Mass spectrometric analysis of stable-isotope-labelled amino acid tracers

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A group of continuous-flow isotope-ratio mass spectrometry (CF-IRMS) techniques that have been introduced recently, promise to improve greatly the analysis of stable isotope tracers in many areas of life science, including human nutrition. These offer the ability to process rapidly large numbers of samples. This is fundamental, especially if techniques developed for clinical research are to be used for diagnosis. The present paper charts the development of CF-IRMS techniques, including GC–combustion isotope-ratio mass spectrometry (GC–C-IRMS; also termed compound-specific isotope analysis) and contrasts this with GC–mass spectrometry (GC–MS), demonstrated by studies of protein metabolism using stable-isotope-labelled amino acid tracers.

The advantages of stable-isotope tracers over radioisotopes in clinical nutrition are now widely recognized. The elements of organic matter (H, C, N, O and S) each possess a suitable non-radioactive rare isotope that can be enriched and incorporated into substrates for *in vivo* studies. These provide ideal tracers to measure rates of intermediary metabolism and to trace metabolic pathways. In terms of the measurement of protein or peptide synthesis, amino acid tracers could potentially be synthesized with any of the minor isotopes ^{2}H, ^{13}C, ^{15}N, ^{18}O or ^{34}S. However, only ^{2}H, ^{13}C and ^{15}N have seen widespread use, as ^{34}S-labelled S amino acids are not yet available commercially and ^{18}O is too readily lost from functional groups by exchange reactions to be of general use. However, the latter has been used to study collagen turnover following incorporation into hydroxyproline *in vivo* (Molnar *et al.* 1985).

In contrast to radioisotopes, stable isotopes have significant natural abundance (Preston, 1992). This natural abundance presents a barrier in terms of the limit of detection of the particular isotope added as tracer. Table 1 lists the natural abundance of ^{2}H (deuterium), ^{13}C and ^{15}N. Furthermore, metabolites have to be separated from all other compounds containing the tracer element to ensure accurate tracer analysis. This additional consideration necessitates quantitative sample preparation chemistry.

Table 1. *Typical variation in stable isotope natural abundance in biological systems and analytical precision obtained in state-of-the-art continuous-flow isotope-ratio mass spectrometry (IRMS) systems.*

(^{2}H precision is estimated by comparison with that obtained by ^{13}C and ^{2}H analysis using dual-batch-inlet IRMS; minimum working enrichment is calculated as ten times the standard deviation of an analysis at natural abundance)

Isotope	Natural abundance (ppm)	Variation in biological systems	Analytical precision (ppm)	Minimum working enrichment (atom %)
^{13}C	11110	150	2	0·002
^{15}N	3660	100	2	0·002
^{2}H	156	50	0·2	0·0002

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Precise and accurate physical techniques are required to measure tracer concentrations of stable isotopes near natural abundance. Although optical spectroscopy has been used as an economical method of analysis at higher enrichments, mass spectrometry (MS) is the preferred technique for the analysis of stable isotopes. The present paper describes the variety of MS techniques that are available to the analyst and discusses the criteria used to select the appropriate analytical technique for studies of protein metabolism in man.

THE ISOTOPE-RATIO MASS SPECTROMETER (IRMS)

The MS analysis of the stable isotopes originated in geochemistry laboratories about 46 years ago (Nier, 1947). The Nier dual-batch-inlet IRMS was introduced specifically for high-precision analysis of the stable isotopes H, C, N, O and S, in the natural environment. The IRMS is essentially a low-resolution, multiple-collector, electron-impact gas source, magnetic-sector MS. This is fitted with a dual-batch inlet and changeover valve to allow alternate introduction of unknown pure gas and reference gas samples into the ion source. These high-purity simple gas samples (CO_2, N_2, H_2 or SO_2) are conventionally produced manually on a remote vacuum line (Preston, 1992). There is little doubt that, 50 years after the introduction of the first IRMS instrument, the most precise measurements of stable isotope abundance will be made using instruments of similar design, albeit improved and automated. The design of the inlet system requires a considerable sample size (>1 μmol) as most of the sample gas is required to maintain optimum conditions in the viscous flow inlet. The large sample size requirement and low throughput has limited IRMS applications in human nutrition to those that can provide large samples with low tracer enrichment, such as urinary endproduct analysis in whole-body protein turnover studies (Preston *et al.* 1984), body fluid analysis in energy expenditure studies (Taggart *et al.* 1991) or analysis of the rate of substrate oxidation to breath CO_2 (Preston & McMillan, 1988). IRMS can detect an enrichment change of <1 part in 10^5 in >1 μmol sample gas. A precision of ± 0·0002 atom % ^{13}C can be obtained routinely (Table 2).

