solving for \( r_{CO2} \) yields the classical Lifson equation (Equation 3) for calculating

### Table 6.2. Daily intakes and body content ²H and ¹⁸O.

<table>
<thead>
<tr>
<th>Isotopes</th>
<th>Intake (mg kg⁻¹ day)</th>
<th>Body content (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>²H</td>
<td>6.9</td>
<td>1.5</td>
</tr>
<tr>
<td>¹⁸O</td>
<td>133.4</td>
<td>68.6</td>
</tr>
</tbody>
</table>

Fig. 6.1. A schematic diagram showing the introduction of ²H₂¹⁸O into the body water and the distribution of ²H and ¹⁸O in water losses and ¹⁸O in expired CO₂.
\[ r_{\text{CO}_2} = 0.5 \times N \times (k_O - k_H) \] (3)

**Assumptions of the $^2\text{H}_2^{18}\text{O}$ Method**

**Constant body water pool sizes**

During the study period, the $^2\text{H}_2^{18}\text{O}$ method assumes no changes in body water pool sizes. This assumption might be appropriate for adults of stable weight, but would not be appropriate for growing infants, children or adolescents. With appropriate corrections for changes in body water pool sizes, daily EE of 1- and 4-month-old formula-fed full-term infants (Wong et al., 1990) and of premature infants (Roberts et al., 1986; Jensen et al., 1992) estimated by the $^2\text{H}_2^{18}\text{O}$ method has been shown to agree within 1% of the energy balance values in the 1- and 4-month-old infants and by indirect calorimetry in the premature infants.
Constant H$_2$O and CO$_2$ fluxes

In weight-stable adults and healthy subjects, the H$_2$O and CO$_2$ fluxes are relatively constant and daily EE estimated by the $^2$H$_2$$^{18}$O method has been shown to be within 2% of the calorimetric values (Klein et al., 1984; Coward et al., 1988; Schoeller, 1988). However, in subjects with acute and chronic illnesses, in subjects recovering from surgery, or in subjects undergoing exhaustive exercise, this assumption could be violated. However, even when H$_2$O and CO$_2$ fluxes are not constant, such as during recovery from surgery (Jones et al., 1988) and heavy exercise (Stein et al., 1987), the isotope method still agrees within 10% of the calorimetric values.

No sequestration of $^2$H and $^{18}$O in metabolites other than H$_2$O and CO$_2$

Sequestration of $^{18}$O is negligible. However, it is documented that $^2$H in body water is incorporated into cholesterol (Wong et al., 1993a) and fat during biosynthesis. Therefore, the assumption that there is no sequestration of $^2$H into metabolites other than H$_2$O and CO$_2$ is not valid. However, except under conditions of excessive lipogenesis or a high rate of weight gain (> 100 g day$^{-1}$), isotope sequestration in humans results in less than 1% error in the daily EE estimate (Haggarty et al., 1991).

H$_2$O and CO$_2$ leaving the body are isotopically identical to body water

Because more energy is required to change liquid H$_2$O containing $^2$H and $^{18}$O to H$_2$O vapour than liquid H$_2$O containing $^1$H and $^{16}$O, at equilibrium, H$_2$O vapour contains 5.5% fewer $^2$H and 1% fewer $^{18}$O than liquid H$_2$O (Halliday and Miller, 1977; Pflug et al., 1979; Schoeller et al., 1986; Wong et al., 1988). When CO$_2$ is allowed to come in contact with liquid H$_2$O, isotope exchange between the oxygen atoms of the CO$_2$ and H$_2$O will take place. If the reaction is allowed to finish, the CO$_2$ will end up with 3.9% more $^{18}$O than the liquid H$_2$O. Therefore, H$_2$O vapour and CO$_2$ leaving the body are not isotopically identical to body water. However, the classical Lifson’s equation (Equation 3) can be modified to correct for the isotope discrimination that takes place during evaporation and during isotope exchange between CO$_2$ and H$_2$O.
No re-entry of the labelled H$_2$O and CO$_2$ into the body

Re-entry of the labelled H$_2$O and CO$_2$ into the body is possible in small animals living in burrows. In humans, re-entry of the labelled H$_2$O and CO$_2$ is most likely when premature infants are cared for using incubators. However, daily EE of premature infants in incubators estimated by the isotope method has been shown to be within 1% of the calorimetric estimates (Roberts et al., 1986; Jensen et al., 1992). Therefore, re-entry of the labelled H$_2$O and CO$_2$ in humans is not likely to affect the accuracy of the isotope method for estimation of EE in free-living subjects.

Equations used in the $^2$H$_2^{18}$O method

Because of the many violations of the assumptions associated with the $^2$H$_2^{18}$O method, the classical Lifson equation has subsequently been modified to account for the differences in isotope dilution spaces of $^2$H ($N_H$) and $^{18}$O ($N_O$), the changes in body pool sizes during growth, and isotope discrimination during evaporation and equilibration. With these corrections, rCO$_2$ is calculated as follows:

$$rCO_2 \text{ (mol day}^{-1}) = \alpha \times ((k_O \times N_O) - (k_H \times N_H))$$

(4)

where $\alpha$ is the correction factor for isotope fractionation and insensible water loss. The constant, $\alpha$, has a value of 0.4556 for infants and 0.4584 for adolescents and adults.

The isotope dilution spaces ($N_H$, $N_O$) are calculated from the dose mixture of $^2$H$_2^{18}$O ($d$) in g given to the subject and the rise in $^2$H or $^{18}$O abundance in the physiological fluid ($E_a$) at time zero using the extrapolation method or at equilibration using the plateau method as follows:

$$N_H \text{ or } N_O \text{ (mol)} = (d \times A \times E_a) / (a \times E_a \times k)$$

(5)

where $A$ is the amount of laboratory water in g used in the dilution of $a$ g of the dose mixture; $E_a$ is the rise in $^2$H or $^{18}$O abundance in the laboratory water after the addition of the dose mixture; $E_a$ is the rise in $^2$H or $^{18}$O abundance in the physiological fluid at time zero or at equilibration; and $k$ is a constant to convert g of H$_2$O to moles and has a value of 18.02.

The rCO$_2$ is converted to EE using the Weir equation (de V. Weir, 1949):

$$EE \text{ (kcal day}^{-1}) = 22.4 \times ((1.106 \times rCO_2) + (3.941 \times rO_2))$$

(6)
where \( rO_2 \) is the oxygen consumption in moles per day and is calculated from the respiratory quotient (RQ) using the relationship:

\[
RQ = \frac{rCO_2}{rO_2}
\]  

(7)

The RQ can be measured by indirect calorimetry or a population RQ can be used. Otherwise, a food quotient (Black et al., 1986) calculated from dietary intakes, with appropriate corrections for changes in body composition, can be used in place of RQ.

### Analytical Methods

#### Dosages of \( ^2H_2^{18}O \)

In most human studies, the isotopes are taken orally. For intravenous administration, the isotopic water can be ultrafiltered for the removal of pyrogen and bacterial contamination (Wong et al., 1991). The \( H_2^{18}O \) at 10 atom % \( ^{18}O \) is suitable for studies in older infants, children and adults. For premature infants and full-term newborns, \( H_2^{18}O \) at 50–95 atom % \( ^{18}O \) is preferred in order to minimize the volume of isotopic water administered. It is crucial to know the exact quantity of \( ^2H_2^{18}O \) given to the volunteer. For oral administration, the bottle containing the isotopic water should be rinsed twice with drinking water, infant formula, breast milk or other suitable dietary fluids. For intravenous doses, the exact weight of the syringe before and after administration of the isotopic water must be determined, or the infusion system must be flushed with saline solution to ensure complete administration.

The optimal dosages of \( ^2H_2^{18}O \) and the duration of the study to obtain the most accurate and precise EE measurements have been described in detail (Schoeller, 1983). The theoretical optimal doses are 0.3 g \( H_2^{18}O \) kg\(^{-1}\) total body water (TBW) and 0.12 g \( ^2H_2O \) kg\(^{-1}\) TBW for all subjects except neonates. For neonates, the optimal doses are 0.4 g \( H_2^{18}O \) kg\(^{-1}\) TBW and 0.16 g \( ^2H_2O \) kg\(^{-1}\) TBW. For adults, TBW can be estimated from body weight assuming approximately 55% of body weight is TBW. For premature infants, older infants, children and youths, TBW can be estimated from body weight using the approximate percentage of TBW relative to body weight as shown in Table 6.3.

The optimal metabolic periods are 3–14 days in neonates and children and 5–28 days in youths and adults (Schoeller, 1983). These metabolic periods represent one and a half to three half-lives of the \( ^2H \) and \( ^{18}O \) isotopes in the respective study groups in order to maintain analytical
precision of the isotope ratio measurements. In practice, 5 days should be sufficient for neonates and 10–14 days should be sufficient for children, adolescents and adults.

Sources of $^{2}\text{H}^{18}\text{O}$

Deuterium oxide at 99.8 atom % $^{2}\text{H}$ and $^{2}\text{H}^{18}\text{O}$ at 10–97 atom % $^{18}\text{O}$ can be purchased from vendors such as Isotec Inc. or Cambridge Isotope Laboratories.

Mass spectrometric analyses

The first gas isotope-ratio mass spectrometer (GIRMS) was built in 1940 (Nier, 1940). The GIRMS instrument consisted of three major components: an ion source, a permanent magnet and a single detector. The vacuum of the mass spectrometer was maintained with a two-stage mercury diffusion pump. The gas sample was introduced into the ion source of the mass spectrometer through a capillary leak in order to eliminate isotope fractionation during the passage of the gas sample from the sample inlet to the ion source. All samples introduced into a GIRMS instrument must be in a gaseous form. For measurement of deuterium abundance, $\text{H}_2$ is used. For oxygen abundance measurements, $\text{CO}_2$ is used. The gas molecules are ionized with electrons in the ion source and

<table>
<thead>
<tr>
<th>Age group</th>
<th>Body weight or age</th>
<th>Total body water (% of body weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preterm$^a$</td>
<td>&lt; 700 g</td>
<td>86</td>
</tr>
<tr>
<td>1000–2000 g</td>
<td>82</td>
<td></td>
</tr>
<tr>
<td>Infants$^b$</td>
<td>At birth</td>
<td>69</td>
</tr>
<tr>
<td>1–24 months</td>
<td>62</td>
<td></td>
</tr>
<tr>
<td>3–10 years</td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>Youths$^c$</td>
<td>11–17 years</td>
<td>56</td>
</tr>
</tbody>
</table>

$^a$Ziegler et al. (1976).
$^b$Fomon et al. (1982).
$^c$Ellis et al. (2000).
the positively charged molecules are propelled by an accelerating potential into the magnetic field where they are resolved into separate ion beams according to their masses. With \( \text{H}_2 \) gas, two charged molecules with masses of 2 (\( ^1\text{H}^1\text{H}^+ \)) and 3 (\( ^2\text{H}^1\text{H}^+ \)) are produced. For \( \text{CO}_2 \), three positively charged \( \text{CO}_2 \) molecules with masses of 44 (\( ^{12}\text{C}^{16}\text{O}^+ \)), 45 (\( ^{13}\text{C}^{16}\text{O}^+ \)) and 46 (\( ^{12}\text{C}^{16}\text{O}^{18}\text{O}^+ \)) are formed. Because the earlier Nier mass spectrometer had only one collector, each positively charged molecule was focused alternately on to the collector by adjusting the ion accelerating voltage. A dual-collector system (Nier, 1947; Nier et al., 1947) was subsequently developed for simultaneous measurements of the ion currents of two isotopic masses. To maximize precision of isotope ratio measurement, a dual inlet system (McKinney et al., 1950) was later incorporated into the Nier-type mass spectrometer for alternate introduction of a sample and a standard gas into the mass spectrometer.

The basic design of the Nier–McKinney-type mass spectrometer persists in GIRMS today. A schematic diagram showing the general configuration of a modern GIRMS is shown in Fig. 6.3. With advances in electronic, vacuum and computer technologies, the entire process of valve sequencing, pressure matching, ion source tuning and focusing, vacuum monitoring, data collection and data analysis is controlled entirely by a computer. Up to six separate collectors are now available on a GIRMS instrument for simultaneous measurements of \( ^{12}\text{C}^{16}\text{O}^+ \), \( ^{13}\text{C}^{16}\text{O}^+ \), \( ^{12}\text{C}^{16}\text{O}^{18}\text{O}^+ \), \( ^{14}\text{N}^{15}\text{N}^+ \) and \( ^{15}\text{N}^{15}\text{N}^+ \). With a split-flight tube design and a separate dual collector system, a modern GIRMS instrument can measure the stable isotopic abundance of hydrogen, carbon, oxygen and nitrogen.

**Sample collection**

Any physiological fluid such as plasma, saliva, urine or breath water vapour can be collected for the determination of fractional turnover rates of \( ^2\text{H} \) and \( ^{18}\text{O} \) and \( \text{N}_\text{H} \) and \( \text{N}_\text{O} \). In most human studies, urine is the preferred sample because it is non-invasive and the easiest to collect. In infants, urine can be collected using cotton balls (Wong et al., 1993b). The urine absorbed by the cotton balls can be expressed with a plastic syringe into an appropriate sample vial. If the samples are not processed immediately for isotope ratio measurements, they should be stored at \(-20^\circ\text{C}\) until ready for analysis. The sample-collection apparatus must be free of moisture in order to avoid dilution of the samples, particularly when only small quantities are available.
Fig. 6.3. Schematic diagram of a gas isotope-ratio mass spectrometer. The entire process of valve sequencing, pressure matching, gas switching, ion source tuning, vacuum monitoring, and data collection and reduction is controlled automatically by a computer.
Sample requirements

For a typical doubly labelled water study, a minimum of three samples (two-point method) is required: one prior to the administration of the $^{2}$H$_{2}^{18}$O, one at the beginning of the study and one at the end of the study following the administration of the tracer. However, the two-point method is suboptimal because if any one of these three samples is contaminated or cannot be analysed for any reason, the results of the entire study are lost. Therefore, the multiple-point method is recommended. In the multiple-point method, a minimum of two post-dose samples at the beginning of the study and two post-dose samples at the end of the study are collected in addition to the baseline sample.

The samples collected must be converted to the gaseous state before they are introduced to the ion source of the mass spectrometer for gas isotope-ratio measurements. For the measurements of $^{2}$H : $^{1}$H ratios in water or physiological fluids, H$_{2}$ gas is the preferred final product. However, CO$_{2}$ is the preferred final product for the measurements of $^{18}$O : $^{16}$O ratios by GIRMS.

In a typical mass spectrometer, 1 ml of H$_{2}$ gas at standard temperature and pressure is sufficient for precise measurements of $^{2}$H : $^{1}$H ratios. This amount of H$_{2}$ is equivalent to 0.8 mg of water. One ml of CO$_{2}$ is also ample for precise $^{18}$O : $^{16}$O ratio measurements. This amount of CO$_{2}$ is equivalent to 2 mg of water. With a proper inlet system and cryogenic trapping devices, however, the amount of CO$_{2}$ required for isotope ratio measurements can be reduced to approximately 3 µl. This adaptation significantly reduces the amount of material needed for isotopic measurements.

Sample preparation for $^{2}$H abundance measurements

The most commonly employed procedure for the conversion of water in physiological fluids to H$_{2}$ is the zinc reduction method (Wong et al., 1992). Water in 10 µl of the sample is reduced to H$_{2}$ according to the equation:

$$H_{2}O + Zn \rightarrow ZnO + H_{2}$$  \hspace{1cm} (8)

using zinc turnings (Biogeochemical Laboratory, Department of Geological Sciences, Indiana University, Bloomington, IN 47405, USA) at 500°C for 30 min in an evacuated reduction vessel. Briefly, the sample (10 µl) is transferred under an atmosphere of N$_{2}$ to a reduction vessel (Fig. 6.4) containing 200–250 mg of zinc turnings. The sample is frozen.
with liquid N2 and the vessel is evacuated. The water is reduced to H2 by heating the zinc turnings to 500°C for 30 min. With biological fluids, the sample must be placed in the wall bubble of the reaction vessel to prevent poisoning of the zinc by the sample matrix. At natural abundances, the deuterium values are accurate to \(-0.2 \pm 1.2\) parts per mille (‰) (mean \(\pm\) SD, \(n = 68\)) and reproducible within 1.2‰ (SD). At 600‰ enrichment levels of deuterium, the values measured from plasma, urine, saliva and human milk samples are accurate to \(-4.3 \pm 4.8\)‰ (mean \(\pm\) SD, \(n = 200\)) and reproducible within 3.2‰ (SD). On cooling to room temperature, the H2 is introduced into a GIRMS for \(^2\)H abundance measurement.

