Review

Stable Isotope Methods for Studies of Mineral/Trace Element Metabolism

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Introduction

Of the 90 chemical elements that occur naturally on earth, in the human food chain, and in the human body, only a small number (C, H, O, N, S) constitute the bulk of organic matter present in the body. The remaining elements, henceforth referred to as "minerals," are present in widely varying concentrations in different organs: many with established functions essential for life (essential elements; e.g., zinc), others with toxic properties (e.g., mercury), some with therapeutic value (e.g., lithium), and many with an as yet uncertain role (e.g., dysprosium) in the biologic processes. While much is known about certain aspects of metabolism of these elements, examples of important aspects that require considerably better insight than is currently available include availability of minerals in infants and children, assessment of trace element status in humans, details of metabolism of such trace elements as selenium and chromium in humans, homeostatic regulation of mineral metabolism in humans, and target organ/cell utilization of minerals. Investigation of these issues generally requires use of isotopic tracers. While radioisotopes have long been effectively used to elucidate some of these issues, especially in the animal models, their use suffers from two important limitations. First, they are radioactive and therefore generally not suitable for use with human subjects. And second, in many instances either the radioactive half-life is not suitable (too short, ^{28}Mg, ^{41}K, ^{64}Cu; too long, ^{65}Zn), or there is no radioactive isotope (lithium). The alternative to radioactive isotopes as metabolic tracers is the use of stable isotopes.

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Increasing applications of stable isotopes as metabolic tracers have been reported.[1,2] In comparison with their radioactive counterparts, several important features of stable isotopes have contributed to their preferential use, especially in metabolic studies with human subjects.[3] Stable isotopes of N, H, C, and O have now achieved the mature status of routine application with an extensive bibliography.[1,2,4] In clear contrast, stable isotopes of minerals/trace elements have not been applied as extensively.[5-7] The reasons for this state of affairs have been discussed previously,[5] but lack of suitable measurement methods has played a prominent role. During the last decade, concerted efforts have been directed toward rectifying this shortcoming.[5,6] As a consequence, further development of the two general methods of stable isotope measurement has received renewed attention: mass spectrometry[8-19] and neutron activation analysis.[5] The former has included ionization methods with electron bombardment of chelates,[18] thermal ionization,[19] and, to a lesser extent, fast atom bombardment[8] (FAB) and secondary ion mass spectrometry[9] (SIMS). A recent development (viz., inductively coupled plasma mass spectrometry[20] [ICP-MS]) has generated considerable interest for these applications because of the realization that it can function as the workhorse for a broad range of minerals and a large variety of applications.[10-17]

Successful applications of stable isotopes as metabolic tracers in studies of mineral metabolism require special insight, which cannot always be gained from similar investigations using stable isotopes of C, H, O, and N. There are major differences both in their measurement and feasibility of use. In this presentation, we will focus on the general question of how to use stable isotopes as metabolic tracers for studies of mineral metabolism, with special focus on their use with human subjects. However, because recent studies have also documented important advantages of these metabolic tracers over their radioactive counterparts

in studies of certain aspects of mineral metabolism using animal models, we will also discuss an example of the recent developments for this purpose.

This subject matter will be presented in the following order: fundamental considerations, measurement of stable isotopes in biologic materials, and selected applications. While appropriate references to literature have been included, the experimental aspects of this discussion have been largely drawn from the work performed in our laboratories and those of our collaborators. No attempt has been made to include all published accounts of the stable isotope approach.

Fundamental Considerations

Insightful application of stable isotopes as tracers requires some understanding of the essential features of tracer experiments as performed *in vivo* in humans (or selected animal models). The main purpose of isotopic tracers is to follow the dynamic/equilibrium state of transport of minerals or their compounds. This may be for the purposes of understanding metabolism of the mineral as it occurs in foods or for other reasons (e.g., as a therapeutic agent) under a broad range of conditions of health and disease. Route of entry for the mineral may be via the alimentary canal, the respiratory system, the integument, or in certain cases by injection. Depending on the route of entry, the mineral may undergo a number of biochemical alterations during which the newly entered "mineral" may exchange with a portion of that present endogenously in the system. At any time after its introduction into the body, the mineral will be present in varying fractions in different organs/body fluids.

The major fundamental difference between a stable isotope and its radioactive counterpart is in the universal presence of the former in all body fluids/tissues and the general absence of the latter (with few exceptions). Therefore, the most basic question in the design of such experiments using stable isotopes is the feasibility of achieving sufficient isotopic enrichment in the fluid/tissue/subcompartment of interest in order to be able to quantify the presence of the tracer with the necessary degree of precision and accuracy. Major factors affecting the degree of isotopic enrichment are the amount of the isotope administered, its efficacy of absorption (except when injected) and retention, its specific compartmental distribution, and the size of the endogenous form in the compartment of interest with which it will exchange.

Data relevant to these issues are given in *Tables 1* through *3*. In *Table 1*, we have summarized the body composition data for trace elements Zn, Cu, and Se in a 70-kg reference man. The following important points become clear. Total body content of elements and, thus, their constituent stable isotopes, vary widely. In addition, the total body fraction of a chemical element present in any anatomic compartment varies markedly both among organs and for different elements. For instance, while some 30% of total body zinc is in the bone, only 0.1% of the element is present in plasma. Similarly, plasma contains 5% of total body copper, a relatively large proportion compared with that for zinc.

Table 1 Selected body composition data relevant to stable isotope tracer investigations

Parameter	Zinc	Copper	Selenium
Daily intake (mg)	10	1	0.10
Amount absorbed (mg)	3.0	0.5	0.08
Total body content (mg)	2300	72	14
Compartmentation (% total body content)			
Bone	30	10	—
Brain	0.9	11	—
Heart	0.4	1	0.3
Liver	2	17	8
Blood plasma	0.1	5	2
Skeletal muscle	60	35	50

Table 2 Quantitative aspects of chemical speciation for iron in humans[a]

Chemical form	Whole body iron (%)
Hemoglobin	60.5
Myoglobin	8.4
Heme enzymes	2.1
Non-heme enzymes	2.6
Ferritin	18.4
Hemosiderin	7.9
Transferrin	0.08

[a] Data calculated from information in ref. 21.

Total body/organ content of an element is not always the overriding issue in the determination of the feasibility of stable isotope tracer investigations. Chemical elements occur in many forms in the body (*Tables 2* and *3*), each with its own characteristic turnover rate. For instance, data in *Table 2* indicate that 60.5% of body iron is present in the form of hemoglobin, while only 0.08% is in the circulating plasma (as transferrin). Similarly, data given in *Table 3* indicate that while muscle contains a major fraction of body selenium, only a small portion of it is in the form of the biologically active selenoenzyme glutathione peroxidase. These issues have important implications in regard to the extent of isotopic exchange that might take place after administration of a suitable dose of stable isotope and play important roles in the determination of the feasibility of stable isotope tracer investigations. Thus, factors other than gross elemental composition of an organ need to be considered in the overall evaluation of exchange reactions between isotopic tracers reaching the organ and any particular form of the element present in that organ or compartment.

Natural isotopic abundances of elements vary over a wide range. Some selected data have been presented

Table 3 Quantitative relationship between organ selenium content and its fraction present as glutathione peroxidase in the rat[a]

Organ	Selenium content (% all tissues analyzed)	Selenium present as GSH-Px (% organ selenium)
Muscle	39.8	5
Liver	31.7	63
Red blood cells	7.9	82
Spleen	1.0	41

[a] Data from ref. 22.

in *Table 4*. In this table, data have been given for gross body content of a 70-kg reference man, the total number of stable isotopes for the element, and the total body content (mg/kg body weight) of the least abundant stable isotope. Thus, the reference man contains 270 mg magnesium/kg, of which 27 mg/kg consists of ^{25}Mg. Similarly, the iron content of the reference man is 57 mg/kg, of which ^{58}Fe constitutes 0.19 mg/kg. These data permit worst-case calculations of the degree of isotopic enrichment that one may be able to achieve under experimentally defined conditions. This is the first required step in determining the feasibility of any stable isotope tracer investigation. In *Table 5*, we have calculated the amount (mg) of the least abundant stable isotope (as 100% enriched) required to increment the natural isotopic abundance of a 70-kg man by 10%, have compared this with the normal daily intake of the element, and have determined the cost of the isotope at current prices for such an experiment.