GC–MASS SPECTROMETRY

In contrast to IRMS, GC–MS, used for many years by organic chemists for structural analysis of volatile organics, can be used to assay stable-isotope enrichment in considerably smaller quantities of material, but at much poorer precision. This is because the instrument was primarily designed to rapidly scan mass spectra using a single detector. Precise and accurate (isotope) ratio measurement over a wide dynamic range from the single detector system is a hard criterion to meet. Furthermore, scanning analysis is inferior to true simultaneous detection as transient changes in ion production and sample pressure will introduce error into isotope-ratio measurement. Finally, the ion ratio of high-mass fragments used for tracer analysis by GC–MS is considerably greater than that of the simple gases used for IRMS analysis. Multiple-collector low-resolution IRMS instruments are inherently more precise and accurate as they give true simultaneous measurement and have detector systems with gain optimized for particular isotope pairs. Thus, GC–MS complements IRMS, both analytical techniques frequently being required in complex experimental protocols. Typical analytical precision for modern

Table 2. *[1-^{13}C]Leucine analysis by various mass spectrometry (MS) systems*

(Tracer dilution reflects the minimum working enrichment in the C compound used for analysis; this contains the labelled carboxyl-C alone, the six C atoms of leucine or the eleven to eighteen C atoms of a volatile derivative, depending on the method of sample gas production; minimum working enrichment is calculated as ten times the standard deviation of an analysis at natural abundance)

	Sample size	Precision (atom % ^{13}C)	Minimum working enrichment (atom % ^{13}C)	Tracer dilution (atom % ^{13}C)	Method
IRMS	>1 μmol	<0·0002	<0·002	0·002	Decarboxylation*
Elemental analyser–CF-IRMS	1 μmol	0·0002	0·002	0·012	Combustion*
Gas injection–CF-IRMS	<1 μmol	0·0002	0·002	0·002	Decarboxylation*
GC–C-IRMS	10 nmol	0·0002	0·002	0·02–0·04	Combustion
GC–MS	<1 nmol	0·05	0·5	0·5	

IRMS, isotope-ratio mass spectrometry; CF-IRMS, continuous-flow isotope-ratio mass spectrometry; GC–C-IRMS, GC–combustion isotope-ratio mass spectrometry.
* Off-line.

GC–MS instruments working in selected ion monitoring mode is ± 0·05 atom % in a sample of ≤1 nmol, if a single heavy isotope is incorporated into a derivatized amino acid (Table 2).

IRMS and GC–MS techniques are thus complementary, many clinical research laboratories having invested in both, but they fail to offer a solution to a frequent problem for the experimenter: how do we analyse small tracer enrichment changes in nanomolar–micromolar components? This is no abstract problem, but is an increasingly frequent requirement in many substrate metabolism studies.

CONTINUOUS-FLOW ISOTOPE-RATIO MASS SPECTROMETRY (CF-IRMS)

About 10 years ago the sample size, sample throughput and manual gas sample preparation limitations of IRMS were significantly reduced with the introduction of the first of the hybrid CF-IRMS instruments for analysis of ^{15}N (Preston & Owens, 1983) and then ^{13}C (Preston & Owens, 1985). This technique has been termed elemental analyser–CF-IRMS, or automatic N and C analyser (ANCA–IRMS). A commercial Dumas combustion analyser was interfaced to an IRMS via a simple capillary leak. Automated analysis of organic and inorganic samples of ≤1 μmol, with precision approaching that of conventional IRMS instruments is now possible (Table 2). Briefly, automatic Dumas combustion analysers introduce samples into a high-temperature catalytic-oxidation furnace. Combustion products are swept by He carrier gas through a Cu reduction stage where nitrogen oxides and O_2 are removed. The remaining CO_2 and N_2 are dried by passage through a chemical water trap. CO_2 can be trapped if only ^{15}N analysis is required, otherwise CO_2 and N_2 are resolved in a packed column GC before being swept into the IRMS ion source. The three IRMS manufacturers (Europa

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Scientific Ltd, Crewe; Finnigan MAT, Bremen, Germany; Fisons Instruments, Nantwich) each produce a range of CF-IRMS instrument configurations.

