Use of stable isotopes and mathematical modelling to investigate human mineral metabolism

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Individuals have varying needs for minerals that are dependent, amongst other things, on their lifestyle, age and genetic makeup. Knowledge of exact individual nutritional requirements should lead to better health, increased quality of life and reduced need for expensive medical care. Bioavailability, nutrient–gene interactions and whole-body metabolism all need to be investigated further if we are to progress towards the goal of defining optimal health and nutritional status. The discussion which follows will critically review the latest developments in the area of metabolism for several of the minerals that are essential for human health: Ca, Zn, Cu and Se. Stable-isotope tracers and mathematical modelling are some of the tools being used to facilitate the greater understanding in uptake, utilisation and excretion of these minerals. Stable isotopes, administered in physiological doses, present little or no risk to volunteers and allow metabolic studies to be carried out in vulnerable population groups such as children and pregnant women. Intrinsic labelling of foodstuffs ensures that the tracer and the native mineral will behave similarly once inside the body. Advances in computing power and software dedicated to solving nutritional problems have made it possible for investigators to use mathematical modelling in their experimental work. Mineral metabolism is ideally suited to a form of modelling known as compartmental analysis, which allows rates of mineral transfer and sizes of mineral stores to be calculated accurately without the need for invasive sampling of body tissues.

Stable isotopes: Mathematical modelling: Mineral metabolism

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Introduction

Most minerals have several naturally-occurring stable isotopes, all with a fixed abundance. In order to follow the metabolism of a mineral, it is necessary to ingest it in an altered form so that it can be distinguished from the naturally-abundant mineral. The form of the ingested mineral can be altered by enriching one of the low-abundance isotopes. This so-called 'tracer' or 'label' is injected and/or taken orally and its appearance in the plasma, urine and faeces can be monitored over several days. The concept of a tracer is important in isotope work. A true tracer is one that does not perturb the system it is investigating. Only radioisotopes can be called true tracers, since they are administered in extremely small quantities. The term 'label' is more appropriate for stable isotopes, since they are usually given in quantities equivalent to the amount received in the normal diet. The word 'tracer' is, however, still used in some stable-isotope studies with the implicit understanding that it will almost certainly perturb the system under investigation. The underlying assumption in this type of work is that the label (or tracer) behaves in the same kinetic way as the naturally-abundant mineral (sometimes called the tracee), otherwise the use of labels to mimic the behaviour of the native mineral is invalid. There must be no discrimination by the body between the two forms of the mineral. Although stable isotopes were the first tracers to be used in nutritional science (Schoenheimer & Rittenburg, 1935), radioisotopes quickly became the standard amongst researchers after the Second World War. It wasn't until the early 1960s that the first mineral study was published using an enriched stable isotope of Fe, ^{58}Fe (Lowman & Krivit, 1963). The first studies using Ca (McPherson, 1965) and Cr (Donaldson *et al*. 1968) followed shortly afterwards, and the late 1970s saw the first Zn and Cu stable-isotope studies performed (King *et al*. 1978). A combination of advances in MS and concern about exposure to ionising radiation led to a growing number of researchers switching to stable-isotope tracers. Several reviews and guides have been written subsequently (Buckley, 1988; Turnlund, 1989, 1991, 1994; Mellon & Sandstrom, 1996). The stable isotopes used in nutrition studies are normally incorporated in food either intrinsically (Janghorbani *et al*. 1981*b*; Weaver, 1985; Fox *et al*. 1991) or extrinsically (Janghorbani *et al*. 1982; Christensen *et al*. 1983; Johnson & Lykken, 1988; Weaver, 1988). An intrinsic label is one in which the stable isotope is incorporated biosynthetically into a plant or animal, whereas an extrinsic label is simply added to the food before ingestion. Nutritional researchers are often interested in knowing how much of a mineral in a food, meal or diet is utilised for normal body functions. This is known as the bioavailability of the mineral. It is not possible to measure the utilisation of minerals directly so absorption and retention are used as indirect measures instead. Stable isotopes are ideal for investigating the absorption and retention of minerals because of the ease in distinguishing the label from the native element. There are two definitions of absorption: apparent and true. Apparent absorption is the quantity of mineral that is absorbed and retained in the body within the experimental time period (usually several days) from a single test meal. True absorption is the quantity of mineral that has been absorbed from a single test meal. True absorption is, by definition, always greater than apparent absorption. Knowledge of the quantity of label given and the subsequent quantity recovered in the faeces is sufficient to calculate the apparent absorption. If the mineral is prone to large endogenous losses, a second label can be given intravenously at the same time as the oral label and both are followed in the faeces. Endogenous losses can then be accounted for and the true absorption of the mineral calculated. This is known as a dual-tracer experiment and is valid if the oral and intravenous (IV) labels behave in the same way kinetically. Dual-tracer experiments can also be used to calculate true absorption from urine and plasma samples.

Recent advances in MS have been accompanied by a massive increase in computing power and software development. This advance has allowed complicated mathematical equations to

be solved quickly. The use of mathematical modelling in nutrition has been advanced since the 1980s in conferences and workshops (Canolty & Cain, 1985; Hoover-Plow & Chandra, 1988; Abumrad, 1991; Siva Subramanian & Wastney, 1995; Coburn & Townsend, 1996; Clifford & Muller, 1998). The vast majority of the work published from those meetings was concerned with compartmental modelling. This type of modelling is especially suited to mineral metabolism, because the kinetics of such systems can be approximated by a small number of distinct compartments connected together to form a deterministic process. Important nutritional information can be provided in a well-designed kinetic study that uses stable-isotope labels in conjunction with compartmental modelling.

Before describing why models might be useful in nutrition, some definitions must be given to clarify the terminology. A compartment is a theoretical construct that may well combine material from several different physical spaces. It is defined as an amount of material that acts as though it is well mixed and kinetically homogeneous, and a compartmental model consists of a finite number of compartments with specified interconnections between them. The interconnections are a representation of the flux of material that is transported from one location to another. By defining a compartmental model in this way, the researcher can reduce a complex metabolic system into a small number of pathways and compartments. The outputs from such a model include the quantity of mineral stored in certain body pools, the rate and extent to which the mineral moves from one pool to another and how long it will remain in the body before being excreted. This output can inform the approach of the nutritionist about status, daily requirements and mineral retention, and only by doing modelling can this sort of information be obtained from human subjects in a non-invasive way. Although compartmental models may seem complicated to the non-mathematician, the following example may help clarify some of the main issues involved.