Reduction of water or water in physiological fluids with zinc turnings eliminates memory effect because each water sample is reduced to H2 with a fresh aliquot of zinc in a separate vessel. The same vessel also serves as the sample bulb for direct introduction of the H2 into the mass spectrometer.

Fig. 6.4. Dimensions of the reduction vessel.
Recently, several mass spectrometer manufacturers have adapted an equilibration technique for the measurement of stable hydrogen isotope ratios in water or water in physiological fluids (Prosser and Scrimgeour, 1995). However, a much larger sample size is required. For readers who are interested in this procedure, the specifications can be obtained from the manufacturers and therefore will not be discussed here.

Sample preparation for $^{18}$O abundance measurements

The H$_2$O–CO$_2$ exchange method is the standard procedure for the preparation of aqueous samples for $^{18}$O:$^{16}$O isotope ratio measurements by GIRMS. A water sample of approximately 1 g is allowed to equilibrate with a specific volume of CO$_2$ of known $^{18}$O content at 25°C for 48–72 h with constant shaking. At the end of the equilibrium, the CO$_2$ is removed from the equilibration vessel and cryogenically purified for mass-spectrometric analysis. The $^{18}$O value of the water sample is calculated according to the procedure of Craig (1957):

$$\delta^{18}\text{O}_{\text{water}} (\%/oo) = \delta^{18}\text{O}_f + \alpha / k \times (\delta^{18}\text{O}_f - \delta^{18}\text{O}_i)$$ (9)

where $\delta^{18}\text{O}_{\text{water}}$ is the $^{18}$O content of the water or physiological fluid; $\delta^{18}\text{O}_f$ and $\delta^{18}\text{O}_i$ are the isotopic composition of $^{18}$O in the CO$_2$ before and after equilibration, respectively; $\alpha$ is the oxygen isotope fractionation factor between CO$_2$ and H$_2$O and has a value of 1.0414 at 25°C; and $k$ is defined as the ratio of $N_{\text{H}_2\text{O}}$ and $2N_{\text{CO}_2}$ where $N_{\text{H}_2\text{O}}$ and $N_{\text{CO}_2}$ are the number of oxygen atoms in the water sample and the CO$_2$, respectively. The precision (SD) of the equilibration method ranges from 0.01 to 0.6%/oo.

An automated H$_2$O–CO$_2$ equilibration system is available from mass spectrometer manufacturers. This system utilizes the difference in pumping speeds between gas molecules and water molecules passing through a capillary to minimize the loss of sample. Thus, there is no need to freeze the water sample before the equilibration vessel is evacuated and CO$_2$ is admitted, or before the CO$_2$ is extracted after equilibration for isotope ratio measurement. In the basic system first described by Roether (1970) and Fairbanks (1982), nine samples (10 g each) can be prepared for analysis simultaneously. Subsequently, we evaluated a commercial system (ISOPREP-18, VG Instruments, Ltd, Cheshire, UK) for its accuracy and precision for $^{18}$O:$^{16}$O isotope ratio measurements on 0.1 g of water and biological fluids (Wong et al., 1987a). A schematic diagram of the ISOPREP-18 system is shown in Fig. 6.5.

With water samples at natural abundances of $^{18}$O, the $^{18}$O:$^{16}$O ratios
Fig. 6.5. Schematic diagram of the ISOPREP-18 H₂O–CO₂ equilibration system.
were accurate to −0.05 ± 0.50‰ (mean ± SD, n = 52) and reproducible within 0.21‰ (SD, n = 52). With 0.1 ml of biological fluid (urine, plasma, saliva, human milk) at 250‰ enrichment level of 18O, an accuracy of −0.32 ± 0.87‰ (mean ± SD, n = 200) and a precision of 0.97‰ (SD, n = 200) were obtained. Up to 48 samples can be accommodated, and isotopic equilibrium between the aqueous sample and CO2 may be attained within 10 h. Each analysis requires approximately 20 min. The entire process in which the vessel is evacuated, CO2 is injected, equilibrated and extracted, and data are collected is controlled automatically by computer.

For very small samples, an alternative procedure is to convert H2O to CO2 with guanidine hydrochloride according to the following reaction (Wong et al., 1987b):

\[
\text{NH}_2\text{C(NH)NH}_2 \cdot \text{HCl} + 2\text{H}_2\text{O} \rightarrow 2\text{NH}_3 + \text{CO}_2 + \text{NH}_4\text{Cl}
\]

Water (10 mg) is transferred to a Pyrex tube containing 100 mg of guanidine hydrochloride. After the water is frozen with liquid N2, the tube is evacuated, sealed, and heated to 260°C for 16 h. After cooling, the tube is opened and heated to 80°C to release NH3 and CO2 from the reaction mixture. The NH3 is removed with 100% H3PO4 and the CO2 is cryogenically purified for mass-spectrometric analysis. A schematic diagram showing an apparatus to convert H2O to CO2 is shown in Fig. 6.6.

An accuracy of 1.16 ± 0.16‰ (mean ± SD) and a precision of 0.28‰ were obtained in saliva, urine, plasma and milk samples with natural abundance of 18O when compared with values obtained by the classical H2O–CO2 equilibration method. For physiological fluids at 250‰ 18O enrichment, an accuracy of −4.70 ± 0.96‰ (mean ± SD) and a precision of 0.88‰ were obtained using the guanidine hydrochloride method. The conversion of H2O to CO2 with guanidine hydrochloride is very appealing because of the small sample size requirement, high precision, lack of memory effect and the ability to prepare batches of samples together. However, the procedure is labour-intensive and should only be used when only a very small sample is available.

Isotope abundance measurements

GIRMS is used to measure the 2H and 18O abundance in the H2 and CO2 samples, respectively. The instrumentation is known as gas isotope-ratio mass spectrometry because all samples entering the ion source of the mass spectrometer must be in a gaseous form such as H2 for 2H abundance measurements and CO2 for 18O abundance measurements. On
entry into the ion source of the mass spectrometer, the H$_2$ or the CO$_2$ gas is ionized by electrons to form positively charged ions of $^1$H$^+H^+$ and $^1$H$^2H^+$ for H$_2$, or C$^{16}$O$_2^+$ and C$^{16}$O$^{18}$O$^+$ for CO$_2$. Because of the difference in ionic masses between these positively charged ions, they are separated.
into two ion beams through a magnetic field. The amounts of $^1$H and $^2$H in the H$_2$ or $^{16}$O and $^{18}$O in the CO$_2$ are directly proportional to the amplified ion beam intensities of the $^1$H$^1$H$^+$ and $^1$H$^2$H$^+$, or $^{16}$O$^{16}$O$^+$ and $^{16}$O$^{18}$O$^+$, as measured by the detectors of the mass spectrometer. These amplified signals are compared with those of the laboratory H$_2$ or CO$_2$ standard and are expressed as the isotope ratios of $^1$H$^2$H : $^1$H$^1$H or $^{16}$O$^{18}$O : $^{16}$O$^{16}$O.

**Units**

The $^2$H and $^{18}$O abundances in the H$_2$ and CO$_2$, respectively, are expressed in delta ($\delta$) per mille ($\text{‰}$) units, which are defined as follows:

$$\delta^aX_{\text{WS}} (\text{‰}) = \left( \frac{R_{\text{sample}}}{R_{\text{WS}}} - 1 \right) \times 10^3 \quad (11)$$

where $a$ is the atomic weight of the heavier isotope ($X$), and $R_{\text{sample}}$ and $R_{\text{WS}}$ are the $^1$H$^2$H/$^1$H$^1$H or $^{16}$O$^{18}$O/$^{16}$O$^{16}$O ratios of the sample and laboratory working standards, respectively. For ease of inter-laboratory comparison, the $\delta^aX_{\text{WS}} (\text{‰})$ values are normalized against two international water standards, Vienna-Standard Mean Ocean Water (V-SMOW) and Standard Light Antarctic Precipitation (SLAP) as follows (Gonfiantini, 1984):

$$\delta^aX_{\text{Normalized}} (\text{‰}) = \left[ \frac{(\delta_{\text{sample-WS}} - \delta_{\text{V-SMOW-WS}})}{(\delta_{\text{SLAP-WS}} - \delta_{\text{V-SMOW-WS}})} \right] \times \delta^o \quad (12)$$

where $\delta_{\text{sample-WS}}$, $\delta_{\text{V-SMOW-WS}}$ and $\delta_{\text{SLAP-WS}}$ are the $\delta^2$H or $\delta^{18}$O values of the sample, V-SMOW and SLAP measured against the laboratory working standard, respectively. The $\delta^o$ has an accepted value of $–55.5 \text{‰}$ for $^{18}$O abundance measurements and an accepted value of $–428 \text{‰}$ for $^2$H abundance measurements. These relative $\delta$ values can be converted to absolute atom % values as follows (Wong and Klein, 1986):

$$\text{Atom \%} = \text{fractional abundance} \times 100 \quad (13)$$

The fractional abundance ($F$) for deuterium is calculated from the per mille value as follows:

$$F = \frac{R}{1+R} \quad (14)$$

$$R = (\delta/1000 + 1) \times R_{\text{V-SMOW}} \quad (15)$$

where $R$ is the $^2$H : $^1$H ratio of the sample, $\delta$ is the normalized ($\delta$H value of the sample and $R_{\text{V-SMOW}}$ is the $^2$H : $^1$H ratio of V-SMOW, which has a
value of 0.00015595 (de Wit et al., 1980). Because oxygen has three isotopes ($^{16}\text{O}, ^{17}\text{O}, ^{18}\text{O}$), the fractional abundance of $^{18}\text{O}$ must be calculated using the following relationships:

$$F = \frac{^{18}\text{O}}{(1 + ^{17}\text{O} + ^{18}\text{O})}$$  \hspace{1cm} (16)

$$^{17}\text{O} = (1 + \delta)^{1/2} \times ^{17}\text{O}_{\text{V-SMOW}}$$  \hspace{1cm} (17)

$$^{18}\text{O} = \left(\frac{\delta}{1000} + 1\right) \times ^{18}\text{O}_{\text{V-SMOW}}$$  \hspace{1cm} (18)

in which $^{17}\text{O}$ and $^{18}\text{O}$ are the $^{17}\text{O}:^{16}\text{O}$ and $^{18}\text{O}:^{16}\text{O}$ ratios of the sample, $\delta$ is the normalized $\delta^{18}\text{O}$ value of the sample, and $^{17}\text{O}_{\text{V-SMOW}}$ and $^{18}\text{O}_{\text{V-SMOW}}$ are the $^{17}\text{O}:^{16}\text{O}$ and $^{18}\text{O}:^{16}\text{O}$ ratios of V-SMOW and have values of 0.000373 (Hayes, 1982) and 0.0020052 (Baertschi, 1976), respectively.

Reference materials

There are several primary and provisional reference materials for $^2\text{H}$ and $^{18}\text{O}$ isotope ratio measurements and they are summarized in Table 6.4. These reference materials can be purchased from the International Atomic Energy Agency, Analytical Quality Control Services, Agency’s Laboratories, PO Box 100, A-1400 Vienna, Austria.

Concluding Remarks

The $^2\text{H}^{18}\text{O}$ method is the only non-invasive procedure currently available for the estimation of total energy expenditure under free-living con-

<table>
<thead>
<tr>
<th>Table 6.4.</th>
<th>Isotopic composition of primary standards and provisional reference materials for $^2\text{H}$ and $^{18}\text{O}$ isotopic ratio measurements.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Standards</strong></td>
<td><strong>Description</strong></td>
</tr>
<tr>
<td>V-SMOW</td>
<td>Standard Mean Ocean Water</td>
</tr>
<tr>
<td>SLAP</td>
<td>Standard Light Antarctic Precipitation</td>
</tr>
<tr>
<td>GISP</td>
<td>Greenland Ice Sheet Precipitation</td>
</tr>
<tr>
<td>IAEA 302A</td>
<td>Water</td>
</tr>
<tr>
<td>IAEA 302B</td>
<td>Water</td>
</tr>
<tr>
<td>IAEA 304A</td>
<td>Water</td>
</tr>
<tr>
<td>IAEA 304B</td>
<td>Water</td>
</tr>
</tbody>
</table>
ditions. Unfortunately, there is currently a global shortage of 10% \( \text{H}_2\text{H}^{18}\text{O} \) and researchers should plan their research project accordingly. If possible, sufficient \( \text{H}_2\text{H}^{18}\text{O} \) to complete the project should be ordered and received prior to the start of the project. Because the \( \text{H}_2\text{H}^{18}\text{O} \) method provides a cumulative estimate on energy expenditure, researchers interested in assessing daily variation in energy expenditure or variation in the intensity of energy expenditure must consider the use of other methods, such as heart-rate monitoring or motion sensors, along with the \( \text{H}_2\text{H}^{18}\text{O} \) method.

**References**


Schoeller, D.A. (1983) Energy expenditure from doubly labeled water: some fun-


Introduction

Obesity is a major health problem in all industrialized countries because of its association with increased risk of hypertension, coronary heart disease, diabetes, cancer and many other medical ailments (Bray, 1985; van Itallie, 1985). The third National Health and Nutrition Examination Survey (Troiano et al., 1995) carried out in the USA indicated that, based on the body mass index (BMI), approximately one in four American children and adolescents is overweight, a condition that is increasing most rapidly among minority children and adolescents. The continuous increases in the prevalence of being overweight or obese among children and youths in the USA, particularly among minority populations, is of great concern because overweight or obese children and youths are at increased risk of becoming overweight or obese adults (Harsha et al., 1987; Rolland-Cachera et al., 1990; Serdula et al., 1993; Whitaker et al., 1997; Mo-suwan et al., 2000; Williams, 2001). Adult obesity has been linked to increased risks of hypertension and diabetes (Bray, 1985; National Institutes of Health Consensus Development Conference Statement, 1985; van Itallie, 1985). In fact, cardiovascular risk factors, such as high blood pressure and high blood concentrations of glucose, lipid and lipoproteins, have been identified in overweight children and adolescents (Chu et al., 1998; Dwyer et al., 1998).

Although BMI has been used extensively as a general index of adiposity to identify children, adolescents and adults who are overweight or
obese, many have expressed concern about its accuracy (Hammer et al., 1991a,b; Pietrobelli et al., 1998; Ellis et al., 1999; Prentice and Jebb, 2001). More accurate estimates of the percentage body fat (%FM) in healthy subjects can be measured indirectly by numerous techniques, including underwater weighing, total-body electrical conductivity (TOBEC), $^{40}$K counting, dual-energy X-ray absorptiometry (DXA), anthropometry, bio-electrical impedance (BIA) and isotope dilution.

Underwater weighing has long been considered the gold standard for the estimation of body fat. The procedure, however, is not applicable to infants and young children. Certainly, the procedure is not suitable for any research subjects who are not comfortable about submerging underwater while holding their breath after emptying their lungs.

TOBEC is a rapid, non-invasive methodology developed for predicting body fatness in humans. The measurement requires only minimum training of the operator and cooperation from the subject. However, TOBEC instruments are no longer manufactured and have been shown to be not suitable for estimating body fat in prepubescent children and children with low body fat (Wong et al., 2000a).

Whole-body $^{40}$K counting also is a non-invasive procedure for estimating body fatness in humans. However, the facility must be shielded from atmospheric radiation, built with materials voided of natural and man-made radiation and supervised by a specialist. Therefore, there are only a few whole-body $^{40}$K counting facilities in the world.

Since the development of DXA in the early 1990s (Pietrobelli et al., 1996), DXA has emerged as one of the most widely accepted methods of measuring body composition in human subjects. The popularity of DXA can be attributed partly to its speed, ease of performance, and low radiation exposure (Njeh et al., 1999). We found that DXA was an appropriate method for estimating body composition in a group of girls because its bias and limits of agreement were independent of age, ethnicity and body fatness (Wong et al., 2002). However, DXA may not be an optimal method for measuring body fatness of an individual girl because her body fatness could be under- or overestimated by 28%.

Underwater weighing, TOBEC, whole-body $^{40}$K counting and DXA methodologies are further limited to use in on-site studies and would not be feasible for field studies or studies involving large number of subjects. The use of skinfold thickness measurements to estimate %FM is particularly appealing in population studies, because this procedure is relatively easy to perform, the measurements are non-invasive and do not involve radiation exposure, the instrument (skinfold calipers) is inexpensive and does not require electrical power to operate and, most important of all, the measurements can be carried out anywhere. Over the years, many skinfold thickness equations have been developed for predicting body
fatness. However, most of these equations were developed using adult
data (Sloan et al., 1962; Durnin and Rahaman, 1967; Wilmore and Behnke,
1970; Katch and McArdle 1973; Durnin and Womersley, 1974; Jackson et
al., 1980) and only two equations were developed specifically for
children and adolescents (Brook, 1971; Slaughter et al., 1988). Furthemore,
most of these equations were based on data collected from
Caucasian subjects. Among the skinfold thickness equations, the
Slaughter, Lohman and Boileau equation (Slaughter et al., 1988) yielded
the closest agreement in %FM (mean difference ± SD = 0.8 ± 5.0 %) when
compared with the four-compartment criterion method (Wong et al.,
2000b). Furthermore, the use of the triceps and calf skinfold thickness in
the Slaughter, Lohman and Boileau equation yielded similar mean differ-
cences (Caucasians –0.3%; African-Americans 1.3%) when compared with
those obtained using the biceps and subscapular skinfold thickness
measurements. More importantly, triceps and calf skinfold measure-
ments are easier to obtain than the biceps and subscapular skinfold
measurements, because the former measurements require minimal
moving of clothing on the upper body, avoiding problems due to
modesty. Our analyses (Wong et al., 2000b), however, indicated that
further refinement of the Slaughter, Lohman and Boileau quadratic equa-
tion is needed in order to improve the accuracy of predicting %FM
among minority populations.