In the context of protein metabolism studies, elemental analyser–CF-IRMS has been used for the ^{15}N analysis of endproducts of N metabolism and to analyse amino acid enrichment in studies of tissue protein synthesis. The latter study was the first demonstration that a single instrument could be used to analyse both high-enrichment, low-concentration precursor amino acids and low-enrichment, high-concentration amino acid samples from protein hydrolysates, separated by HPLC (Fearon *et al.* 1991). Finally, simultaneous ^{15}N and ^{13}C analysis has been demonstrated recently in whole proteins (Brookes *et al.* 1991).

GAS ANALYSIS BY CONTINUOUS-FLOW ISOTOPE-RATIO MASS SPECTROMETRY

As the function of the elemental analyser is to automatically produce and purify simple gases in a He carrier, the principle was readily adapted to CF-IRMS analysis of breath CO_2 from a whole-body protein turnover study (Preston & McMillan, 1988). Exhaled breath samples were injected into the elemental analyser before the gas purification stage, where samples were dried, CO_2 was purified by GC and swept into the IRMS. This manual procedure led to the development of a dedicated automated gas analyser (Prosser *et al.* 1991).

As with amino acid tracer analysis by elemental analyser–CF-IRMS, amino acids have first to be separated quantitatively before analysis by preparative GC or HPLC. Purified amino acids labelled with ^{15}N can undergo combustion by elemental analyser without tracer dilution. When amino acids are labelled with a single (carboxyl) C atom, the sensitivity of the assay is considerably improved by cleaving the carboxyl-C for analysis, to avoid the tracer dilution by all the unlabelled C atoms during combustion. Gas injection CF-IRMS has been used to analyse CO_2 derived by cleavage of the carboxyl-C of [1-^{13}C]leucine separated from protein hydrolysates in studies of tissue protein synthesis (Brookes & Milne, 1991).

COMPOUND-SPECIFIC ^{13}C ANALYSIS: THE INTRODUCTION OF GC–COMBUSTION ISOTOPE-RATIO MASS SPECTROMETRY

The advantages of on-line separation of metabolites in terms of sample throughput, sample size reduction and reduced labour costs spurred the development of this final form of CF-IRMS. Earlier experiments had been conducted with GC–MS instruments acting as detectors for CO_2 and N_2 produced by on-line combustion of components separated by packed column GC systems (Matthews & Hayes, 1978). Volatile samples separated by high-resolution capillary GC are subjected to combustion in an on-line capillary Dumas combustion train and the simple gas products are dried and presented in a He carrier to the MS ion source. As the combustion product with the highest concentration, ^{13}C analysis in CO_2 derived from essential oils was first demonstrated in 1984 (Barrie *et al.* 1984). GC–C-IRMS has since been used for analysis of alkanes (Rieley *et al.* 1991), amino acids (Silfer *et al.* 1991) and carbohydrates (Tissot *et al.* 1990). In the context of protein metabolism studies, GC–C-IRMS analysis of [1–^{13}C]leucine has been compared recently with analysis of the carboxyl-C cleaved by reaction with ninhydrin

following off-line separation by preparative GC (Yarasheski *et al.* 1992). The new technique compared favourably in terms of sample size and ease of preparation, but derivatization for GC–C-IRMS analysis added five further unlabelled C atoms to a molecule containing a single labelled and five unlabelled C atoms. The product of combustion was, thus, only one-eleventh as enriched as the carboxyl-C, in this example. In terms of limit of tracer detection GC–C-IRMS analysis compares unfavourably with off-line separation and combustion of the underivatized compound (one-sixth of atoms labelled) or cleavage and analysis of the carboxyl-C (Table 2).

^{15}N ANALYSIS BY GC–COMBUSTION ISOTOPE-RATIO MASS SPECTROMETRY

In contrast to ^{13}C analysis, N_2 gas produced by combustion of amino acids derivatized using most common agents does not suffer dilution by unlabelled N atoms. However, a number of technical problems result in these apparent advantages being hard to realize in practice. First, the N content of a typical derivatized amino acid may be about 12–18-fold less than the C content. Furthermore the product of combustion, N_2, produces only about one-thirtieth the partial pressure of CO_2. This calculation assumes complete conversion of organic N to N_2, which is a greater technical challenge than quantitative CO_2 production, as the former requires oxidizing followed by reducing chemistry (to convert NO to N_2) whereas the latter simply requires oxidation. Furthermore, a leak in the complex stages of the capillary gas preparation system severely compromises analysis against an already significant background signal.