The data demonstrate a number of important characteristics inherent in the method of stable isotope tracers. First, compared with the daily intakes of the element, the doses of the stable isotopes required to achieve 10% enrichment in the isotopic content of an adult man do constitute physiologically relevant amounts. Experiments could readily be designed to accommodate the need for addition of such amounts to the diet in ways that do not disturb the daily intake of the element. However, a much more serious issue is related to the costs associated with some of these applications. It is clear from the data that even well-funded research programs could not justify costs involved with studies of Mg, K, Ca, or Fe under the conditions of these calculations. Therefore, it is essential that ways be explored to reduce the cost factor by one to three orders of magnitude, depending on the specifics involved.

The cost analysis presented above constitutes the worst case, sometimes by a large margin. For the majority of minerals, the administered isotope is not likely to exchange with a major portion of the body's content within an experimentally practical length of time. For instance, only 1% of total body calcium is present outside the bone matrix, so a 10% enhancement of the readily exchangeable calcium pools requires two to three orders of magnitude less enriched isotope than given in *Table 5*. Similarly, only approximately 10% of total body zinc is likely to exchange with an administered dose of the stable isotope within a week, so that if the aims of the experiment were to measure the magnitude of the exchangeable pool of zinc, the cost would be reduced by an order of magnitude. For some elements, such as potassium, the major portion of body pool is available for exchange and for these, the cost is an important limitation. This highlights the need to find ways to reduce the amount of required isotope.

The factor 10% enrichment was chosen as the minimum isotopic enrichment required for an acceptable outcome of pool size measurements, in relation to the present-day achievable measurement precision. This is an important issue that needs further elaboration. Measurement of exchangeable pool size of an element requires administration of a suitable dose of the enriched stable isotope followed by measurement of the enriched isotope ratio ($R_{a/b}$) after completion of isotopic exchange. The expression for the calculation of the exchangeable pool size (W_e) is

$$W_e = k/(R_{a/b} - R^\circ_{a/b}) \quad (1)$$

where k is the constant and $R^\circ_{a/b}$ is the baseline ratio.

The major question that arises is the relationship between the achievable precision of the isotope ratio measurement (σ/R) and the consequent expected uncertainty in W_e (σ/W_e). A general expression relating σ/W_e to σ/R can be derived

$$\sigma/W_e = \sqrt{2a^2(R/R^\circ)^2} \quad (2)$$

Table 4 Stable isotope composition of selected chemical elements[a]

Element	Total body content (mg/kg)	No. of stable isotopes	Total body content of least abundant isotope (mg/kg)
Mg	270	3	^{25}Mg, 27
K	2000	2	^{41}K, 138
Ca	14000	6	^{46}Ca, 0.46
Fe	57	4	^{58}Fe, 0.19
Cu	1.1	2	^{65}Cu, 0.34
Zn	33	5	^{70}Zn, 0.20
Se	0.2	6	^{74}Se, 0.0017

[a] Calculated from data given in ref. 23.

Table 5 Dose requirements for a 10% increment in isotopic content of the reference man

Element	Daily intake (mg)	Assumed total utilization (fraction of dose)	Dose (mg)	Cost ($/dose)
Mg	500	0.5	380	3200
K	3000	1.0	970	19400
Ca	1000	0.5	6.4	42000
Fe	10	0.1	13	1490
Cu	2	0.5	5.0	10
Zn	10	0.3	5.0	390
Se	0.1	0.5	0.02	5

Table 6 Consequences of changes in achieved isotope enrichment and measurement precision to expected uncertainty of pool size calculations

Measurement precision	$R/R°$	Constant in Eq. 2(a)	σ/W_e
1%	1.1	0.1	0.16
	1.05	0.2	0.29
0.1%	1.1	0.01	0.016
	1.05	0.02	0.030
	1.01	0.10	0.14

where a is a constant relating the precision of isotope ratio measurement to the extent of achieved isotope enrichment:

$$\sigma_1/R = \sigma_2/R° = a(R-R°)/R° \quad (3)$$

From the expression presented in Eq. 2, one can readily calculate the consequences of various permutations between the two important factors: degree of uncertainty in the estimation of W_e and measurement precision of isotope ratios. Some illustrative calculations have been summarized in *Table 6*. The results are important in the design of isotopic tracer studies. For instance, if we can measure the two isotope ratios (R, $R°$) with precision of 1% and can afford to enrich the pool by 10%, we can expect an uncertainty of 16% in our estimate of the exchangeable pool size. If, on the other hand, we can afford the cost of isotope for only 5% enrichment, we face two alternatives. First, if our measurement precision cannot be improved beyond 1%, we will have an uncertainty of 29% in our estimate. Second, improving the measurement precision by an order of magnitude (to 0.1%) would permit us to estimate the size of the exchangeable pool with an uncertainty of only 3.0%. These calculations serve the important purpose of indicating where the investment should go: in paying for the isotope or in achieving better measurement precision. They further illustrate the importance of aiming for as good a precision in the measurement of isotope ratios as is practical, regardless of the cost factor.

Measurement of Stable Isotopes in Biologic Materials

Isotopic tracer studies require two types of analyses. In some instances (e.g., incorporation of ^{58}Fe in circulating red blood cells,[10]) measurement of only the ratio of two or more isotopes is sufficient. However, in most instances, it is necessary to perform quantitative isotopic analysis. The latter could be performed by combining isotope ratio measurements with total elemental analysis. But, this method is not as desirable as direct measurement of isotopic content because of the added error of the method used for elemental analysis. The most accurate approach to quantitative isotopic analysis is based on the concept of *in vitro* stable isotope dilution (SID).[12-17]

Two general methods have been used for the measurement of stable isotopes in biologic materials: neutron activation analysis and mass spectrometry. The former method is selective by its nature and can only measure certain stable isotopes.[5] For example, of the six stable isotopes of selenium, three can be routinely measured with this method. Similarly, of the five stable isotopes of zinc, only three can be measured. Of the four stable isotopes of iron, two can be quantified with neutron activation analysis. In addition, in order to perform neutron activation analysis for the majority of minerals/trace elements requires a nuclear reactor with 1 to 10 megawatt power output. These reactors are of limited availability. Furthermore, successful application of neutron activation analysis requires a sufficient level of training in analytic radiochemistry. This is generally not available to the users. For these reasons, neutron activation analysis has proven of limited accessibility for stable isotope tracer studies.

In contrast to neutron activation analysis, mass spectrometry should, in principle, be capable of isotopic measurements of all stable isotopes. However, considerable difficulty has been encountered in the past in the application of mass spectrometry to the required analyses and this has, in general, been related to the difficulties of ion production rather than mass analysis. The earlier attempts using chelate mass spectrometry have generally not been successful.[6,18] The classic method of thermal ionization mass spectrometry (TI-MS) has proven of uneven capability.[6,19,24-27] For instance, for measurements of isotope ratios for lead[25] and calcium[24] the method has proven the most suitable, providing the highest degree of measurement precision, albeit slow, sample throughput. For other elements, such as magnesium, it may also prove to be the most precise, although no working procedures are as yet available for these applications. However, for those, such as zinc and copper as applied to biological

materials, it presents a modest level of measurement precision,[6,26-28] not consistent with its potential. Yet, for others, like iron, it has not proven successful, despite considerable effort. For a bi-isotopic element, such as potassium, differential evaporation from the sample all but precludes its use for precise measurement of the ratio $^{41}K/^{39}K$.

Despite its potential ability for very precise measurements of isotope ratios, TI-MS is not likely to find widespread acceptance for these applications for the following reasons: requirements for very high level of chemical separations, slow sample throughput, difficulties associated with production of sufficient ion beam intensities, production of stable ion beam, the need for an extra stable isotope to correct for differential isotope evaporation effects, and, finally, the high cost of instrumentation and lack of available expertise.