BIA is an alternative to skinfold thickness equations for estimating
body composition. The BIA instrument is portable and is not very expen-
sive. Therefore, it is a rather useful piece of field equipment for body-
composition measurements. Since the subject must be in a supine
position for the installation of the electrodes, the method might not be
applicable to studies involving a large number of subjects.

Another alternative is the isotope dilution method. Tritium oxide
has long been the isotopic tracer of choice. Its usage, however, has
diminished dramatically because of ethical concerns due to the
exposure of subjects to radioactivity. Subsequently, deuterium oxide
(\(^2\text{H}_2\text{O}\)) or \(^{18}\text{O}\)-labelled water (\(\text{H}_2^{18}\text{O}\)) are the two isotopic tracers
of choice because both \(^2\text{H}\) and \(^{18}\text{O}\) are stable isotopes of hydrogen
and oxygen, respectively, and they do not emit any harmful radiation.
Both \(^2\text{H}_2\text{O}\) and \(\text{H}_2^{18}\text{O}\) have been used extensively in studies with
preterm infants, newborns, children, adolescents, and pregnant and lac-
tating women without any adverse effects. Furthermore, the isotope dilu-
tion procedure can be carried out anywhere, the isotopic tracer can be
administered to many subjects at the same time and the samples collected
for \(^2\text{H}\) or \(^{18}\text{O}\) assays can be shipped easily to the appropriate analytical
site.

The primary purpose of this chapter is to present the theory of the
isotope dilution method, the equations used in the calculation, the ana-
lytical procedures and the limitations.
Theory of the Isotope Dilution Method

The use of the isotope dilution method to estimate body composition is based on the assumption that fat-free mass (FFM) has relatively constant water content (TBW) with negligible water associated with fat stored in adipose tissue. Because TBW of the FFM can be measured accurately with the dilution principle, the mass of the FFM can be estimated as follows:

$$\text{FFM (kg)} = \text{TBW} / H$$

(1)

where $H$ is the hydration of the FFM. The commonly used value for $H$ is 0.73. Because hydration of FFM has been shown to be much higher among preterm infants, newborns and children and the value changes with age, the use of a single hydration value for FFM in infants and children is not appropriate and will be discussed later in the chapter.

The dilution principle states that when a known amount of tracer ($D$ moles) is added to a pool of water, after thorough mixing, the amount of tracer can be calculated from the volume of the water ($V$ litres) and the concentration of the tracer ($[D]$ moles l$^{-1}$) based on the following relationship:

$$D \text{ (moles)} = V \times [D]$$

(2)

Since $D$ is known and $[D]$ can be measured, Equation (2) can be rearranged to determine $V$.

$$V = D / [D]$$

(3)

The volume can then be converted to TBW (kg) if the density of water, $d$ (kg l$^{-1}$), is known.

$$\text{TBW} = V \times d$$

(4)

After converting TBW to FFM using Equation (1), FM (kg) is simply the difference between body weight ($Wt$ kg) and FFM.

$$\text{FM} = Wt - \text{FFM}$$

(5)
Analytical Methods

Dosages of \( ^2\text{H} \) and \( ^{18}\text{O} \)

In most human studies, the \( ^2\text{H}_2\text{O} \) or \( \text{H}_2^{18}\text{O} \) are administered orally. For intravenous administration, the isotopic water must be ultrafiltered for the removal of endotoxin and bacterial contamination (Wong et al., 1991). Approximately 0.04 g or ml kg\(^{-1} \) body weight of \( ^2\text{H}_2\text{O} \) at 99.8\% \( ^2\text{H} \) or 0.6 g or ml kg\(^{-1} \) body weight of \( \text{H}_2^{18}\text{O} \) at 10\% \( ^{18}\text{O} \) are routinely used for the determination of TBW. It is crucial to know the exact quantity of \( ^2\text{H}_2\text{O} \) or \( \text{H}_2^{18}\text{O} \) given to the volunteer. For oral administration, the bottle containing the isotopic water should be rinsed twice with drinking water, formula, breast milk or suitable dietary fluids. For intravenous doses, the exact weight of the syringe before and after administration of the isotopic water must be known, or the syringe can be flushed several times with saline solution to ensure complete administration. Because the highly enriched \( ^2\text{H}_2\text{O} \) is hydroscopic and only a very small amount of \( ^2\text{H}_2\text{O} \) is needed for TBW measurement, the \( ^2\text{H}_2\text{O} \) should be diluted five- to tenfold with water in order to minimize weighing error. Usually a batch of diluted \( ^2\text{H}_2\text{O} \) is prepared when multiple subjects of similar body weight are to be studied. As long as the diluted \( ^2\text{H}_2\text{O} \) is stored in a tightly sealed container and refrigerated, the diluted tracer should last for a long time.

Sources of \( ^2\text{H} \) and \( ^{18}\text{O} \)

Deuterium oxide at 99.8 atom \% \( ^2\text{H} \) and \( \text{H}_2^{18}\text{O} \) at 10-97 atom \% \( ^{18}\text{O} \) can be purchased from isotope manufacturers such as Isotec Inc. or Cambridge Isotope Laboratories.

Mass spectrometric analyses

The most accurate and precise instrumentation for the measurements of \( ^2\text{H} \) and \( ^{18}\text{O} \) content in physiological fluids is gas isotope-ratio mass spectrometry (GIRMS). The instrumentation has been described in detail in Chapter 6 and will not be repeated here. Basically, all samples introduced into the mass spectrometer must be in a gaseous form. For measurements
of deuterium abundance, H\textsubscript{2} is used. For oxygen abundance measurements, CO\textsubscript{2} is used.

**Sample collection**

Any physiological fluid such as plasma, saliva, urine or breath water vapour can be collected for the determination of TBW. If H\textsubscript{2}\textsuperscript{18}O is used as the tracer, expired CO\textsubscript{2} can also be used. Generally, urine is the preferred sample, because it is non-invasive and the easiest to collect. In infants, urine can be collected using cotton balls (Wong et al., 1993). The urine absorbed by the cotton balls can be expressed with a plastic syringe into an appropriate sample vial. If the samples are not processed immediately for isotope ratio measurements, they should be stored at \(-20^\circ\text{C}\) until ready for analysis. When physiological fluids are the samples of choice, the sample-collection apparatus must be free of moisture in order to avoid dilution of the samples, particularly when only small quantities are available.

**Sample requirements**

For a typical TBW measurement, a minimum of two samples is required (the equilibrium method): one prior to administration of the \(\text{H}_2\text{O}\) or H\textsubscript{2}\textsuperscript{18}O and one after 1–6 h following the administration of the tracer. With plasma, saliva, breath water vapour, and breath CO\textsubscript{2}, the tracers should reach isotopic equilibrium in 3 h (Wong et al., 1988). With urine, the post-dose sample should be collected at least 5–6 h after administration of the tracer. To eliminate concern about the exact time at which the isotopic tracer has reached isotopic equilibrium, the ‘zero-time extrapolation method’ can be employed. With the zero-time extrapolation method, multiple post-dose samples are collected following the administration of the tracer over a period of 5–10 days. The isotope decay curve is then extrapolated to time zero or the time when the isotopic tracer was administered. The isotopic enrichment of \(2\text{H}\) or \(18\text{O}\) at time zero is then used to calculate TBW.

In a typical mass spectrometer, 1 ml of H\textsubscript{2} gas at standard temperature and pressure is sufficient for precise measurements of \(\text{H}^2\) : \(\text{H}\) ratios. This amount of H\textsubscript{2} is equivalent to 0.8 mg of water. One ml of CO\textsubscript{2} is also ample for precise \(\text{O}^{18}/\text{O}^{16}\)-ratio measurements. This amount of CO\textsubscript{2} is equivalent to 2 mg of water. With a proper inlet system and cryogenic
trapping devices, however, the amount of CO₂ required for isotope-ratio measurements can be reduced to approximately 3 µl. This adaptation significantly reduces the amount of material needed for isotopic measurements.

Sample preparation for ²H abundance measurements

The most commonly employed procedure for the conversion of water in physiological fluids to H₂ is the zinc reduction method (Wong et al., 1992). Briefly, the sample (10 µl) is transferred under an atmosphere of N₂ to a reduction vessel containing 200–250 mg of zinc turnings (Biogeochemical Laboratory, Department of Geological Sciences, Indiana University, Bloomington, IN 47405, USA). The sample is frozen with liquid N₂ and the vessel is evacuated. The water is reduced to H₂ by heating the zinc turnings to 500°C for 30 min.

\[ H₂O + Zn \rightarrow ZnO + H₂ \] (6)

With biological fluids, the sample must be placed in the wall bubble of the reaction vessel to prevent poisoning of the zinc by the sample matrix. At natural abundances, the deuterium values are accurate to −0.2 ± 1.2‰ (mean ± SD, n = 68) and reproducible within 1.2‰ (SD). At 600‰ enrichment levels of deuterium, the values measured from plasma, urine, saliva and human milk samples are accurate to −4.3 ± 4.8‰ (mean ± SD, n = 200) and reproducible within 3.2‰ (SD). On cooling to room temperature, the H₂ is introduced into a GIRMS for ²H abundance measurement.

Reduction of water or water in physiological fluids with zinc turnings eliminates memory effect because each water sample is reduced to H₂ with a fresh aliquot of zinc in a separate vessel. The same vessel also serves as the sample bulb for direct introduction of the H₂ into the mass spectrometer.

Sample preparation for ¹⁸O abundance measurements

The H₂O–CO₂ exchange method is the standard procedure for the preparation of aqueous samples for ¹⁸O/¹⁶O isotope ratio measurements by GIRMS. A water sample of approximately 1 g is allowed to equilibrate with a specific volume of CO₂ of known ¹⁸O content at 25°C for 48–72 h
with constant shaking. At the end of the equilibrium, the CO₂ is removed from the equilibration vessel and cryogenically purified for mass-spectrometric analysis. The ¹⁸O value of the water sample is calculated according to the procedure of Craig (1957):

\[
\delta^{18}O_{\text{water}} (\text{‰}) = \delta^{18}O_i + \alpha/k \times (\delta^{18}O_i - \delta^{18}O_f) \tag{7}
\]

where \( \delta^{18}O_{\text{water}} \) is the ¹⁸O content of the water or physiological fluid; \( \delta^{18}O_i \) and \( \delta^{18}O_f \) are the isotopic compositions of ¹⁸O in the CO₂ before and after equilibration, respectively; \( \alpha \) is the oxygen isotope fractionation factor between CO₂ and H₂O and has a value of 1.0414 at 25°C; and \( k \) is defined as the ratio of \( N_{\text{H}_2\text{O}} \) and \( 2N_{\text{CO}_2} \) where \( N_{\text{H}_2\text{O}} \) and \( N_{\text{CO}_2} \) are the number of oxygen atoms in the water sample and the CO₂, respectively. The precision (SD) of the equilibration method ranges from 0.01 to 0.6‰.

An automated H₂O–CO₂ equilibration system is available from mass spectrometer manufacturers. This system utilizes the difference in pumping speeds between gas molecules and water molecules passing through a capillary to minimize the loss of sample. Thus, there is no need to freeze the water sample before the equilibration vessel is evacuated and CO₂ is admitted, or before the CO₂ is extracted after equilibration for isotope-ratio measurement. In 1987, we evaluated a commercial system (ISOPREP-18, VG Instruments Ltd, Cheshire, UK) for its accuracy and precision for ¹⁸O:¹⁶O isotope-ratio measurements on 0.1 g of water and biological fluids (Wong et al., 1987). With water samples at natural abundances of ¹⁸O, the ¹⁸O:¹⁶O ratios were accurate to \(-0.05 \pm 0.50\text{‰}\) (mean ± SD, \( n = 52 \)) and reproducible within 0.21‰ (SD, \( n = 52 \)). With 0.1 ml of biological fluid (urine, plasma, saliva, human milk) at 250‰ enrichment level of ¹⁸O, an accuracy of \(-0.32 \pm 0.87\text{‰}\) (mean ± SD, \( n = 200 \)) and a precision of 0.97‰ (SD, \( n = 200 \)) were obtained. Up to 48 samples can be accommodated, and isotopic equilibrium between the aqueous sample and CO₂ may be attained within 10 h. Each analysis requires approximately 20 min. The entire process in which the vessel is evacuated, CO₂ is injected, equilibrated and extracted and data are collected is controlled automatically by computer.

### Isotope abundance measurements

On entry into the ion source of the mass spectrometer, the H₂ or the CO₂ gas is ionized by electrons to form positively charged ions of \(^1\text{H}^+\) and \(^2\text{H}^+\) for H₂ or \(^{16}\text{O}_2^+\) and \(^{16}\text{O}^{18}\text{O}^+\) for CO₂. Because of the difference in ionic masses between these positively charged ions, they are separated
into two ion beams through a magnetic field. The amounts of $^1\text{H}$ and $^2\text{H}$ in the $\text{H}_2$ or $^{16}\text{O}$ and $^{18}\text{O}$ in the $\text{CO}_2$ are directly proportional to the amplified ion beam intensities of the $^{1}\text{H}^{+}$ and $^{1}\text{H}^{2+}$ or $^{16}\text{O}_{2}^{+}$ and $^{16}\text{O}^{18}\text{O}^{+}$ as measured by the detectors of the mass spectrometer. These amplified signals are compared with those of the laboratory $\text{H}_2$ or $\text{CO}_2$ standard and are expressed as the isotope ratios of $^{1}\text{H}^{2+}:^{1}\text{H}^{1+}$ or $^{16}\text{O}^{18}\text{O}+:^{16}\text{O}^{2+}$.

### Units

The $^2\text{H}$ and $^{18}\text{O}$ abundances in the $\text{H}_2$ and $\text{CO}_2$, respectively, are expressed in delta (δ) per mille (‰) units, which are defined as follows:

$$\delta^a X_{\text{WS}} \text{ (‰)} = \frac{R_{\text{sample}}}{R_{\text{WS}}} - 1 \times 10^3 \quad (8)$$

where $a$ is the atomic weight of the heavier isotope ($X$), and $R_{\text{sample}}$ and $R_{\text{WS}}$ are the $^{1}\text{H}^{2+}:^{1}\text{H}^{1+}$ or $^{16}\text{O}^{18}\text{O}+:^{16}\text{O}^{2+}$ ratios of the sample and laboratory working standard, respectively. For ease of interlaboratory comparison, the $\delta^a X_{\text{WS}}$ (‰) values are normalized against two international water standards, Vienna-Standard Mean Ocean Water (V-SMOW) and Standard Light Antarctic Precipitation (SLAP) as follows (Gonfiantini, 1984):

$$\delta^a X_{\text{Normalized}} \text{ (‰)} = \left[ \frac{\delta_{\text{sample-WS}} - \delta_{\text{V-SMOW-WS}}}{\delta_{\text{SLAP-WS}} - \delta_{\text{V-SMOW-WS}}} \right] \times \delta^0 \quad (9)$$

where $\delta_{\text{sample-WS}}$, $\delta_{\text{V-SMOW-WS}}$, and $\delta_{\text{SLAP-WS}}$ are the $\delta^2\text{H}$ or $\delta^{18}\text{O}$ values of the sample, V-SMOW, and SLAP measured against the laboratory working standard, respectively. The $\delta^0$ has an accepted value of $-55.5$‰ for $^{18}\text{O}$ abundance measurements and an accepted value of $-428$‰ for $^2\text{H}$ abundance measurements. These relative $\delta$ values can be converted to absolute atom % values as follows (Wong and Klein, 1986):

$$\text{Atom %} = \text{fractional abundance} \times 100 \quad (10)$$

The fractional abundance ($F$) for deuterium is calculated from the $\delta%\text{oo}$ value as follows:

$$F = R/(1+R) \quad (11)$$

$$R = (\delta/1000 + 1) \times R_{\text{V-SMOW}} \quad (12)$$

where $R$ is the $^2\text{H}:^1\text{H}$ ratio of the sample, $\delta$ is the normalized $\delta^2\text{H}$ value.
of the sample, and \( R_{V \text{-SMOW}} \) is the \(^2\text{H}: ^1\text{H} \) ratio of V-SMOW, which has a value of 1.5595 \( \times 10^{-4} \) (de Wit et al., 1980). Because oxygen has three isotopes (\(^{16}\text{O}, ^{17}\text{O}, ^{18}\text{O} \)), the fractional abundance of \(^{18}\text{O} \) must be calculated using the following relationships:

\[
F = \frac{18R}{1 + 17R + 18R} \tag{13}
\]

\[
17R = (1 + \delta)^{1/2} \times 17R_{V \text{-SMOW}} \tag{14}
\]

\[
18R = \left( \frac{\delta}{1000} + 1 \right) \times 18R_{V \text{-SMOW}} \tag{15}
\]

in which \( 17R \) and \( 18R \) are the \(^{17}\text{O}: ^{16}\text{O} \) and \(^{18}\text{O}: ^{16}\text{O} \) ratios of the sample, \( \delta \) is the normalized \( \delta^{18}\text{O} \) value of the sample, and \( 17R_{V \text{-SMOW}} \) and \( 18R_{V \text{-SMOW}} \) are the \(^{17}\text{O}: ^{16}\text{O} \) and \(^{18}\text{O}: ^{16}\text{O} \) ratios of V-SMOW and have values of 3.73 \( \times 10^{-4} \) (Hayes, 1982) and 2.0052 \( \times 10^{-3} \) (Baertschi, 1976), respectively.

