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STABLE ISOTOPE METHODS FOR NUTRITIONAL INVESTIGATION

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INTRODUCTION

Throughout the 1950s and 1960s when radioactive tracer administration for biomedical studies was most active, nutritionists remained principal users of nonradioactive, stable isotope tracers for clinical investigation. There were several reasons: *(a)* the dynamics of nutrient interactions with body fuel stores were first revealed with stable isotope tracers (248); *(b)* deuterium oxide

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dilution was a safe and simple means of estimating total body water for body composition analysis; *(c)* stable ^{15}N is the only convenient nuclide for tracing body nitrogen dynamics; and *(d)* radiotracer use was prohibited in populations of malnourished children where urgent questions of pathophysiology and therapy awaited a quantitative data base (211). Even so, limited availability of stable nuclides and the relatively tedious methods compared with those in use for radiotracer measurement limited growth of stable isotope tracer use for human investigations.

In the last decade this situation has changed drastically. Stable nuclides, produced principally at Los Alamos and Oak Ridge National Laboratories, are now available in quantity. In fact, last year's sales of highly enriched ^{13}C exceeded the entire world's supply of this pure nuclide 10 years ago. In addition, the coincident microelectronics "revolution" helped make available a range of simple-to-operate, computer-controlled mass spectrometry systems capable of sensitive, precise quantitation of stable isotope tracers in biological materials. Furthermore, investigators have realized that stable isotope tracer methodology offers certain advantages over radiotracer techniques other than the generally appreciated ethical reasons for nonradioactive isotope usage. These advantages, discussed elsewhere (8, 11), include the ability to measure both substrate content and isotope enrichment (20); the capacity to determine easily the intramolecular location of the tracer label(s) (179); the ability to study the same individual repeatedly in order to assess intrasubject variance (13), natural progression, or the effects of intervention (47); and the ability to use several stable isotope tracers simultaneously in the same individual to gain more comprehensive insight into multiple metabolic fuel interactions (21, 37, 80, 102, 122, 196, 198, 231, 232, 301). Finally, recent large-scale synthesis of stable isotopically labeled amino acids (110, 161, 162) and demonstration that complex nutrients can be intrinsically labeled with nonradioactive tracers (24, 81, 111, 116, 211, 253) have opened potential avenues for clinical investigation of nutrient digestion and bioavailability to an extent not previously possible.

Recent reviews (8, 12, 91, 185, 298, 308–310), symposia (141, 147–149, 287, 307), and bibliographies (142–146) have chronicled progress of the stable isotope field in the last decade. The magnitude of this progress prohibits a comprehensive review. This chapter, then, focuses on recent stable isotope studies of nutrient transport and metabolism in man; related animal work, radiotracer precedents, and promising allied stable isotope approaches are acknowledged where appropriate. This emphasis on human investigation restricts discussion of other nutritionally significant topics, and the reader is referred elsewhere. These topics include *(a)* agricultural use for soil and fertilizer research, for assessment of plant protein turnover, and for following nitrogen cycles (71, 97–99, 238); *(b)* tracing food sources of various organisms

(56, 63, 219, 220, 261); *(c)* attempts to infer the dietary habits of ancient man (36, 249, 272); *(d)* detection of adulteration of natural food products (59); *(e)* definitive or reference quantitative analysis of a diverse range of nutrients, metabolic intermediates, hormones, vitamins, and related compounds of nutritional interest (16, 17, 35, 39, 55, 96, 131, 160, 163, 208, 274, 281, 293, 305); and *(f)* quantification of membrane receptor synthesis, regulation, and degradation by density gradient centrifugation (57, 72, 108, 151–153, 155, 224, 225, 233).

Similarly, this review restricts discussion of valuable and proven biochemical techniques that have, as yet, had limited quantitative applications in the field of human nutrition and metabolism. Perhaps the most promising of these is nuclear magnetic resonance (NMR) spectroscopy, which not only can locate and quantify certain stable isotopic species in complex physiological matrixes, even entire tissues, but also can assess the neighboring intramolecular or local cellular environment of the tracer (1, 31, 32, 34, 40, 41, 83, 109, 186, 190, 201, 234, 257). Furthermore, the possiblility that in vivo biochemical events may soon be visualized using stable isotope tracers and NMR imaging (23) offers exciting potential for the future.

INSTRUMENTATION

Because radionuclides can be incorporated into compounds with high specific activities compared with the very small natural in vivo radioactive background, virtually "weightless" radioisotope tracers can be administered experimentally. Stable isotopes, by their very nature, are already present in living systems and must be administered and measured in "excess" of their natural abundance backgrounds (Table 1). Thus the key element in stable isotopic tracer investigations is the ability of the instrumentation to distinguish small amounts of excess enrichment on top of the natural abundance isotopic background.

In tracer studies the stable isotopic composition is specified as an "atom %" or "mole %",—i.e. the mole fraction of compound that is isotopically labeled, expressed as a percentage (7, 229, 239). Because a wide variety of different labels is possible for any compound, and because different instrumental techniques measure different types of stable isotopic enrichment, the type of label is also stated with the "atom %" designation. For example, the amino acid glycine has two carbon atoms. If 90 out of 100 glycine molecules were labeled with ^{13}C in the first carbon, then the resulting material would be 90 atom % [1−^{13}C]glycine, 1.1 atom % [2−^{13}C]glycine (i.e. natural abundance, see Table 1), and 45.5 atom % ^{13}C to denote the stable isotopic enrichment at the first carbon, at the second carbon, and over the entire molecule, respectively.