Considerable effort has recently been directed toward other methods of ion generation, leading to availability of such instruments as FAB mass spectrometry (FAB-MS) and SIMS. While some work has been reported in connection with these devices for specific application to stable isotope tracer studies of minerals[8,9,28] the full potential and limitations of these devices have not yet been documented. Therefore, judgment of whether such devices will prove anything more than of limited potential is premature.

In the early 1980s, ICP sources were shown to be able to provide a source of high efficiency ion production for many chemical elements.[20] After successful coupling of an ICP ion source with a quadrupole MS, a new mass spectrometric technique, ICP-MS, was born. The first commercial instruments based on this technique became available in 1984. The investigations performed thereafter, focussing on the issue of whether this method will provide a general capability for stable isotope measurements in specific reference to stable isotope tracer studies of minerals/trace elements, have provided an exciting outlook.[29,30] Despite the short time interval, this method has now been developed for, and applied to, metabolic investigations of minerals/trace elements of widely different characteristics: Li,[14] Mg,[31] K,[32] Fe,[33,34] Cu,[13] Zn,[11,13] Se,[16] and Br.[15] Based on available information concerning ion production and ion beam intensities, it is clear that this method can be developed further to provide a general isotope measurement method for the majority, if not all, of the bi- and poly-isotopic chemical elements. Sufficient information is now available to be able to draw general conclusions about the measurement capabilities and potential limitations of this method for studies of mineral metabolism. A brief discussion of the salient features of this method as applied to the precise and accurate measurement of stable isotopes in biologic materials derived from metabolic investigations appears below. The emphasis of this discussion will be on providing an overview of the method, and some of its important experimental parameters, whose understanding is necessary for an insight into its potential utility for stable isotope tracer investigations. More detailed treatment of technical aspects of the instrumentation and chemical procedures can be found elsewhere.[35,36]

Principles of inductively coupled plasma mass spectrometry

An ICP-MS consists of two major subsystems: an ICP and a mass spectrometer (MS). The former acts as the ion source for the latter. A schematic of this system is given in *Figure 1*.

An ICP consists of a continuous stream of argon gas flowing through a confined region, into which radiofrequency (rf) power is directed. The energy from rf power causes ionization of argon atoms, creating an argon plasma (ionized gas). For the purposes of this discussion, the major feature of the argon plasma is its high temperature (approximately 7,000°K). When a sample is introduced into the argon plasma, the chemical elements of the sample are subjected to this high temperature and, depending on their ionization potential, are ionized mostly into singly charged species (M^+). The degree of ionization efficiency varies for different chemical elements, but is generally high *(Table 7)*. Therefore, the argon-plasma acts as an efficient source of ions for the majority of chemical elements.

There are several methods for introducing samples into the argon plasma.[35] For use with biologic materials and isotopic measurements, the most convenient is sample introduction as a solution (pneumatic nebulization; PN) or, in certain cases, as gas (hydride generation; HG). When sample is introduced as solution (PN), it is carried into a stream of argon gas (nebulizer gas) as liquid droplets produced from the movement of the gas by the orifice of the nebulizer. This results in production of droplets of varying sizes (10 to 100 μm). Large droplets cannot be passed into the plasma because they will extinguish it. Thus, a chamber (spray chamber) is placed between the nebulizer and the torch. The larger particles coalesce in the spray chamber and are directed into the waste container, while the smaller ones are carried into the torch. With the present pneumatic nebulizers, this is an inefficient process and only a small fraction (<5%) of the analyte solution

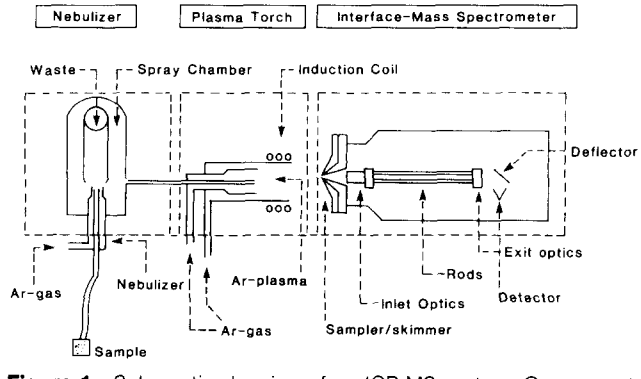

Figure 1 Schematic drawing of an ICP-MS system. Components are not drawn to scale. For more information on instrument design, refer to refs. 20, 35, and 36.

Table 7 Representative values of ionization efficiency for argon plasma[a]

Category	Ionization potential (eV)	Ionization (%)
Alkali elements: Li, Na, K	4.3–5.4	100
Alkali earths: Mg, Ca	6.1–7.6	99
Transition elements: Cr, Fe, Cu, Zn	7.7–9.4	75–98
Selenium	9.8	33
Br	11.8	5
Cl	13.0	0.9

[a]Refer to Ch. 10 in ref. 35.

is carried into the torch, where its constituent elements are ionized with high efficiency.

There are several alternative methods to pneumatic nebulization, none of which is at present standard on the commercial systems. Of these, introduction as gas is in principle very efficient and could lend itself relatively easily to sample solutions resulting from tracer investigations. There are a number of approaches to production of gaseous samples, but one that has proven successful for specific application to isotopic tracer studies is based on the hydride generation principle.[37] In this method, the element of interest is converted to its corresponding hydride by the reaction with nascent hydrogen which is generated from reaction of $NaBH_4$ with HCl. The resultant gas is separated from the liquid and carried into the torch with argon gas. This method is limited to those elements that can form volatile hydride: As, Bi, Ge, Pb, Sb, Se, Sn, and Te (refer to ref. 35, Ch. 13). Therefore, its scope of application is relatively limited. Other methods of converting the element of interest into a gaseous stream, such as use of volatile chelates, need to be explored for other elements, such as chromium and zinc.

Ions produced from the elemental constituents of the sample are carried through the sampler/skimmer orifices into the mass spectrometer, where each ion of interest is focussed sequentially by the combined actions of the rf/DC voltages of the quadrupole mass analyzer onto the ion detector. The mass spectral region of interest can be scanned incrementally to produce a record of ion beam intensity as a function of mass number (a mass spectrum) or the instrument can be programmed to sequentially record the individual ion beam intensities for selected regions (peaks), thus performing quantitative analysis. A typical mass spectrum for a solution containing Fe, Zn, Cu, and Se is shown in *Figure 2*. In the measurement of isotope ratios, which is the principal feature of ICP-MS, the present instruments can perform sequential analysis only, using peak hopping procedures.[20,32,36] Because of the many factors that might influence the overall ion beam intensity during the time necessary for the two measurements needed for a ratio, this method of data acquisition could be an important limitation in the achievement of measurement precision. If methods could be developed to permit simultaneous measurement of two or more ion beam intensities (as is done in high-precision isotope ratio mass spectrometry with thermal ionization or gas source analyzers) this could prove to be an important development in pushing the precision capabilities of the device beyond its present state.

In principle, the ion beam intensities recorded are proportional to the concentration of the isotope of interest in the analyte solution. In practice, however, many factors, instrumental and chemical, introduce such uncertainties that ion beam intensities may not be directly proportional to isotopic concentrations. In contrast, because the effect of these unknown factors is almost the same for all isotopes of any given element, they generally do not affect isotope ratios. Therefore, this method provides an accurate means for the measurement of isotope ratios for any bi- or polyisotopic element (also see discussion under *Accuracy of Isotopic Analyses*).

Analytic performance of inductively coupled plasma mass spectrometry

For the purposes of stable isotope tracer studies, sufficient insight into the following analytic features of ICP-MS is necessary: background intensities, sensitivity, interferences, precision of isotope ratio measurements, instrument memory, sample throughput, chemical separation procedures, and accuracy of isotopic analysis. These are briefly discussed below.