### Reference materials

The isotopic composition of several primary and provisional reference materials for \(^2\text{H} \) and \(^{18}\text{O} \) isotope ratio measurements are summarized in Table 7.1. These reference materials can be purchased from the International Atomic Energy Agency, Analytical Quality Control Services, Agency’s Laboratories, PO Box 100, A-1400 Vienna, Austria.

### TBW calculation

In practice, a dose dilution is performed to determine the exact \(^2\text{H} \) or \(^{18}\text{O} \) content in the dose rather than using the enrichment data provided by the manufacturer. Therefore, TBW is calculated using the following equation rather than Equation (4):

\[
\text{TBW (kg)} = \frac{d \times A \times E_a}{a \times E_d \times 10^3} \tag{16}
\]

where \( d \) is the dose of \(^2\text{H}_2\text{O} \) or \( \text{H}_2^{18}\text{O} \) in g; \( A \) is the amount of laboratory water in g used in the dose dilution; \( a \) is the amount of \(^2\text{H}_2\text{O} \) or \( \text{H}_2^{18}\text{O} \) in g added to the laboratory water in the dose dilution; \( E_a \) is the rise in \(^2\text{H} \) or \(^{18}\text{O} \) abundance (\( \% \text{oo} \)) in the laboratory water after the addition of the isotopic water; \( E_d \) is the rise in \(^2\text{H} \) or \(^{18}\text{O} \) abundance (\( \% \text{oo} \)) in the post-dose sample using the equilibrium method or the zero-time enrichment
using the zero-time extrapolation method. The density of TBW is assumed to be 1.0 kg l⁻¹.

**Limitations**

The use of the isotope dilution method for the estimation of body composition makes several assumptions: (i) the analytical errors of the isotopic measurements have minimal effect on the body composition estimates; (ii) the hydration of FFM is constant; (iii) the volume of the water remains unchanged during the equilibration period; and (iv) the loss of isotopic tracer during the equilibration period is negligible.

**The analytical errors of the isotopic measurements have minimal effects on the body composition estimates**

The accuracy and precision of the isotope ratio measurements are summarized in Table 7.2 (Wong et al., 1987).

As shown in Table 7.2, the ²H and ¹⁸O isotope ratio measurements by GIRMS are very accurate and precise. The effect of analytical errors on TBW or FFM estimation is demonstrated in Fig. 7.1. As shown in the figure, TBW or FFM can be estimated with an error of less than 1.5%, regardless of the age of the study subjects, when the conventional dosages of ²H₂O (40 mg kg⁻¹ body weight) or H₂¹⁸O (600 mg kg⁻¹ body weight at 10% ¹⁸O) are employed.

However, the error on FM estimated using the isotope dilution method

<table>
<thead>
<tr>
<th>Standards</th>
<th>Description</th>
<th>δ²Hᵥ-SMOW (‰)</th>
<th>δ¹⁸Oᵥ-SMOW (‰)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V-SMOW</td>
<td>Vienna-Standard Mean Ocean Water</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>SLAP</td>
<td>Standard Light Antarctic Precipitation</td>
<td>−428.0</td>
<td>−55.5</td>
</tr>
<tr>
<td>GISP</td>
<td>Greenland Ice Sheet Precipitation</td>
<td>−189.7</td>
<td>−24.8</td>
</tr>
<tr>
<td>IAEA 302A</td>
<td>Water</td>
<td>506.2 ± 4.7</td>
<td></td>
</tr>
<tr>
<td>IAEA 302B</td>
<td>Water</td>
<td>992.3 ± 13.5</td>
<td></td>
</tr>
<tr>
<td>IAEA 304A</td>
<td>Water</td>
<td>252.9 ± 2.6</td>
<td></td>
</tr>
<tr>
<td>IAEA 304B</td>
<td>Water</td>
<td>503.3 ± 5.5</td>
<td></td>
</tr>
</tbody>
</table>
varies depending on the age of the study subjects and can be as high as 6% in full-term infants and 24% in preterm infants (Fig. 7.2). To minimize the effect of analytical errors on FM estimation, the dosage of 2H2O or H218O can be increased. For example, if the dosage is increased fivefold, FM of preterm infants can be estimated with an error of less than 5%.

### Hydration of FFM is constant

The hydration of FFM has been assumed to be 73%. A hydration of FFM of 73% might be appropriate for healthy adolescents and adults, but would not be applicable to preterm infants, full-term infants and young children. For example, hydration of FFM as high as 89% has been reported for preterm infants weighing less than 1000 g (Ziegler et al., 1976). For preterm infants weighing more than 1000 g, the hydration of

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**Table 7.2.** Analytical errors of 2H and 18O measurements by gas isotope-ratio mass spectrometry.

<table>
<thead>
<tr>
<th>Sample</th>
<th>δ2H ± SDa (‰)</th>
<th>Accuracy (‰)</th>
<th>n</th>
<th>δ18O ± SDb (‰)</th>
<th>Accuracy (‰)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine</td>
<td>317.4 ± 10.7</td>
<td>3.2</td>
<td>50</td>
<td>250.79 ± 2.02</td>
<td>+0.02</td>
<td>50</td>
</tr>
<tr>
<td>Plasma</td>
<td>326.4 ± 7.1</td>
<td>3.8</td>
<td>50</td>
<td>257.86 ± 3.11</td>
<td>–0.50</td>
<td>50</td>
</tr>
<tr>
<td>Saliva</td>
<td>329.4 ± 14.0</td>
<td>2.5</td>
<td>50</td>
<td>254.55 ± 4.21</td>
<td>–0.30</td>
<td>50</td>
</tr>
<tr>
<td>Milk</td>
<td>331.4 ± 3.8</td>
<td>3.5</td>
<td>50</td>
<td>259.69 ± 5.46</td>
<td>–0.51</td>
<td>50</td>
</tr>
</tbody>
</table>

*aPrecision of single-sample measurement = 1.4‰.

*bPrecision of single-sample measurement = 0.02‰.

---

Fig. 7.1. Effect of analytical errors on TBW or FFM estimation. The analytical errors are taken to be 3.2‰ for 2H and 0.32‰ for 18O.
FFM varies between 83 and 88%. As shown in Fig. 7.3, the hydration of FFM in newborns is much higher than 73% and changes with age (Fomon et al., 1982). The most dramatic change in hydration of FFM occurs between birth and 6 months of age.

Because FFM is obtained by dividing TBW by the hydration of FFM, the error of FFM estimated using the isotope dilution method is directly proportional to the error of the hydration of FFM. Therefore, if the hydration of FFM is within 5 units of the true value, the error on FFM would not exceed 5%. However, when the hydration of FFM is inaccurate by up to 1 unit of the true value, FM estimated using the isotope dilution method could be inaccurate by as much as 20% among preterm infants.

![Fig. 7.2. Effect of analytical errors on FM estimation. The analytical errors are taken to be 3.2‰ for ²H and 0.32‰ for ¹⁸O.](image1)

![Fig. 7.3. Changes in hydration of FFM from birth to 10 years of age.](image2)
and 6% among full-term infants (Fig. 7.4). Therefore, when using the isotope dilution method to estimate body composition, particularly body fatness, the appropriate hydration of FFM must be used.

The volume of water remains unchanged during the equilibration period

For healthy children, adolescents and adults, the assumption is appropriate. The assumption, however, would not be applicable to very low-birthweight-infants (<1500 g birthweight) because they lose a large amount of body water after delivery.

Therefore, the extrapolation method is recommended in order to account for the rapid changes in body water during equilibration period. Certainly, if blood samples can be used, the equilibration method would be applicable assuming the postdose sample can be collected within two hours. The changes in body water within the 2-h equilibration period would be minimal.

The loss of isotopic tracer during the equilibration period is negligible

In adults, the loss of the isotopic tracer during an equilibration period of 6 h is less than 2%. However, in preterm infants with high metabolic rate,
approximately 5% of the isotopic tracer can be lost after a 6-h equilibration period. As shown in Fig. 7.5, the isotopic tracer can be diluted by dietary water, atmospheric water, and metabolic water generated during the oxidation of protein, carbohydrate and fat. The isotopic tracer also can be eliminated from body water in the form of urine, breath water vapour, sweat, respiratory CO$_2$ if H$_2^{18}$O is used as the tracer, and during the synthesis of organic materials. Dilution of the isotopic tracer by dietary water, metabolic water, and atmospheric water during the equilibration period would lead to significant overestimation of TBW as shown in Fig. 7.6.

The data in Fig. 7.6 were calculated based on the theoretical amounts of water and CO$_2$ generated from the oxidation of carbohydrate, fat and protein (Lusk, 1976); the $^2$H and $^{18}$O content of physiological fluids and expired CO$_2$ (Wong et al., 1988); the estimated rates of dietary water intake, atmospheric water inhalation, metabolic water produced and water output in adults (Schoeller and van Santen, 1982), in infants (Coward et al., 1982; Butte et al., 1988 and in preterm infants (Roberts et al., 1986); the $^{18}$O content of atmospheric oxygen (Dole et al., 1954); and the $^2$H and $^{18}$O content of atmospheric water vapour, carbohydrate, fat and protein (Bricout, 1979; Schoeller et al., 1986). As shown in Fig. 7.7, an overestimation of 1% in TBW would lead to an error of approximately 6% in FM estimated among term infants but over 20% in preterm infants.

The zero-time extrapolation method based on multiple samples would minimize the error introduced by the dilution of the isotopic tracer, because the rate of disappearance of the isotopic tracer reflects both the loss and dilution of the tracer during the equilibration period.
Concluding Remarks

Although there is a global shortage in the supply of $10\% \text{H}_2\text{O}^{18}$, $2\text{H}_2\text{O}$ is readily available and the cost of $2\text{H}_2\text{O}$ is significantly lower than $\text{H}_2\text{O}^{18}$. Similar to many other procedures, the major costs associated with the isotope dilution method are personnel and analytical measurements. As long as the users are aware of the limitations of the methodology, the isotope dilution method offers a simple, indirect procedure for the esti-

![Fig. 7.6. Overestimation of TBW due to the dilution of the $2\text{H}_2\text{O}$ or $\text{H}_2\text{O}^{18}$ by dietary water, metabolic water and atmospheric water during a 6 h equilibration period.](image1)

![Fig. 7.7. Effect of a 1% overestimation of TBW on FM estimated in preterm infants, term infants, 6-month-old infants, 12-month-old infants and adults.](image2)
mation of body composition and the procedure can be carried out anywhere and on a large number of study subjects.

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Rolland-Cachera, M.-F., Bellisle, F., Deheeger, M., Pequignot, F. and Sempe, M. (1990) Influence of body fat distribution during childhood on body fat distri-


Introduction

Several aspects of glucose homeostasis are unique to infants and children. Throughout gestation, glucose is transported from the maternal circulation across the placenta to meet a substantial proportion of the glucose and energy needs of the fetus. Thus, it is not surprising that there is no evidence of fetal glucose production (Kalhan et al., 1979). However, hepatic glucose production (gluconeogenesis and glycogenolysis) is well established within hours of birth both in term and very premature infants (Bier et al., 1977b; Frazer et al., 1981; Bougnères et al., 1982; Patel and Kalhan, 1992; Sunehag et al., 1993, 1996b; Tyrala et al., 1994).

Glucose is the principal substrate for the metabolism of the brain, which utilizes ~ 20 times more glucose per 100 g of tissue than fat or muscle tissue (Bier et al., 1977b; Haymond and Sunehag, 1999). In newborns, the brain accounts for 12% of the body weight (Bier et al., 1977b; Gruenwald and Minh, 1960). As a consequence, glucose utilization by the brain accounts for about 90% of their total glucose utilization (Fig. 8.1). From the newborn period to adulthood, the brain-to-body weight ratio decreases from ~ 12 to 2% (Bier et al., 1977b; Haymond and Sunehag, 1999). This results in a linear decrease in brain glucose utilization up to an age of ~ 9 years or a body weight of ~ 30 kg at which a plateau corresponding to a 40% brain glucose utilization, i.e. 830 μmol min⁻¹ (Fig. 8.1) is reached.

During periods of fasting, the glucose needs must be met by glucose produced from endogenous fuel stores, i.e. via glycogenolysis and gluco-
neogenesis from lactate, glycerol and amino acids (Fig. 8.2). Since infants and small children have smaller substrate stores, their blood glucose falls faster than in adults. In the fed state, glucose is provided via the food, but it can also be given intravenously or be produced via gluconeogenesis from parenteral lipid and amino acid substrate (Fig. 8.2).

Disturbed glucose homeostasis can have serious consequences. Hypoglycaemia, resulting in neuroglycopaenia, leads to seizures, unconsciousness, mental retardation and death if not treated (Siesjo, 1988).

![Graph showing glucose utilization by the brain from the newborn period to adulthood](from Endocrinol. Metab. Clin. North Am. 28, 663–694, 1999, with permission).

**Fig. 8.1.** Glucose utilization by the brain from the newborn period to adulthood (from Endocrinol. Metab. Clin. North Am. 28, 663–694, 1999, with permission).

Endogenous glucose sources:
- Glycogen
- Lactate, glycerol and amino acids

Glycogenolysis

Gluconeogenesis

Exogenous glucose sources:
- Feeding
- Parenteral glucose
- Parenteral lipid (glycerol) and amino acids

**Fig. 8.2** Endogenous and exogenous sources of plasma glucose.
Therefore, it is crucial to identify promptly the cause of hypoglycaemia in every case so appropriate treatment can be offered. Conversely, hyperglycaemia is frequently observed in premature infants receiving parenteral glucose and may result in urinary loss of glucose, osmotic diureses, dehydration and hyperosmolarity associated with an increased risk of cerebral bleedings (Dweck and Cassady, 1974; Cowett et al., 1979; Lilien et al., 1979; Stonestreet et al., 1980; Wu et al., 1990). After the newborn period, diabetes is the principal cause of hyperglycaemia and it is associated with similar acute complications as described above for premature infants (except for cerebral bleedings), but also with long-term microvascular complications such as neuropathy, nephropathy, retinopathy and cardiovascular disease (Nathan, 1993).

Measurements of glucose concentration are important but provide no mechanistic information about the metabolic events leading to either hypo- or hyperglycaemia. Hypoglycaemia can, for example, be the result of decreased inflow of glucose in the glucose pool (glucose appearance rate), but it can also be due to increased glucose utilization (glucose disappearance rate) (Haymond and Sunehag, 1999). Similarly, hyperglycaemia can be caused by increased inflow and/or decreased utilization (Haymond and Sunehag, 1999) (Fig. 8.3).

To distinguish between these metabolic events requires dynamic measures of glucose appearance and disappearance, gluconeogenesis and glycogenolysis. Dynamic measures of glucose metabolism in infants and children can be obtained using stable isotope and gas

\[ \text{Steady state euglycaemia} \quad R_a = R_d \]

\[ \text{Hyperglycaemia} \quad R_a > R_d \]

\[ \text{Hyperglycaemia} \quad R_a < R_d \]

\[ \text{Dynamics of hypo- and hyperglycaemia: the sugar bowl.} \quad R_a = \text{plasma glucose appearance rate; } R_d = \text{plasma glucose disappearance rate (from Endocrinol. Metab. Clin. North Am. 28, 663–694, 1999, with permission).} \]
chromatography–mass spectrometry (GC-MS) techniques, which have high sensitivity (picomoles of substrate can be measured accurately) and precision.