In tracer studies, the quantity of interest is the isotopic enrichment in excess of the naturally occuring isotopic component. For radioisotope tracer studies,

Table 1 Organic stable isotopes available for biological tracer studies

Element and isotope	Naturally occurring abundance (atom %)	Isotopic enrichment of commercial material (atom %)
^{1}H	99.986	
^{2}H	0.014	99.9
^{12}C	98.916	
^{13}C	1.084	99
^{14}N	99.634	
^{15}N	0.366	99
^{16}O	99.758	
^{17}O	0.038	60
^{18}O	0.204	95
^{32}S	95.018	
^{33}S	0.750	
^{34}S	4.215	90
^{36}S	0.017	

the standard unit of measurement is specific activity. For stable isotope tracers, which have no "activity," the equivalent unit of measurement is "atom % excess" or "mole % excess." The natural abundance isotopic contribution to a mono-labeled species is simply the natural abundance of the isotope in question (Table 1). For the above example of $^{13}C-$labeled glycines, subtracting the natural abundance contributions gives the enrichments 88.9 atom % excess [$1-^{13}C$]glycine, 0 atom % excess [$2-^{13}C$]glycine, and 44.4 atom % excess ^{13}C. Likewise, 4.0 atom % [^{15}N]glycine would become 3.63 atom % excess ^{15}N. Because the natural abundances of the minor isotopes are small, the probability of having molecules labeled naturally at multiple sites is vanishingly small (7). For example, the natural abundance of [$^{15}N,1-^{13}C$]leucine is 0.004 atom %, making the difference between atom % and atom % excess [$^{15}N,1-^{13}C$]leucine negligible.

To appreciate the instrumentation required to measure stable isotopic enrichments, the dilution of an administered stable isotope tracer in vivo has to be appreciated. Consider the case for studying amino acid or protein metabolism using a stable isotopically labeled (e.g. ^{15}N or ^{13}C) amino acid tracer infused intravenously for a short period (4–8 hr) (12, 179, 182). The infused tracer will be diluted in the blood with unlabeled amino acids entering the free amino acid pool via the diet and via body protein breakdown. Conversely, free amino acids (including the labeled amino acid tracer) will leave the system via catabolism to CO_2 and urea and via protein synthesis (285). The dilution of label occurs in stages (Figure 1): *(a)* The labeled amino acid is infused slowly into the system

($\sim$ 1–10 μmol $kg^{-1}hr^{-1}$) as a tracer dose such that the plasma amino acid enrichment rarely exceeds 2–5 atom % excess for the amino acid infused (an initial 20–50-fold dilution). *(b)* Depending upon the metabolic pathways available to the amino acid tracer, stable isotopic enrichment will be transferred to other plasma amino acids (e. g. transfer of ^{15}N from leucine via transamination to glutamate and alanine), adding an additional 2–50-fold dilution. *(c)* Because only one ^{15}N-labeled amino acid is typically infused while all twenty amino acids are catabolized to urea and ammonia, the ^{15}N enrichment in urinary urea and ammonia will reflect a total dilution of 200–1000 (Figure 1). *(d)* For the case of a ^{13}C-labeled amino acid tracer infusion, the $^{13}CO_2$ produced by tracer oxidation will be severely diluted ($\sim$ 10,000-fold) with the large volume of CO_2 produced from the oxidation of carbohydrate, fat, and other amino acids (182). *(e)* Finally, because the duration of amino acid tracer infusion is short compared with the slow turnover rate of most of the body's different proteins, only a small amount of label will be incorporated into protein (a dilution of 1000 or more).

The possibilities of stable isotope label dilution range from as little as 20-fold for the amino acid infused in the free amino acid pool to greater than 10,000-fold in the end products of protein metabolism and in protein itself. No single

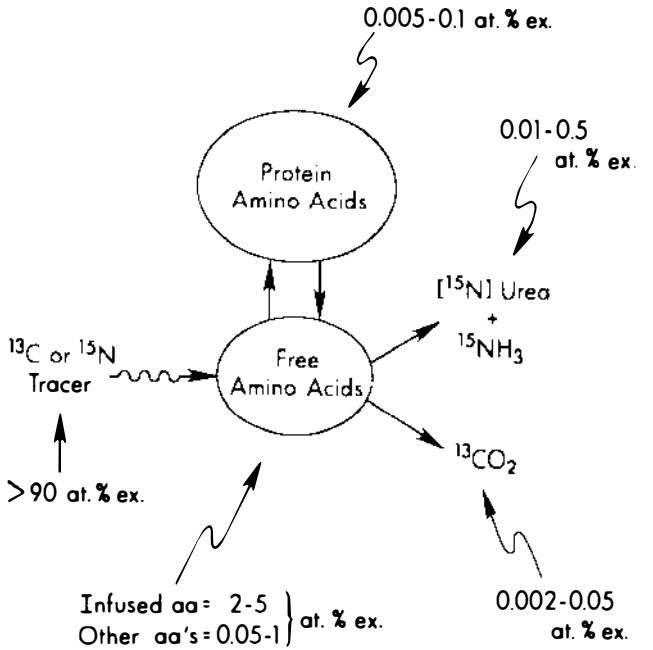

Figure 1 Approximate dilutions of amino acid stable isotope tracer in vivo. The tracer is infused into the plasma compartment.

analytical technique can routinely measure this range of isotopic dilution in a wide range of compounds with the accuracy and precision required for physiological studies. The same analytical considerations apply to the study of carbohydrate or fat metabolism using stable isotope tracers. The primary instrumental technique for the precise quantitation of stable isotopic enrichment has been, and probably will continue to be, mass spectrometry. Although other analytical approaches are noted in the appropriate applications to follow, in the remainder of this section we discuss the different types of mass spectrometers currently used to measure the wide range of tracer isotope dilutions encountered.