Consider the two-compartment model shown in Fig. 1, which shows the compartments and the pathways with the direction of transfer of material. Associated with each pathway is a rate constant which is conventionally written as k (i,j) which denotes transfer of material from compartment 'j' to compartment 'i' per unit time. The type of compartment models which are usually found in nutrition are described by constant-coefficient ordinary first-order differential equations. The basis of these equations is that the rate constants do not alter over the course of the experiment, and by applying the conservation of mass to the system under study one can solve the resultant series of equations. If we denote the mass of material in compartment 1 as P (for plasma) and that in compartment 2 as E (the 'rest' of the body), the system can be described as:

$$\frac{d\mathrm{P}}{d\mathrm{t}} = k_{1,2} \times \mathrm{E} - \left(k_{2,1} + k_{0,1}\right) \times \mathrm{P},$$

$$\frac{d\mathrm{E}}{d\mathrm{t}} = k_{2,1} \times \mathrm{P} - k_{1,2} \times \mathrm{E}.$$

To solve these equations, we need to sample one of the two compartments so that the variables ($k_{0,1}$, $k_{1,2}$ and $k_{2,1}$) can be fitted to the resultant data. It is often the case that the only compartment we can sample is the plasma. This limits the number of 'other' compartments we can use in our model because we will not be able to uniquely identify the variables that make up the resultant equations. This is called *a priori* identifiability and is used to identify, given noise-free data, the number of compartments a model may have in order that all its variables will have a single solution. Often investigators have an in-depth knowledge of the metabolism of a particular mineral and want to include many compartments to reflect this. Any model that is not *a priori* identifiable must be carefully evaluated and the results from it used cautiously. The fitted
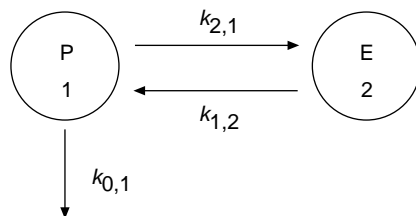
Fig. 1. Example of a two-compartment model. The two compartments are plasma (P) and the 'rest' of the body (E). ⟶, Direction of transfer of material between compartments with associated rate constant in the form k(i,j) where transfer is from j to i.

variables from a model should always be quoted with their calculated precision so that an assessment of the quality of the model can be made. This is called *a posteriori* identifiability, and plays an important part in drawing nutritional conclusions from the modelling of real data. The *a posteriori* identifiability is dependent on good quality data and the correct choice of model structure for the system under investigation. Our two-compartment model is *a priori* identifiable. If the data from the plasma samples are of sufficient quality and they support a two-compartment rather than, say, a one- or three-compartment structure then the model should be *a posteriori* identifiable. A compartment model is, therefore, constrained by the type of data collected, the location it is collected from and the statistical considerations involved in fitting experimental data to models.

Closely linked with compartmental modelling is empirical modelling. This is usually applied to data generated by multiple sampling of the plasma over a few hours after an IV label has been administered. Data are plotted semilogarithmically and then fitted to an exponential equation of the form:

$$y(t) = \sum_{i=1}^{n} A_i \exp\left(-a_i t\right),$$

where n is an integer between 1 and 4 which is determined on fitting the data, A_i and a_i are coefficients also determined on fitting. At most, only four exponential equations can be determined from plasma sampling for reasons discussed in Landaw & DiStefano (1984). Kinetic variables of interest such as transit time (the time an average molecule spends in plasma during a single transit), plasma residence time (the total time an average molecule spends in plasma before irreversible loss), the recycling number (the total number of times an average molecule recycles through plasma before irreversible loss), mean sojourn time (the total time an average molecule spends in the body, after introduction into the plasma, before irreversible loss) and the total traced mass in the system can all be computed from the fitted variables. The drawback to empirical models is that they tell you nothing about mineral absorption, endogenous losses or bioavailability. This type of modelling also makes the assumption that the mineral introduced into the plasma via an IV injection behaves in the same kinetic way as the mineral that appears in the plasma through the oral route. It cannot be assumed that a mineral introduced into the body by different methods will be transported in the plasma in the same way. If the transport mechanism is different then the clearance rate will also be different and the kinetic behaviour will not be the same. In modelling a nutritional system the oral route of label administration is preferred because this is how we consume minerals from food in our everyday lives. Due to

these considerations, compartmental models are much more widely used than empirical models in mineral research, and this is reflected in the models cited in the present review.

A comprehensive literature exists on both compartmental and empirical modelling (Atkins, 1969; Shipley & Clark, 1972; Anderson, 1983; Carson *et al*. 1983; Green & Green, 1990; Wolfe, 1992; Jacquez, 1996; Wastney *et al*. 1999) and they are both well-grounded theoretically. Dedicated software tools such as SAAMII (1997) allow non-mathematicians to set up compartmental and empirical models without the need to define the differential equations that underpin them.

The present review focuses on the metabolism of Cu, Ca, Zn and Se and cites specific examples from the literature where modelling with stable isotopes has been carried out in human volunteers.

Copper

Background

Cu is essential for man and is involved in many enzyme systems in the body (Linder, 1991). It is a difficult element to study because it only has two stable isotopes (^{63}Cu 69·2 % and ^{65}Cu 30·8 %), which means that oral and IV labels cannot be given simultaneously. Endogenous losses play a significant role in Cu metabolism (Turnlund, 1998) and are usually quantified by IV dose in conjunction with faecal monitoring (Turnlund *et al*. 1998). There is potential to measure endogenous losses by ingesting an oral isotope and examining appearance of label once all the unabsorbed isotope has been excreted. Extrapolation back to the time of isotope administration can allow a correction for endogenous losses in the collected stools to be made. Cu exists in two distinct forms in the plasma; the non-caeruloplasmin (CP) or albumin-bound fraction is the form in which the Cu appears in the plasma following a meal (Linder, 1998). Most of this form is rapidly converted to the CP form in the liver. This process creates a problem in modelling Cu metabolism because it is difficult to separate and measure accurately the quantity of Cu in the two forms (Buckley *et al*. 1996). The last Committee on Medical Aspects of Food Policy report on dietary reference values (Department of Health, 1991) stated that there were no adequate data on human Cu requirements, and they were unable to derive estimated average requirements or lower recommended nutrient intakes with sufficient confidence. Also, there is no reliable biochemical measure of Cu status, nor is it understood what factors affect Cu bioavailabilty.

