Uptake rate measurement of some amino acids on normal and treated yeast cells to xenobiotics using ^{14}C labelled amino acid

Nurhajati Tanudjojo, Pringgo Soedigdo, and Soekeni Soedigdo

Abstract

Benzo(α)pyrene (BP) and 7,12—dimethylbenz(α) anthracene (DMBA) are potent carcinogens for mammals, which are able to affect the normal metabolic processes. The influence both of BP and DMBA to the transport rate of individual ^{14}C labeled amino acids (^{14}C-lysine; ^{14}C-valine; ^{14}C-leucine or ^{14}C-tyrosine) in yeast Saccharomyces cerevisiae strain A3 were studied by introducing about one μCi (37 kBq) of individual ^{14}C labeled amino acid into 30 ml liquid ethanol media that contained BP (0.001% v/v) or DMBA (0.001% v/v), then followed by inoculating a known concentration of yeast suspension in such a manner to get the initial optical density (OD) of new cultures were about 0.10. Uptake rates were determined at certain intervals after inoculation, using a liquid scintillation counter. The results show that BP had the tendency to increase the uptake rate while DMBA showed a reversed effect on the use of amino acids. It was also found that tyrosine was absorbed faster than valine as well as leucine and this was different with the result reported by the former investigators.

Key words: uptake rate, amino acid, yeast xenobiotic

Introduction

Several agents that cause cancer in human beings have been studied extensively. Benzo(α)pyrene (BP) and 7,12—dimethylbenz(α)anthracene (DMBA) are polynucleated hydrocarbons that belong to potent chemical carcinogens [1, 2]. So far many studies on the physiological influences of BP and DMBA have been generally carried out in mammals. Much research has been done, but the mechanism of carcinogenesis in mammals is not clear. The effect of BP and DMBA in unicellular eucaryotes has been studied [3]. However, the results from this study could not explain clearly all the problems found in carcinogenesis.

We studied the influence of BP and DMBA on the uptake rate of some amino acids in yeast. For its growth and regeneration, uptake of amino acids by the yeast *Saccharomyses cerevisiae* from the media is proceeded by active transport. Permeases facilitate the active transport of various amino acids [4]. The uptake rate of individual amino acid from the media or wort* is different [4, 5]. Based on the uptake rates, Pierce [6–8] divided the amino acids in wort into four groups. The most readily absorbed was the A group that consists of glutamic acid, aspartic acid, asparagine, glutamine, serine, threonine, lysine, and arginine, followed by the B group that consists of valine, methionine, leucine, isoleucine, and histidine, then followed by the C group that consists of glycine, phenylalanine, tyrosine, tryptophan, alanine, and ammonia and the slowest one was D group containing only proline.

According to Weisburger et. al. [9] when carcinogens enter an organism, they are metabolized and become the ultimate carcinogens that react with DNA. The adduct could result in mistranscription in mRNA formation. Since mRNA is a protein template, this matter would further cause the mistranslation in protein synthesis [10, 11]. The last process could probably give rise to the change in the structure of either the cell membrane or the cell wall.

It is supposed that the presence of BP and DMBA in the culture media of yeast would be able to inhibit or to change the amino acid uptake pattern of yeast. We studied the uptake of some amino acids, which

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* Wort is a sweet mixture of malt and water, which, when it is chemically changed by fermentation, will become beer.

represented each A, B, and C group by yeast using a radioactive tracer method.

Materials and methods

The yeast used in this experiment was *Saccharomyses cerevisiae* strain A3 obtained from Biochemistry Subdivision, Bandung Instititute of Technology and had been adapted to grow on 1% liquid ethanol media. BP and DMBA were obtained from Fluka (Buchs, Switzerland), while the ^{14}C labeled amino acids were from the Radiochemical Center Amersham (Buckinghamshire, UK). The A, B, and C groups of amino acids each were represented by ^{14}C-lysine, ^{14}C-valine, ^{14}C-leucine, and ^{14}C-tyrosine. Picofluor from Packard (Downer Grove, Ill., USA) was used as a liquid scintillation cocktail. All the chemicals used under this experiment were analytical grade reagents.

The Beckman liquid scintillation counter (Beckman, Irvine, Calif., USA, LS 7550, No. Series 7775082) was used to count the radioactivity. Separation of yeast (from residual media) was carried out using a portable centrifuge. Inoculations and sampling out inoculates at various intervals were done in the sterile Laminair Airflow Bench (Clemco, Australia) and an Edmund Buhler shaker (Dreieich, Germany) was used to activate yeast regeneration during incubation.

Regeneration

BP treated yeast group

A sample of 0.3 cm^3 of 0.1% BP solution was put into an Erlenmeyer flask (125 ml) containing 30 ml of 1% liquid ethanol media which consisted of 1% KH_2PO_4, 1% pepton, and 1% ethanol (v/v). The volume of 10^{-2} cm^3 (~ 37 kBq or 1 μCurie) of one of the ^{14}C labeled amino acids (^{14}C-lysine) was added to the media, followed by inoculation of ethanol yeast which was taken from the stock suspension to get the initial optical density of 0.100. The culture was shaken at the speed of 180 rpm and incubated at room temperature. After 0, 5, 15, 25, 35, and 45 hours of inoculation, about 2 ml of the inoculate was taken from the culture and centrifuged to separate the yeast from the media. The supernatant was used to determine the radioactivity found in the residual media. The same treatment was done for the other ^{14}C-labeled amino acids on the 5th, 10th, and 15th regeneration.

DMBA treated yeast group

The step and the sequence of work was the same as in the BP treated yeast group. In this group DMBA replaced BP.

Counting and calculation

A 0.5 cm^3 sample of supernatant of each culture was added to the counting bottles each of which contained 4.5 cm^3 of the liquid scintillation cocktail. The mixture was shaken in order to mix it thoroughly, then counted using a liquid scintillation counter. Each sample was counted for 5 minutes and repeated 3 times. The counts obtained in counts per minute (cpm) were transformed into desintegrations per minute (dpm).