Isotope Ratio Mass Spectrometry

The classical dual-inlet dual-collector isotope ratio mass spectrometer (IRMS) is ideally suited for measuring very low enrichments in the easily prepared end products of metabolism, urea and CO_2 (90). The instrument derives its sensitivity for determining very small differences in isotopic enrichment ($\sim$ 0.0001 atom % excess) *(a)* by measuring only small gas molecules (N_2, H_2, and CO_2 for ^{15}N, ^{2}H, ^{13}C, and ^{18}O enrichments), *(b)* by simultaneously measuring both the major and minor isotope-produced ions with a set of ion-collectors (e. g. mass 44 for $^{12}CO_2$ and 45 for $^{13}CO_2$), and *(c)* by always measuring the isotope ratio of the sample gas against the isotope ratio of a reference gas of known isotopic content via periodic switching with a dual-gas-inlet system (7, 87, 193, 242). Although IRMS has been under continuous evolution since the 1930s, the instrument has been tedious to use. With the recent application of the microcomputer to instrument control, the current systems can now operate automatically (242). At present, the limitation of the IRMS technique is not the instrument, but the sample preparation procedures.

Because IRMS measures small gas molecules placed in an inlet system of finite volume, determination of isotopic enrichment involves converting μmole amounts of compound in pure form to the proper gas. The end products of metabolism, typically with low levels of isotopic enrichment, are therefore ideal candidates for isotopic measurement by IRMS: Carbon dioxide can readily be trapped from expired air in mmole amounts (241); urea and ammonia can be isolated from urine and converted to N_2 (usually via reaction of ammonium salt with alkaline hypobromite) (230, 262). In contrast, few circulating metabolites can be isolated routinely in pure form in suitable amounts for IRMS. For example, leucine from approximately 30 ml of plasma must be isolated from all the other N-containing compounds for measurement of leucine ^{15}N by IRMS.

Techniques Ancillary to IRMS

Although IRMS can measure smaller differences in isotopic enrichment than non-mass-spectrometric techniques, the full sensitivity of IRMS is not always

required for biological tracer studies. Three non-mass-spectrometric techniques, each specific for a different single element, exist and are competitive with IRMS for ease of sample preparation and measurement. *(a)* The oldest of these is the falling-drop method for determining the deuterium content of water by measuring the density increase in water as ^{2}H replaces ^{1}H (134, 194, 250). Using less than 0.5 ml of water, this amazingly simple method can detect 0.002 atom % excess of ^{2}H. *(b)* Nitrogen-15 can be measured separately from ^{14}N using the small differences in the frequency of emitted light of $^{14}N_2$ (297.7 nm), $^{14}N^{15}N$ (298.3 nm), and $^{15}N_2$ (298.9 nm) (68, 128, 202, 235, 265). The emission is obtained by placing a small amount of N_2 gas in a sealed glass tube in a radio-frequency or microwave cavity. The emission spectrometer is a low-cost instrument compared to the IRMS. However, the emission method has only modest sensitivity for ^{15}N ($\sim$ 0.01 atom % excess), requiring that more ^{15}N-labeled amino acid be given to measure urinary urea or ammonia ^{15}N enrichments than would be required by IRMS. *(c)* A technique that has not seen much application, but that has a potentially important future, is infrared absorption spectroscopy for measurement of $^{13}CO_2$ (105, 130). Using a tunable diode-laser as the infrared light source and spectrophotometric or photoacoustic detection, $^{13}CO_2$ enrichments as low as 0.002 atom % excess ^{13}C can be measured directly from small-volume expired air samples. Thus this instrument is ideally suited for ^{13}C-related breath tests.

Gas Chromatography–Mass Spectrometry

During the 1960s, the technique of interfacing a gas chromatograph with a mass spectrometer was perfected, but more than a decade passed before computer control of gas chromatography–mass spectrometry (GCMS) evolved the technique of selected ion monitoring (SIM) for quantitation of isotopic enrichment in a wide range of biochemicals (84, 85, 107, 138–140, 270, 288, 291). The strengths of SIM-GCMS are the ability of the gas chromatograph to resolve individual components in a complex mixture and the ability of the mass spectrometer to generate ions nearly unique to the compound being measured. Because most biological molecules are too polar to pass through the gas chromatograph directly, they must first be chemically modified ("derivatized") to block the polar groups (150, 18). A wide variety of derivatives can be prepared for most classes of compounds, and the type of derivative chosen can add additional selectivity to the GCMS assay.

Isotopic enrichment is determined either from the ion produced by the entire derivatized molecule or from a suitable fragment ion. The low-resolution mass spectrometers routinely employed for SIM-GCMS can distinguish and quantitate the isotopic combinations that produce ions of different masses (e.g. [^{15}N]leucine, [^{15}N,1$-^{13}C$]leucine, and [5,5,5$-^{2}H_3$]leucine), but not isotopic combinations having the same mass (e.g. [^{15}N]leucine, [1$-^{13}C$]leucine,

[2−^{13}C]leucine, or [2−^{2}H]leucine) (9, 101, 177). The exception to the latter case is the selection of fragment ions containing only some of the original atoms in the molecule (177, 179).