Studies

In contrast to the other main minerals of nutritional interest, very few papers have been published that attempt to model Cu metabolism in man using stable isotopes, although work has been done using animal models (Buckley, 1991; Dunn *et al*. 1991). These studies were used as the basis for the human model of Scott & Turnlund (1994*a*) in which five young men took part in a 90-d intervention trial. The study was divided into three metabolic periods: adequate Cu (1·68 mg/d for 24 d), low Cu (0·785 mg/d for 42 d) and high Cu (7·53 mg/d for 24 d), with the Cu (as a CuSO$_4$ solution) added to the diet during all three metabolic periods. A solution containing 392 µg ^{65}Cu was infused on days 7, 49 and 73 of the study and an oral dose ingested on days 13 (1600 µg), 31 or 32 (1020 µg), 55 or 56 (1020 µg) and 79 (7660 µg). The model consists of five compartments, two delays and two excretion routes (Fig. 2). Only data from plasma
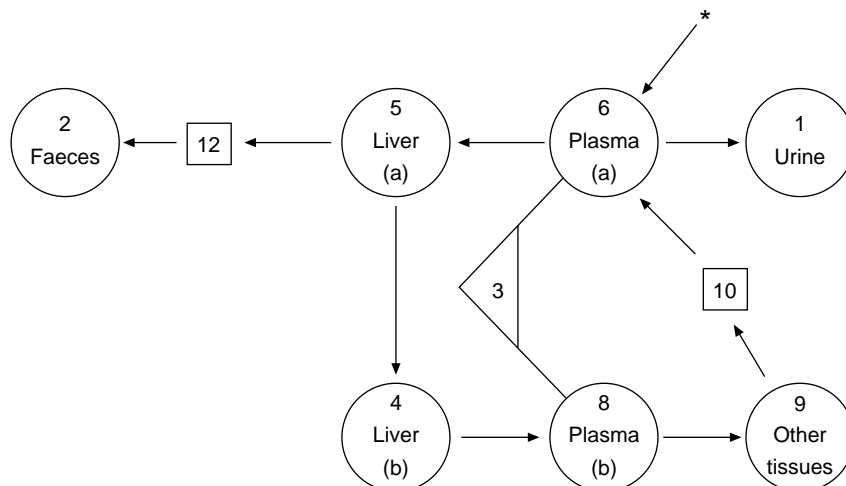
Fig. 2. Compartmental model of copper metabolism in adult men. The compartments are liver, plasma, urine, faeces and other tissues. (□), Delays; (△), sum of the indicated compartments; *, site of ^{65}Cu input; ➤, direction of transfer of material between compartments. Plasma samples were taken from compartment 3. (From Scott & Turnlund 1994a; reproduced with permission.)

samples were used to fit the rate constants in the compartment model. Although two plasma compartments were specified, no attempt was made to quantify the separate Cu-containing plasma fractions. According to the investigators, no single set of variables could be found to fit all the data, so the oral and IV data were fitted separately for each metabolic period. No theory was given as to why the different routes of tracer administration might lead to dissimilar kinetics. The model predicted that the half-life of Cu in the compartment identified with the non-CP form was approximately 40 min. This value is higher than that found in the only other two studies that have attempted to measure the half-life of non-CP-bound Cu. Buckley *et al.* (1996) reported lower half-lives (8·7 and 12·3 min) in two volunteers after IV infusion with a ^{65}Cu label. These values agreed with those of D'Addabbo *et al.* (1971) of 8·5 and 10 min for the half-life of ^{64}Cu in two subjects after IV injection.

Although Scott & Turnlund (1994a) did not include urinary data in their model, this should not influence the accuracy since it is well known (Turnlund *et al.* 1990) that urinary excretion makes up a very small percentage of the total endogenous excretion and is relatively constant. The lack of any faecal data is a drawback. It is thought that as much as 4·5 mg/d of endogenous Cu may be secreted into the gastrointestinal tract (Linder, 1998). Since the average dietary intake is approximately 1mg/d, it is evident that most of the secreted Cu is reabsorbed to maintain zero balance. Turnlund *et al.* (1998) found that about 20 % of the absorbed Cu from a meal is eventually lost in the faeces. Any model of Cu metabolism should include this excretion pathway if it is to be physiologically relevant. The Scott & Turnlund (1994a) model incorporates a biliary excretion path that predicts that about 0·15 mg/d will be excreted with moderate Cu intake (1·68 mg/d) and Turnlund *et al.* (1998) found a similar experimental value. However, a re-absorption pathway should be included in any future model to account for known physiology.

Despite the limitations of their model, the findings from Scott & Turnlund (1994a) generated important information. The total body Cu content was predicted to be between 75 and 98 mg compared with 49–118 mg reported in previous literature (Davis & Mertz, 1987; Linder, 1991). The model also predicted that 4·5 % of the total body Cu was in the plasma compared with literature estimates of 2–6 %. Of the plasma Cu, 62–72 % was estimated to be CP-bound.

This estimate is comparable with research by Wirth & Linder (1985), who found that approximately 60 % was CP-bound, whilst Owen (1982) estimated that 90 % of plasma Cu was bound to CP. The route of administration was found to influence some of the rate constants. This finding is a clear indication that the body handles oral and IV Cu differently and calls into question the use of IV tracers to quantify re-excretion of dietary Cu.

The only other stable-isotope paper that contains a compartmental model of human Cu metabolism is that by Buckley (1996). This model contains ten compartments and sixteen rate constants. The variables are derived from literature values and known human physiology of Cu. The model is used to simulate how different dietary levels of Cu might influence endogenous excretion and, ultimately, Cu levels in the liver, muscle and other tissues. The paper points out a very useful and largely overlooked use of compartmental modelling in predicting what might theoretically happen during an experiment. This information can be used to optimise the design of the subsequent study in areas such as sampling times, dose size and required precision of MS measurements.

Future work

Future work on designing models of Cu metabolism should include the collection of faecal samples, a longer period of blood sampling and the separation of CP- and non-CP-bound Cu fractions in the plasma. An extended period of blood sampling will allow the appearance of labelled CP to be fully quantified. Also, the re-absorption pathway from the gut should be included; it must be present as the body reuses most of the very large amount of Cu excreted in bile and pancreatic juices.

Care must also be exercised in the choice of route for label administration. Two possible reasons why IV Cu may be metabolised differently are: (1) the chemical form administered; (2) the rate of administration. Both these factors could cause the IV Cu to be bound and transported differently in the plasma from Cu in a meal. If it is demonstrated that IV and oral Cu behave differently, the use of IV dosing must be restricted to studies where information on parenteral feeding is required.

Studies on mineral bioavailability should be carried out using intrinsically-labelled foods in conjunction with a controlled Cu diet. This procedure has a dual purpose: first, that a physiological dose in the correct chemical form is consumed; second, the volunteers will be in a steady-state and any resultant model will relate directly to the quantity of Cu they have received over several days. In other words, transient confounding effects will be minimised.

At present, dietary intervention studies are used to investigate mineral bioavailability. Used in conjunction with a compartmental model, intervention studies could yield much more information about the homeostatic control points in the body. It should be possible to see which body stores of Cu are mobilised during a shortage of dietary Cu and, conversely, where Cu is stored and at what rate in times of plenty. Knowledge of this sort will enable more appropriate dietary recommendations to be made.

Zinc

Background

Zn is the second most abundant trace element in man and is essential for the transmission of the genetic message, cell division, cell differentiation and growth. There are more than 300

enzymes containing Zn and it is essential for the maintenance of membrane stability. The use of stable isotopes in the study of Zn metabolism in human subjects began in the early 1980s (Jackson *et al*. 1984). Several reviews (Krebs *et al*. 1995; Wastney *et al*. 2000) deal specifically with Zn metabolism, whilst others focus on status (King, 1990). The driving force behind the use of modelling in Zn research is mainly due to the lack of a good biochemical status indicator. Zn status has been estimated from the plasma Zn concentration, but this value is known to vary markedly due to stress, infection and glucocorticoids (Cousins, 1989). Although erythrocyte metallothionein has been suggested as a useful status marker (Grider *et al*. 1990, Sullivan *et al*. 1998), more studies are needed to evaluate its usefulness. Zn requirements have traditionally been estimated from the difference between Zn intake and a measurement of obligatory losses. This is another area where modelling input can be helpful in identifying rates of transfer between body pools.