The calculation of the uptakes was done as follows:

$$A_u = \frac{A_p - A_l}{OD_l - OD_p} \cdot \frac{1}{T}$$

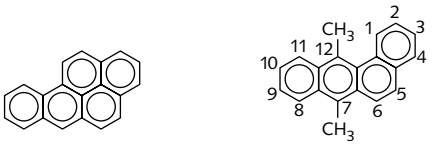
where:

A_u = radioactivity of amino acid uptake rate /0, 10 OD/hour,
A_p = radioactivity of the previous amino acid uptake rate /0, 10 OD/hour,
A_l = radioactivity of the latter amino acid uptake rate /0, 10 OD/hour,
OD_p = the previous optical density of yeast inoculate,
OD_l = the latter optical density of yeast inoculate,
T = length of time between the previous and the latter intervals.

Results and discussion

The molecular structures of BP and DMBA are shown in figure 1. Figure 2 shows the ethanol yeast regeneration curve. The lag phase was the period roughly between zero to five hours, the exponential phase was between 5 to 20 hours, 20 to 30 hours was the early stationary phase and the late stationary phase was the period over 35 hours. Samples were taken from the yeast culture following the sequence of its regeneration growth phase.

The uptake rate of the ^{14}C-lysine of the three groups of yeast is shown in figure 3. During the lag phase (0 to 5 hours of incubation), the decrease of the labeled amino acids radioactivity in the residual media was significant. At the next interval, however, they increased, while the yeast population was higher at this interval.



Benzo(α)pyrene (BP)

7,12-dimethylbenz(α)anthracene (DMBA)

FIG. 1. Molecular structure of BP and DMBA

This indicated that during the lag phase the rates of the uptake were not stable yet. It was supposed that the decreased radioactivity in the media was not simply caused by the uptake, but that there were other factors involved. The stickiness of the outer cell wall could lead to the attachment of ^{14}C-lysine molecules onto the cell wall. By further incubating while shaking, the molecules were unattached from the media, which increased the radioactivity of the media, so that at 5 to 15 hours the uptake rates were reduced.

For control and BP yeasts, the uptake rates began to appear at 5 to 15 hours and were increasing at 15 to 25 hours, while for DMBA yeast, the rate was slower. At the early stationary phase (25 to 35 hours) the uptake rate began to decrease for BP yeast up to the late stationary phase (35 to 45 hours). For control yeast, the uptake rate decreased during the late stationary phase, however, the uptake rate for the DMBA yeast increased. These phenomena could probably be related to the metabolic processes of each group of yeasts.

Similar uptake patterns were indicated for ^{14}C-valine and ^{14}C-leusine (figs. 4 and 5), however, for ^{14}C-tyrosine (fig. 6), pyramidal shapes of uptake rate were shown on the three groups of yeasts, and the peak was found at the incubating interval of 15 to 25 hours.

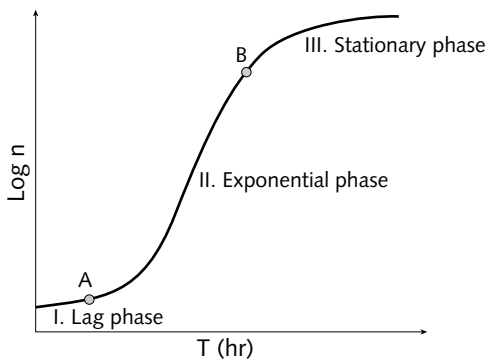


FIG. 2. Yeast regeneration curve

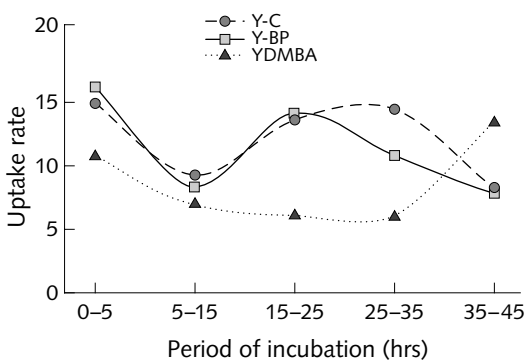


FIG. 3. Uptake rate of 14C-Lys of yeasts at various periods of incubation (dpm/0.10 OD/h)

Figure 7 shows the uptake rates of the four ^{14}C-labeled amino acids on the control yeast. Through the sum of the all intervals uptake rate it could be stated that the fastest to be absorbed was ^{14}C-lysine, then followed by ^{14}C-valine, ^{14}C-tyrosine, and ^{14}C-leucine. The same pattern was applied for BP and DMBA yeasts (figs. 8 and 9). As illustrated in the figure 3 to 9, even though the speed of each amino acid uptake fluctu-

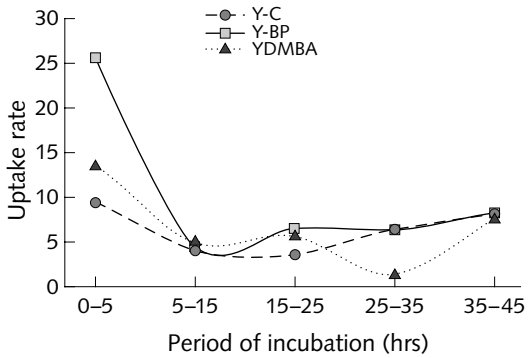


FIG. 4. Uptake rate of 14C-Val of yeasts at various periods of incubation (dpm/0.10 OD/h)