In contrast to IRMS, SIM-GCMS has a tremendous ability both to distinguish types of labeled substrates and to measure a wide range of isotopes. Furthermore, SIM-GCMS requires little actual material (nmol or pmole amounts). For example, the free amino acids can be isolated from 100 μl plasma samples and derivatized in batches of 30 or more in one day, and the isotopic enrichments can be measured by SIM-GCMS the next day (177, 182). Known amounts of multiply deuterated compounds can also be added directly to the physiological samples prior to any sample extraction step and be used as isotopic internal standards for quantitation of substrate levels simultaneous with the isotopic enrichment measurement (20, 101, 184). Because the multiply deuterated analog is chemically more similar to the substrate being measured than the conventional internal standard, accuracy, precision, and sensitivity of substrate quantitation are usually superior by SIM-GCMS.

The only significant limitation of SIM-GCMS is that it lacks sensitivity for detecting isotopic enrichment below ~ 0.1 atom % excess. SIM-GCMS is the technique of choice for measurement of almost any infused stable isotopically labeled substrate in plasma, because isotopic enrichment levels are not a problem. SIM-GCMS has also been used with more limited success to follow the transfer of label from the infused substrate to other metabolites (58, 102, 177, 178, 180, 312).

IRMS and SIM-GCMS have complimentary strengths and weaknesses: IRMS measures very small isotopic differences in readily purified compounds, and SIM-GCMS measures modest isotopic differences in metabolites obtainable only in small amounts in complex matrixes. However, neither technique is suited for routine measurement of stable isotope tracer incorporation into slow-turnover products, e.g. individual protein-bound amino acids. An intermediate, compromise approach for measuring these types of species is isotope-ratio-monitoring gas chromatography–mass spectrometry (IRM-GCMS). This technique combines advantages of both IRMS and SIM-GCMS. In IRM-GCMS the gas chromatographic effluent is catalytically combusted to N_2, CO_2 and H_2O prior to entering a modified isotope ratio mass spectrometer for determination of ^{13}C or ^{15}N enrichment (181). IRM-GCMS has improved sensitivity for isotopic enrichment over SIM-GCMS, yet retains gas chromatographic selectivity and a small sample size requirement. Thus the three mass spectrometric techniques, IRMS, SIM-GCMS, and IRM-GCMS, provide methods for measuring stable isotopic enrichment routinely through almost any metabolic pathway in the body.

END PRODUCT METHODS

"Naturally Labeled" Tracers

Carbon isotopes are fractionated to a small degree during biochemical processes (259). For example, plants that fix CO_2 via photosynthesis by the Calvin C_3 pathway (216) contain relatively less ^{13}C than plants utilizing the C_4 Hatch-Slack dicarboxylic acid pathway (95). Glucose derived from cane and corn falls into the latter category. Because the diet of Europeans is derived from a C_3 plant food chain, their $^{13}CO_2$ enrichment rises when a corn-sugar meal is given. Thus the relatively ^{13}C enriched corn sugar is "naturally labelled" (154). Use of this effect has allowed an extensive series of end product studies of glucose metabolism using "shelf" glucose as the tracer. These investigations have quantified glucose oxidation during a conventional 100 g oral glucose tolerance test (157, 158, 195) and compared the values with those estimated from indirect calorimetry (60). The effects of obesity (159, 221), diabetes (157–159), and exercise (212–214, 222, 223) on glucose substrate oxidation have been measured as well. Because the diet of Americans is derived largely from a C_4 plant food chain, this "naturally-labeled" approach is not possible here. However, the same effect can be obtained by adding a small amount of highly enriched ^{13}C-glucose to natural glucose.

Recently, a unique alternative approach to "naturally-labeled" carbohydrate tracer studies has been developed by Shulman et al (258). After first maintaining subjects on formulas of low natural abundance ^{13}C, these investigators demonstrated that one-month-old infants can utilize dietary starch for energy by measuring increased breath $^{13}CO_2$ following ingestion of naturally high abundance ^{13}C corn cereal.

For the purposes of the discussion of gastrointestinal breath tests to follow it is important to emphasize that natural isotopic fractionation, used to advantage above, must be taken into account when designing $^{13}CO_2$ oxidation protocols employing ingestion of nutrients of different natural ^{13}C contents. Schoeller et al (244) have extensively evaluated and tested meal composition effects on expired natural $^{13}CO_2$ "background" variation.

Tests of Gastrointestinal Function

Noninvasive evaluations of gastrointestinal functions by means of expired gas analysis are now commonplace. The most routine clinically employed test, breath hydrogen excretion (67, 174, 200, 207), is somewhat analogous to the naturally labeled glucose example above. The only source of breath hydrogen is that generated by intestinal bacterial digestion of unabsorbed foodstuffs. Thus the hydrogen atoms of the nutrients themselves serve as a natural tracer for the diagnosis of malabsorption syndromes.

An alternative and extremely fruitful approach for assessing malabsorption of specific nutrient fuels primarily in the pediatric age group has been the $^{13}CO_2$ breath test (5, 106, 137, 245), which measures the increase in expired $^{13}CO_2$ (or lack thereof) following ingestion of an appropriate ^{13}C-labeled substrate. A major contribution of this field has been the study of lipid absorption replacing the alternative diagnostic evaluation that requires collection and analysis of 72 hr fecal fat samples. By evaluating the combined results of $^{13}CO_2$ breath excretions following administration of [^{13}C] triolein, [^{13}C] trioctanoin, and [^{13}C] palmitic acid, fat malabsorption due to pancreatic insufficiency can be differentiated from that due to intestinal mucosal disease or bile salt deficiency (289, 290). Similarly, intestinal bacterial overgrowth and ileal dysfunction can be recognized following cholyl-[1,2$-^{13}C_2$] glycine administration (243, 260). Finally, McLean et al (173) recently showed that lactose absorption is nearly complete in the small intestine of formula-fed full-term infants. It seems clear that critical use of noninvasive functional tests of this sort will allow important information on nutrient digestion and assimilation to be obtained for the first time in the growing human.