Studies

There have been numerous compartmental models developed using radioisotopes, two of which (Foster *et al*. 1979; Wastney *et al*. 1986) were used as the basis for the stable-isotope model of Scott & Turnlund (1994*b*). They studied the effect of three different dietary levels of Cu on Zn metabolism in five adult men. Fig. 3 shows a schematic representation of their model. Two liver compartments, two erythrocyte compartments and five other tissue compartments were used to fit the data. It is highly unlikely that such a detailed model could be developed using stable-isotope data. The experiment was conducted over 90 d and divided into three metabolic periods
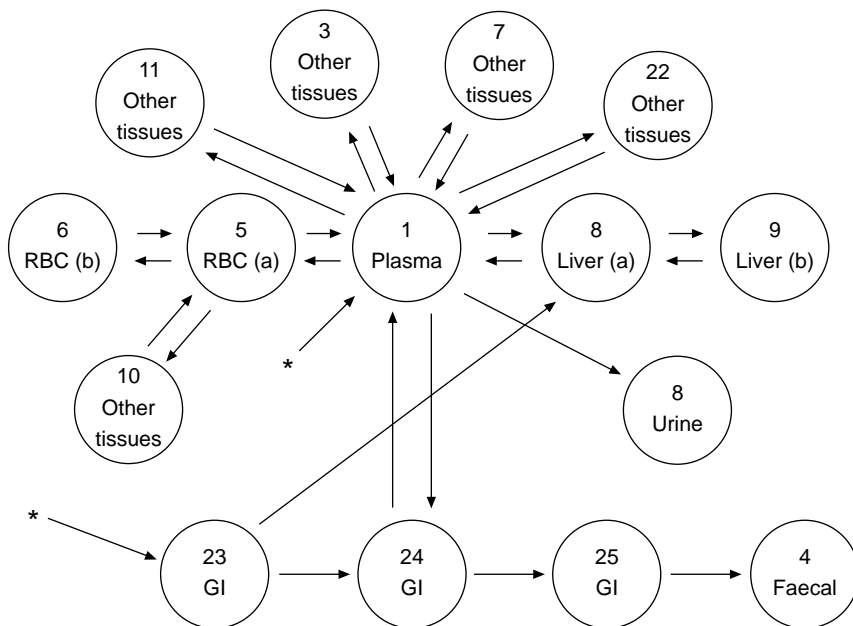

Fig. 3. Compartmental model of zinc metabolism in adult men analysed using the CONSAM program (Foster *et al*. 1989). The compartments are plasma, erythrocytes (RBC), liver, other tissues, gastrointestinal tract (GI) and faeces. *, Sites of isotope input; →, direction of transfer between compartments, a rate constant of the form $L_{(i,j)}$ (transfer from j to i) being associated with each transfer (not shown). (From Scott & Turnlund, 1994*b*; reproduced with permission.)

consisting, nominally, of medium, low and high Cu intakes. Zn intake was kept constant at approximately 11 mg/d over the study period. Oral [67]Zn was given on days 13, 31, 55 and 79. IV [70]Zn was given on days 7, 49 and 73. Blood, urine and faecal samples were collected on the study days and for several days thereafter. On fitting the data it was found that excretion into the gastrointestinal tract (rate constant L(24,1)) depended on route of administration and was lower after the IV dose than the oral dose. All other rate constants, however, showed no effect of route of administration. The main finding from the study was that only three of the rate constants were affected by the different levels of Cu in the metabolic periods. These were: L(1,24); L(12,1); L(24,23). All other rate constants were not affected by dietary Cu level and their values did not differ by more than 4 % from those found by Wastney *et al*. (1986). Compartment masses were also in agreement with those predicted by Foster *et al*. (1979) and Wastney *et al*. (1986). Total body Zn was calculated to be in the range 1800–2300 mg, of which 95 % was found in the 'other tissues' compartments. Predictions for the total Zn in the liver compartments ranged from 66 to 80 mg, and that in the erythrocytes was estimated to be about 30 mg. It should be noted that half the predicted rate constants have CV of more that 50 %. Particularly uncertain (CV in excess of 1000 %) are the predicted values for the rate of return of Zn from the other tissue compartments (3, 7 and 22) to the plasma. Since these compartments probably represent bone and skeletal muscle, an investigation of several months is needed to predict accurately the rate of return of label from these slowly-turning-over compartments. This factor indicates that a simpler model would have been more appropriate for this study. Another shortcoming, as pointed out by the authors, was that only a few blood samples were taken after administration of the oral doses. This procedure would not have allowed proper characterisation of the initial appearance of the label in the plasma. It is, therefore, quite difficult to say whether the oral and IV doses are handled in the same way by the body. In a separate study Wastney *et al*. (1991) used the same model structure as shown in Fig. 3 and found that a [70]Zn oral dose of 1·99 mg gave very similar kinetic results to an oral dose of [65]Zn (a radioisotope) given at the same time. This finding is important, since one of the criticisms of stable-isotope research is that the dose sizes are too large and therefore they do not act as true tracers.