Background considerations. Because of the high temperatures involved with argon plasmas, argon gas it-

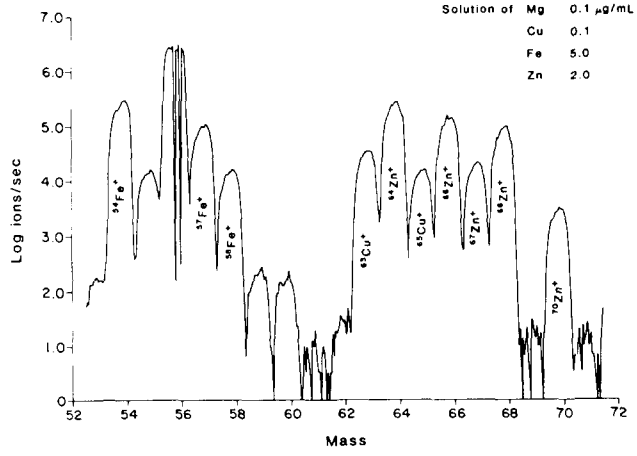

Figure 2 Mass spectrum of a solution containing (µg/ml): Mg (0.1), Cu (0.1), Fe (5.0), and Zn (2.0).

Table 8 Examples of argon plasma backgrounds generated when deionized water is introduced with the pneumatic nebulizer system

Isotope of interest	I_b (c/s)[a]	Concentration equivalent[b] (μg/ml)	Blood plasma concentration (μg/ml)
^{6}Li	42 ± 2	0.00056	—
^{7}Li	396 ± 5	0.00034	—
^{24}Mg	58 ± 21	0.000021	20
^{25}Mg	14 ± 4	0.000039	
^{26}Mg	30 ± 12	0.000069	
^{54}Fe	8,240 ± 58	0.13	1
^{57}Fe	629 ± 24	0.026	
^{58}Fe	234 ± 6	0.072	
^{63}Cu	58 ± 12	0.00019	1
^{65}Cu	22 ± 3	0.00015	
^{67}Zn	22 ± 3	0.0023	1
^{68}Zn	77 ± 8	0.0017	
^{70}Zn	56 ± 3	0.036	
^{74}Se	60 ± 9	0.034	0.1
^{77}Se	50 ± 7	0.0033	
^{82}Se	30 ± 3	0.0017	
^{79}Br	245 ± 12	0.025	3
^{81}Br	4,684 ± 103	0.48	

[a] I_b is the ion beam intensity recorded with deionized water.
[b] Concentration equivalent refers to the concentration of the element of interest of natural isotopic composition corresponding to the recorded I_b.

self, any impurities present in the gas, as well as the matrix constituents of the sample solution will produce a complex ion beam background. This background creates one of the major limiting factors in the application of the method to stable isotope tracer studies. When the method is used for elemental analysis, this is not as serious a problem because one can select stable isotopes which present a high signal to background ratio. This is not generally possible for stable isotope tracer studies, as the choice of stable isotopes is dictated in part by the need to use stable isotopes of low natural abundance and in part due to the requirement for two to three stable isotopes. Therefore, a sufficient understanding of this problem and development of procedures to avoid any potential problems is a necessary aspect of analytic chemistry of this method. Selected data on the relative intensities of ion beam background generated in argon plasma when deionized water is used as the sample solution with pneumatic nebulizers are given in *Table 8*.

In this table, we present the recorded ion beam intensities obtained when deionized water is nebulized into the system and have converted these observed background intensities to the corresponding elemental (natural composition) concentrations. In addition, we list normal concentrations of human blood plasma which generally constitute the most limiting sample size in such studies. Examination of the data clearly demonstrates the known[38] uneven background across the mass spectrum. But, more importantly, the data also show that while background ion intensities could pose some problems (e.g., ^{74}Se), these are not expected to become limiting except in a few special circumstances.[29]

Sensitivity. The intensity of the ion beam produced from the isotope of interest at its prevalent concentration in the biologic matrix in comparison with the background intensity is one of the most important initial criteria determining whether ICP-MS can be suitable for any given application. As can be inferred from the data presented in *Table 8*, the least-abundant stable isotopes of elements generally present sufficient ion beam intensity to permit quantitative analysis, even in the most limiting sample size. However, it is important to bear in mind the fact that in these applications, sensitivity should be considered in relation to the required precision of isotope ratios for the specific isotope pair involved. In order to be able to achieve measurement precision $\leq 1\%$, it is necessary to acquire a minimum of 10,000 counts in the lesser of the two ion beams.

Interferences. Two potentially significant types of interferences are likely to arise with ICP-MS: isobaric interferences and interferences from matrix constituents. The quantitative significance of these depends strongly on the specifics of any given situation and they need to be considered individually.

Isobaric interferences occur when an isotope of an element different from that of interest occurs within the same nominal value of m/z (mass-to-charge ratio). Examples of this type of interference include ^{58}Ni in the measurement of ^{58}Fe,[3,29] a serious problem when fecal samples are involved.[12] Resolution of this interference requires a chemical separation procedure that selectively removes iron from nickel in fecal digest.[12]

A second type of interference that is encountered in working with biologic matrices is related to the complex nature of molecular composition of these materials. The digest resulting from wet ashing of a biologic material contains a large variety of compounds, mostly inorganic, in concentration ranges covering orders of

magnitude. For example, while the concentration of bromine in human plasma is approximately 3 μg/ml, that for sulfate is 30 μg/ml. Similarly, the bromine and sulfate concentrations in human urine, which vary depending on dietary intake, may be approximately 5 and 2,000 μg/ml, respectively. In the measurement of $^{81}Br/^{79}Br$, presence of sulfate in the solution presents an interference at m/z = 81, presumably due to $H^{32}S^{16}O_3^+$.[15] This species presumably originates from decomposition, in the argon plasma environment, of SO_4^{2-} present in the sample. Because concentration of sulfate is disproportionately higher in urine than in plasma, insufficient removal could cause a serious measurement error in urine.[15] Based on the known complex composition of biologic matrices, these types of interferences could pose a serious problem in the accurate measurement of stable isotopes with ICP-MS.

Precision of isotope ratio measurements. Application of stable isotopes in tracer experiments differs from that for radiotracers in an important basic way due to the presence of the tracer isotope in the natural material. Thus, a basic consideration that must be resolved prior to the establishment of its feasibility in any given application is the relationship between the achievable degree of isotope enrichment under the dosing conditions relevant to the experiment and the precision with which the excess enrichment can be determined. The isotope excess in any given organ, tissue, or fluid compartment at a time after dosing is determined using the method of *in vitro* stable isotope dilution, where a known amount of highly enriched isotope (different from the *in vivo* tracer) is spiked into the sample to be analyzed and two isotope ratios are measured. This requires that the element of interest possess at least three stable isotopes that can be measured (if the element is only bi-isotopic, a variation of this method can still be used). The expressions used for the calculation of isotope excess from the *in vivo* tracer are:

$$W_r = W_s/(R_{s/r} - R^\circ_{s/r}) \quad (4)$$

$$W_r^* = W_r(R_{t/r} - R^\circ_{t/r}) \quad (5)$$

where W_r, W_s, and W_t^* are the weights of reference, spike, and excess *in vivo* tracer in the spiked sample, respectively; $R_{s/r}$ and $R_{t/r}$ are the estimated ratios (wt/wt) for the spiked to reference and tracer to reference isotopes, respectively; and $R^\circ_{s/r}$ and $R^\circ_{t/r}$ are the corresponding baseline ratios.