The ultimate precision of IRMS analysis depends on the number of sample molecules that enter the MS ion source (Preston, 1992). The optimum working of the MS with low 'abundance sensitivity' (that is, the overlap of the major ion beam into the minor ion beam collector due to ion and/or molecule collisions) is compromised by too high carrier gas pressure. Thus, the sample loading has to be optimized; this could potentially compromise GC resolution. Baseline separated peaks are essential in GC–C-IRMS, as considerable isotope fractionation can occur by chromatographic resolution of heavy and light molecules (Matthews & Hayes, 1978). Thus, isotope ratios have to be measured over the full width of resolved peaks. The final performance of the instrumentation thus depends on a number of factors, not least of which is good chromatography, a process which starts with obtaining reproducible high yields while purifying and derivatizing complex biological samples.

THE SURRC GC–C-IRMS APPARATUS FOR ^{15}N ANALYSIS

Fig. 1 shows the SURRC GC–C-IRMS development system for ^{15}N analysis, including component details. The system uses wide-bore (0·53 mm) capillary-column GC as a compromise between high sample:carrier gas value and good GC resolution. Volatile amino acid derivatives are injected into the GC where they are resolved into individual components. The 7·5 ml/min He carrier flow sweeps amino acid samples via an automatic solvent bypass valve (valve 1; Fig. 1) into a capillary combustion stage. CO_2 and water are removed in a liquid N_2 trap. Sample N_2 is swept into the IRMS ion source via a second GC column which resolves N_2 from any CO formed by poor combustion and removes trace impurities. The $^{m}/_{z}$ $^{29}/_{28}$ isotope ratio is calculated by integrating data

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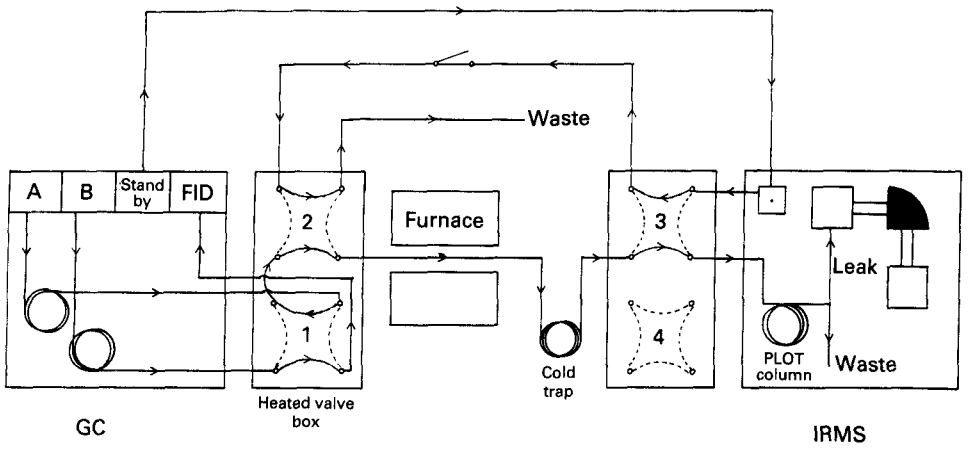

Fig. 1. The Scottish Universities Research and Reactor Centre GC–combustion-isotope-ratio mass spectrometry (IRMS) development system for ^{15}N. The GC is an Ai model 93 (Ai, Cambridge) fitted with two 0·53 mm i.d. bonded phase columns (injectors A and B) and a flame ionization detector (FID). The analytical column for *tert*-butyldimethylsilyl-amino acids is a 30 m long 0·53 mm i.d. 1·5 μm film DB-5 column (J&W Scientific, Folsom, California, USA). The automated, heated valve for solvent diversion (1) is a C4UWT VALCO unit (VICI, Switzerland). Valve 2 is a manual version of the same valve. The furnace is 300 mm long and contains a 2 mm i.d. stainless-steel combustion tube packed with 200 mm CuO granules (part B1051; Elemental Microanalysis, Okehampton), held at 750°. The cold trap consists of three turns of 0·75 mm i.d. stainless-steel held at liquid N_2 temperature. Standby valve (3) and spare valve (4) are both VALCO model C4UWP units operating at room temperature. The porous layer open tubular (PLOT) column is a 30 m × 0·53 mm i.d. GS-MOL column (J&W Scientific). The Tracermass mass spectrometer (Europa Scientific Ltd, Crewe) is controlled by proprietary software.

across the full width of each peak. Fig. 2 shows the major beam ($^{m}/_{z}$ 28) from amino acids purified and derivatized from blood plasma. The sample injected contained 25 nmol cycloleucine as internal standard. Typical analytical precision achieved so far is ±0·002 atom % ^{15}N at natural abundance.