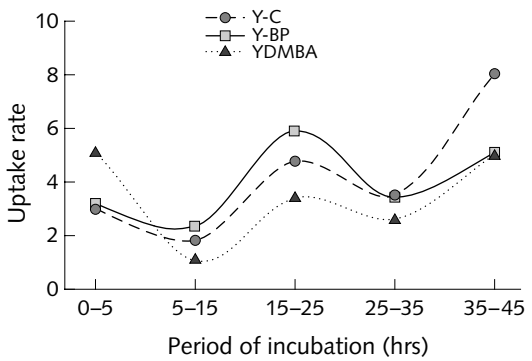


FIG. 5. Uptake rate of 14C-Leu of yeasts at various period of incubation (dpm/0.10 OD/h)

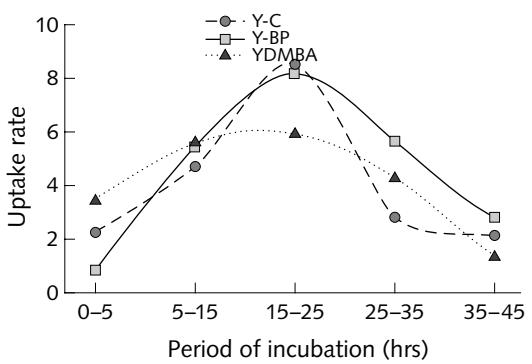


FIG. 6. Uptake rate of 14C-Tyr of yeasts at various period of incubation (dpm/0.10 OD/h)

ated along all intervals, in each group of yeast (control and treated) ^{14}C-tyrosine was absorbed faster than ^{14}C-leucine. But if the uptake rate was considered on the effective intervals of yeast growth rate (exponential up to the early stationary phases) and comparison was done beyond the uptake rate of ^{14}C-lysine, the tendency to increase the uptake of amino acids was shown by BP yeast, while DMBA yeast showed the reverse effect (fig.10). Also, ^{14}C-tyrosine was absorbed faster than ^{14}C-valine as well as ^{14}C-leucine. This phenomenon differs to that found by Pierce [7–9].

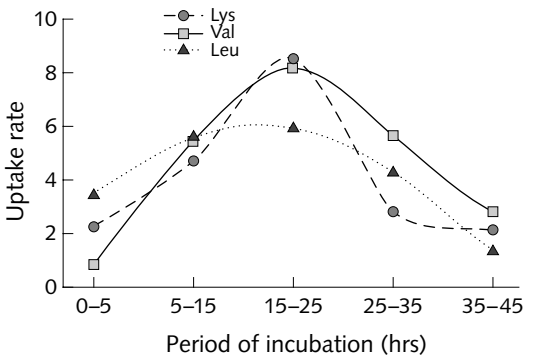


FIG. 7. Uptake rates of 14C-amino acids of control yeast at various periods of incubation (dpm/0.10 OD/h)

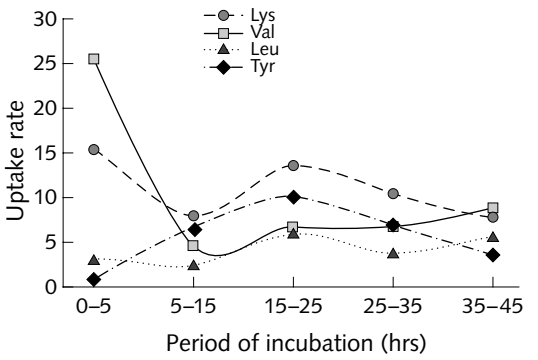


FIG. 8. Uptake rates of 14C-amino acids of treated yeast with BP at various periods of incubation (dpm/0.10 OD/h)

Conclusion

The uptake rates of amino acids by yeasts fluctuated during each generation period. The uptake of amino acids appeared consistently between the transition and early stationary phases. Tyrosine was absorbed relatively faster than valine and leucine which was different from that stated by Pierce [7–9]. It was supposed that BP and DMBA led to different effects on the cell membrane as well as the cell wall, so the uptake rate patterns for various amino acids of BP and of DMBA yeasts were different from that of the control.

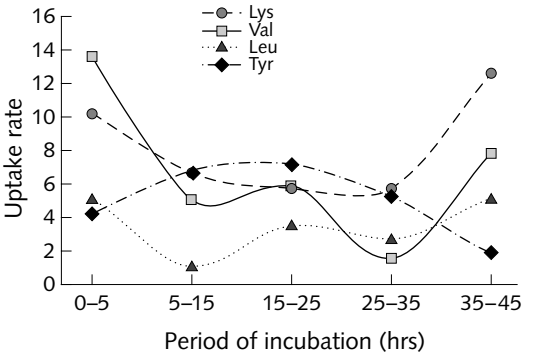


FIG. 9. Uptake rates of 14C-amino acids of treated yeast with DMBA at various periods of incubation (dpm/0.10 OD/h)

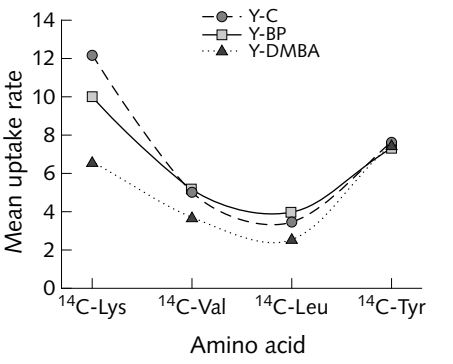


FIG. 10. Mean uptake rates of 14C-amino acids (dpm/0.10 OD/h) of normal and treated yeast to BP and DMBA during 5–35 hours of incubation

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Selenium determination in whole human blood by radiochemical neutron activation analysis: preliminary results in Argentina

Sonia Edith Hevia, María Luz Pita Martín de Portela, and Sara Manuela Resnizky

Abstract

In Argentina there are no values regarding the content selenium in food or its effect on nutritional status. Neutron activation analysis and a radiochemical separation scheme for selenium has been adjusted for blood samples from healthy adult individuals. Fasting whole blood was collected, lyophilized, packed, and irradiated in a nuclear reactor. Selenium was determined by a radiochemical separation. The method was validated using certified reference materials. The selenium concentrations (mean value ± SD) from five pools and from 22 individual samples were 0.070 ± 0.020 µg/ml and 0.071 ± 0.014 µg/ml, respectively, ranging between 0.047 to 0.105 µg/ml. These preliminary results show values close to those published for the population living in areas with adequate selenium intake, but lower values than those published for the USA population. However, it will be necessary to carry out nutritional status studies in other areas of Argentina, taking into account the geographic and topographic diversity of the country.