An important issue is the magnitude of the uncertainty (σ/W_t^*) resulting from the measurement uncertainties present in the parameters of Eq. 5. This can be determined from considerations of the propagation of errors:

$$\sigma/W_t^* = \{(\sigma^2/W^2_s) + (\sigma^2_{s/r} + \sigma^{\circ 2}_{s/r})/(R_{s/r} - R^\circ_{s/r})^2 + (\sigma^2_{t/r} + \sigma^{\circ 2}_{t/r})/(R_{t/r} - R^\circ_{t/r})^2\}^{1/2} \quad (6)$$

In *Table 9*, estimates of σ/W_t^* have been summarized for two cases of achievable level of isotope enrichment for studies of selenium using ^{74}Se as the *in vivo* tracer. The higher levels of isotope enrichment taken are realistic values observed in studies with human subjects for urine or plasma.[39] The lower level has been used to illustrate the impact in cases in which such low levels of enrichment might be involved. There is a wide latitude in the achieved value of $R_{82/77}$, depending on the amount of spike added to the sample. We have taken the value of 3.00 (200% of the natural amount in the sample) for this example. In these calculations, we have assumed a precision of 1% for W_s. The data show an important point. If the level of isotope enrichment that is achieved in an experiment is 100% of the natural ratio ($R_{t/r} = 2R^\circ_{t/r}$), as is readily achieved for human plasma,[39] or higher (human urine[39]), measurement precision for the isotope ratios of 1% (achieved routinely[16]) is sufficient to permit conduct of experiments. On the other hand, if the level of isotope enrichment was only 10% of the natural ratio, a precision of 1% would not be sufficient to permit accurate studies, but achievement of 0.1% measurement precision (not routinely obtained) would be necessary. These issues are analogous to the previous discussion of errors (*Table 6*).

Table 9 Relationship between achieved level of isotope enrichment, measurement precision, and the resultant overall uncertainty

Achieved isotope excess (% baseline)	Precision of R, R°	
	1%	0.1%
100	0.024	—
10	0.144	0.020

At the present stage of its development, ICP-MS is capable of providing measurement precision at the level of 1% or somewhat better for many isotope pairs.[10-17] However, 0.1% level of precision, while achievable in principle,[20] has not yet been reached for routine analyses involving a large number of samples.

Instrument memory. A major advantage of ICP-MS relates to its high sample throughput. Depending on the number of isotopes to be analyzed and the specifics of sample introduction method, 50 to 100 solutions can be analyzed during a given operating day. Isotope ratios of samples derived from stable isotope tracer studies will typically vary by an order of magnitude. Thus, it is important to understand how long one needs to allow between any two sequential samples before residual from the previous analyte solution has been reduced to a satisfactory level. The overall "memory effect" of ICP-MS varies for the two sample introduction systems discussed here (PN and HG) and may also vary for different chemical elements. Details of these have been discussed previously.[14,37]

Sample throughput. Total time required for complete analysis of an analyte solution for the purpose of isotope ratio measurements is approximately 5 to 10 minutes, depending on such specifics as the sample introduction system used or the number of isotopes to

be analyzed. Therefore, during a regular work-day of 8 to 10 hours, approximately 6 to 7 hours can be effectively spent on data acquisition. This then permits complete analysis of at least 50 analyte solutions. Experience over several years shows that this is in fact the sample throughput performance of the instrument. This sample throughput is consistent with achievement of isotope ratio measurement precision of 0.5% to 1%, but not 0.1%.

Chemical separation procedures. As indicated earlier, some degree of chemical separation is necessary for accurate measurement of isotope ratios. The extent of separations needed varies for different elements and various matrices.[10-17] Practical methods for routine application have now been developed for Li,[14] Mg,[17] Fe,[10,12] Cu,[13] Zn,[11,13] Se,[16] Br,[15] and K (Janghorbani, unpublished data). A summary of the overall procedures that are now available is illustrated in *Figure 3*.

These procedures, while differing in detail for each element or matrix, have certain common features. A representative subsample is oxidized with a combination of inorganic acids/H_2O_2. The resultant digest is then subjected to a modest level of chemical separation devised specifically for the isotopes of interest and the particular matrix involved. The details of this part of the scheme are important to the success of the measurement. For instance, for the measurement of isotope ratios of iron in blood, a single-step precipitation with ammoniumpyrrolidindithiocarbamate (APDC) has proven sufficient, while this will not be adequate for the ratio $^{58}Fe/^{57}Fe$ in feces. For the latter case, it is necessary to separate nickel content which can be done effectively using NH_4OH as a nickel-complexing agent. While precipitation with APDC has proven sufficient for zinc, in certain matrices (muscle) erroneous ratios were obtained for copper (apparently due to sulfate interference). This problem was resolved when an ion exchange step was added to the scheme.

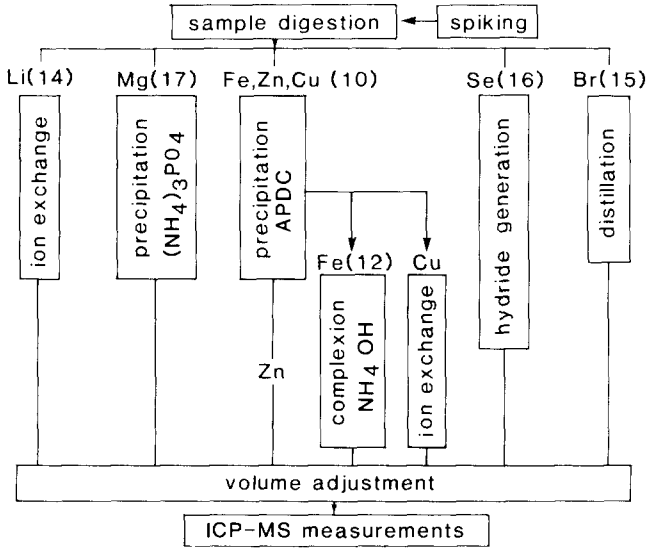

Figure 3 Generalized scheme for sample preparation for ICP-MS. For more details, consult the refs. given in the figure (10, 12, and 14–17).

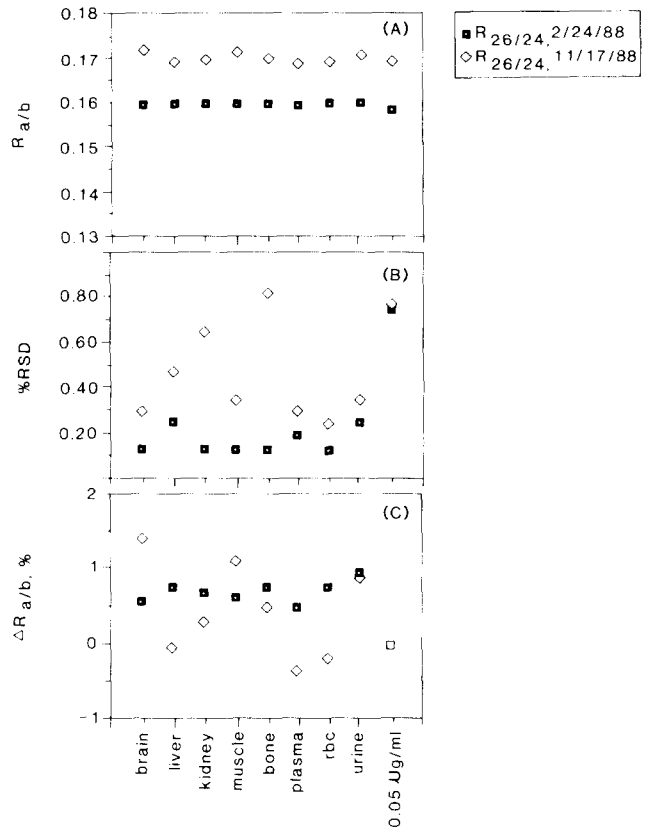

Figure 4 Intermatrix equivalency of measured ion beam intensity ratios for natural magnesium for the ratio $^{26}Mg/^{24}Mg$. For similar data on $^{25}Mg/^{24}Mg$, refer to ref. 16.

After the chemical separation step, excess acids are removed, volume is adjusted to provide optimal count rates for the isotopes of interest, and the solutions are ready for mass spectrometric analysis.

The scheme shows that the extent of chemical separations needed is relatively modest. This is an important feature of ICP-MS compared both with neutron activation analysis and thermal ionization mass spectrometry.