Further improvement to precision is largely limited by the MS sensitivity of the development system. Continuing development work is concentrating on optimizing the catalyst conditions to reduce a small memory effect and improve catalyst lifetime, especially for samples that may be derivatized with silylation agents which gradually poison the catalyst.

CHOICE OF THE APPROPRIATE ANALYTICAL TECHNIQUE

If we wish to measure the protein fractional synthetic rate (FSR) of a particular protein or group of proteins, we can roughly estimate tracer enrichment over time in the protein in question. This can aid protocol design and the choice of optimal analytical technique. A crude rate of incorporation of a tracer amino acid can be estimated from literature values of FSR and precursor enrichment for a given tracer amino acid, be it delivered by infusion or by flooding dose. For designing experimental protocols in tracer studies a 'minimum working enrichment' can be taken as ten times the standard deviation of an analysis at natural abundance.

If short-half-life proteins with a high FSR are being measured, there is little doubt that

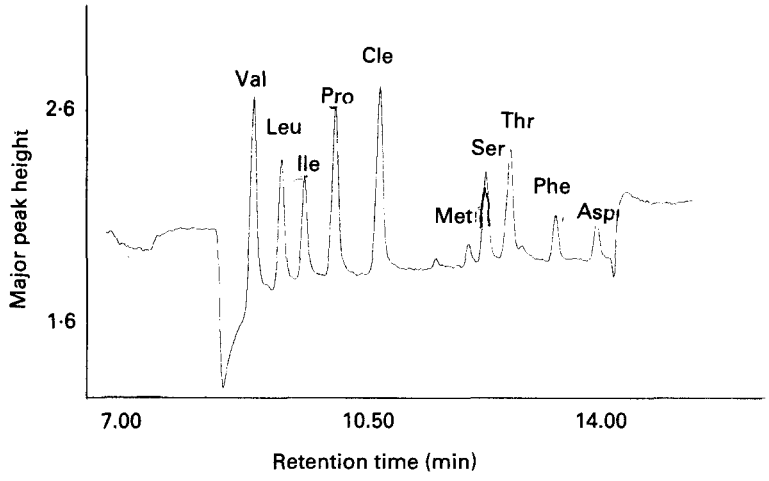

Fig. 2. The mass spectrometer major beam ($^{m}/_{z}$ 28) output for an amino acid sample, derivatized as *tert*-butyldimethylsilyl derivatives and injected into the Scottish Universities Research and Reactor Centre GC–combustion-isotope-ratio MS (IRMS) system. The amino acids valine, leucine, isoleucine, proline, cycloleucine (25 nmol internal standard; retention time 10·7 min), methionine, serine, threonine, phenylalanine and aspartate are shown in order of retention time. The two baseline minima reflect the operation of the solvent dump valve (see Fig. 1).

GC–MS will give the highest sample throughput and smallest sample requirement. It is also possible that some future studies will use liquid chromatography–mass spectrometry (LC–MS) to follow the incorporation of stable-isotope-labelled amino acids directly into peptides, but this is outwith the scope of the present paper. GC–MS is always likely to be the method of choice for precursor enrichment analysis. However, for protein hydrolysates, the minimum working enrichment of a singly-labelled amino acid tracer is quite poor at 0·5 atom % (ten times the precision level in Table 2). If ^{2}H is the tracer atom used, then GC–MS is the only analytical technique currently available (excluding off-line separation, vacuum pyrolysis and IRMS analysis, which is very labour-intensive for large sample numbers).

For ^{15}N-labelled amino acid tracers, when it is more widely available, GC–C-IRMS will be preferable to off-line separation and elemental analyser–CF-IRMS analysis in terms of throughput and sample size. It should also be noted that as all CF-IRMS techniques are element-specific, analysis of ^{15}N–^{13}C tracer studies will be more definitive compared with GC–MS analysis.