Stable Isotopes

Isotopes are chemically identical atoms with different weight (mass) due to different numbers of neutrons, e.g. $^{11}$C, $^{12}$C, $^{13}$C, $^{14}$C. Some of these isotopes are radioactive and decay to other compounds while giving off radiation (e.g. $^{11}$C and $^{14}$C), while others do not and are, therefore, denoted stable (e.g. $^{12}$C, $^{13}$C). A number of stable isotopes have been found to be useful in human investigations since they are non-radioactive and naturally occurring and can, therefore, be used without risk in individuals of all ages, even very premature infants (Bier, 1987, 1990; Bougnères, 1987; Wolfe, 1992; De Meer et al., 1999). Table 8.1 depicts the stable isotopes most commonly used in metabolic studies. Since substrates (glucose, amino acids, fatty acids, etc.) labelled with stable isotopes are metabolically equivalent to the corresponding unlabelled substrates, they can be used to trace the metabolism of these substrates in vivo (Bier, 1987, 1990; Bougnères, 1987; Wolfe, 1992; De Meer et al., 1999). Substrates labelled with stable isotopes with high isotopic purity (98–99 atom %) are commercially available. The most commonly used stable labelled carbohydrate compounds are displayed in Table 8.2.

When a stable labelled substrate is infused over time, the labelled molecules mix and eventually equilibrate with the unlabelled molecules and a condition of approximate steady state is reached. At steady state, substrate concentrations and the ratio between labelled and unlabelled molecules (i.e. tracer : tracee) in the pool remain constant. This means that

Table 8.1. Natural abundance of commonly used stable isotopes. The corresponding natural isotope is given within parentheses.

<table>
<thead>
<tr>
<th>Isotope</th>
<th>Natural abundance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^2$H (1H)</td>
<td>0.015</td>
</tr>
<tr>
<td>$^{13}$C (12C)</td>
<td>1.11</td>
</tr>
<tr>
<td>$^{15}$N (14N)</td>
<td>0.36</td>
</tr>
<tr>
<td>$^{17}$O (16O)</td>
<td>0.037</td>
</tr>
<tr>
<td>$^{18}$O (16O)</td>
<td>0.204</td>
</tr>
</tbody>
</table>
the rate of entry and loss of both labelled and unlabelled substrates are equal. Under these circumstances the entry rate of the labelled substrate is known and thus, the entry and loss of the unlabelled substrate can be calculated (Bier, 1987, 1999; Bougnères, 1987).

**Gas Chromatography–Mass Spectrometry**

Tracer : tracee ratios are measured by GC-MS (Fig. 8.4). In preparation for this analysis, the substrate (molecule) to be analysed undergoes a process called derivatization (Bier, 1987). The purpose of this process is to form a complex molecule, often an ester, with a lower boiling point (i.e. more volatile).

**Gas chromatograph (GC)**

The derivatized molecule is vaporized at high temperature in the injector of the gas chromatograph and is subsequently carried through a capillary glass column to the source of the mass spectrometer by an inert gas (helium, hydrogen, nitrogen or methane) under pressure. In the column, the molecules to be analysed are separated from other components of the sample by a temperature-regulated interaction between the molecule in the carrier gas (the mobile phase) and the stationary phase coating the inner surface of the column (Bier, 1987) (see below). Capillary columns provide high efficiency and good peak separation, which is important in metabolic studies, where the sample size is small. The stationary phase is a liquid with a high boiling temperature and must be as inert as possible.

**Table 8.2.** Commonly used stable isotope tracers of glucose, derivatization procedures and GC-MS analyses.

<table>
<thead>
<tr>
<th>Labelled substrate</th>
<th>Derivative</th>
<th>Ionization mode</th>
<th>Column</th>
<th>Masses</th>
<th>Carbons included in the fragment</th>
</tr>
</thead>
<tbody>
<tr>
<td>[1-13C]glucose</td>
<td>Penta-acetate</td>
<td>PCI</td>
<td>17/1701</td>
<td>331; 332</td>
<td>C1–C6</td>
</tr>
<tr>
<td>[U-13C]glucose</td>
<td>Penta-acetate</td>
<td>GC-C-IRMS</td>
<td>17/1701</td>
<td>13CO2/12CO2</td>
<td>C1–C6</td>
</tr>
<tr>
<td>[6,6-2H2]glucose</td>
<td>Penta-acetate</td>
<td>PCI</td>
<td>17/1701</td>
<td>242–244</td>
<td>C2–C6</td>
</tr>
<tr>
<td>[1-2H]glucose</td>
<td>Penta-acetate</td>
<td>PCI</td>
<td>17/1701</td>
<td>331–333</td>
<td>C1–C6</td>
</tr>
</tbody>
</table>
Fig. 8.4. Principles of gas chromatography–mass spectrometry (see text for details).
to prevent chemical interaction with the analytes. GC columns are labelled with letters (referring to the manufacturer) and numbers (referring to the chemical structure of the stationary phase).

**Mass spectrometer (MS)**

The GC effluent is introduced into the mass spectrometer via the GC-MS interface, in which a separator diverts the carrier gas from the MS, thus reducing the pressure (to preserve the vacuum in the ion source) and concentrating the substrate. There are different kinds of separators (jet, effusion, membrane separators), but all are based on the difference in physical properties between the carrier gas and the sample.

**Ion source**

Following the separation, the sample enters the ion source in the mass spectrometer (Fig. 8.4), where the molecule is ionized either by a direct bombardment of electrons (electron ionization mode, EI) or by reaction with methane ions generated by electrons bombarding methane gas molecules (chemical ionization, CI). In both cases the electrons are emitted from a heated filament and attracted towards a positively charged plate (the anode).

Employing the EI mode, a molecule passing through this beam of electrons is hit by electrons, resulting in removal of an electron from the molecule, thus generating a ‘molecular ion’:

\[
\text{Molecule} + e \rightarrow \text{Molecule}^+ + 2e.
\]

Due to the excess energy added to the molecule, it is broken apart into fragments. At a given electron energy, e.g. the conventional 70 eV for EI, a particular molecule will fragment in a very characteristic fashion.

When chemical ionization (positive or negative) is used, methane gas (reagent gas) is introduced into the ion source. When the methane molecules are hit by electrons, various methane ions (\(\text{CH}_5^+, \text{H}^+, \text{CH}_3^+, \text{C}_2\text{H}_5^+\) and \(\text{C}_3\text{H}_5^+\)) are generated, which, in turn, react with and ionize the sample molecules. Since the energy applied to the ions generated in the CI mode is lower than that produced in the EI mode, CI ionization results in larger fragments of the analyte than EI ionization.

The ions to be analysed (whether generated in the EI or the CI mode) are propelled out of the ion source by a relatively low repeller potential towards a number of focus lenses. A large potential over these lenses accelerates the ions to the mass analyser of the mass spectrometer.
For negative chemical ionization (NCI), the repeller and lens voltages are reversed compared with positive chemical ionization (PCI).

**Mass analyser**

There are two types of mass analysers, quadrupole and magnet sector instruments (the magnet mass analyser will be discussed below in connection with isotope ratio mass spectrometry).

The quadrupole serves as a mass filter. It consists of four parallel rods. Both a radio frequency and a DC voltage are applied to the rods, thus generating an electrodynamic field, i.e. a sinus wave with variable amplitude and frequency. The magnitudes of the radio frequency and the DC voltage determine the mass of the ions which can pass through the filter. Ions which successfully traverse the quadrupole are directed towards an electron multiplier (the detector). When the ions strike the metal surface of the electron multiplier, numerous electrons are discharged from the surface. These electrons hit the interior of the multiplier resulting in the release of additional electrons, thereby amplifying the signal 10⁵–10⁷-fold. The amplification of the signal is primarily determined by the energy and the mass of the analysed ion.

Finally, a computer is interfaced with the mass spectrometer to collect and process the signal data from the multiplier. The computer is also used to control GC, source and mass analyser.

**Gas chromatography–combustion–isotope ratio mass spectrometry (GC-C-IRMS)**

This technique measures the ratios of $^{13}$CO₂ and $^{12}$CO₂ or $^{15}$N to $^{14}$N in a sample. The GC principle is similar to that described above for GC-MS, but the GC is not connected directly to the MS but to a combustion oven, where the sample is oxidized at high temperature (940°C for carbon and 980°C for nitrogen) resulting in CO₂ (mass 44) and N₂ (mass 28), respectively. To assure complete oxidation of C to CO₂ without any potential contribution from N₂O (mass 44) and to achieve optimal production of N₂, i.e. removing remaining N₅O₃ and any potential CO (mass 28), the sample is then subjected to reduction at 600°C in a reduction oven.

Following the combustion, the N₂ and CO₂ gases, respectively, are directed into the vacuum of the ion source via a dual inlet system with a switch valve that permits alternate inflow of the sample and the reference gas. Thus, the sample and the reference gas can be analysed almost simultaneously. Since the mass spectrometer conditions are exactly the same
during analysis of reference gas and of sample, and the ratios between the $^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$, respectively, of the reference gas are known, the corresponding absolute ratios in the sample can be measured. As in the GC-MS instrument, a filament emits electrons that ionize the sample molecule; the ions are directed through a number of focusing lenses to the mass analyser. In the IRMS instrument, the analyser consists of a curved electromagnet (or a permanent magnet), which deflects the ions according to their mass (lighter masses are deflected more than heavier ones). The labelled and unlabelled ions are then collected simultaneously in separate Faraday’s coupes (double or triple collectors). When the ion beams hit the collector, a measurable ion current is discharged, and the ratio between the currents for the various masses is directly related to the isotope ratio.

**Derivatization Procedures for Glucose Analyses**

Several different derivatization procedures for glucose have been described. The dibutane-boronate (Wiecko and Sherman, 1976; Bier et al., 1977b) and di-O-isopropyledene (Hachey et al., 1999) derivatization procedures are relatively laborious and are not commonly employed but are useful in certain situations. In contrast, the penta-acetate derivatization procedure (Argoud et al., 1987) is simple and can be completed in 1–2 h. It can be used for analyses using both EI and PCI GC-MS as well as GC-C-IRMS. An additional advantage is that it requires only a small amount of plasma (50 µl or less) and, therefore, it is very useful for studies of glucose metabolism in infants and children.

**Penta-acetate derivative**

Plasma is deproteinized with ice-cold acetone (acetone : plasma = 4 : 1). The supernatant is separated from the pellet by centrifugation (3000 r.p.m. at 4°C) and dried under nitrogen (or vacuum). The drying is crucial for successful derivatization since any remaining water will destroy the derivatization process. Acetic anhydride/pyridine 2 : 1 (50 µl to 50 µl of plasma) is added and the sample (capped) is either heated at 60°C for 10 min or left at room temperature overnight. The derivatized sample can be analysed directly with GC-MS or dried under a stream of dry nitrogen at room temperature and reconstituted in ethyl acetate and then analysed by GC-MS employing the EI or PCI mode (see below) or GC-C-IRMS (see below).
**GC-MS and GC-C-IRMS Analyses of Glucose**

**GC column**

The chemical structure of the stationary phase determines the polarity of the GC column. For non-polar molecules (i.e. those including only carbons and hydrogens), a non-polar column should be chosen, while polar molecules, which also primarily contain carbons and hydrogens but also nitrogen, oxygen, phosphorus, sulphur, etc., require a more polar column. As mentioned above, GC columns are labelled with letters representing the manufacturer, e.g. SP, HP, and a number referring to the chemical structure of the stationary phase, e.g. 1, 5, 17 and 1701. For carbohydrates, medium polar GC columns (e.g. nos 17 and 1701) provide very good separation and peak sharpness. The stationary phase of the no. 1701 column consists of 14% cyanopropylphenyl and 86% dimethylpolysiloxane, and the no. 17 column, 50% phenyl and 50% methyl siloxane. The no. 17 column separates the $\alpha$ and $\beta$ isomers of glucose, while 1701 merges them.

**GC-MS or GC-C-IRMS**

Glucose labelled with $^{13}$C can be analysed by GC-MS as well as GC-C-IRMS, while GC-MS is the best choice for deuterated substrates, although some new GC-C-IRMS instruments are purported to measure deuterium enrichment in organic samples.

**Ionization mode**

Glucose can be analysed in both the EI and the PCI mode. Using the penta-acetate derivative, EI results in various fragments containing four to six of the six carbons in the glucose molecule (Biemann et al., 1962). Some of these fragments generate peaks that are too small (i.e. have too low abundance) to be measured accurately, or have a low mass within a range that contains many peaks from solvent, derivatization agents and other substrates, which might interfere with the fragment under analysis. A useful EI fragment from the penta-acetate derivative is mass (unlabelled compound) 242, in which carbon 1 is cleaved off. In the PCI penta-acetate fragment with mass 331 (unlabelled molecule), all six carbons in the glucose carbon skeleton are preserved, and at this high mass there are no confounding peaks derived from solvent or derivatization agents. Thus, performing mass isotopomer analysis during infusion of [U-
13C]glucose (see below), which requires analysis of the entire glucose molecule, the PCI penta-acetate fragment is the method of choice. If only EI is available, the dibutane-boronate (Wiecko and Sherman, 1976; Bier et al., 1977b) and di-O-isopropylatedene derivatives (Hachey et al., 1999) generate fragments containing all six glucose carbons.

In cases where both a [1-13C]- and a [6,6-2H2]glucose tracer are administered, it is advantageous to measure the [6,6-2H2]glucose enrichment by GC-MS employing the EI mode and using the 242 fragment, which cleaves off carbon 1, thus preventing any effects of the [1-13C]glucose tracer. The [1-13C]glucose enrichment can be analysed by GC-C-IRMS, which provides a measure of only the 13C glucose enrichment without any influence from the [6,6-2H2]glucose tracer (Table 8.2).

Optimizing the instrument performance

Before starting sample analyses, it is important to determine the status and optimize the performance of the instrument using tuning, natural abundance and standard curves.

1. Using a calibration fluid that generates well-defined peaks for each of the ionization modes, the voltages of the components (e.g. repeller, focus lenses, electron multiplier) of the mass spectrometer are adjusted so that sensitivity, peak shape, resolution etc. are optimized (tuning).

2. Knowing the chemical structure of the fragment to be analysed and the natural abundance of its components (Table 8.1), a theoretical value for natural abundance can be calculated, e.g. the structural formula for the penta-acetate PCI fragment of glucose (base mass M + 0 = 331) is C14H19O9.

\[
\text{Natural abundance for mass 332 (i.e. } M + 1) \text{ would be } (14 \times 1.11) + (19 \times 0.015) + (9 \times 0.037)\% = 16.158\%. \text{ The natural abundance of } M + 2, M + 3, \text{ etc. can be calculated using standard statistical formulae or commercially available software. The natural abundance of } M + 1, M + 2, \text{ etc. in a baseline blood sample or an unlabelled standard should not differ by more than } \pm 0.5\% \text{ from the theoretical values. Greater differences indicate that the performance of the instrument is not optimal or that there is contamination of the sample with labelled substrate.}
\]

3. Standard curves: one standard curve for each tracer used should always be analysed with the samples. These curves are used to correct for instrument deviations. Standard solutions containing carefully weighed mixtures of labelled (tracer) and unlabelled (tracee) substrate are prepared and their concentration determined by an independent method. Standards with tracer:tracee ratios covering the range of the tracer:tracee ratios expected in the samples are prepared and analysed daily with the samples of unknown tracer:tracee ratios. The standard curve must be absolutely linear, i.e. have an r² of at least 0.99; in other
words, it can be represented by the equation \( y = ax + b \), where \( y \) is the measured value, \( x \) is the correct value, \( a \) is the slope and \( b \) the intercept. The slope should be close to 1 and the intercept close to natural abundance. Since the instrument measures the tracer : tracee ratio of standards and samples equally, the corrected value can be derived from the standard curve equation.

**Use of Stable Isotopes and GC-MS and GC-C-IRMS in Studies of Carbohydrate Metabolism in Humans**

**Measurement of turnover rates of glucose**

In the fed state, the major part of plasma glucose is derived from the meal, while in the fasted state, glucose is produced from endogenous stores via glycogenolysis and gluconeogenesis (Fig. 8.2). During infusion of a glucose tracer (Table 8.2), the tracer is diluted by unlabelled substrate from the feeding, glucose infusion and/or from glucose produced via glycogenolysis and gluconeogenesis. When the tracer has equilibrated with unlabelled glucose (the tracee) and a condition of approximate steady state is achieved, the entry rate of glucose (labelled and unlabelled) into the plasma pool equals the disappearance rate of glucose (labelled and unlabelled) from the pool. Since the infusion rate of labelled glucose (tracer) is known, the plasma rates of appearance (\( R_a \)) and disappearance (\( R_d \)) of unlabelled glucose can be calculated using the following equation (Bier et al., 1977a):

\[
\text{Total glucose } R_a = R_d = \left( \frac{E_{\text{infusate}}}{E_{\text{plasma}}} \right) \times I \tag{1}
\]

where \( E_{\text{infusate}} \) is the enrichment \([\text{tracer}/(\text{tracer} + \text{tracee})]\) of the infused tracer; \( E_{\text{plasma}} \) is the enrichment \([\text{tracer}/(\text{tracer} + \text{tracee})]\) of the tracer in the plasma at a steady state and \( I \) is the infusion rate of the tracer.