Key words: selenium, neutron activation analysis, human blood

Introduction

Selenium is an essential trace element in humans, with a narrow range between deficiency diseases and toxicity [1]. The marked geographic variation in concentration of selenium in soil accounts for the large variation in the selenium content in food between countries and between different areas in the same country [2]. Hence, selenium nutritional status differs according to the selenium in the food. Often, there is a significant correlation between the selenium in the food and the selenium status of humans and animals [3].

There are seleniferous areas in Venezuela, but there is lack of information from all other countries. Based on indirect studies carried out in cattle it has been postulated that selenium deficient areas could exist in Buenos Aires [4]. However, there are a few values regarding the selnium content of foods [5], but none regarding human nutritional status or the content of selenium in the soil.

Problems with methodology to determine selenium account for the lack of information on this subject. Neutron activation analysis is a useful technique for determining trace elements [6]. A radiochemical separation scheme for selenium, previously developed at our laboratory, has been adjusted for blood samples. Therefore, we applied this method to determine the level of selenium in healthy humans from Buenos Aires.

Objectives

We evaluated blood selenium levels in a group of healthy adults living in Greater Buenos Aires to obtain information regarding their selenium nutritional status.

Materials and methods

Subjects

Blood samples from healthy adults (5 whole blood pools and 22 individual samples from 5 females and 17 males), aged 18 to 40 years, were collected with EDTA and prepared for selenium determination.

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Sample preparation

Whole blood samples (2 ml) were lyophilized and weighed. Selenium standards were prepared by weighing drops of a diluted selenium solution on ash-free filter paper and air dried. The samples and standards were pelletized, packed in polyethylene bags and aluminum foil and irradiated for 50 hours at the 40 Kw RA-1 reactor (Constituyentes Atomic Center, Buenos Aires, Argentina), at an approximate thermal neutron flux of 2 10^{12} cm^{-2} seg^{1}.

After irradiation and a 2 to 3 week cooling period, the samples and standards were digested with nitric acid using a microwave oven and Parr bombs. Then sulfuric acid and selenium (1 mg) and mercury (100 mg) carriers were added, and they were heated until nitric acid was entirely removed. Finally, they were diluted to 5 N in sulfuric acid, and HgS was precipitated by additioning hydroxilamine clorhydrate (10%) and thioacetamide solution (10%). Selenium coprecipitated with HgS. The precipitates were allowed to stand overnight, then filtered and prepared for measurement using adhesive polyethylene foil.

Measurement of selenium

Selenium was determined using ^{75}Se (t ½ = 120 d) with a Hiper Pure Germanium detector (30% efficiency, resolution 1.8 keV for the 1332 ^{60} Co peak) coupled to a multichannel buffer module. The concentration was calculated using software developed at our laboratory. The method was validated using certified reference materials, horse kidney (IAEA H-8) and animal blood (IAEA A-13). The results were in accordance with the certified values (table 1).

Results and discussion

The selenium concentrations of the five whole blood pools and 22 individual samples are shown in table 2. The mean value for the five whole blood pool was not different from that of the individual data, nor was there any difference between mean values for males and females. Ranges were 0.047 to 0.105 μg/ml for males and 0.055 to 0.072 μg/ml for females.

TABLE 1. Selenium concentrations in certified reference materials

	Mean selenium concentration ± SD (ppm)	
	Experimental	Certified value
IAEA H-8 horse kidney	4.4 ± 0.2	4.67 (4.37–4.97)
IAEA A-13 animal blood	0.22 ± 0.06	0.24 (0.15–0.31)

Selenium was considered a mere environmental toxicant, but since about the mid-twentieth century it has been recognized as an essential micronutrient. While selenium is an essential nutrient, it can be toxic, therfore it is important to know the selenium status of soils, animals, and human beings [2].

There is a wide range of natural dietary selenium in different parts of the world. In South America, there are seleniferous areas in Venezuela and in the waters of the Orinoco river. There are high-selenium shales in parts of the Andes mountains accounting for some high selenium foods in Venezuela, but there are no data from other South American countries including Argentina [4]. In Chile egg yolks from five different geographic areas are being analyzed for selenium [7].

Selenium status can be assessed by measuring selenium levels in serum or whole blood or by measuring the indirect indicator activity of glutathione peroxidase. Ruksan and Zenelli prepared a map of selenium-deficient areas in Argentina, based on glutathione peroxidase levels in cattle grazing in areas where "Enteque seco" is endemic [4]. This disease is an enzootic syndrome produced by *Solanum malaecoxilum*. However, certain nutritional factors and infections can lead to a decrease in the glutathione peroxidase activity as do low selenium levels [8]. Ruksan and Zenelli also believed that there are selenium toxic areas in the provinces of Córdoba and San Luis [4]. These hypotheses were not confirmed through the determination of the selenium content in the soil.

There are no national food composition data for selenium in Argentina. Hack reported some data on the selenium content of wheat grain harvested in five areas of the Province of Buenos Aires from 1993 to 1995. These results showed a wide variation, with a mean value and ranges similar to the German wheat grain [5]. Table 3 shows the values for the provisional content of selenium in eggs and wheat in the United States [9], published values for selenium for eggs in Chile [7] and for wheat in Argentina [5].

Analysis of blood samples from individuals from different regions showed a good correlation between their intake of selenium from food and selenium levels in their blood. Whole blood selenium concentration, unlike serum selenium levels is an index of long-term selenium status and does not fluctuate from day to day.