For 1-^{13}C-labelled tracers, GC–C-IRMS will give the highest throughput of the smallest sample, but the minimum working enrichment will be 0·02–0·04 atom %, dependent on the derivative used. For the best sensitivity, off-line amino acid separation and decarboxylation gives a minimum working enrichment of 0·002 atom % ^{13}C.

MULTIPLE-ATOM TRACERS

It has been assumed that we are limited to stable-isotope-labelled amino acids that are currently widely available, at reasonable cost. As this field continues to grow and the demand for tracer molecules increases, stable-isotope-tracer catalogues will become as large as their radioactive equivalents. Should U-^{13}C-labelled amino acids become widely

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available, then GC–C-IRMS will effectively become six times more sensitive (in the case of leucine) and could be used in preference to off-line separation and decarboxylation of the 1-^{13}C label. The minimum working enrichment would then be 0·003–0·007 atom % ^{13}C, depending on the volatile derivative used for GC–C-IRMS analysis. However, if a multiple-atom tracer is chosen, it will be important to compare the limit of detection of GC–C-IRMS with that of GC–MS. When the mass of the tracer molecule is several units higher than the tracee molecule, the background of the GC–MS fragment pattern is dramatically reduced and the limit of tracer detection improves correspondingly. Calder *et al.* (1992) have recently shown that, with careful sample preparation and control of instrumental variables, [$^{2}H_5$]phenylalanine derived from protein hydrolysates can be measured by GC–MS at 0·005 atom % excess. Their precision of ± 0·0003 atom %, giving a minimum working enrichment of 0·003 atom % represents a 167-fold improvement over the analysis of [^{2}H-]phenylalanine. Thus, this is very similar to that estimated for GC–C-IRMS analysis of U-^{13}C-labelled amino acids. At the time of writing, the ready availability of ^{2}H-labelled tracers makes their use and analysis by GC–MS more practical, although it should be noted that the potential for trace isobaric interference leading to overestimation of tracer enrichment is of much greater concern in GC–MS than in GC–C-IRMS.

Finally, Table 1 shows the minimum working enrichment of ^{2}H, assuming the technical challenge of ^{2}H analysis by CF-IRMS, currently being pursued by instrument manufacturers, can be met. This would provide about an order of magnitude improvement in minimum working enrichment over ^{13}C analysis, due to the low ^{2}H natural abundance. A CF-IRMS instrument for ^{2}H analysis would thus provide the lowest detection limit for stable-isotope-tracer use.

SUMMARY

The present paper has emphasized the need for good sample preparation procedures when working with stable isotope tracers. Considerably greater interchange of experience would help to improve the quality of sample workup in laboratories active in this field and to facilitate the use of these techniques by a wider community. In an ideal world, the lack of tracer availability should not be the determining factor in the choice of analytical technique. Increased stable-isotope-tracer usage in future should result in a greater range and availability of tracer molecules, at reduced cost. The development of a CF-IRMS analyser for ^{2}H analysis would greatly improve the situation as this promises a tenfold improvement of detection limit using tracer molecules that are readily available.

STABLE ISOTOPE NATURAL ABUNDANCE VARIATIONS

It was argued previously that the natural abundance of the stable isotopes ultimately limits the sensitivity of analysis of added tracers. The natural abundance of each isotope is not constant. Natural abundance variations are found throughout the natural environment. These are determined by a variety of physical, chemical and biological factors. The alteration of chemical bond strength caused by the presence of a heavy isotope causes a fractionation effect.

Studies of the natural abundance of ^{13}C and ^{15}N in foodstuffs have revealed significant differences that can be used to trace dietary sources of animals, including man, in present

day populations and anthropological studies (the latter largely by analysis of bone collagen; Schwarcz & Schoeninger, 1991). These authors cite reports of natual abundance variations in individual amino acids. Hobson *et al.* (1993), studying avian tissues, report an alteration in the natural abundance of ^{15}N as an index of reduced nutrient intake. We have frequently noted a difference in ^{15}N natural abundance in the endproducts of N metabolism, urinary urea and ammonium in our clinical studies of whole-body protein turnover (T. Preston & D. C. McMillan, unpublished results). Stable-isotope natural-abundance variations can potentially yield dynamic inferences from static measurements. With the advent of GC–C-IRMS we have a technique that promises measurement of ^{15}N and ^{13}C natural abundance in amino acids and other metabolites in sample sizes and throughput that was hitherto impossible. The measurement of the causes of variation of stable isotope natural abundance in the living body presents a fascinating opportunity for study that could possibly lead to novel methods of nutritional assessment.

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