A subsequent stable-isotope study by Lowe *et al*. (1997) used a much simpler model (Fig. 4). Only six compartments are estimated from the stable-isotope labels, although a seventh is included (compartment 7) because it was calculated by another method. Six healthy women were recruited and placed on a constant Zn diet (7 mg/d) for 7 d before the study day and 7 d subsequent to it. Label administration was a simultaneous oral ([67]Zn) and IV ([70]Zn) dose followed by blood, urine and faecal sampling for up to 11 d afterwards. The model was developed on the basis of known physiology and the minimum number of compartments required to adequately fit the data. The resulting model was *a priori* identifiable according to the GLOBI program (Saccomani *et al*. 1994). This means that all the variables had a single possible solution. It was also *a posteriori* identifiable, meaning that all variables were estimated with good precision from the experimental data. Compartments 2, 3 and 7 have not been named explicitly, but the authors speculate that compartments 2 and 3 are some combination of liver, erythrocytes and kidney. The mass of compartment 7 was estimated by assuming it contained Zn stored in the rest of the body (skeletal muscle and bone). This value could not be calculated from the stable isotope data because the label was not returned from it within the 7 d study period. Previous radioisotope studies (Foster *et al*. 1979; Wastney *et al*. 1986) have estimated skeletal muscle and bone to contain approximately 95 % of the body Zn. Griffin *et al*. (2000) compared results from this model with those from the same model but with an added erythrocyte compartment that exchanges with the plasma compartment. Body-weight-corrected Zn pool masses were found to be significantly greater ($P < 0.05$) in children than in adults.
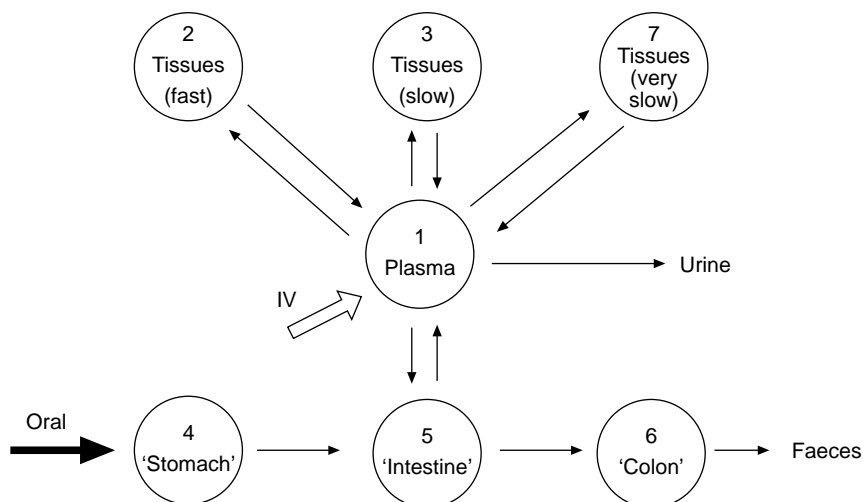
Fig. 4. 'Tracer' compartmental model. Although compartment 7 (Tissues (very slow)) is indicated, the rate constant L(1,7) and compartment mass (M_7) could not be resolved from the data and were estimated by other means (see p. 303). →, Direction of transfer between compartments; IV, intravenous. (From Lowe *et al.* 1997; reproduced with permission.)

The most recent multi-compartmental model is that developed by Miller *et al.* (1998) using two oral labels (^{67}Zn, ^{68}Zn) and one IV (^{70}Zn) label. Blood, urine and faeces were collected and a constant daily diet was adhered to over the course of the experiment. As shown in Fig. 5, fourteen compartments were identified as contributing to Zn metabolism over the 9 d study period. This structure was not shown to be *a priori* identifiable, but was shown to be *a posterori* identifiable. As well as the tracer data, use was made of information from the published literature on tracee excretion, absorption, circulation and storage. A large part of the paper is taken up with an in-depth explanation of the rationale behind the model. One volunteer's data are thoroughly examined and the stages through which the experimenter must go in order to fit a model to the data are examined in detail. The compartments that are included as part of the rapidly-exchanging Zn pool (EZP) are indicated in Fig. 5. The criterion for inclusion was defined as any compartment with IV label enrichment equal to that in the plasma (± 25 %) by 72 hours after label administration. This readily exchangeable zinc accounts for only about 5 % of the total body Zn but is thought to be involved in the known physiological functions of the element. An estimate of the EZP for subject 1, based on the compartmental model, was 141 mg. An alternative method (Miller *et al.* 1994), which uses a measure of the plasma or urine enrichment, calculated the EZP to be 180 mg, an overestimation of about 28 %.

The EZP can, as mentioned earlier, be calculated without the need for a full compartmental model. This was first shown by Miller *et al.* (1994) who demonstrated that plasma or urine enrichment data could be used to estimate the EZP. By assuming that the pools that make up the EZP can be combined (after 48 h) into one 'EZP compartment', a single exponential function can be fitted to either plasma or urine enrichment data collected 2 d after IV or oral administration. An additional requirement with oral enrichment data is that the absorption of the dose must be known. Enrichment in this case is defined as:

$$\text{EZP enrichment} = \text{mass of isotope label in EZP/total EZP mass.}$$

Fig. 5. Compartmental model of zinc metabolism. The colon represents a delay element. *, Compartments included in the exchangeable Zn pool (EZP); →, direction of transfer between compartments; RBC, erythrocytes; exch, exchanging; intest, intestine. (From Miller *et al.* 1998; reproduced with permission.)

By plotting the natural logarithm ($\log_e$) of the EZP enrichment *v.* time (for data collected 2 d after oral dosing), a straight line is produced which is an indication that labelled Zn is being cleared out of a single compartment (the EZP). By extrapolating the straight line back to the time of label administration, the size (mass) of the EZP can be calculated. Several assumptions are made in this type of modelling: (1) the loss of isotopic label from the EZP occurs at a mono-exponential rate from the time of label administration; (2) the label is homogeneously mixed with the EZP during the measurement period. These two assumptions lead to an inherent overestimation of EZP. Miller *et al.* (1994) found this overestimation to be about 24 % when they fitted their data to a compartmental model (Wastney *et al.* 1986). The subsequent paper (Miller *et al.* 1998) found an overestimation of about 28 %. If this error is consistent then it is not an impediment to the use of this type of calculation for predicting the EZP size. The method has been used by several investigators (Sian *et al.* 1996; Watson *et al.* 1999). Other workers have also calculated EZP size using compartmental modelling (Fairweather-Tait *et al.* 1993; Lowe *et al.* 1993).

Future work

Future work would benefit from a study design incorporating a controlled diet before and during the experimental period. Studies using intrinsic labels of Zn should also be encouraged. There is no definitive evidence that oral and IV Zn are handled in the same way by the body. The effect of size of IV dose and rate of infusion should be examined in relation to the subsequent appearance in plasma, urine and faeces. There is also some evidence that the size of the

oral dose affects the quantity that is absorbed due to competition with endogenous Zn (Sian *et al*. 1993). Care must be taken in study design to minimise the quantity of oral dose if it is to be given in a meal.

There have been few studies involving pregnant or lactating women. There is an increased need for Zn during these periods, and the results from one study led the researchers to suggest that the extra Zn comes from bone resorption of the mother (Moser-Veillon, 1995). Unfortunately, this study assumed that all the Zn in the skeleton was available for resorption. In fact, only the trabecular bone can be broken down rapidly and this accounts for just 20 % of the overall skeleton. Estimates of bone resorption, using stable isotopes, are prone to large error so future pregnancy and/or lactation studies must be well planned.

Calcium

Background

Ca is a mineral with a long history of metabolic studies using radio- and stable-isotope tracers. This is hardly surprising given its important role in human physiology in areas such as cell signalling and bone health. Almost 99 % of the Ca in human subjects resides in the skeleton, where bone is a dynamic part of the body and is in constant flux. Ca homeostasis must be closely regulated because of the role of Ca in cell signalling. Thus, when the body is in need of Ca, bone is broken down and the Ca released into the circulation. Between the ages of 20 and 40 years, bone loss is approximately equal to bone growth, so that net bone remains constant. The situation is somewhat different for post-menopausal women. Bone is lost at an increasing rate and may therefore result in osteoporosis. Bone density measurements and bone biomarkers cannot detect subtle changes in bone dynamics that might indicate the onset of osteoporosis (Marcus *et al*. 1999). Bone biomarkers are subject to high variability and are not a sensitive index of changes in Ca metabolism resulting from nutritional factors. Stable-isotope tracers and mathematical modelling can provide an accurate picture of Ca kinetics and can give some estimation of bone turnover. The double-isotope technique is useful for Ca absorption studies since the oral and IV forms of Ca have been shown to follow similar kinetics once in systemic circulation. When this technique is used in conjunction with balance studies, a wide range of Ca kinetics and body pool sizes can be determined. Precise blood sampling and complete urine and faecal collections need to be made and samples analysed for the Ca from the naturally-abundant source (both endogenous and dietary) and the oral and IV labels.