The rate of glucose produced from the body stores (GPR), i.e. from glycogenolysis and gluconeogenesis, is calculated as follows:

\[
\text{GPR} = \text{total glucose } R_a - \text{exogenous glucose} \tag{2}
\]

where exogenous glucose includes tracer and all infused or ingested glucose. As mentioned above, this model is based on steady-state conditions, but the model also requires that the following assumptions be fulfilled:

1. There is a sufficient infusion rate of the tracer to achieve tracer : tracee ratios that can be measured with high accuracy and precision, i.e. for GC-
MS, no tracer:tracee ratios should fall below 0.7–0.8% and for GC-C-IRMS, not below 0.5%.

2. There is sufficient duration of the isotope infusion to achieve equilibration of the glucose pool. Using the above equation under conditions where a ‘true’ isotopic steady state has not yet been achieved will result in overestimation of plasma glucose appearance (disappearance) rate. The duration of tracer infusion required to reach tracer/tracee equilibrium is dependent on several factors such as the glucose turnover rate, e.g. in a newborn infant, the turnover rate is three times that of an adult. Thus, steady state is achieved faster in infants compared with adults. To reach steady state faster the constant infusion is usually primed. Usually a priming dose corresponding to 60–100 min of constant infusion is used in normoglycaemic subjects (blood glucose ~ 5 mM), while in diabetics with high blood glucose concentrations, a larger priming dose is required to reach equilibrium at a corresponding time (Heath, 1990). In a recent study we have addressed the impact of tracer duration on glucose turnover rates in healthy adults using a dual-tracer approach ([1-13C]glucose and [6,6-2H2]glucose) (Tigas et al., 2002). We demonstrated that with a priming dose corresponding to 60 min tracer infusion, independent of the tracer chosen, a 2.5 h infusion would overestimate glucose turnover by 26–35% compared with a 14.5 h infusion. Increasing the duration of the tracer infusion to 5 h corrected 80% of this error (Tigas et al., 2002).

3. There are no isotope effects, i.e. the tracer and tracee are metabolically equivalent.

4. There is no recycling of the tracer via glycogen storage and subsequent release or via the Cori cycle. Potential recycling is dependent on the choice of tracer. Thus, using glucose labelled with deuterium in the carbon-6 position ([6,6-2H2]glucose), the deuterium label is lost in the pyruvate–oxaloacetate and the malate–fumarate steps of the TCA cycle and, therefore, the [6,6-2H2]glucose can be regarded a non-recycling tracer (with regard to recycling in the Cori cycle). The [6,6-2H2]glucose tracer can, however, potentially recycle through glycogen. When [1-13C]glucose is used, the 13C label can recycle both via glycogen and the Cori cycle, resulting in a false increase in plasma 13C enrichment and subsequently underestimation of glucose Ra (Kalhan et al., 1980). In a recent study, we demonstrated that, even during a 14.5 h infusion, any recycling of [6,6-2H2]glucose or [1-13C]glucose via glycogen was negligible. There was, however, a small recycling of [1-13C]glucose via the Cori cycle (Tigas et al., 2002). Although the [U-13C]glucose tracer is recycled via the Cori cycle, the likelihood that two uniformly labelled three-carbon units will combine and form a new uniformly labelled glucose molecule is negligible and, thus, [U-13C]glucose can also be regarded a non-recycling tracer.
If steady state cannot be achieved during a primed constant rate tracer infusion, glucose turnover can be calculated using non-steady-state equations as described by Steele et al. (1956) and modified by De Bodo et al. (1963). However, these equations require an accurate estimate of the glucose pool and the fraction of this pool impacted over time. This is particularly complicated when a large glucose bolus is used, as for example when a stable labelled intravenous glucose tolerance test is performed. The limitations of Steel’s equations have been discussed by Cobelli et al. (1990).

Glucose production rates in newborn infants

Using [6,6-2H2]glucose and [1-13C]glucose and isotope dilution methods, we and others have demonstrated that:

1. Term healthy newborn infants produce glucose to maintain normoglycaemia within a few hours of birth before any feedings (Bier et al., 1977b; Kalhan et al., 1980; Sunehag et al., 1996b) at rates (on a per kg body weight basis) about three times (~ 30 µmol kg⁻¹ min⁻¹) those reported from normal adults after an overnight fast (~ 11 µmol kg⁻¹ min⁻¹) (Bier et al., 1977b; Haymond and Sunehag, 1999). In one of these studies both the [6,6-2H2]glucose and the [1-13C]glucose were used to determine the impact of recycling of the [1-13C]glucose tracer on measures of glucose Ra (Kalhan et al., 1980). The investigators reported that glucose carbon recycling overestimated the glucose turnover rate by 3–20% (Kalhan et al., 1980).

2. Newborn infants of diabetic mothers studied within the first hours of life before any feedings had 20% lower glucose turnover rates (production and utilization) than those born to non-diabetic mothers (Sunehag et al., 1997).

3. Very premature infants, i.e. with gestational ages below 29 weeks, can produce glucose at the same rate as term infants within the first day of life (Bier et al., 1977b; Sunehag et al., 1993, 1994; Tyrala et al., 1994). Since it is well known that in fetuses of the same gestational age, enzymes regulating glucose production are not activated, the reported results imply that the birth process itself activates these enzymes.

Glucose production rates in children

Using [6,6-2H2]glucose, Bier et al. (1977b) demonstrated that glucose production as a function of total body weight increases in a linear fashion up to a weight of 25–30 kg, after in a linear which it gradually approaches a plateau of 830 µmol min⁻¹ where it remains throughout adulthood. In a recent study, we have determined glucose production rates using [1-13C]glucose in healthy, non-obese, pre-pubertal children between the ages of 7 and 9 years and adolescents between the ages of 13 and 16 years following an overnight fast (Sunehag et al., 2001b). We demonstrated that:
(i) using a paired study design, measures of glucose turnover are highly reproducible in both children and adolescents (a difference of 10% could be detected in five subjects); and (ii) the pre-pubertal children had significantly higher glucose turnover rates (on a per kg body weight basis) than adolescents (~20 vs. 13 µmol kg⁻¹ min⁻¹). These values are in good agreement with our previous results (Bier et al., 1977b).

Although glucose turnover rates have been measured with high accuracy using stable isotope-GC-MS technique for 30 years, estimating its two components, gluconeogenesis and glycogenolysis, has been a challenge for many investigators and, in the past, many approaches have been tried in animal and human adult research. Measures of gluconeogenesis are complicated by inflow, loss and exchange of carbons in the TCA cycle reactions (Fig. 8.5).

Glycogenolysis can be estimated directly by nuclear magnetic resonance (NMR) but not using GC-MS, while gluconeogenesis can be estimated by GC-MS but not NMR. Thus, using NMR, gluconeogenesis must be calculated by subtracting rates of glycogenolysis from rates of glucose production (which must be measured by GC-MS). In contrast, using GC-MS glycogenolysis is calculated by subtraction. The method using NMR requires expensive and specialized equipment, which is generally not available, and the subject must be moved to the unit housing the instrument and must lie still for hours (limiting its use in infants and children). The method has been widely published and the reader is referred to these publications.

![Fig. 8.5. Schematic of the gluconeogenic and glycogenolytic pathways.](image-url)
Measurements of gluconeogenesis

Various approaches to measure gluconeogenesis using stable isotopes have been tried in the past. Those pertinent to the paediatric population are discussed below.

Estimating the gluconeogenic contribution from individual substrates (tracing a labelled gluconeogenic precursor, e.g. glycerol, alanine, or lactate, to the product, glucose)

Based on the appearance of $^{13}$C in glucose following infusion of [2-$^{13}$C]glycerol, we and others (Bougnères et al., 1982; Fjeld et al., 1992; Patel and Kalhan, 1992; Sunehag et al., 1996b, 1997) have demonstrated that in fasting term infants studied during the first day of life, glycerol accounts for about 10–20% of glucose production (measured by isotope dilution of [6,6-$^{2}$H$_{2}$]glucose). In addition, using the same study design, we demonstrated (Sunehag et al., 1996a) that very premature infants (< 28 weeks) receiving only a small amount of intravenous glucose (1/3 of their normal turnover rate) are capable of converting glycerol to glucose during the first day of life. Frazer et al. (1981), showed in term AGA (appropriate for gestational age) and SGA (small for gestational age) infants studied during their first 8 h of life that about 10% of $^{13}$C from infused [2,3-$^{13}$C$_{2}$]alanine appeared in blood glucose, implying that newborn infants are also capable of using amino acid carbon for glucose production in the immediate perinatal period.

Collectively, these results clearly demonstrate that gluconeogenic key enzymes are activated within a few hours of birth independent of gestational age. However, the information provided is limited because: (i) only one substrate at a time can be studied, and (ii) tracer dilution in the TCA cycle is not taken into account.

Recently three new stable isotope methods with the potential to measure gluconeogenesis accurately have been described. Each of them has, however, both strengths and weaknesses. The three methods are:

1. Mass isotopomer distribution analysis (MIDA) of plasma glucose during infusion of [U-$^{13}$C]glucose (Tayek and Katz, 1997; Katz and Tayek, 1998; Haymond and Sunehag, 2000) (Fig. 8.6).
2. MIDA during infusion of [2-$^{13}$C]glycerol (Hellerstein and Neese, 1992; Neese et al., 1995; Hellerstein et al., 1997) (Figs 8.7 and 8.8)
3. Ingestion (or infusion) of deuterium oxide and measurement of deuterium enrichment in glucose carbon 5 or glucose carbon 6 (Kalhan et al., 1995; Landau et al., 1995, 1996) (Fig. 8.9).
This method (Fig. 8.6) was described by Katz and Tayek (Tayek and Katz, 1997; Katz and Tayek, 1998) but has been recently modified by Haymond and Sunehag (2000). Briefly, infused \([U-^{13}C_6]\)glucose undergoes glycolysis and yields \([U-^{13}C_3]\)pyruvate, which in turn generates oxaloacetate.

**[U-^{13}C]glucose MIDA**

This method (Fig. 8.6) was described by Katz and Tayek (Tayek and Katz, 1997; Katz and Tayek, 1998) but has been recently modified by Haymond and Sunehag (2000). Briefly, infused \([U-^{13}C_6]\)glucose undergoes glycolysis and yields \([U-^{13}C_3]\)pyruvate, which in turn generates oxaloacetate.
Figure 8.8. Precursor–product relationship using [2-\(^{13}\text{C}\)]glycerol MIDA. Distribution of unlabelled, singly and doubly labelled glucose molecules at unlabelled/labelled triose-phosphate ratios of 50 : 50 and 90 : 10, respectively.

labelled with \(^{13}\text{C}\) in either \(\text{C1}–\text{C3}\) or \(\text{C2}–\text{C4}\). Oxaloacetate is subsequently converted to phosphoenolpyruvate (PEP) directly or following the reactions in the TCA cycle. PEP generated directly from oxaloacetate will be labelled with \(^{13}\text{C}\) in all three carbons (triply labelled). Tracer dilution due

Figure 8.9. \(^2\text{H}_2\text{O}\) method: relationship to the gluconeogenic pathway (glucose carbon 6 pathway shown in grey and glucose carbon 5 pathway in white).
to entry of unlabelled carbon from acetyl-CoA and amino acids (glutamine in particular via α-ketoglutarate), loss of carbon via CO$_2$, and exchange of carbon in the fumarate–oxaloacetate cycle, results in singly and doubly labelled oxaloacetate and subsequently PEP molecules. Thus, glucose generated from these singly, doubly and triply labelled PEP molecules will be labelled by $^{13}$C primarily in one (M + 1), two (M + 2) or three (M + 3) of its carbons, and if two labelled triose phosphate molecules combine, there will be a very small number of molecules labelled in four (M + 4) or five carbons (M + 5) (Fig. 8.6). M + 1, M + 2, M + 3, M + 4 and M + 5 of glucose are labelled isotopomers of glucose, which can only be generated via the gluconeogenic pathway. Since the likelihood that [U-$^{13}$C$_6$]glucose (M + 6) will be formed by recombination of two M + 3 molecules is extraordinarily small, the M + 6 of glucose in the plasma space represents non-metabolized labelled glucose. The isotopomer pattern of plasma glucose, i.e. the fraction of each isotopomer, is determined by GC-MS after correction for natural abundance and contribution from the tracer. These corrections can be manually calculated (Fernandez et al., 1996). This is laborious, but software packages are available (Fernandez et al., 1996). It is of great importance that the infusion rate of the tracer is sufficient to achieve enrichments that can be measured with high accuracy by GC-MS for each individual (M + 0 to M + 6) isotopomer. Thus, at steady state, the enrichment of the largest isotopomer, i.e. the M + 6, should not be below ~ 6%. The fraction of gluconeogenic molecules (% GNG molecules) can be calculated from the isotopomer pattern of plasma glucose using the following equation:

\[
\% \text{GNG molecules} = \frac{5 \Sigma M_i / 6 \Sigma M_i}{5 \Sigma M_i / 6 \Sigma M_i} (3)
\]

where $\Sigma M_i$ is the labelled glucose molecules generated via gluconeogenesis (M + 1 to M + 5), and $\Sigma M_i$ is all labelled glucose molecules.

However, this would represent only a minimal estimate of gluconeogenesis because of the tracer dilution in the TCA cycle. This dilution can be corrected by comparing the $^{13}$C isotopomer pattern in lactate to that of glucose, as described by Tayek and Katz (1997) and by Katz and Tayek (1998). This model requires analysis of plasma lactate enrichments (Hachey et al., 1991), i.e. an additional derivatization and GC-MS analysis are needed (Tayek and Katz, 1997). In addition, lactate enrichments measured in a peripheral vein may not be representative of that in portal blood. Therefore, we have published an alternative method to correct for TCA cycle dilution (Haymond and Sunehag, 2000). According to this model, the TCA cycle dilution is represented by the ratio of $^{12}$C/$^{13}$C in the labelled molecules of glucose that could only be derived via gluconeogenesis (glucose M + 1 to M + 5). Thus, the uncertainties and extra work associated with lactate analyses are avoided.
Gluconeogenesis as a fraction of glucose $R_a$ is the product of gluconeogenic molecules and the dilution factor:

$$\text{Fractional GNG} = \left( \frac{\sum M_i}{\sum M_j} \right) \times \left( \frac{\sum^{12} C_i}{\sum^{13} C_i} \right)$$

Glucose $R_a$ is calculated from the $[U-^{13}C]$glucose enrichment (M + 6) using isotope dilution, as described above. The rate of gluconeogenesis is then calculated from the product of fractional gluconeogenesis and glucose $R_a$.

$[2^{-13}C]$glycerol MIDA

This method was described by Hellerstein et al. (1992, 1997) and by Neese et al. (1995). The infused $[2^{-13}C]$glycerol is subsequently converted to dihydroxyacetone phosphate (which is continuously involved in interconversion with glyceraldehyde-3-phosphate), thus providing singly labelled three-carbon units to the triose phosphate pool. Glucose (product) formed by combination of two three-carbon units (i.e. two triose-phosphates) (precursor) can be unlabelled, singly or doubly labelled (Fig. 8.7). As shown in Fig. 8.8, the mass isotopomer distribution (the fractions of M + 0, M + 1 and M + 2) is dependent on the enrichment of the precursor pool according to the generic formula for the formation of a biopolymer, $(a + b) \times (a + b) = a^2 + 2ab + b^2$.