Protein Turnover

Prior to the development of SIM-GCMS, tracing the flow of nitrogen in the free amino acid pool (Figure 1) was extremely difficult, and the methods to study whole-body protein metabolism in man were directed toward measuring the excretion of ^{15}N label in the end products of protein metabolism. Rittenberg and coworkers (236, 237, 263) described the first simple noninvasive method of this type: a single dose of [^{15}N] glycine was administered and the time course of ^{15}N excretion in urinary urea was followed. Because this method used a simple compartmental analysis (a rapid-turnover metabolic N pool and a slower-turnover body urea pool) that could not account for differences in end-product kinetics from different ^{15}N-labeled amino acids (285, 302–304), the method has seen little clinical application.

In 1969, twenty years after Rittenberg et al's work, Picou & Taylor-Roberts demonstrated that a stochastic approach, which does not require invocation of distinct compartments (255), could be used to measure whole-body protein dynamics (211). In their method, [^{15}N] glycine is administered as repetitive doses until the ^{15}N enrichment reaches an isotopic steady state, or "plateau," in urinary urea. The whole-body protein turnover rate is estimated from the dilution of ^{15}N in urea, and the whole-body protein systhesis rate is assumed to be the difference between protein turnover and catabolic rates (the latter being the total urinary N excretion rate). This stochastic end-product method has become a benchmark method and has been used to study the effects of protein intake, malnutrition and fasting (15, 78, 204, 211, 264, 285, 295), age (74, 77, 205, 296, 311), and burn-injury or surgical trauma (49, 135) on protein metabolism in man.

Any whole-body protein-turnover measurement will be based on a method containing underlying assumptions that are potential important sources of error (285). Because the Picou & Taylor-Roberts end-product method has been widely used, most of the method's inherent assumptions have been tested in man in a variety of ways: by direct comparison with other methods of measuring whole-body protein metabolism (77), by testing different routes of isotope administration and doses (211, 266, 271, 285, 286), and by investigation of the pattern of ^{15}N labeling in the different free amino acids (180). The present philosophy is that this end-product method appears valid for measuring relative changes in whole-body protein metabolism in unstressed adult subjects where the transfer of glycine ^{15}N to other amino acids and the separate end-products, urea and ammonia, have been studied (180).

The biggest single drawback to the continuous administration of [^{15}N]glycine is the long period required to equilibrate the ^{15}N in the free amino acid pool and in urinary urea ($\sim$ 60 hr for adult man) (180). Waterlow and his colleagues have developed a faster method using a single dose of [^{15}N] glycine and stochastic analysis of the fraction of ^{15}N dose appearing in urinary ammonia and urea over 9 hr (66, 286). This method has considerable potential for rapid evaluation of human protein dynamics. However, using ammonia as an end product instead of urea gives a significantly different estimate of protein turnover, and it is not clear whether ^{15}N in ammonia, urea, or an average of the two should be used (66).

Recently, a new end-product method using compartmental analysis has been developed for measuring whole-body protein dynamics. The method uses either *(a)* a pulse injection of [^{15}N] alanine followed by a pulse injection of urea ^{15}N 48 hr later (169, 218), or *(b)* simultaneous pulse injections of [^{15}N] alanine and [^{13}C] urea (14). The former requires collection of urine for 72 hr, the latter for 24 hr. In either case, the method considers both the ^{15}N appearing in urinary ammonia and in urinary urea and solves a four-compartment model (two urea pools and fast- and slow-turnover metabolic N pools). The principal drawbacks are the long and tedious urine collection schedule and access to a sophisticated computer program (218), but the advantage is a detailed kinetic analysis of protein dynamics from noninvasive end-product measurements.

KINETICS OF INDIVIDUAL METABOLITES

Carbohydrates

Because carbohydrate is a prominent fuel source and maintenance of normoglycemia is a crucial homeostatic function, measurement of glucose kinetics (9, 126) was one of the earliest applications of SIM-GCMS stable isotope tracer work. Much of this work was directed toward the pediatric age group where

prior information was limited until glucose production was measured in the newborn infant (10, 124) and throughout childhood (10), the onset of gluconeogenesis in the human neonate determined (69, 122), and three-carbon sources for neonatal new glucose production quantified (20, 69, 122). Furthermore, knowledge of glucose homeostasis in the infant was extended through measurement of maternal glucose production rates in the third trimester, and at term, in normal and in diabetic pregnancies (123, 127). Using this framework of normal physiological data, disturbances in glucose homeostasis were measured in infants of diabetic mothers (125), and in children with severe undernutrition (132, 133), cyanotic congenital heart disease (103), Duchenne Muscular Dystrophy (104), and Maple Syrup Urine Disease (104).

Nonradioactively labeled glucose has proven to be a useful tracer in adult studies as well. By tracer priming the bicarbonate pool with $NaH^{13}CO_3$ (3), Wolfe, Burke, and their coworkers (27, 299) were able to measure conveniently glucose oxidation responses during clinically significant rates of intravenous glucose infusion. Using [6,6−$^2H_2$] glucose tracer, Clutter et al (38) determined the increment in circulating plasma epinephrine level required to produce an increase in hepatic glucose production. Finally, the combined use of [U−^{13}C] and [6,6−$^2H_2$] glucose tracers, allowed Robert et al (232) to evaluate carbohydrate homeostatic function in healthy elderly subjects. This investigation showed that glucose production was slightly decreased in elderely adults but that glucose oxidation, glucose carbon recycling, and hepatic regulation of glucose output in response to exogenous glucose were the same as seen in the young adult. Thus reduced glucose tolerance in the elderly is clearly not the result of an hepatic but rather of a peripheral defect(s) in glucose regulation, a conclusion supported by the related data of DeFronzo (54).