Accuracy of isotopic analyses. A number of issues are involved in the establishment of accuracy of isotopic analysis. These have been discussed previously.[10,12-17] An important component of these is the accuracy with which the relevant isotope ratios in samples resulting from *in vivo* tracer experiments can be measured. An example of the level of accuracy that can be expected from this technique is given in *Figure 4*, which illustrates the equivalency of these measurements for a number of matrices for stable isotopes of magnesium. Data given in *Figure 4A* show that the ion beam intensity ratios vary for any given analyte solution measured on different days, and highlight the need for appropriate standards. Data given in *Figure 4B* show that measurement precision is independent of the nature of the matrix. And, finally, data summarized in *Figure 4C*

indicate the equivalency of the measured ion beam intensity ratios for all the matrices, as compared with a standard solution of magnesium.

Measurements made with ICP-MS reflect recorded ion beam intensities assigned to any value of m/z. In addition to various interferences, as discussed above, two other factors could result in ion beam intensity ratios different from true isotope ratios. On the one hand, as yet poorly understood matrix effects may modify the background signals recorded at the m/z of interest. At present, the only effective method for correcting for this is by actual analysis of a "complete sample blank," a solution resulting from processing of deionized water as close to the sample as is possible. The second complicating factor relates to argon-plasma/mass spectrometer effects leading to an effective mass discrimination, so that the recorded ion beam intensity ratio is not identical with the true isotope ratio. This effect is expected to be larger for lighter elements.[40] While, in principle, appropriate correction algorithms could be applied[40] so that the measured ion beam intensity, corrected for background, could be converted to the expected true ratio, a much more practical approach is based on calibration of the recorded ion beam intensity ratios against their true isotope ratios using a set of "stable isotope calibration standards." These standards are made by the appropriate combination of natural elements with progressive increments of highly enriched spikes for the isotopes of interest.[10,12-17] The standards are then analyzed with the samples of interest and calibration plots are constructed. A typical set of stable isotope calibration plots is shown in *Figure 5*. These data were obtained from a set of solutions whose $^{82}Se/^{77}Se$ and $^{74}Se/^{77}Se$ ratios had been altered in a known manner, without major changes in the solution concentration of ^{77}Se. The recorded intensity data for ^{77}Se (b1,b2) show that there was a significant drift in intensities during measurement. This is also reflected in the intensities recorded for the incrementally spiked isotopes ^{74}Se and ^{82}Se (a1,a2). However, the measured ion beam intensity ratios (c1,c2) show a high degree of linearity with respect to the true isotope ratios.

We have examined the overall accuracy of quantitative isotopic analyses by the application of *in vitro* SID to several materials of natural isotopic composition and comparison of the results with independent elemental analyses. Some of our results have been summarized in *Table 10;* other data have been given elsewhere.[10,12-17] The experience from these studies indicates clearly that the precision/accuracy of overall isotopic analysis is determined mostly by the precision/accuracy of the measurements of isotope ratios.

Applications of Stable Isotope Tracer Methods

Stable isotopes have a wide range of application. Some of these have been reported previously.[5,7,18,19,29,30] In this section, we present a selected number of examples of applications that have been explored with this approach.

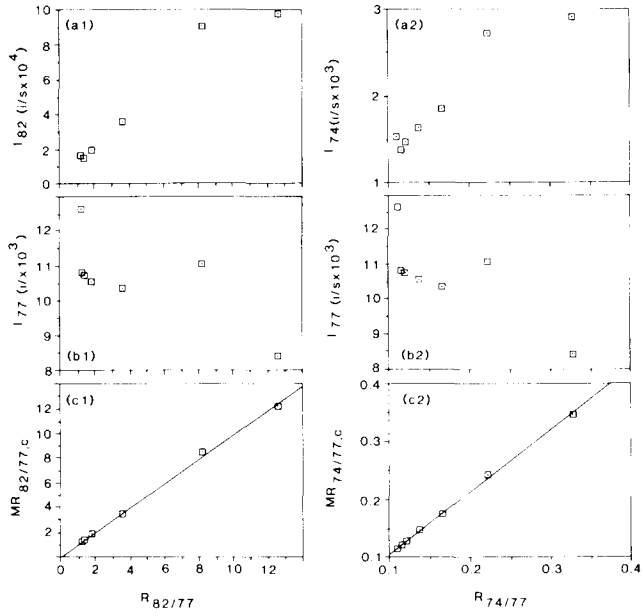

Figure 5 An example of stable isotope ratio calibration for $^{82}Se/^{77}Se$.[16] Various measured parameters are plotted against the true isotope ratio for $^{82}Se/^{77}Se$ or $^{74}Se/^{77}Se$ in synthetic standards. I_a, recorded ion beam intensity for isotope of interest; $MR_{a/b,c}$, recorded ion beam intensity ratio for isotope a to b, corrected for blank; and $R_{a/b}$, true isotope ratio expressed on weight basis.

Studies of dietary availability

The major focus of recent applications of the stable isotope tracer method has thus far been on dietary availability of minerals.[6,18,19,33,34,41-53] These studies have been performed with a number of minerals/trace elements: Ca,[41] Mg,[42] Fe,[33,34] Cu,[49,51] Zn,[43,44,47,51] Pb,[45] and Se.[46,50] Here, we have illustrated this line of investigation by two examples: availability of iron in infants,[33,34] and exchangeability of dietary pools for zinc.[44]

Dietary availability of iron in infants. Iron deficiency is a leading nutritional disorder among infants and children both in industrialized and developing countries.[33,54] Development of strategies for prevention of iron deficiency requires knowledge of its absorption. Use of radioiron is not warranted for these studies.

There are two general approaches to the experimental determination of gastrointestinal absorption of a nutrient.[55] One approach is based on the estimation of the amount of unabsorbed isotopic tracer appearing in stools after administration of the label in an appropriate manner. We have referred to this as the "fecal isotope balance" procedure. This method is applicable only if absorption is reasonably high (at least 10%). For iron, absorption of non-heme iron could be substantially lower. Under these conditions, "fecal isotope balance" is not applicable to this problem.

The second approach is based on the appearance of the ingested isotopic label in an appropriate compartment. The accepted approach to the measurement of iron absorption is the "hemoglobin incorporation"

Table 10 Accuracy of *in vitro* stable isotope dilution

Matrix	Spike	Measured isotope	Elemental concentration (μg/g or ml)	Comparison[a] (μg/g or ml)
Serum	^{6}Li	^{7}Li	24.5 ± 0.3	25.0 (added)
	^{82}Se	^{77}Se	0.098 ± 0.001	0.135 ± 0.008 (HG/AA-S)
Urine	^{6}Li	^{7}Li	25.1 ± 0.3	25.0 (added)
	^{82}Se	^{77}Se	0.0473 ± 0.0003	0.0489 ± 0.0003 (HG/AA-S)
SRM 1577a (bovine liver)	^{57}Fe	^{54}Fe	192 ± 2	194 ± 20
	^{65}Cu	^{63}Cu	156 ± 2	158 ± 7
	^{67}Zn	^{68}Zn	120 ± 1	123 ± 8
	^{82}Se	^{77}Se	0.697 ± 0.002	0.69 ± 0.01
	^{26}Mg	^{24}Mg	617 ± 4	600 ± 15

[a]Natural Li added to sample, Li content prior to this addition was undetectable; HG/AA-S, hydride generation/atomic absorption spectrophotometry; all comparison data for SRM 1577a are certified values.