The principles for $[2^{-13}C]$glycerol MIDA calculations of gluconeogenesis have been described in detail by Hellerstein et al. (1992, 1997) and by Neese et al. (1995). Briefly, based on the GC-MS measures of the M + 0, M + 1 and M + 2 ion current ratios using the penta-acetate derivative of glucose: (i) the excess M + 1 and M + 2 are calculated by subtracting natural abundance (obtained from the baseline sample); (ii) excess M + 2/excess M + 1 is calculated; (iii) the triose-phosphate pool enrichment (p) is calculated using a polynomial equation including the M + 2/M + 1 ratio (Hellerstein et al., 1992, 1997; Neese et al., 1995); (iv) a value corresponding to 100% gluconeogenesis (the asymptotic value = A) is determined from a second polynomial equation also including the M + 2/M + 1 ratio; and (v) fractional gluconeogenesis (% of glucose $R_a$) is calculated from the ratio of excess M + 1/A (Hellerstein et al., 1992, 1997; Neese et al., 1995). This method is very error sensitive. Thus, to ensure reliable data, it is crucial that: (i) natural abundance in the unlabelled standard and the baseline plasma sample should be very close to the theoretical value (at most ± 0.5% difference); (ii) both M + 1 and M + 2 are > 0.5%; and (iii) p is between 9 and 20% (Hellerstein et al., 1992, 1997; Neese et al., 1995). To fulfil (ii) and (iii) requires that the
[2,13]glycerol tracer be infused at a substrate rather than a tracer rate (25–50% of the glycerol turnover rate). In addition, a second tracer is needed for measurement of glucose Ra, e.g. [U-13C]glucose infused at a low rate, i.e. ~0.1–0.4 µmol kg⁻¹ min⁻¹ (depending on expected turnover rate). At these infusion rates, potential M + 1 and M + 2, which could interfere with those derived from the [2,13C]glycerol tracer, are negligible.

Deuterium enrichment at glucose carbons 5 and 6

This method was described by Landau et al. (1995, 1996) and Kalhan et al. (1995). Ingested (or infused) deuterium oxide equilibrates with body water, and subsequently deuterium exchanges with the hydrogens at various stages of glucose metabolism, e.g. with the hydrogens at the methyl carbon of pyruvate (carbon 3) and the hydrogen at carbon 2 of glyceraldehyde-3-phosphate (Fig. 8.9). When glucose is formed from pyruvate, the pyruvate carbon 3 with its attached hydrogen/deuterium atoms becomes carbon 6 of glucose (Fig. 8.9), while carbon 2 of glyceraldehyde-3-phosphate with its attached hydrogen/deuterium becomes carbon 5 of glucose (Fig. 8.10). Thus, it was theorized and we demonstrated (Sunehag et al., 1999a) that deuterium enrichment in glucose carbon 6 represents gluconeogenesis from pyruvate (which does not include the contribution from glycerol) and the deuterium enrichment at glucose carbon 5 represents total gluconeogenesis, i.e. including the contribution from glycerol (Figs 8.9 and 8.10) (Landau et al., 1996). While deuterium incorporation at glucose carbon 5 and 6 represents only gluconeogenesis, the deuterium enrichment at glucose carbon 2 reflects total equilibration of deuterium in body water (Fig. 8.10). This latter measure can be replaced by a measure of deuterium enrichment in plasma water or urine.

Animal data have indicated that humans can safely ingest deuterium oxide enriched to 1%, even for prolonged periods (Peng et al., 1972). To achieve a body water enrichment of 1% would require ingestion of 10 g deuterium oxide kg⁻¹. If this amount were ingested as a bolus, it would result in reversible but severe vertigo. We have used a dose of 3–5 g kg⁻¹ (resulting in a body water enrichment of 0.3–0.5%) distributed over 6–8 h in both children and adults without any side effects. A body water enrichment of this low magnitude would result in deuterium enrichment in glucose carbons 5 and 6 below the limit of accuracy for usual GC-MS measurements. However, Kalhan et al. (1995) and Landau et al. (1995) have described a method to overcome this problem. For measurements of deuterium enrichment in glucose carbon 6, carbon 6 with its attached hydrogens and hydroxyl group is cleaved off using periodate, thus generating formaldehyde. When formaldehyde is reacted with ammonium, hexamethylenetetramine (HMT), C₆H₁₂N₄, is generated. Each of the
Fig. 8.10. Schematic of proton transfer during gluconeogenesis (grey) and glycogenolysis (white).
six carbons in HMT (with their attached hydrogens) is derived from carbon 6 of glucose, resulting in HMT enrichments fivefold higher than that of glucose carbon 6. The fivefold increase instead of the theoretical sixfold is the result of incomplete proton exchange between body water and the methyl hydrogens of pyruvate. The HMT can be analysed directly by GC-MS without further derivatization. The HMT enrichments are converted to the corresponding glucose carbon 6 enrichments using a standard curve. Since glucose labelled with only one hydrogen at carbon 6 is not available commercially, a standard curve prepared from [1-2H1]glucose converted to sorbitol is used (Muntz and Carrol, 1960; Landau et al., 1995). Sorbitol has an alcohol group at positions 1 and 6, and consequently HMT can be made from both. Since only one is labelled, the enrichment for each standard must be divided by two. Due to the incomplete equilibration between the protons in body water and those at carbon 3 of pyruvate, the slope of the standard curve is \( \sim 5 \).

Measurement of deuterium enrichment in glucose carbon 5 is an extremely tedious procedure involving about 50 steps (Landau et al., 1996). Principally, xylose is formed and HMT is prepared from carbon 5 in the same fashion as described above for carbon 6 of glucose. The HMT enrichments are converted to the corresponding glucose carbon 5 enrichments using a standard curve prepared from D7-glucose. Since the equilibration between the protons in body water and that at carbon 2 of glyceraldehyde-3-phosphate is complete, the enrichment in HMT will be sixfold higher than that of glucose carbon 5, i.e. the slope of the standard curve should be \( \sim 6 \).

HMT is analysed employing the EI mode. A non-polar GC column (No. 5) with thick film (1.0 \( \mu \)M) provides excellent chromatographic resolution.

Gluconeogenesis as a fraction of glucose \( R_a \) can be calculated as follows:

\[
\text{Gluconeogenesis from pyruvate} = \frac{2H_{C6}}{2H_{\text{body water}}} \tag{5}
\]

where \( 2H_{C6} \) is deuterium enrichment in glucose carbon 6 and \( 2H_{\text{body water}} \) is deuterium enrichment in body water. Total gluconeogenesis is determined by:

\[
\text{Total gluconeogenesis} = \frac{2H_{C5}}{2H_{\text{body water}}} \tag{6}
\]

where \( 2H_{C5} \) is deuterium enrichment in glucose carbon 5.

Deuterium enrichment in plasma water or urine can be measured by gas isotope-ratio mass spectrometry (see Chapter 7).

The deuterium oxide glucose carbon 6 method is thought to underestimate gluconeogenesis as a result of incomplete equilibration of the
deuterium in body water and the hydrogens at pyruvate carbon 3, and because the contribution of glycerol is not included. In contrast, the [U-13C]glucose MIDA, [2-13C]glycerol MIDA and the deuterium oxide glucose carbon 5 method are reported to reflect total gluconeogenesis.

Table 8.3 depicts the advantages and disadvantages of each of these latter three methods.

<table>
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<td>TCA cycle dilution makes calculations somewhat more complicated.</td>
<td>Precursor pool estimated (not measured directly). Requires single pool assumption. Potential hepatic glycerol zonation may lead to under-estimation of gluconeogenesis. Substrate amount of tracer needed. Sensitive to analytical errors.</td>
<td>Straightforward concept. TCA cycle dilution must not be accounted for.</td>
</tr>
<tr>
<td>Requires only very small sample volumes, therefore can be used in all subject populations. Very simple sample preparation. Rapid sample throughput. One analytical measurement gives complete data. Despite higher isotope cost, lower total cost compared with the deuterium oxide glucose C5 method. Brief isotope retention. Does not partition glycerol fraction of gluconeogenesis.</td>
<td>Requires only very small sample volumes, therefore can be used in all subject populations. Very simple sample preparation. Rapid sample throughput. Two analytical measurements required for complete data. Despite higher isotope cost, lower total cost compared with the deuterium oxide C5 method. Brief isotope retention. Partitions glycerol fraction of gluconeogenesis.</td>
<td>Requires large blood sample volumes (precludes its use in infants and small children). Laborious sample preparation. Slow sample throughput. Three analytical measurements required for complete data. High personnel cost resulting in high total cost. Long isotope retention. Does not partition glycerol fraction of gluconeogenesis.</td>
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Comparison of the three methods

Although there are controversies regarding which of these methods provides the most accurate estimates of gluconeogenesis, each of them may be uniquely useful depending on the study design and population since: (i) in infants and small children the sample volume that can be withdrawn is limited; (ii) a study including numerous samples requires simple sample preparation; and (iii) there may be use of other tracers in the same study. Therefore, we compared these methods in nutritional studies in infants, children and adults under identical conditions.

The [U-13C]glucose MIDA, [2-13C]glycerol MIDA and the deuterium oxide glucose carbon 6 methods were compared in three groups of prematurely born infants receiving total parenteral nutrition providing glucose at a reduced rate (50% normal glucose turnover rate) (Sunehag et al., 1999a). Our results demonstrated that [U-13C]glucose and the [2-13C]glycerol MIDA provided virtually identical estimates of gluconeogenesis, while the deuterium oxide glucose carbon 6 estimate was significantly lower (Fig. 8.11). Using [2-13C]glycerol MIDA, the gluconeogenic contribution from glycerol and non-glycerol sources could be partitioned, demonstrating that the deuterium oxide glucose carbon 6 estimate corresponded very well to the non-glycerol contribution as measured by the [2-13C]glycerol MIDA. As mentioned above, this is precisely what should

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**Fig. 8.11.** Comparison of [U-13C]glucose MIDA, [2-13C]glycerol MIDA and 2H2O with measurement of deuterium enrichment in glucose carbon 6 in three groups of comparable very premature infants receiving total parenteral nutrition. Adding the contribution from glycerol (as measured by [2-13C]glycerol MIDA) to the 2H2O glucose carbon 6 estimate would result in equal estimates of gluconeogenesis using all three methods.
be expected since the deuterium oxide glucose carbon 6 does not include the contribution from glycerol. In conclusion, in infants, both \([U^{13}C]\text{glucose}\) and \([2^{13}C]\text{glycerol}\) MIDA are useful tools for measuring total gluconeogenesis, while deuterium oxide with measurements of the deuterium enrichment at glucose carbon 6 would underestimate gluconeogenesis by the contribution from glycerol. Thus, under conditions where glycerol is a major gluconeogenic substrate (e.g. in individuals receiving parenteral lipids), the deuterium oxide glucose carbon 6 method would significantly underestimate gluconeogenesis. The deuterium oxide glucose carbon 5 method, which does include the contribution from glycerol, requires large blood sample volumes, and its use is therefore precluded in premature infants.

We have, however, compared the \([U^{13}C]\text{glucose}\) MIDA with the deuterium oxide glucose carbon 5 methods in healthy young adults following a 66 h fast. Preliminary results demonstrated that the two methods estimate gluconeogenesis equally well (Sunehag et al., 2001a).

In a recent publication, Kalhan et al. (2001) used the deuterium oxide glucose carbon 6 method and reported that, in newborn term infants 24–48 h of age, gluconeogenesis from pyruvate, i.e. not including the contribution from glycerol, accounted for about 30% of glucose production. We measured gluconeogenesis from pyruvate using the deuterium oxide glucose carbon 6 method in a group of eight children (aged 8–16 years) following an overnight fast. The subjects were studied on two occasions following a week of identical diets i.e. 60% carbohydrate, 25% fat and 15% protein (Sunehag et al., 2001b). We found that: (i) measures of gluconeogenesis were highly reproducible (a 10% difference could be detected with a sample size of nine subjects, and a 25% difference with only four subjects) (Sunehag et al., 2001b); (ii) gluconeogenesis from pyruvate accounted for about 50% of glucose production (Sunehag et al., 2001b).

We have also investigated the impact of high carbohydrate/low fat diet (60% carbohydrate, 25% fat and 15% protein) versus low carbohydrate/high fat diet (30% carbohydrate, 55% fat and 15% protein) on measures of gluconeogenesis in adolescents (Sunehag et al., 1999b). Our results demonstrated that, after an overnight fast, gluconeogenesis measured by the deuterium oxide glucose carbon 6 method accounted for 55 ± 3% (high carbohydrate diet) and 58 ± 2% (high fat diet) of glucose production, i.e. the dietary macronutrient content did not significantly affect gluconeogenesis (Sunehag et al., 1999b).

To our knowledge, there is no other published report on gluconeogenesis in healthy children (after the newborn period). Only one single study has reported data on gluconeogenesis in Kenyan children with falciparum malaria using \([2^{13}C]\text{glycerol}\) MIDA, but this study did not include any healthy controls (Dekker et al., 1997).
Summary

Stable isotopes and GC-MS are useful tools in studies of the dynamics of carbohydrate metabolism in infants and children. Stable isotopes are non-radioactive and naturally occurring, and substrates labelled with stable isotopes are metabolically equivalent to the corresponding unlabelled compounds. They can therefore be used without risk in children. GC-MS, GC-C-IRMS and IRMS have very high precision and sensitivity and require only small amounts of plasma (with the exception of the deuterium oxide glucose carbon 5 method for measuring gluconeogenesis). This is of particular importance in studies of infants and small children in whom only limited amounts of blood can be withdrawn. Additionally, various tracers can be combined in the same study. Thus, carbohydrate, amino acid and lipid metabolism, as well as their interaction, can be measured simultaneously. It has to be pointed out, however, that using a multi-tracer design requires in-depth knowledge about substrate metabolism to avoid interaction between the tracers, thus confounding the results.

References

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Introduction

Cholesterol metabolism has long been of interest to investigators working in the field of coronary heart disease, but its importance has increased in recent years with the publication of many interventional and prospective studies showing that reduction of serum cholesterol will lower the incidence of coronary heart disease (Gould et al., 1998). While this has been an extraordinary achievement, it has had the unintended effect of focusing almost all attention on plasma cholesterol and low-density lipoprotein (LDL) concentrations to the exclusion of other potentially important aspects of cholesterol physiology. In contrast to plasma lipoprotein concentration and kinetic studies, whole-body cholesterol metabolism is concerned with the overall production, absorption, turnover and elimination of cholesterol. Plasma cholesterol is a relatively small component of the overall cholesterol economy (Fig. 9.1). The total body cholesterol content is about 73 g, whereas plasma cholesterol is only 7 g (Goodman et al., 1980). Circulating cholesterol is part of a single rapidly exchanging pool, which contains an additional 17 g in liver, red blood cells and a portion of all soft tissues. The remainder of body cholesterol, about 49 g, is found in slowly equilibrating pools throughout the body. It is apparent that there is a great deal of physiological information about cholesterol that cannot be obtained by studies of plasma cholesterol alone.

Whole-body cholesterol metabolism can be defined mathematically as well as physiologically. Mathematically, plasma cholesterol kinetics
has often been fitted to a traditional two-compartment (Goodman and Noble, 1968) or three-compartment model (Goodman et al., 1973; Samuel and Lieberman, 1973). Data are generated by the intravenous administration of cholesterol tracer followed by plasma cholesterol sampling for 10-40 weeks. Figure 9.2 (solid line) shows the average decay of plasma tracer over 40 weeks in humans. However, both two- and three-compartment models are not completely satisfactory for use in patient studies. The two-compartment model is somewhat inaccurate because the data

Fig. 9.1. Body cholesterol pools. Data are from Goodman et al. (1980). The mass of the slow pools cannot be determined exactly by isotopic methods.

Fig. 9.2. Turnover of cholesterol tracer in human plasma after intravenous injection (Ostlund, 1993). The solid line is calculated from reported values, whereas the open and closed circles are derived from two theoretical datasets in which some parameters of a three-pool model differ from each other by 45–81%.
are more complex than can be accounted for by two compartments. Unfortunately, while the three-compartment model can account for the data, it cannot be solved with precision, i.e. even with multiple plasma samples over many months, the kinetic constants associated with the second and third compartments cannot be calculated uniquely and many quite different solutions can be found that all fit the data equally well (Ostlund, 1993). In Fig. 9.2, the open and solid circles are from two theoretical data sets that fit the solid decay curve very well but differ by 45–81% in kinetic constants. Thus, the three-pool model is not useful for most physiological studies that involve small numbers of subjects. Figure 9.3 shows an alternative minimal model of whole-body cholesterol metabolism that overcomes some of these difficulties. The rapidly exchanging pool 1 has a mass of about 24 g with a daily turnover rate of 4.6%. While rapid with respect to other cholesterol pools, this turnover is still quite slow with respect to most other metabolic processes. The daily cholesterol production rate of about 1100 mg comprises about 85% endogenous production and 15% dietary absorption. The forward rate constant for transfer from the rapid cholesterol pool to slow pools is 8.4% day\(^{-1}\) and the reverse rate constant is 4.1% day\(^{-1}\). Pools 2 and 3, which both turn over slowly, are not distinguished. These kinetic constants can be determined with a fractional standard deviation of 3–12% after 10 weeks of sampling. The exact mass of the slow cholesterol pools cannot be determined by any current tracer method since it is possible.