Fatty Acids and Cholesterol

Cholesterol synthesis was one of the earliest problems to which stable isotope tracer methodology was applied. Thus Goldwater & Stetten (79) and Popjak (215) showed that cholesterol in the animal fetus was virtually entirely derived from fetal de novo synthesis and not from maternal dietary intake. Later, the effects of dietary cholesterol intake (206) and estrogen treatment effects (129) on cholesterol synthesis in the rat were evaluated in similar fashion. In man, first London & Rittenberg (168) and then Taylor et al (273) were able to estimate cholesterol synthesis and turnover by incorporation of deuterium from body water enriched with ^2H_2O. Most recently, Ferezou et al (65) by combined administration of deuterated and tritiated cholesterol, [^{14}C] mevalonate, and [^{13}C]acetate, were able to study rates of various processes involved in cholesterol absorption, synthesis, and incorporation into serum lipoproteins in man.

In a similar fashion, de novo synthesis of fatty acids has been estimated by measuring deuterium incorporation from ^2H_2O (268, 279). Emken et al (62) fed deuterated trioleine and trielaidin to volunteers to assess the relative incorporation of *cis* and *trans* fatty acid isomers (of the kind produced by hydrogenation of vegetable oil) into plasma, erythrocyte, and platelet lipids. By complexing [1−^{13}C] palmitic acid to human serum albumin (300), Bougneres et al (20) quantified free fatty acid transport rates in the human newborn, and Galster et al (70) estimated the circulating plasma epinephrine threshold for lipolysis and showed that this effect occurred at plasma epinephrine levels lower than those required to produce hepatic glycogenolysis (38).

Amino Acids

Until SIM-GCMS was developed, the use of stable isotopes in nutritionally related research was limited primarily to the ^{15}N protein end-product methods discussed previously, while the radioisotopes were used to measure in vivo kinetics of individual metabolites (285). Recent advances in SIM-GCMS altered this situation since it is now easier to measure isotopic enrichment in circulating free amino acids with a stable isotope tracer than with a radiotracer (177). Thus whole-body metabolite kinetics using [1−^{13}C] leucine (27, 80, 182, 183, 192, 196–198, 227, 231), [2−^{15}N] lysine (48, 88, 196, 198), labeled alanines (69, 102, 231), [^{15}N] glycine (74, 180, 231), [5−^{15}N] glutamine (76), and [$^{15}N_2$] urea (297, 301) have now been measured for a wide range of protein and energy intakes and pathophysiological states.

The interconversion rate of one metabolite into another can be determined by infusing simultaneously the tracers corresponding to the two metabolites. For example, by infusing [^{15}N] leucine simultaneously with the appropriately labeled alanine (to measure the whole body alanine production), the rate of leucine N conversion to alanine production can be estimated (6, 102). The simultaneous infusion of [*phenyl*−$^2H_5$] phenylalanine and [1−^{13}C] tyrosine tracers allows measurement of phenylalanine and tyrosine kinetics as well as the rate of phenylalanine conversion to tyrosine (37). Of course, the double tracer infusion technique has also been used extensively with radioisotopes. However, a technique that is rarely used with radioisotope tracers is the placement of different stable isotope labels on the same tracer molecule. A multi-labeled tracer has the distinct advantage of being able to define simultaneously individual rates of different metabolic steps. Consider the case of an infusion of di-labeled L−[^{15}N,1−^{13}C] leucine: Transamination, the first step in leucine catabolism, effectively removes the di-labeled species (the ^{15}N is lost to a large transaminating pool of nitrogen) and a ^{13}C labeled α−keto-isocaproate ([1−^{13}C] KIC) is formed. The [1−^{13}C] KIC can either be reaminated to form a [1−^{13}C] leucine or be decarboxylated, releasing $^{13}CO_2$. All of these stable isotopically labeled species ([^{15}N,1−^{13}C] leucine, [1−^{13}C]

leucine, and $^{13}CO_2$) can be measured and the separate whole-body rates of leucine deamination to KIC, KIC amination to leucine, and KIC decarboxylation can be determined (179).

Any leucine label-combination that is removed after the reversible transamination step can be used as a tracer of whole-body leucine flux (e.g. [1−^{13}C] leucine or [5,5,5,−$^{2}H_3$] leucine (101, 182), but any label-combination that is removed by transamination (e.g. [^{15}N] leucine or [2−^{2}H] leucine) follows the fate of the leucine nitrogen—a fate that adds an additional component to the flux (179). In contrast, the nitrogens of lysine are disposed at the same rate as the carbon skeleton, such that [2−^{15}N] lysine can be used to measure the whole body lysine flux. These examples illustrate the important point that the biochemistry of the chosen label must be consistent with the biological processes being studied.

The tracer (stable or radioisotope) infusion method for measurement of whole-body metabolite fluxes has inherent assumptions and limitations that are potential sources of error (285). However, experience gained with a particular tracer helps to improve the model. Such is the case for the [1−^{14}C]-or [1−^{13}C] leucine tracer where the problem of intracellular versus extracellular partitioning of label has been recognized using plasma [1−^{13}C] KIC as an index of intracellular leucine enrichment (80, 183).