Table 11 Extent of ^{58}Fe enrichment in infant's blood following administration of a single oral dose of ^{58}Fe-ferrous sulfate[a]

Subject	Weight ratio of $^{58}Fe/^{57}Fe$ on day					Increase[b] (%)
	−42	−14	+14	+42	+70	
3166	0.1473	0.1475	0.1898	0.1893	0.1856	28
3343	0.1440	0.1455	0.1686	0.1686	0.1736	18
3378	0.1418	—	0.1547	0.1607	0.1619	12
3382	0.1458	0.1468	0.1573	0.1574	0.1570	7.5
3384	0.1432	0.1434	0.1811	0.1793	0.1778	25
Mean ± 1SD	0.1444 ± 0.0022	0.1458 ± 0.0018				

[a]A single dose (1.44 mg ^{58}Fe, 1.95 mg Fe) was administered with 84 mg sodium ascorbate, 400 mg sucrose, and one drop of cherry flavoring 2 hours away from either feeding. Infants' age was 126 days on day of isotope administration (day 0).
[b]Mean percent increase for the three postdosing samples, compared with the mean of days −42 and −14.

method, as applied extensively to studies with adult subjects.[21] This method involves administration of a suitable dose of radioactive iron and the determination of the extent of incorporation in the circulating hemoglobin 14 days after its administration. From the knowledge of appearance of radioactivity in blood, blood volume of the subject, and the assumption that a constant fraction of absorbed radioactivity was incorporated into the circulating red blood cells at 14 days,[21] its absorption is estimated.

The initial issue in relation to whether or not such an approach can be taken with stable isotopes of iron is the question of the degree of isotope enrichment that can be achieved after a physiologically relevant dose of the stable isotope ^{58}Fe has been administered to the infant.[10] An example is presented to illustrate this important point. Assume that a 4-month-old infant weighing 7 kg has a blood volume of 65 ml/kg and a hemoglobin concentration of 11 g/dl. This infant's circulating hemoglobin mass will be 50 g, the circulating iron mass will be 173.5 mg, and the circulating mass of ^{58}Fe will be 0.559 mg. If 1 mg of ^{58}Fe is administered, 10% of the dose is absorbed, and all of the absorbed dose is incorporated into circulating erythrocytes, the resultant isotope enrichment will be 17.9% above the baseline value of 0.1475. Data on the achieved level of isotopic enrichment for a number of infants have been summarized in *Table 11*. More extensive data have been given previously.[33]

Data given in *Table 11* were obtained for infants who received a single dose of ^{58}Fe-ferrous sulfate (1.44 mg ^{58}Fe, 1.95 mg Fe) between feedings at the age of 126 days.[33] The data show that pursuant to this protocol, circulating hemoglobin was enriched to the extent of 7.5% to 28% over the baseline ratio. Comparing this level of enrichment with the theoretic calculations of feasibility[10,33] shows that under the conditions of the protocol reported here, this method should provide data with a level of uncertainty due to the analytic measurement method within the 5% to 20% range. In other words, an estimated value of 10% absorption has an analytic component of uncertainty of approximately 0.1 (10% ± 1%). Therefore, the method should be able to distinguish clearly between iron absorption of 10% and either 8% or 12% (± 2% SD).

The data also indicate that while the present measurement precision is sufficient to permit studies of absorption around 10%, the current methods do not permit evaluation of iron absorption when it is much less than 5% with a high degree of accuracy, or when it

is desirable to administer much less of the enriched isotope. This experience demonstrates the desirability of improving measurement precision of the method beyond its present capabilities.

Exchangeability of dietary pools of zinc. Gastrointestinal regulation constitutes a major mechanism for the maintenance of body economy of some minerals, but not others. Examples of the former are zinc, copper, and iron; an example of the latter is selenium. Two components of gastrointestinal regulation may be evoked: absorption and endogenous secretions. Many factors could potentially influence absorption. An important factor about which little reliable information is available is the relationship between the chemical form of the mineral and its absorption.

Studies related to the effect of chemical forms of minerals on absorption under the conditions reflecting the fate of endogenous dietary mineral are difficult to design due to lack of information about the specific chemical forms of the mineral that occur in natural foods. A practically feasible approach to this problem is that provided by the approach of biologic labeling of foods with a suitable isotopic tracer,[56-59] as illustrated in *Figure 6*. This approach has proven successful in the elucidation of heme/non-heme components of dietary iron absorption[21] using the radiotracer method.

In order to pursue studies of biologically labeled foods, it is necessary first to investigate whether suitable experimental protocols can be devised to label foods of interest with a sufficient degree of isotopic enrichment in order to be able to perform such studies in humans.[56-59] This is especially important when using stable isotopes, because of the potential high costs involved. A number of such feasibility experiments have now been completed with zinc and selenium, establishing that sufficient isotope enrichment can be achieved for poultry meat and zinc,[56] soybeans and zinc,[59] poultry meat and selenium,[57] and, finally, hen's egg and selenium[58] to permit the conduct of realistic human protocols. An example of the degree of isotope enrichment achieved for zinc in soybeans and chicken meat is given in *Table 12*.

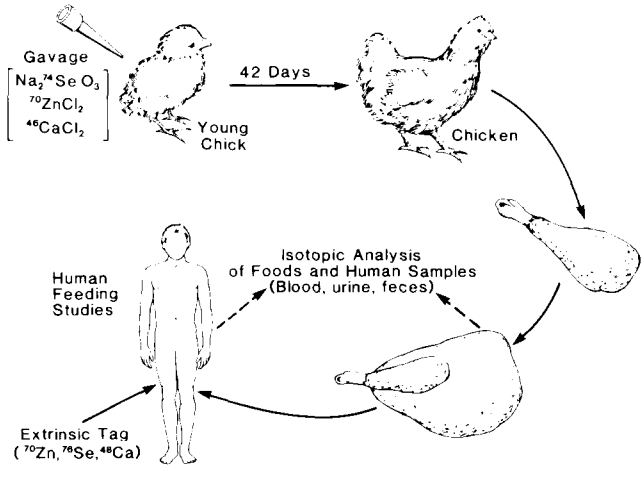

Figure 6 A general approach illustrating biologic labeling and use of human foods for studies of mineral absorption.[56-59]

Table 12 Isotopic contribution to total zinc intake of a subject consuming ^{70}Zn-labeled soybean or ^{68}Zn-labeled chicken[a]

Mixed chicken meat (300 g)	
^{68}Zn	2,200 μg
Zn	5,200 μg
Whole soybeans (100 g)	
^{70}Zn	600 μg
Zn	1,600 μg

[a] Data from refs. 56 and 59.

Table 13 Fractional absorption of extrinsic and intrinsic zinc consumed simultaneously by adults[a]

Period I	
Protein source, chicken	
Total zinc intake, 11.7 mg	
F_e	0.46 ± 0.06
F_i	0.57 ± 0.06
Ratio	0.79 ± 0.06
Period II	
Protein source, chicken/soy	
Total zinc intake, 10.1 mg	
F_e	0.46 ± 0.06
F_i	0.57 ± 0.05
Ratio	0.79 ± 0.05
Period III	
Protein source, chicken	
Total zinc intake, 6.7 mg	
F_e	0.66 ± 0.04
F_i	0.72 ± 0.04
Ratio	0.91 ± 0.03

[a] Data from ref. 44.
F_e, fractional absorption of extrinsic label; F_i, fractional absorption of intrinsic label.

These data demonstrate that when 300 g of labeled meat is given to an adult subject, it provides 5.2 mg total zinc and 2.2 mg *excess* ^{68}Zn. In comparison, when ^{70}Zn-labeled soybean is given (100 g), the contributions will be 0.6 mg *excess* ^{70}Zn and 1.6 mg total zinc. The amounts of *excess* isotope provided by these foods is sufficient to permit conduct of successful studies.[56,59]

An example of the application of biologically labeled foods is the study of comparative absorption of intrinsic/extrinsic zinc.[44] In this study, biologically labeled chicken meat (^{68}Zn) was given simultaneously with $^{70}ZnCl_2$ to healthy young adults under variable but controlled conditions of total zinc intake. Some results from this study have been summarized in *Table 13*.

The data from this experiment demonstrate that while a highly significant linear correlation is present between absorption of intrinsic and extrinsic tags of zinc,[44] the intrinsic label may be somewhat better absorbed. This would be analogous to higher absorption of heme compared with non-heme iron. However, the difference may disappear as the total amount of extrinsic zinc present in the lumen of the gut is reduced

(period I versus III, *Table 13*). Additionally, replacement of animal protein with vegetable protein may not adversely influence absorption of either label (period I versus II, *Table 13*). Regardless of the details of these interpretations, the results of this and other similar experiments demonstrate that the intriguing issues of dietary availability of minerals/trace elements can be resolved directly in human subjects with the use of safe and non-invasive methods based on non-radioactive stable isotopes.