TABLE 2. Selenium levels in the blood of the study population

	Mean values ± SD μg/ml
Whole blood pools (n = 5)	0.070 ± 0.020 (0.050–0.100)
Total group (N = 22)	0.071 ± 0.014 (0.047–0.105)
Males (n = 17)	0.072 ± 0.016 (0.047–0.105)
Females (n = 5)	0.066 ± 0.008 (0.055–0.072)

Number in brackets are ranges.

Longnecker et al. demonstrated that selenium blood levels reflect usual dietary selenium intake. They found a basal blood selenium level of 0.196 μg/ml in healthy, white males aged 22 to 64 years, with a selenium intake of 80 μg/day; after feeding them a piece of bread containing 206 μg of selenium daily for two weeks, their selenium blood level significantly increased and remained fairly constant for the 52 weeks of treatment and returned to the baseline level 12 weeks after the treatment ended [10]. Hence, selenium concentrations in whole blood may reflect usual selenium intake.

The mean values of the Argentinian group studied, those for the areas with a high incidence of Keshan's disease, and those with variable (high, low, or normal) selenium intakes are shown in figure 1 [3, 8].

From this preliminary study we concluded that this methodology will allow to determine the selenium nutritional status of humans and cattle in different parts of Argentina. Blood selenium levels in this group of Argentinian healthy adults, living in Greater Buenos Aires, were similar to those published for the population living in areas with an adequate selenium intake. However, it will be necessary to carry out collaborative studies to determine the nutritional status in other areas of Argentina, taking into account the geographic and topographic diversity of the country. Therefore, these results should not be extrapolated to the whole country.

TABLE 3. Selenium content of eggs and wheat in the United States, eggs in Chile, and wheat in Argentina

	Selenium (μg/100 g)		
	USA	Chile	Argentina
Egg white	17.6 ± 7.6	79 ± 41	
Egg yolk	45.2 ± 10.7	81 ± 43	
Wheat grain	70.7 ± 14.2		42 ± 23 (1994/95 harvest) 29 ± 4 (1993/94 harvest)

Sources: ref. 5, 7, 9.

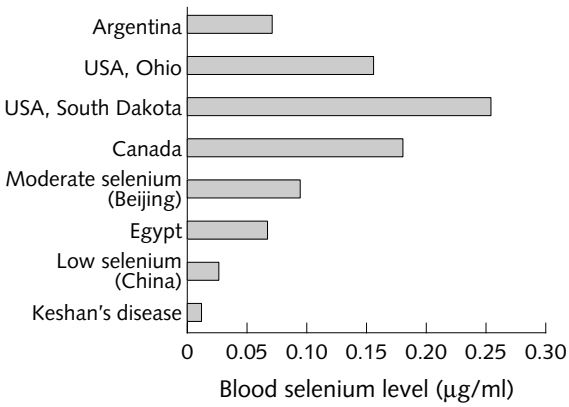

FIG. 1. Selenium levels in blood from Argentina and worldwide

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Stable isotope techniques in human nutrition research: concerted action is needed

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Abstract

Use of stable isotopes in applied nutrition has been a developing field for over 60 years. The past 20 years has seen improvements in computer and mass-spectrometer technology that has opened up even greater possibilities in the understanding of human metabolism. While improvements in technology can bring great opportunities, it can also cause problems if there is no consensus among the stable isotope user-community on standardization of new techniques and methods. Users of stable isotopes have traditionally been split into two groups; those who work with heavy isotopes (e.g., ^{58}Fe, ^{70}Zn) and those who work with light isotopes (e.g., $^{2}H_{2}{}^{18}O$). Standardization issues have been addressed by the light isotope users and awareness of this type of problem is starting to emerge within the heavy isotope community.

Key words: nutrition, stable isotopes, mass spectrometry, harmonization, SIGN

Background

In the latter part of the twentieth century, stable isotope techniques became indispensable tools for human nutrition research. Their use now permits researchers to explore aspects of basic and applied nutrition *in vivo* that are otherwise impossible to assess. Stable isotope labels can be used to trace the absorption and utilization of minerals and trace elements in the human body [1]. With isotopically labeled water we can assess parameters of body composition and energy expenditure [2] and substrates labeled with ^{2}H or ^{13}C allow us to measure the activity of selected enzyme pathways and gastrointestinal function to determine functional consequences of altered nutrition [3]. As valuable as such information is for basic and applied nutrition research, it is often challenging to obtain meaningful data using these techniques. Specialists from various scientific disciplines including nutritionists, food scientists, analytical chemists, and mass spectrometrists have to work together, each contributing their expertise.

In 1999 and 2000 meetings were held at the International Atomic Energy Agency (IAEA, Vienna, Austria) to assess the current situation and future directions for the application of isotopic techniques in nutrition research. IAEA plays an active role in the promotion and dissemination of isotopic techniques in applied research, including human nutrition research. IAEA projects, managed by both the Department of Nuclear Applications and the Department of Technical Cooperation, in the field of human nutrition range from small research projects, through training to national and regional projects especially focused on monitoring nutritional interventions.

At the 2000 consultants meeting, the consensus was that there is a need to improve data quality [4]. Comparability of data between laboratories is often limited as techniques are sophisticated and sample analysis, as well as calculations, are challenging. Sources of bias are multiple and require more than simple underpinning of the work with quality control by the use of reference materials. Considering the likely expansion of stable isotope techniques, concerted action is needed to ensure comparability of results and data quality. This refers in particular to applications in which data

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generated by stable isotope techniques may influence political decisions, e. g., within the framework of governmental led dietary advice and food fortification programs. The workshop "Stable isotope users group in nutrition: opportunities, perspectives and harmonization," held on Monday 27th August 2001 at the 17th International Conference on Nutrition under the sponsorship of IAEA tackled this problem.