In contrast to the continuous infusion protocol, the single-dose administration of stable isotopically labeled tracers to measure the kinetics of individual metabolites in man has had only limited success. IRMS has been used to measure whole-body glycine (292), urea (170, 282), and creatine (210) kinetics; SIM-GCMS has been used to measure alanine and glycine kinetics (156, 312). Two drawbacks exist: *(a)* IRMS can measure the disappearance of label over several orders of magnitude, but measurement of tracer enrichment in plasma constituents by IRMS is extremely tedious. *(b)* Although SIM-GCMS is ideally suited to measure tracer enrichment in plasma constitiuents, it has a limited dynamic range for measuring isotopic enrichment. Therefore, to resolve two exponential decay components using GCMS, ^{15}N-labeled amino acid doses producing initial plasma enrichments of > 50 atom % excess have been used (156, 312).

Direct Measurement of Protein Synthesis

Because the specific activities of amino acids in individual proteins are much easier to measure than the corresponding amino acid stable isotopic enrichments, most protein synthesis measurements have been made with radioisotopes. However, four different stable isotope approaches have been developed for measuring protein synthetic rates in man, using a continuous infusion of tracer: *(a)* The simplest method is the constant infusion of [^{15}N] glycine and measurement of total incorporation of ^{15}N in specific protein by isolating the

protein and converting it to N_2 gas (267). The principal drawback to this method is that the ^{15}N from glycine is transferred at different rates to many amino acids, each of which is incorporated into protein. Thus, the actual precursor ^{15}N enrichment is unknown and may shift with physiological changes. *(b)* The second approach is to infuse a ^{15}N-labeled amino acid that transfers only a small fraction of its ^{15}N to other amino acids during metabolism —e.g. L−[2−^{15}N] lysine. This tracer has been used to measure synthesis rates of albumin and sarcoplasmic and myofibrillar protein in man (88). However, during the 20 hr that the lysine tracer is infused, ^{15}N is transferred to glutamate and arginine, contributing an additional and unknown amount of ^{15}N to the protein. *(c)* A scheme for determining albumin synthesis rates using [^{15}N] glycine to label hepatic arginine removes two of the above problems by measuring specifically both the precursor enrichment (estimated from the urinary urea ^{15}N) and the product (the arginine guanidino−^{15}N enrichment in albumin) (75). *(d)* The last method uses the incorporation of ^{13}C from an infusion of [1−^{13}C] leucine to measure muscle protein synthetic rates by isolating and decarboxylating the protein leucine for measurement of the resulting $^{13}CO_2$ by IRMS (226, 227). Although the former methods use ^{15}N tracer infusions of 20–60 hr, the [1−^{13}C] leucine method requires only a 7-hr infusion and, in addition, obtains whole-body leucine kinetic information from plasma leucine ^{13}C and expired $^{13}CO_2$ enrichments.

BODY WATER AND ENERGY EXPENDITURE

Accurate determination of total body water is important in a variety of clinical situations for estimation of body composition. Total body water has been determined in animals and man for almost 50 years by isotopic dilution of deuterated, tritiated, or (more recently) ^{18}O-labeled water (51, 89, 194, 240, 247, 250). The method is simple: A tracer dose of labeled water is given orally and allowed to equilibrate for 2–4 hr before a sample of physiological fluid is obtained. The dilution method has been verified by direct desiccation of small animal carcasses (51) and for several different types of fluid: serum, urine, and saliva (89, 240, 247).

Both ^2H_2O and $H_2{}^{18}O$ measure more than just body water. In the case of ^2H_2O, the deuterium also exchanges with labile body hydrogen in protein and in other constituents. The amount of exchange has been computed theoretically (52) and tested experimentally (51). The overestimate of body water from hydrogen exchange appears to be in the range of 2–3 % (247). The water ^{18}O, on the other hand, rapidly exchanges with the body bicarbonate pool after a dose of $H_2{}^{18}O$ is given. Thus total body water can also be measured noninvasively from the ^{18}O enrichment in expired CO_2 (247). The error due to water

oxygen loss via expired CO_2 is insignificant over the short period required for isotopic equilibrium to be reached.

The observation that body water is continually diluted with molecular oxygen uptake and CO_2 output for respiration (164, 166) has led to the development of a novel method for measuring whole-body energy expenditure. The disappearance of ^2H enrichment in water with time after administration of ^2H_2O is primarily due to turnover of body water, but the disappearance of ^{18}O enrichment in water after administration of $H_2{}^{18}O$ is due to the turnover of body water plus CO_2 production (165, 199). Thus both the average CO_2 production and total energy expenditure can be determined from the differential disappearance rates of ^2H and ^{18}O. For small animals the minimum period of study is about 2 days (165) versus a 13-day period for humans (246).

TOTAL BODY NITROGEN

While proton activation techniques taking advantage of the ^{15}N $(p,\alpha\gamma)^{12}C$ reaction can be used to measure nitrogen-15 content of tissue samples (175, 228), two neutron activation methods have recently proven of great value in quantifying total body nitrogen. Even though these methods measure the principal stable isotope of nitrogen, ^{14}N, they do so by neutron bombardment. Thus the subject does receive a small radiation dose on the order of 30–50 mrem or about that of a routine chest X-ray. The first method employed the ^{14}N $(n,2n)$ ^{13}N reaction (18a, 43, 203). Because ^{13}N decays by positron emission and not through release of a characteristic gamma-ray, the above reaction lacks specificity, and interfering reactions may contribute nearly 20% of the total signal. Nevertheless, using this method, Burkinshaw et al (29) measured total body nitrogen in 91 subjects with an estimated standard error of about 4% for a single determination. As expected, total body nitrogen was closely related to fat-free mass and total body potassium in these healthy individuals.