**Fig. 9.3.** A minimal model of cholesterol metabolism (Ostlund, 1993). Pool 1 is the rapidly exchanging cholesterol pool consisting of plasma and liver cholesterol and a portion of the cholesterol in other organs. Pools 2 and 3, which cannot be easily resolved experimentally, are more slowly mixing pools found in many organs. \(L(0,1)\) is the first-order rate constant for cholesterol removal from the body, and the forward and reverse rates are kinetic constants for transport of cholesterol between the rapid and slow pools. Arrows indicate cholesterol input from diet and endogenous biosynthesis.
that there is direct secretion of cholesterol into them. The very slow nature of cholesterol turnover can be appreciated and is emphasized by the finding that injected cholesterol radioactive tracer is still detectable in the plasma a year after intravenous administration (Samuel et al., 1972).

Cholesterol kinetics can also be viewed as a physiological model, as shown in Fig. 9.4 (Ostlund, 2002). Dietary cholesterol of 300 mg day\(^{-1}\) mixes with 1000 mg of endogenous biliary cholesterol to form an intestinal pool from which 56% of the cholesterol is absorbed (Bosner et al., 1999). The key points of regulation of whole-body cholesterol metabolism are associated with cholesterol entry or exit. Dietary cholesterol consumption and endogenous biosynthesis together account for the cholesterol production rate or rate of cholesterol entry into the body. Cholesterol biosynthesis is downregulated as the amount of absorbed cholesterol increases (Grundy et al., 1969) and is increased when cholesterol absorption is reduced by feeding phytosterols (Gylling et al., 1999). Although small amounts of cholesterol are converted to steroid hormones or desquamated in skin cells, cholesterol is almost quantitatively eliminated in the stool, either as unabsorbed intestinal cholesterol or bile acids. The key points of metabolic control for cholesterol elimination are bile acid synthesis, biliary cholesterol secretion and reabsorption of cholesterol in the intestine. It can be seen that cholesterol absorption is important, whether or not the diet contains cholesterol.

There are no definitive data relating whole-body cholesterol metabolism to coronary heart disease, and such studies are needed. Some workers have reported no relationship of the mathematical parameters of cholesterol models to lipoprotein levels (Palmer et al., 1986), whereas others have found an inverse relationship between high-density lipoprotein (HDL) cholesterol and slowly exchanging cholesterol pools (Miller, 1987). However, these studies were small and the patients highly selected.

**Fig. 9.4.** A physiological model of whole-body cholesterol metabolism (Ostlund, 2001).
and possibly not representative of the population. Moreover, there are no studies relating whole-body cholesterol metabolism to coronary heart disease itself, and cholesterol absorption measurements have often been omitted.

**Synthetic Cholesterol Tracers**

In many stable isotope applications, the labelled tracer is introduced in a quantity designed to enrich the pool of natural material by approximately 1–5% and detection is performed with gas chromatography–mass spectrometry (GC-MS). This level allows ready determination of the tracer:tracee ratio. However, if cholesterol tracer were given to achieve an enrichment of 1%, it would involve administration of 700 mg, an amount that would be clinically unsafe and economically unfeasible. Thus, the principal analytical problem in cholesterol metabolism is the large natural cholesterol pool that forms the background noise level above which the signal of the cholesterol tracer must be read. As shown in Fig. 9.5, natural cholesterol is a mixture of isotopomers due largely to natural abundance $^{13}C$. The $m/z$ 386 base mass of cholesterol comprises 74% of the isotopomers but appreciable amounts are found in higher masses and $m/z$ 390 ($M+4$) still accounts for one part in 300 of natural cholesterol. Since tracer detection at dilutions of 1/2500 or more is desired, tracers have usually been multiply labelled with either deuterium or $^{13}C$ to give products increased by 5–8 mass units. In this way, the natural background of high-mass isotopomers is greatly reduced. Representative tracers (see Fig. 9.6 for numbering) are:

- $[2,2,3,4,4,6,7,7-2H_{8}]$cholesterol (Ferezou et al., 1981b),
- $[25,26,26,27,27,27-2H_{7}]$cholesterol (Lutjohann et al., 1993; Jones et al., 2000),
- $[26,26,27,27,27-2H_{6}]$cholesterol (Bosner et al., 1993; Lutjohann et al., 1993),
- $[2,2,4,4,6-2H_{5}]$cholesterol (Bosner et al., 1993; Lutjohann et al., 1993),
- $[23,24,25,26,27-13C_{5}]$cholesterol (Ostlund and Matthews, 1993).

Cholesterol labelled with $^{18}O$ has also been studied as a potential tracer for cholesterol metabolism (Hudgins et al., 1988). Labelling in the side chain and in the sterol nucleus are equally acceptable. Deuterated cholesterol is usually preferred because they are much less expensive than those made with $^{13}C$ and appear to be metabolized identically to cholesterol with retention of the label after passage through the gastrointestinal tract and absorption into the plasma (Lutjohann et al., 1993; Bosner et al., 1999). Before use, all sterol tracers need to be adequately solubilized by
warming in oil or by emulsification, since cholesterol crystals are extremely energetically stable and dissolve in bile salt solutions only over many days (Mufson et al., 1972).

**Analytical Methods**

There are two general types of mass spectroscopic methods for determining cholesterol enrichment, GC-MS or liquid chromatography–mass spectrometry (LC-MS), in which cholesterol is kept intact or subjected to controlled fragmentation before measurement, and isotope ratio mass spectrometry (IR-MS), where the cholesterol molecule is converted to simple compounds such as CO₂ or H₂ before analysis. GC-MS is most commonly used and has the advantages of high chromatographic resolution of sterols and high throughput. Gas chromatography can be done with most commercial columns but the best resolution of sterols with subtle structural differences is achieved with relatively polar stationary phases such as methylphenyl or trifluoropropylmethyl polysiloxane. The latter is useful for the separation of Δ⁵-sterols from corresponding 5α-reduced stanols (Ostlund et al., 2002).

Negative ion mass spectrometry is an especially sensitive method for detection of cholesterol with more dynamic range for comparing small amounts of tracer with large amounts of natural material (Ostlund et al., 1996). As little as 1–10 fg is detectable. Cholesterol is extracted from plasma as crude neutral sterols and then derivatized with pentafluorobenzoyl chloride to give cholesteryl pentafluorobenzoate (Fig. 9.6). Under the standard conditions of electron ionization mass spectrometry,
cholesterol is fragmented into characteristic ions that reveal the sterol structure. However, with the much more gentle conditions of methane chemical ionization, the cholesterol molecule remains essentially intact and the principal signal obtained is the molecular anion at $m/z$ 580 (Fig. 9.7). In negative ion mass spectrometry in general, and in the case of

Fig. 9.6. Structure of cholesterol and cholesteryl pentafluorobenzoate.

Fig. 9.7. Mass spectrum of cholesteryl pentafluorobenzoate (Ostlund et al., 1996).
cholesterol in particular, the instrument running conditions are critical. Small air leaks, detectable with SF$_6$ gas, may eliminate almost all the signal. The methane pressure and ion source temperature are also critical and should be investigated using injected cholesteryl pentafluorobenzoate itself as the test material. For sample analyses, it is desirable to inject as much sample as possible, and the large amount of cholesterol in plasma makes this feasible, but with high sample loads that still give satisfactory chromatography a false signal peak is sometimes observed at all monitored masses. Figure 9.8 shows that this artefact, quantified by the peak area at the unrelated peak of m/z 700, can be reduced by controlling the methane flow with little effect on the cholesterol signal measured at m/z 581 (M+1). It is often desirable to acquire data at a mass higher than M to facilitate measurement of the very large peak of natural cholesterol.

IRMS is an alternative method with several advantages with respect to cholesterol metabolism. In this technique, enrichment in circulating
Cholesterol is determined on purified cholesterol after combustion to CO$_2$ or reduction of combustion to hydrogen gas (Hudgins et al., 1988; Jones et al., 1993). The principal positive feature is that the isotope ratios can be measured to a very high precision, sometimes as much as $10^{-5}$, or 20-fold that of GC-MS. The presence of isotopomers in natural material is not as important, and tracers do not have to be heavily labelled to be detectable over the natural cholesterol background. The principal drawback of IRMS has been the rather laborious preparation of samples for analysis and small sample throughput. However, purification of the sample by gas chromatography with online combustion and/or reduction is being developed rapidly and workable methods have been reported for cholesterol (Gremaud et al., 2001). Although online analyses tend to be less sensitive than traditional offline IRMS sample preparation and standard instrumentation, the online technique seems very promising.

**Cholesterol Biosynthesis**

Deuterated water incorporation was used to measure cholesterol biosynthesis in animals in one of the first metabolic turnover studies ever performed (Rittenberg and Schoenheimer, 1937) and has been employed successfully up to the present time (Taylor et al., 1966; Peng et al., 1973; Jones et al., 1993, 1999). For human studies, about 50 g of deuterated water is given orally and plasma is taken for analysis at baseline and after 24 h. The enrichment of plasma water is at an equilibrium level after 1 h and remains nearly constant over 24 h, whether or not water losses are replaced by feeding labelled drinking water. Cholesterol is isolated by chromatography and its hydrogens pyrolysed to water and reduced to hydrogen gas, which is in turn analysed by IRMS. Some workers have given larger doses of deuterated water and used GC-MS instead of IRMS as the method of detection (Diraison et al., 1997). Of the 46 hydrogens in cholesterol, about 22 are derived from water with the rest being from unlabelled carbon precursors. The fractional rate of cholesterol biosynthesis is calculated from the average enrichment of body water and cholesterol. The absolute rate of cholesterol biosynthesis requires knowledge of the mass of the rapidly mixing body cholesterol pool or its estimation from published data. Measurement of cholesterol biosynthesis can be performed over shorter time periods but its diurnal variation with a peak at night in humans needs to be taken into account. Substantial differences in cholesterol biosynthesis can be measured while on unsaturated vs. saturated dietary fats (Jones et al., 1994).
Long-term studies of cholesterol turnover require the intravenous administration of cholesterol tracer. Since cholesterol is insoluble in water, it must be solubilized in a biocompatible form that is sterile, non-pyrogenic and does not result in deposition of microparticulates in the reticulo-endothelial system. The most widely used method has been to combine 15–100 mg cholesterol tracer in ethanol with lecithin-stabilized intravenous triglyceride emulsions such as Intralipid® (Ferezou et al., 1981a; Bosner et al., 1999). Tracer is dissolved in ethanol at 20 mg ml$^{-1}$, passed through a solvent-resistant 0.2 µm filter, and tested for sterility by culture in thioglycollate broth and for pyrogenicity with the limulus assay. Tracer solution and Intralipid are warmed to 37°C and ethanolic tracer solution is added dropwise to the Intralipid with mixing. After cooling to room temperature the mixture is passed through a 1.2 µm particulate filter and the final concentration of tracer is determined by mixing it with natural cholesterol. When stored at −12°C the intravenous tracer is stable for a month and can be prepared in large batches. It is given intravenously by injecting into a freely flowing infusion of saline followed by washing the syringe with saline.

When 100 mg of tracer was infused the enrichment in plasma cholesterol could be followed for more than 60 days by IRMS (Fig. 9.9) (Ostlund and Matthews, 1993). The calculated cholesterol kinetic parameters were...
identical to those obtained with $^{14}$C cholesterol injected at the same time. Intravenous cholesterol equilibrates with biliary cholesterol after several days and the enrichment in stool can be used to estimate biliary cholesterol secretion (Ferezou et al., 1982).

**Cholesterol Absorption**

Cholesterol absorption can be measured either as percentage absorption (percentage of intestinal cholesterol that is absorbed) or absolute absorption (mg of intestinal cholesterol absorbed). Both give unique information. Percentage cholesterol absorption is most closely related to the intrinsic function of the intestine and is useful for assessing events occurring within the intestine, such as the inhibition of cholesterol absorption by plant sterols (Ostlund et al., 1999b). Percentage absorption varies considerably with physiological processes and is often informative with respect to the regulation of those processes. Absolute absorption is important in determining the feedback effect of cholesterol on cholesterol biosynthesis and LDL receptor level. Absolute cholesterol absorption is the result of several processes including biliary cholesterol secretion, as well as the efficiency of intestinal absorption, and tends to be regulated to a constant value. Thus, it may vary less with physiological manipulations than percentage absorption.

Percentage cholesterol absorption can be measured by determining either the amount of oral tracer absorbed into plasma or the amount that is not absorbed and is subsequently excreted in the stool. Plasma methods are based on the pharmacological concept that absorption can be determined by giving one labelled tracer intravenously and another orally (Zilversmit and Hughes, 1974). The ratio of the isotopes in plasma is compared with that administered and will be unity if absorption is complete. The purpose of the intravenous isotope is to quantify the endogenous cholesterol pool into which the absorbed oral isotope is diluted. In a typical study, 30 mg of cholesterol-d$_6$ is given orally dissolved in vegetable oil in a test meal and 15 mg cholesterol-d$_6$ is given intravenously (Bosner et al., 1993, 1999; Jones et al., 2000). Appearance in plasma is not immediate but rather requires about 3 days for complete absorption and equilibration with plasma cholesterol. Cholesterol tracer appears to remain in the intestine for some time and is partially secreted into chylomicrons during at least three subsequent meals (Beaumier-Gallon et al., 2001). The ratio of isotopes in plasma can be measured quite accurately as a ratio and is read from a standard curve such as that of Fig. 9.10, which compares mass spectroscopic area ratios with $m/z$ 585 (oral tracer) with $m/z$ 586 (intravenous tracer) on the $y$ axis to mole ratios.
on the x axis. The contributions of natural cholesterol to these peaks, measured as the ratio of $m/z$ 585 : $m/z$ 581 or $m/z$ 586 : $m/z$ 581 in a baseline sample are about 10% of the area due to the isotope and are subtracted before calculations are performed (Bosner et al., 1999). Percentage cholesterol absorption is determined by dividing the plasma isotope ratio by the administered ratio and multiplying by 100. The average cholesterol absorption in sterol-poor test meals in normal subjects is 56%. The values are highly reproducible in the same subject under standard conditions, but vary among subjects from 30 to 85%, suggesting that there is a prominent genetic or lifestyle component contributing to cholesterol absorption (Bosner et al., 1993).

Percentage cholesterol absorption is reduced when as little as 150 mg of cholesterol is included in the test meal so that both phytosterol and cholesterol content of the test meal must be controlled (Ostlund et al., 1999a). Since the rapidly mixing cholesterol pool does not change over short time periods, it has been possible measure differences in percentage cholesterol absorption between two test conditions by using a single oral isotope and comparing its plasma concentration after the two absorption tests (Ostlund et al., 1999b).

Percentage cholesterol absorption can also be measured by faecal recovery methods (Ferezou et al., 1982; Lutjohann et al., 1993). Cholesterol-d$_6$ or cholesterol-d$_7$ are given at a dose of 3 mg TID for a week. A non-absorbable plant sterol recovery marker, $[5,6,22,23-$2H$_4]$sitostanol or $[2,2,4,4,6-$2H$_5]$sitosterol, is also included. At the end of the period, a non-quantitative stool sample is analysed. Percentage cho-

Fig. 9.10. Standard curve for dual-isotope cholesterol absorption studies (Bosner 1999). The filled circles represent pure tracers and the open circles depict tracers diluted 1/2500 in natural plasma sterols.
cholesterol absorption is calculated as the ratio of cholesterol: plant sterol in the stool divided by that administered multiplied by 100. This method requires continuous feeding, but it has the potential advantage of measuring cholesterol absorption over time rather than at a single time point. Since cholesterol is degraded by colonic bacteria, the degradation products (coprostanol and coprostanone) are also measured, and labelled cholesterol metabolites are combined with labelled intact cholesterol for calculation of the ratio of labelled stool cholesterol: plant sterol.

Little work on absolute cholesterol absorption has been done using stable isotopes. Nevertheless, the critical feature of such studies is the labelling of endogenous cholesterol by intravenous infusion and of dietary cholesterol by oral labelling followed by analysis of stool cholesterol enrichment after equilibration of plasma cholesterol and biliary cholesterol. Preliminary work has shown that biliary cholesterol secretion can be measured in this way with stable isotopes and that, in addition to dietary cholesterol, the intestinal secretion of unlabelled cholesterol needs to be considered and accounts for about 13% of faecal cholesterol (Ferezou et al., 1982).

Future Work

Stable isotope techniques suitable for cholesterol metabolic studies are well established. Several weaknesses in current knowledge need to be addressed in future work. The possibility of a direct link between whole-body cholesterol metabolism and coronary heart disease independent of circulating lipoprotein levels needs to be researched further. Factors that control cholesterol absorption, a critical variable that varies widely in the population, need to be better understood. The exact pathways for metabolism and transport of HDL cholesterol, and its excretion from the body through biliary secretion and conversion to bile acids, need better definition with respect to heart disease incidence. Finally, the transporters that are likely to be responsible for cholesterol absorption need to be studied with respect to cholesterol absorption and metabolism.

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