Studies of dietary availability of minerals investigated with the stable isotope approach are now numerous. These studies have included investigations of the absorption of Zn,[43,47] Cu,[48] Fe,[33,34,49] Se,[46,50] Pb,[45] Ca,[41] and Mg[42] in a number of subject groups, including infants,[33,34,41,51] women,[50] adult men,[26,53] and the elderly.[47,49] The wide range of problems explored to date is only an indication of the major impact this method will have on such studies in the future.

Turnover studies

Stable isotopes have long been used to investigate turnover of proteins and amino acids, with ^{15}N as the isotopic probe.[60] Little has been done in this poorly understood area of mineral metabolism with stable isotopes.[26,39,61] We have recently begun an investigation of some aspects of selenium turnover and will provide a brief summary of the findings. The reader is referred to the original literature for more detail.[39,46,61]

The studies completed to date with stable isotopes of selenium have begun to indicate the complex nature of its metabolism in humans.[39,52,53,61,62] Important differences in absorption of different forms of selenium,[52,53] the body's ability to retain the absorbed dose,[52,62] and heretofore unrecognized interactions with other nutrients[61] have now been demonstrated in the human for this trace element. An example of the previously unrecognized interaction between selenium and vitamin C (ascorbic acid) is given here, to illustrate the added dimension to the experimental investigation of trace element metabolism that can be gained with the use of stable isotope tracers.[61]

Two groups of healthy adult volunteers were placed on natural foods that are low in ascorbic acid, so that their daily intake of the vitamin was determined to be 20 mg. The menu for this study depended primarily on cereals as the source of protein. As a result, daily selenium intake was substantially higher than would result from self-elected diets for the geographic area of the study (*Table 14*). One group received vitamin C supplement as two 500-mg pills that were consumed 30 minutes before breakfast and dinner (AA-S). The ascorbic acid-supplemented group then received a single dose of $^{74}SeO_3^{2-}$ on day 10 of the study and was followed for an additional 14 days in order to determine absorption and retention of the label. The ascorbic acid-restricted group (AA-R) received the same label, but on day 30 of the experiment, and was also followed for an additional 14 days.

The results given in *Table 14* show that the normal parameters of selenium balance were not different between the two groups. The two groups consumed 305 ± 41 and 294 ± 63 μg selenium/d for the AA-S and AA-R groups, respectively. Daily urine excretion for the two groups was 110 ± 8 and 117 ± 4 μg/d, respectively. Fecal excretion for the AA-R group was somewhat higher than for the AA-S group, leading to somewhat more positive balance (163 ± 9 versus 121 ± 9) for the AA-S group. Plasma concentration of selenium was not different for the two groups.[61]

In contrast to the normal indices of selenium balance, there were major differences in the metabolism of the labeled selenite between the two groups (*Table 14*). Absorption of selenite was significantly reduced in the AA-R group compared both with the AA-S group and the expected normal absorption of selenite in similar subjects.[46] The major difference between the two groups was in their ability to retain the absorbed label. The 7-day retention of the AA-R group was only 73% of that for the AA-S group. The corresponding value for 14 days was 54%, indicating a major effect of tissue ascorbic acid depletion on the retention of absorbed selenite. While the reasons for the observed differences are not clear, considerations of the known reductive pathway[63] for metabolism of selenite permit speculation that tissue ascorbic acid may be involved in this normal reduction of selenite to precursor (HSe^-) for incorporation into selenoproteins and other forms with high body retention indices. However, regardless of the actual reasons for these observations, these interactions are observable only when isotopic methods are used, clearly demonstrating the power of this approach.

Table 14 Selenium balance and isotopic parameters in relation to tissue ascorbic acid status[a]

	Selenium balance			Isotopic data		
		Excretion			Retention (% absorbed dose)	
Group	Intake	Urine (μg/d)	Feces	Absorption (% dose)	7-day	14-day
AA-S	305 ± 41	110 ± 8	32 ± 5	75 ± 0.3	60 ± 4	50 ± 4
AA-R	294 ± 63	117 ± 4	56 ± 2	50 ± 6	44 ± 6	27 ± 7

[a] From ref. 61.
AA-S, ascorbic acid supplemented; AA-R, ascorbic acid restricted.

Table 15 Isotopic enrichment in plasma of rats given a single-day dietary replacement with ^{65}Cu[a]

Time (days)	$R_{65/63}$	
	+Cu	−Cu
−7	0.461 ± 0.004	
0	0.463 ± 0.003	0.463 ± 0.002
2	0.702 ± 0.037	1.581 ± 0.070
7	0.557 ± 0.007	0.917 ± 0.044
14	0.474 ± 0.002	0.664 ± 0.014

[a] Unpublished data.

Investigations with animals

Use of stable isotopes as tracers has so far been emphasized for application to studies with human subjects. However, these approaches can also be effectively used to address metabolic issues in animals. This is especially useful with large animals for which disposal of radioactive carcasses is difficult or with small laboratory animals for which suitable radiotracer methods are not available.

An example of the feasibility of such experiments is given in *Table 15* for future studies of copper transport. In this preliminary experiment, two groups of adult rats (250 g) were placed on a copper-deficient diet and given either deionized water (−Cu) or deionized water containing 20 μg/ml copper (+Cu). On day zero of the experiment alone, drinking water was replaced for both groups with deionized water containing 20 μg/ml ^{65}Cu.

Data summarized in *Table 15* show the extent of isotopic enrichment in the plasma of the two groups. In the +Cu group, the natural isotope ratio (0.463 ± 0.003) was increased to 0.702 ± 0.037 at 48 hours, an increase of 52%. At 14 days after dosing, the plasma ratio was still elevated significantly (2.4%). In the −Cu group, the ratio increased by 240% on the second day, and was elevated 43% on day 14 after dosing. These data clearly demonstrate that even for an isotope like ^{65}Cu, whose natural abundance is relatively high, animal model protocols could prove effective in the investigation of those aspects of their metabolism which could not be addressed without the use of isotopic tracers. Similar studies have also been performed with other minerals, such as magnesium[31] and selenium.[64] The approach with stable isotopes is especially desirable when the issues of transport of the label relative to the endogenous mineral are of interest (e.g., measurement of metabolic pool sizes). Understanding of many issues of mineral metabolism requires the use of multiple-isotope feeding concepts.[64] These protocols cannot be designed with radiotracers, but can readily be designed with stable isotope approaches.[64] Thus, sophisticated experiments, focussing on such issues as comparative turnover of endogenous/exogenous forms of minerals, can now be performed. This may become known as one of the major features of this approach.

Conclusions

The substantial body of literature concerning this subject that has accumulated since the first report by Lowman and Krivit in 1963[65] attests to the considerable interest this subject has generated. The stable isotope approach now constitutes an important experimental tool for the studies of mineral metabolism. Compared with the potential breadth of its applications, the method has not yet reached its full potential. However, recent advances in the measurement methodology have paved the way for a true "quantum leap" in applications of the approach.

To date, the major focus of research has been on measurement methods and applications to gastrointestinal absorption, with a few groups focussing on post-absorptive metabolism. This area holds the key to important advances in our understanding of mineral metabolism. With the availability of the more appropriate measurement methods, this important general area could now be explored.

In-depth understanding of mineral metabolism cannot be fully gained from studies with human subjects alone. Judicious use of animal models is necessary.[64] Stable isotopes have important features, not present with radiotracers, that would permit investigations such as the metabolic turnover of endogenous minerals under various dietary conditions, including modulation of mineral intake itself.[64] Thus, stable isotopes will also prove to be important tools for studies with such systems.

Acknowledgments

The authors acknowledge the collaborative nature of some of the data given in this report and refer the reader to the original manuscripts for their specifics. They also gratefully acknowledge discussions with Dr. R. F. Burk during the course of the work.

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