Studies

A recent paper by Wastney *et al*. (1996) examined the differences in Ca kinetics between adolescent girls and young women. Healthy adolescent (*n* 14) and adult (*n* 11) subjects were given a controlled diet with Ca intakes of approximately 1300 mg/d for 7 d before receiving oral (^{44}Ca, 98·5 % enrichment) and IV (^{42}Ca, 93·6 % enrichment) doses. Blood was sampled at frequent intervals for the next few hours and then daily for 14 d. All urine and faeces were also collected for 2 weeks. The model is shown in Fig. 6. Compartment 1 contains the vascular space and some of the extracellular fluid, compartment 2 is soft tissue and compartment 3 contains exchangeable Ca in bone. The rate constants between the three compartments were derived using the IV plasma data. One possible limitation of this work is that the initial volume

of distribution in compartment 1 could not be well determined and was fixed at ten, one and five times vascular space in various volunteers. Neer *et al.* (1967) found that the size of the sampled compartment (compartment 1) was approximately four times the vascular space. They speculated that this factor was due to Ca penetration of capillary walls and/or adsorption to endothelial surfaces.

The rate of endogenous faecal excretion (V_f) was calculated from the cumulative appearance of the IV dose in the faeces. The rate of urinary excretion (V_u) and dietary faecal excretion (V_F) were both calculated from the appearance of naturally-abundant Ca in the urine and faeces respectively. Knowledge of V_u and V_F, along with the daily intake of Ca (V_i) permits the calculation of Ca balance ($\Delta = V_i - (V_F + V_u)$). The rate of bone deposition of the IV label can be estimated by fitting the IV plasma data to this model. The rate of bone deposition of the naturally-occurring Ca (V_{0+}) can then be calculated from knowledge of the mass of Ca in compartment 3. The rate of bone resorption (V_{0-}) cannot be measured directly from stable-isotope data, since it takes several months for dietary Ca laid down in the bone to be returned to the plasma. Instead, V_{0-} must be calculated by difference ($\Delta = V_{0+} - V_{0-}$). Calculations of this type indicate that, in comparison with adult women, adolescent girls resorb more Ca from their skeleton (1177 *v.* 542 mg/d), deposit more Ca in their bone (1459 *v.* 501 mg/d) and therefore retain more Ca (282 *v.* –41 mg/d). A loss of Ca of 41 mg/d, as estimated for women, would eventually lead to detrimental structural changes in the skeleton and increase the risk of fracture in later life. It is essential that the error or uncertainty is quoted with such calculations so that other investigators can evaluate the precision of the data.

A structurally-identical model to that shown in Fig. 6 was employed by Smith *et al.* (1996) to calculate Ca kinetics. Based on a similar sampling protocol to that of Wastney *et al.* (1996), but with the addition of saliva sampling and much smaller dose sizes, the study only used two volunteers (one male, one female) and did not include a controlled diet, either before or during the study. Balance could not, therefore, be guaranteed, and all results were based on an estimated
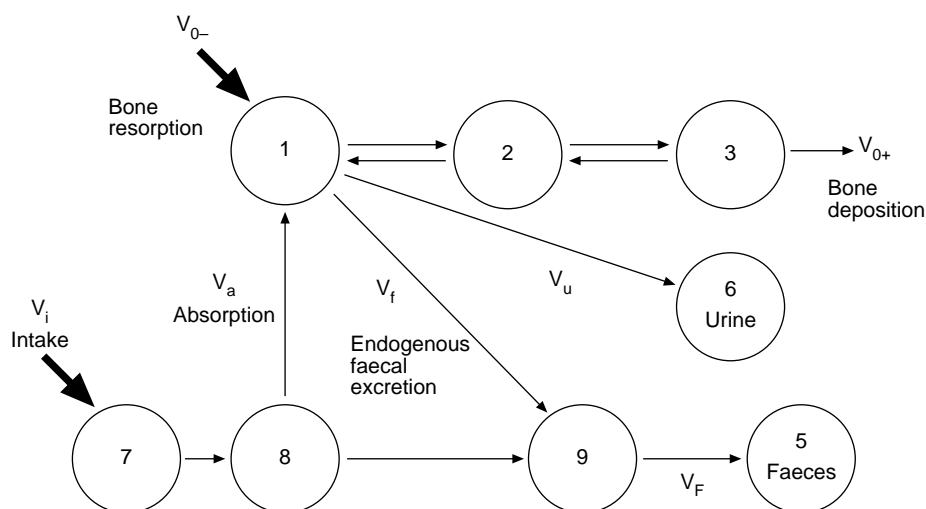

Fig. 6. Model for calcium metabolism in women. The notation of the transport rates are after that of Aubert *et al.* (1963). The compartments are shown together with their compartment number. →, Movement between compartments; ➤ entry of Ca via the diet (V_i) or bone resorption (V_{0-}); V_a, rate of Ca absorption; V_{0+}, deposition in bone; V_u, urine excretion; V_f, endogenous faecal excretion; V_F, faecal excretion. (From Wastney *et al.* 1996; reproduced with permission.)

daily Ca intake for both volunteers. It was shown that the small doses still gave measurable enrichment using high-precision thermal ionisation MS. This led to the conclusion that saliva samples could be used alongside blood samples to calculate Ca kinetics. Again, because no error or uncertainties were quoted in the paper, the use of smaller doses cannot be properly evaluated.

Similar techniques have been used by Abrams *et al.* (1992, 1994). The later study (Abrams, 1994) found a very high bone Ca deposition rate (160 mg/kg per d) amongst thirteen very-low-birth-weight infants fed a high-Ca diet. The earlier paper (Abrams, 1992) used an empirical model of Ca kinetics, in conjunction with urine and plasma data, to derive an equation containing three exponential terms.

The total exchangeable pool size and the rate of bone absorption (V_{0+}) can be calculated from a combination of the variables of the previous equation and the rate of urinary (V_u) and endogenous faecal excretion (V_f).

Of the thirteen volunteers, ten were children (aged 10 months–14 years) and three were adult women (aged 22–33 years). The three adult volunteers had a significantly lower ($P < 0.001$) V_{0+} (10 mg/kg per d) compared with the children (81 mg/kg per d) and a significantly lower ($P < 0.001$) total exchangeable pool size (76 mg/kg) compared with the children (279 mg/kg).