Concept of the workshop

Organized by Dr. Thomas Walczyk and Dr. Andy Coward with the support of IAEA, this workshop brought together experts and other stable isotope users for both light isotope techniques (macronutrients and vitamins) and heavy isotope techniques (mineral and trace elements). Experts from both fields were asked to reflect on the current situation to initiate discussions among the participants to identify specific needs for future action.

Dr. Andy Coward reported on the current status of standardization for the different light isotope techniques that are in routine use and stressed the need for harmonization of these techniques to ensure data comparability in the literature. Dr. Dale Schoeller described some recent advances in light isotope techniques and pointed out the need for interdisciplinary collaboration between nutritionists and mass spectrometrists/analytical chemists in this field of research. Dr. Tom Preston described the existing SIGN network (Stable Isotopes in Gastroenterology and Nutrition) that was established in Europe as a forum for consensus building and standardization of light isotope techniques.

For the mineral and trace element techniques, Dr. Thomas Walczyk highlighted the different sources of bias in the design and conduction of stable isotope studies, sample preparation, mass spectrometric analysis, and data evaluation techniques. Dr. Jack Dainty looked critically at current practice in the transformation of mass spectrometric data into nutritionally meaningful results, the lack of uncertainty estimates in generated data, and the need for an agreed set of defined terms in stable isotope research. Finally, Dr. Judith Turnlund presented available techniques for isotopic analysis of minerals and trace elements in biological samples and compared the use of inductively coupled plasma mass spectrometry (ICP-MS) and thermal ionization mass spectrometry (TIMS) for copper isotopic analysis in human experiments.

The Vienna workshop was the first meeting to bring together these two groups and was attended by more than 80 delegates. Here we report the main points that were addressed by the experts and the outcomes of the discussions from the floor.

A history of interdisciplinary collaboration

Stable isotope research in nutritional sciences serves as an example of how collaboration between individual scientists in diverse disciplines can lead to significant scientific advances. Few examples of this are more illustrative than the development and application of the doubly-labeled water method to the measurement of human energy expenditure.

The doubly-labeled water method was born out of a collaboration between Nathan Lifson, a physiologist, and Alfred O. Nier, a physicist. This collaboration spanned a period of 15 years beginning in the late 1940s. Both were on the faculty of the University of Minnesota, but members of different departments with no obvious interdepartmental ties. Dr. Lifson was investigating the source of oxygen in respired CO_2. Dr. Nier, a pioneer of mass spectrometry, was investigating the isotopic nature of elements and designing instruments for isotopic purification and measurement. Neither alone could readily perform the studies to determine the source of oxygen in respired CO_2, but Dr. Nier could obtain and even produce ^{18}O for use as a tracer and measure the ^{18}O abundance in CO_2, while Dr. Lifson provided the timely scientific question and designed the animal experiment. This collaborative demonstrated that the oxygen in respired CO_2 came from isotopic equilibration of the body water of the mice used in the study [5], through what is now known to be the action of carbonic anhydrase.

The results of this simple collaborative experiment, however, led to a far more interesting idea. Dr. Lifson hypothesized that isotopic equilibration between the oxygen in CO_2 and that in water would produce a differential turnover of the oxygen and hydrogen in body water. The oxygen turnover would be a measure of water and CO_2 flux through the body, while the hydrogen turnover would be a measure of water flux through the body. The difference would therefore be a measure of CO_2 flux and this would provide a non-invasive measure of energy expenditure through the application of the principles of indirect calorimetry.

Continued interdisciplinary collaboration soon proved the hypothesis and validated it under a variety of conditions [6–8] but application to human studies came only 30 years later [9, 10]. It is unlikely that the delay was at all related to limitations on the availability of ^{18}O labeled water or isotope ratio mass spectrometers that were capable of measuring small ^{18}O enrichments. Both items were commercially available during most of the period between when Lifson proposed the human studies and when they were finally performed by others. Rather, the delay appears to be due to the absence of a collaboration between persons with the expertise in mass spectrometry and persons with an expertise in human energy metabolism.

This example illustrates nicely that progress in stable isotope techniques in human nutrition research depends largely on the will of nutritionists as well as mass spectrometrists to cross borders and to share their expertise. While nutritionists became aware over the past two decades of the possibilities that stable isotope techniques offer, mass spectrometrists are less familiar with this specific application of their tools. This leaves the initiative to the nutritionists to build up contacts to the mass spectrometric community. Obviously, such collaborations are difficult to establish and compromises often have to be made in practice either on the mass spectrometric or the nutritional side. As methods are highly sophisticated, such compromises often result in methodological imperfections and, finally, in bias. Harmonization and standardization of techniques could alleviate this problem. Experts in the different scientific fields involved in such studies can contribute their expertise to the definition of guidelines and recommendations that can help others to improve data quality, to minimize the risk of methodological artefacts and, based on a commonly agreed consensus, to improve data comparability.

Sources of bias in stable isotope studies

To apply stable isotope techniques successfully, potential sources of error in the study design, the experimental part, sample analysis, and data transformation have to be identified beforehand and, as far as possible, suitable measures taken for bias control. Because human stable isotope studies are complex, there are many potential sources of bias in addition to those having their origin in purely analytical sources of error (see table 1). While sources of bias in the analytical part and in the calculations can be identified and eliminated before running the human experiment, sources of bias in the study design and the experimental part are often hidden or difficult to assess. At best they become visible only after the study has been completed. For example, isotope doses have to be estimated before an experiment can start but whether the isotope dose has been sufficient to obtain meaningful results remains an open question until the samples are analyzed. Thus the successful application of stable isotope techniques depends on the expertise of the team of scientists running the experiment.