The more promising neutron activation technique for total body nitrogen determination is based on the $^{14}N(n,\gamma)^{15}N$ reaction (94, 188, 276, 294). The emitted gamma rays have a characteristic radiation energy that can be measured for specific determination of nitrogen. Hydrogen also emits a characteristic but different gamma radiation on neutron capture. Vartsky et al (277) devised the clever approach of using body hydrogen as the normalizing internal standard for absolute nitrogen measurements since hydrogen constitutes 10% of body mass and counting efficiency variations are essentially the same for nitrogen as for hydrogen. Furthermore, utilizing total body potassium measurements and representative body composition relationships in muscle and nonmuscle tissue, one can also estimate the muscle and nonmuscle components of the fat free body mass (4, 28, 45), an approach that has given values in good agreement with those obtained by direct chemical analysis (172).

The prompt gamma-ray analysis method has already proven useful and reliable for total body nitrogen measurements in several hundred subjects. While there was reasonably good correlation between total body nitrogen and potassium in normal subjects (61, 172), this was not necessarily the case in ill subjects. Total body potassium assessment seriously overestimated body nitrogen loss in individuals with various types of cancer (44, 61) and was a poor predictor of body nitrogen in malnourished patients (187). Although only recently available for clinical evaluation, prompt gamma-ray quantitation of total body nitrogen has already proven useful in assessing body nitrogen changes in diabetics (283) and in parenterally nourished subjects (46, 189). Its routine application in conjunction with body water, body potassium, and balance estimates should greatly enhance our understanding of physiological and pathophysiological alterations in body N.

INORGANIC ELEMENTS

Intrinsic stable isotope tracer enrichment of natural foodstuffs (111, 116, 253) offers a particularly promising approach toward clarifying the difficult area of inorganic micronutrient absorption and metabolism. Understandably, significant effort has gone into methods development over the last decade. The principal analytical techniques include atomic absorption spectroscopy (50, 209), activation analysis (19, 42, 73, 119, 112–115, 118, 254), thermal ionization mass spectrometry (30, 82, 120, 306), and selected-ion-monitoring mass spectrometry of volatile metal chelates (86, 121, 191, 252). Numerous other less-generally-employed approaches are available—e.g. the use of magnetic susceptability to quantify human hepatic iron stores (22).

Activation analysis has been the most commonly employed recent analytical approach for quantifying inorganic stable isotopes in biological samples (Table 2). However, when activation analysis has been compared with mass spectrometric measurements of the same element, the latter have proven superior (119, 120, 251, 252, 275) with a relative precision about ± 1% versus a coefficient of variation of up to ± 10% obtained by activation analysis.

It is not surprising, then, that the last few years have seen mass spectrometry literature citations approaching those for activation analysis in human stable isotope tracer studies of inorganic element metabolism. Activation analysis has been used to study calcium metabolism in children and adults (92, 112, 269), iron absorption in young men (114), the effects of oral contraceptive use on iron, copper, and zinc absorption in young women (136), the plasma appearance of ^{74}Se after oral doses (117), and in vivo compartmental analysis of sodium metabolism (42). Similarly, mass spectrometry has also been used to quantify iron, copper, and zinc absorption in man (121, 275) as well as to

Table 2 Recent analytical approaches for quantifying selected stable inorganic elements in biological samples

Element	Method[a]	Reference(s)
Fluorine	PAA	256
Sodium	NAA	42, 43, 64
Magnesium	NAA	53, 64, 112, 119
	VMC-MS	86, 251–253
Aluminum	NAA	64
Phosphorus	NAA	43, 64
Potassium	NAA	64, 176
	TI-MS	82
Calcium	NAA	43, 64, 93, 112, 119
	PAA	176
	VMC-MS	86
	TI-MS	306
Titanium	PAA	176
Vanadium	NAA	64
Chromium	VMC-MS	278
Manganese	NAA	64
	PAA	176, 284
Iron	NAA	33, 64, 114, 115, 118, 119, 136, 171
	PAA	176
	VMC-MS	86, 121, 191
	TI-MS	275
Cobalt	PAA	176, 284
Nickel	PAA	176
	VMC-MS	86
Copper	NAA	119, 136
	PAA	176, 284
	VMC-MS	25, 86, 121
Zinc	NAA	113, 115, 116, 118, 119, 136, 167
	PAA	284
	VMC-MS	86, 121
	TI-MS	120, 275
Selenium	NAA	111, 119
	PAA	176
Rubidium	PAA	176
Strontium	PAA	64, 176
Cadmium	PAA	64, 176
Iodine	NAA	2, 280
Lead	PAA	176, 284
	TI-MS	217

[a] Abbreviations: NAA = neutron activation analysis; PAA = proton activation analysis; VMC-MS = mass spectrometry of volatile metal chelates; TI-MS = thermal ionization mass spectrometry.

evaluate magnesium recovery in urine, feces, and plasma following a dose of $^{26}MgCl_2$ (252), and to analyze lead kinetics in healthy men (217).

The difficulties of stable isotope approaches in the study of human mineral metabolism have been discussed recently (119, 251). Nonetheless, rapid recent progress in methodology is promising for answering pertinent questions of human micronutrient metabolism.

CONCLUSION

Stable isotope tracers have now been used safely in human research for almost 50 years. Although in this review we have principally dealt with recent selected human applications, the number of listed citations (the great bulk from the last decade) attests to the active growing interest in use of these materials to solve important problems in clinical nutrition. The increasing supply and decreasing cost of highly enriched stable nuclides, the consistent progress in instrument development, the numerous practical advantages of stable isotope tracer methods, and the ethical acceptability of nonradioactive tracer investigations in humans of all ages assures the continued expansion of this already diverse field.

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