Future work

^{41}Ca is naturally present in minute quantities in nature (approximately 10^{-15} of all Ca is ^{41}Ca). It has a half-life of 104 000 years and so is not a true stable isotope. Futhermore, it is not a radioisotope, but instead is defined as a semi-stable or cosmogenic isotope. However, it can be used as a safe tracer in human subjects because its radiation risk is extremely low. The drawback to using it is that the resulting human samples must be analysed on an accelerator MS, which are not found in many centres in the world, and the cost per sample analysis is relatively high. Subjects need to consume the ^{41}Ca label a few months before any intervention study is contemplated so as to allow the ^{41}Ca to exchange completely with trabecular and cortical bone. Several papers have been published in this area (Elmore *et al.* 1990; Freeman *et al.* 1995, 1997, 2000). The basic premise is that the rate of bone resorption can be measured from urine samples approximately 100 d after a ^{41}Ca dose. By this time the ^{41}Ca urine concentration will be declining very slowly with time. This indicates that all the ^{41}Ca is coming exclusively from the skeleton. A dietary intervention at this point will reveal whether the ^{41}Ca concentration changes and therefore indicates if the intervention increases or decreases bone resorption. This technique obviously has potential for pharmaceutical companies manufacturing 'anti-osteoporosis' drugs, but is particularly appropriate as a tool for investigating dietary interventions where bone biomarkers are not sensitive enough and need to be monitored for lengthy periods. Another benefit of using ^{41}Ca is that all volunteers are labelled for life. A study with a combination of a stable-isotope dose, a ^{41}Ca tracer and a controlled diet should be able to answer many of the interesting kinetic questions about Ca.

Selenium

Background

Se has six naturally-occurring stable isotopes and exists in food as both organic and inorganic forms. The predominant dietary form is reported (Reilly, 1996) to be selenomethionine

(SeMet), which is organic and is readily absorbed, but inorganic Se is also well absorbed. Retention of Se is higher when the element is introduced as SeMet rather than as inorganic selenite (Milner, 1990) and this difference has been found to be related to the incorporation of SeMet into non-specific cellular proteins (Sunde, 1990). Its importance in human nutrition has grown markedly since the discovery that Se is a vital component of glutathione peroxidase (Rotruck *et al*. 1973). Stable isotopes of Se have been used since the early 1980s (Janghorbani *et al*. 1981*a*), and much of the subsequent work has sought to ascertain if a good status indicator of biologically-active Se can be found. There are three selenocompounds in the plasma (Deagen *et al*. 1993), but none of them can be used as status indicators because they either contain SeMet (albumin-bound Se) or are saturable over the nutritional range of intake (glutathione peroxidase, selenoprotein P). SeMet is not thought to be biologically active since much of it is stored in body proteins and its release into the metabolically-active pool will be slow.

Studies

The first use of stable isotopes of Se in conjunction with mathematical modelling was reported by Janghorbani *et al*. (1984). They calculated the apparent body pool size in a group of volunteers who were given a 200 µg oral dose of ^{74}Se as selenite. All faeces and urine were collected for 45 d, thereby allowing body retention of the labelled dose to be calculated during the study period. Body retention over time was found to fit a double exponential equation that yielded half-lives of approximately 2 and 162 d for the two body pools. The authors proposed that the first pool (half-life 2 d) might relate to the Se kinetics of plasma and liver and the second pool (half-life 162 d) may be Se stored in the deeper pools such as the muscle and bone. The apparent body pool size was calculated to be approximately 10 mg, which corresponded well with autopsy data (Schroeder *et al*. 1970) where whole-body Se was estimated to be in the order of 14·6 mg in a similar population group.

In a more recent paper Martin *et al*. (1988) renamed the apparent body size pool to be the selenite-exchangeable metabolic pool. Janghorbani *et al*. (1990) suggest that there are predominantly two pools of Se in the body, SeMet and selenite. The SeMet-containing pool acts as a storage site for Se whereas the selenite-exchangeable metabolic pool carries out all the physiological functions. They have proposed that the selenite-exchangeable metabolic pool be used as an indicator of Se status, since this pool is present in the whole body and not just the plasma.

The first compartmental model of Se developed using stable isotopes was that of Patterson *et al*. (1989). They fed 200 µg enriched ^{74}Se as selenite and collected blood, urine and faeces for 12 d thereafter. They developed a complicated model that included four plasma pools, a liver and pancreas pool and a tissue pool (Fig. 7). Multiple plasma pools were thought necessary since more than one peak was seen in volunteers' plasma and urine data. The model has eighteen rate constants and is *a priori* unidentifiable. No information is given as to the uncertainty of the rate constants, so it is not possible to speculate as to whether it is *a posteriori* identifiable. However, some interesting findings emerge from the model. The mean residence times (the average amount of time a molecule spends in a pool or system before irreversibly leaving) in the four plasma pools are 0·02, 0·18, 0·74 and 9·6 d respectively. The authors speculate that plasma pool 1 could either be newly-absorbed Se that has not been removed by the liver in the first-pass or a rapidly-turning-over pool in the liver. It is suggested that pool 2 may be material absorbed from the enterocyte into the lymphatic system and transported in chylomicrons or VLDL. The kinetics of pool 3 indicates that the Se found here is probably contained in glutathione peroxidase and/or selenoprotein P. The Se in pool 4 comes from the large tissue pools. The mean residence time of
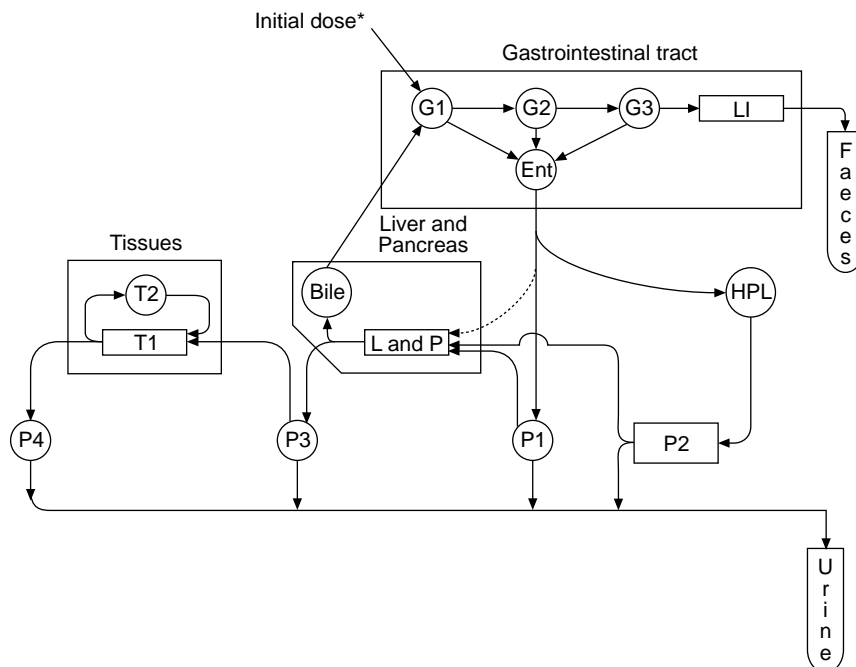
Fig. 7. Model for selenite metabolism. *→, Site of oral tracer administration; →, movement of selenium; (▢), delays; G1, G2, G3, gut compartments; ENT, enterocytes; HPL a compartment in the hepato-pancreatic subsystem or lymphatic system; L and P, liver and pancreas; LI, large intestine; T1, T2, peripheral tissues, (largely skeletal muscle); P1–P4, four kinetically-distinct plasma compartments. (From Patterson *et al.* 1989; reproduced with permission.)

the liver–pancreas subsystem was 20 d and that of the tissues was 221 d. The development of this model is examined in a paper (Patterson & Zech, 1992) given as part of a symposium on the application of models to the determination of nutrient requirements.