It is, however, technically impossible to eliminate all sources of bias in a stable isotope study. The human body is too complex to be described by a set of simple equations. Assumptions are necessary as are ethical and practical considerations. However, simplifications can limit data comparability if no consensus exists among stable isotope users. This becomes obvious when looking at current practice in human mineral and trace ele-

TABLE 1. Selected sources of bias in human experiments involving stable isotope techniques

Study part	Source of bias	Potential cause of bias
Study design	Choice of method	Method might not be suitable to answer question
	Isotope doses	Insufficient isotopic enrichment of sample material
Subjects	Number	Effect is hidden by interindividual variations
	Inclusion/exclusion criteria	Interferences with measured parameter
Isotope administration	Isotopic labeling	No isotopic equilibration between native element and isotopic label in the gut or body
	Oral dose	Incomplete intake of isotopic label
	Intravenous dose	Incomplete transfer; metabolism of oral and isotopic label differs
	Subject control	No fasting before/after isotope administration; incomplete sample collection by the subject
Sampling	Time schedule	Sampling started too early, too short, or was not long enough
Sample preparation	Contamination	Over- or underestimation of isotopic label in sample
	Homogenization	Isotopic composition of sample is not representive
	Isotope effects	Systematic changes in isotopic composition
Mass spectrometry	Multiple	Multiple
Data evaluation	Choice of model	Unjustifiable assumptions/simplifications
	Data transformation	Unjustifiable assumptions/simplifications
	Data modeling	Technique by which data are fitted to metabolic model
	Uncertainty estimate	Not all sources of analytical and physiological relevance have been considered

ment absorption experiments. Often, the term "stable isotope tracers" is used instead of "stable isotope label." The term "tracer" implies that stable isotopes can be followed and quantified in the human body or in its excreta directly, similar to radiotracers. However, this is not the case, as the stable isotopes in a stable isotope tracer are physically indistinguishable from the natural element except that they differ from the natural element in their isotopic abundance. This means that quantification of stable isotope tracers in any material can only be done indirectly via the induced alteration of the natural isotopic abundances of the element. This principle is known as the "isotope dilution concept" and needs calculations to obtain the amount of tracer present in a sample/tracer mixture.

Calculations can be simplified substantially by assuming that the stable isotope tracer is monoisotopic, i.e., that the respective stable isotope is enriched to 100%. To date, evaluation techniques range from ignoring all other isotopes in a stable isotope tracer to concepts where corrections are made to compensate finally for these inaccuracies [11, 12]. Mathematically derived calculation techniques which consider the specific isotopic composition/atomic weight of the stable isotope label from the beginning and that allow proper uncertainty statements at the end have been published recently [13]. However, calculations are much more complex, especially when more than one isotopic label is used.

Any assumption that is made for simplifying the calculations can be considered a compromise for facilitating data evaluation. The introduced bias depends on the chosen calculation method and can be significant when looking at the final nutritional conclusions to be drawn. Thus, the user decides about data quality and, finally, data comparability by the choice of the data evaluation technique.

A critical look on current scientific practice

Harmonization or standardization of methods can certainly improve data quality and data comparability but we must never forget that there has to be a balance between the push of technology and the pull of a need. Indeed it is unlikely that great enthusiasm will evolve for standardization approaches unless there is clear evidence for a need in biology, medicine, and nutrition. Ideally, standardization is an academic exercise for a well-defined process leading from method development and method validation to the application and the production of useful data. But nothing is quite so simple in practice.

The need for information leads to the development of a new method which produces first data that may or may not be useful. Over-enthusiasm and pressure to publish new results as soon as possible may lead to the premature application of the technique. Method validation is done in parallel to the application as it is more prestigious to publish data of limited quality than a thorough methodological evaluation. Fortunately, limits in data quality are soon recognized, improvements are made by different groups in parallel and, after a few years, several modified techniques are in use but as data are generated differently, comparison of results can be difficult. Experiments done in the past or by other groups have to be repeated to unify findings. This is clearly a poor use of resources, especially when stable isotope techniques are involved. In the most extreme cases, a poorly validated or improperly applied technique may even result in wrong conclusions. This can be detrimental not only for scientific progress but also for public health, for example, when stable isotope techniques are used to evaluate governmental led food fortification programs and political decisions are influenced by the results.

It can take years until the lack of data comparability and data quality becomes visible and affects the scientific work of the community. If this is the case and sufficient enthusiasm is generated a process of standardization takes place, sometimes only by informal or unofficial consensus. This is not an entirely satisfactory situation but at least in one light isotope application this has been done successfully. In 1988 a workshop was held in Clare College, Cambridge, to seek a consensus view on the technical aspects of applying the doubly labeled water method for energy expenditure [2]. This action was supported by the IAEA and has strengthened the status of the method. Similarly, the use of ^{13}C-urea for the detection of *Helicobacter pylori* infection is now largely standardized.

Standardization of stable isotope techniques in human nutrition research

Any approach to standardize stable isotope techniques in human nutrition research has to be based on the thorough analysis of possible bias sources in the study design, the experimental part, the analysis, and the data translation. When sources of bias are identified their impact on data quality has to be evaluated and common consensus has to be achieved on the relevant sources of bias, bias sources that are negligible and where bias control is limited because of practical reasons. After prioritizing the relevant sources of bias, possible techniques for bias control need to be discussed among the users and a common decision has to be made on specific guidelines and recommendations.
Some efforts have already been made towards standardization of stable isotope techniques in human nutrition research. We would suggest that a consensus should exist on:

» How to administer the stable isotope dose.

» How to prepare samples for isotopic analysis.
» How to perform the mass spectrometric analysis.
» How to correct mass spectrometric data for measurement bias.
» How to use isotopic reference materials.
» How to translate mass spectrometric data into nutritionally relevant information.
» Which metabolic model to use and, if required, how to best fit data to the model.
» How to calculate overall measurement uncertainty, including the contributions of analytical and physiological variance and the impact of these on dose requirements.
» How to calculate the sizes of biases consequent on model imperfections.
» How data should be reported in the scientific literature.