A study by Ducros *et al.* (1997) gave an oral dose (100 µg) of labelled selenite (^{74}Se, 98·2 % enriched) to three groups of women. All the volunteers lived in the same area and were divided into free-living elderly (aged 64–82 years), institutionalised elderly (aged 68–82 years) and a comparison group of young women aged 31–40 years. The aim of the study was to investigate whether age and/or lifestyle influenced Se exchangeable pool size. Blood samples were taken periodically for 6 months and a two-compartment model was found to fit the resulting data. Compartment 1 had a mean pool size that ranged from 619 µg for the institutionalised elderly to 835 µg for the young adult. This first pool is approximately three times larger than the plasma pool (native Se plasma concentration multiplied by plasma volume) and has a half-life of approximately 1 d. Compartment 2 had a mean pool size range of 1767 µg for the institutionalised elderly and 2473 µg for the young adult. The average half-life is approximately 30 d. Compartment 1 has similar kinetics to the third plasma pool identified by Patterson *et al.* (1989). As blood samples were only taken from 1 h post-dose, no pools could be identified that turn over in less than 2 h. The second compartment has a much shorter turnover time than the tissue compartment identified by Patterson *et al.* (1989). The authors suggest that this compartment may represent label, with a slow turnover, released from the peripheral tissues. They conclude that ageing has no effect on Se status.

A SeMet model was published by Swanson *et al*. (1991) and was based on six adults consuming an oral dose of 200 µg [74]Se as L-SeMet. It was developed from the selenite model of Patterson *et al*. (1989) but contains some differences. A second tissue pool was connected between the third and fourth plasma pools. A pathway to the liver and pancreas system from the fourth plasma pool was also required and the first pass effect was much greater in comparison with the selenite model. The turnover times of both models are given in Table 1.

Table 1 shows that organic Se is metabolised quite differently from inorganic Se. A major difference is that whole-body turnover is much slower with the SeMet form of Se. This difference is attributed to re-utilisation of the organic form via the pathway from the fourth plasma pool to the liver–pancreas subsystem. This pathway does not exist in selenite metabolism and, consequently, the whole-body turnover time is faster. This is in contrast to the major storage areas of the body, where we find that the turnover of SeMet is much faster than that of selenite. In a paper comparing the two models, Patterson *et al*. (1993) conclude that the ability to redistribute Se is beneficial if the body's requirements suddenly change. They also comment, however, that at high dietary intakes, re-utilisation of SeMet could result in excessive tissue accumulation and toxicity.

Future work

The extent to which these models may be related to the real world has been limited due to a lack of studies incorporating intrinsic labels in the test meals. Even when intrinsic labels have been used, no speciation work has been carried out to determine what proportions of the different forms of Se are present in the food. The use of Se exchangeable pool size, as a measure of status, merits further investigation through an intervention study. This would help determine whether changes in dietary intake were proportionately reflected in changes in the rapidly-exchangeable pools.

Conclusions

In conjunction with stable isotopes, mathematical modelling allows the nutritional investigator to probe the human metabolism of most minerals. Body pool sizes, kinetics and residence times can all be calculated from enriched plasma, urine and faecal samples to build up a picture of the dietary fate of a mineral. As improvements are made in MS technology, smaller doses will be required to investigate the system, which should reduce the chances that the steady-state is perturbed. If bioavailability is an issue then intrinsic labels, although more expensive, must be

Table 1. Comparison of turnover times for models of selenite (Patterson *et al*. 1989) and selenomethionine (Swanson *et al*. 1991)

(Mean values and standard deviations)

	Turnover time (d)			
	Selenite		Selenomethionine	
	Mean	SE	Mean	SE
Plasma	2·0	0·1	0·6	0·1
Liver and pancreas	20	5	2·4	0·3
Tissues	221	33	73	3
Whole body	147	13	363	21

more widely used in the future, since the form in which they enter the body will be the same as that of the native mineral. The use of the IV route of administration must be examined critically for indications that the label is not handled in the same way by the body as oral doses. Of the four minerals discussed in the present article, only studies involving Ca can really claim to have been fully validated using both oral and IV tracers. Until proven otherwise, the use of IV with other minerals must be viewed with some caution.

Although the discussion focused on Ca, Cu, Se and Zn, the techniques described are broadly applicable to all the other minerals, provided they have at least two stable isotopes. Fe is the only major mineral where compartmental modelling has had no real impact. This is for two reasons. First, questions about bioavailability and requirements can be answered without modelling. Second, serum ferritin measurements are a good status indicator and therefore pool sizes are not needed. Essential nutrients such as β-carotene and folate have been labelled with stable isotopes and fed to human subjects (Burri & Park, 1998; Clifford *et al*. 1998) and this area is likely to be one of increasing expansion.

More biologists should be encouraged to use modelling in their nutritional work. The Internet can be a source for encouragement and improvement, with many models already existing in cyberspace (Wastney *et al*. 1998).

Although stable isotopes are expensive to buy and exacting to analyse, they are the only genuinely safe way to investigate human metabolism. Compartmental modelling has been used in many scientific fields with distinction and is based on rigorous mathematical theory. The combination of the two approaches has yielded some very exciting research during the past 20 years and, it is hoped, will continue to do so for some years to come.

Acknowledgements

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Digestive Physiology of Pigs

Edited by **J E Lindberg** and **B Ogle**,
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Readership: Lecturers, researchers and students in animal nutrition and animal physiology.

This book brings together edited and revised papers presented at the 8th Symposium on Digestive Physiology in Pigs held in Uppsala in June, 2000. It contains more than 100 papers from leading scientists from around the world in this subject area.

Among other features it contributes to the development of the the science relating to the effects of nutrition on gut physiology. It also creates a platform for future research, that will increase knowledge of how to optimize the nutrition of the pig and to help prevent diet-related gastrointestinal conditions.

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- Development and function of the gastrointestinal tract
- Possible interactions between nutrition and gut function
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- Dietary and animal related factors affecting digestion and digestive secretions
- Influence of the gut microflora on the digestive processes
- Influence of nutrition on the gut microflora
- Role of the gut microflora in the prevention of disease