By using this check-list, the standardization status of a set of some procedures that have emerged from the research field and are now used for applied work is shown in table 2. The table omits many methods in research use but applying the check-list for most of them will show that they are often not close to consensus standardization. To date, only the doubly-labeled water technique for energy expenditure and the ^{13}C-urea breath test for *Heliobacter pylori* infection are standardized procedures.

Contrary to the light isotope techniques no attempts have been made to standardize any of the stable isotope techniques used in mineral and trace element research. Underlying principles, i.e., fecal monitoring, urinary monitoring, plasma appearance (including kinetic modelling techniques), and tissue retention of the administered isotopic label are applied uniformly but there is no common consensus on the listed standardization criteria. This refers in particular to mass spectrometric analysis. In the past, isotope ratio measurements have been performed in mineral and trace element nutrition research primarily by thermal ionization mass spectrometry (TIMS) which is well known as a robust but laborious technique that allows the measurement of isotope ratios at high absolute and relative accuracy. This has changed with the advent of inductively coupled plasma mass spectrometry (ICP-MS). Although not originally designed for isotope ratio but multi-element analysis, second generation (high-resolution) and third generation (multicollector) instruments have become powerful tools for isotopic analysis that are ideally suited for applications in nutrition research [14]. Sample throughput is much higher when compared to TIMS and reproducibilities in isotope ratio measurements that can be achieved by multicollector-ICP-MS are mostly as good and for some elements even better than for TIMS. However, isobaric interferences and instrumental isotope fractionation effects limit the accuracy in isotopic analysis by ICP-MS [15], in particular for iron and calcium, which makes accurate isotope ratio measurements at high precision a technical challenge even for a skillful operator. Quality control guidelines and recommendations for isotopic analysis by ICP-MS are, therefore, urgently required to limit the risk of generating artefacts that are nearly impossible to identify later from published data at current publication practice.

Standardization by collaboration

Standardization approaches will have an impact on data quality and comparability if they are adopted by

TABLE 2. Current status of some stable isotope methods used in nutrition research

Method	Standardization status
(^{2}H, ^{18}O) techniques	
Energy expenditure	Good level of standardization
Body composition (^{2}H)	No formal standards but these could easily be achieved
Breastmilk intake	No formal standards but these could easily be achieved
^{13}C Breath tests	
^{13}C-urea (*Helicobacter pylori*)	Good level of standardization
^{13}C-octanoate (gastric emptying)	Some standardization, could be improved
Nutrient assimilation	Some standardization, could be improved
Gut transit times	Some standardization, could be improved
Liver function	No significant standardization
Any GC/Combustion/IRMS method	Uniform approaches but no standardization
Most GC/MS methods	Uniform approaches but no standardization
Any mineral and trace element technique	Uniform approaches but no standardization

GC/MS, gas chromatography/mass spectrometry; IRMS, isotope ratio mass spectrometry.

the entire community that uses the respective technique, but to stand a good chance of success the drive towards it had to come at the right time. Opportune moments may be difficult to recognize but we would suggest that there should be the concurrent existence of a clearly identified need for the information derived from the methodology, a substantial body of expertise and experience, some clear indication that unofficial consensus already exists, and a clear willingness of participants to collaborate irrespective of the existence of research funds to do it.

A network of stable isotope users that fulfill the above criteria has existed in Europe since 1993. However, it is currently limited to users of light isotope techniques in biomedical research. Initially the work was funded by the European Union as a BIOMED Concerted Action (Biomed 1 Project PL93-1239) and was focused primarily on the development of breath tests for clinical application [3]. It now continues with a more diverse agenda (see *http://www.med.rug.nl./sign/*) and operates as SIGN (stable isotopes in gastroenterology and nutrition). The cluster program consists of four parts: gastrointestinal transit (gastric emptying and intestinal transit), digestion, absorption, and subsequent metabolism in health and disease, gastrointestinal mucosal integrity and pathology, and fermentation and colonic metabolism. Currently, the membership represents 22 institutes in 10 countries and annual meetings have provided a forum for discussion of new work, ideas, and problems.

No such network exists for stable isotope techniques in mineral and trace element nutrition research. However, the existing network could be theoretically extended worldwide to be an umbrella for all users of stable isotope techniques in nutrition research. Although light isotope applications and heavy isotope applications in nutrition research differ not only in the underlying methodological principles but also in the mass spectrometric techniques employed, synergies may develop. Both user groups have problems in common for which a consensus is required. Terminology serves here as an excellent example. "Bioavailability" of a nutrient is a key term that is used by both communities. However, both within and between groups this term is used differently as no commonly agreed definition exists. Scientists in vitamin research use "bioavailability" and "absorption" of a nutrient synonymously. In mineral and trace element research, "bioavailability" is commonly used to describe the amount or fraction of an ingested nutrient that is not only absorbed but also retained by the human body and finally used for physiological function. Other examples could be added. Obviously, this can cause confusion in scientific discussions and points to the need to agree on a common terminology.

Another example for a common interest is the supply and the quality of stable isotopes. For the light elements the recent crisis in the supply of ^{18}O and its unstable price have troubled the user community [16] but apart from this supply it is not a serious issue and the commercial manufacturers have responded to needs, albeit sometimes slowly. For minerals and trace elements, a lot of stable isotope users are not aware that certain isotopes are sold mostly to nutritionists. This is an enormous opportunity as it allows the users to request commonly from stable isotope distributors and manufacturers the production of batches of isotopic labels of defined quality that are dedicated to human nutrition research.

Finally, both user communities suffer from current publication practice. Stable isotope studies in humans can be considered a true interdisciplinary challenge in which nutritionists, mass spectrometrists, and analytical chemists have to work closely together to generate meaningful data. Analytical techniques and mathematical algorithms are sophisticated and can be considered a science in itself. Despite this, nutritional and biomedical journals do not always publish methods and measures of quality control in sufficient detail to make methodological issues clear. Furthermore, primary data sets, e.g., the isotope ratios of the enriched samples/isotopic labels and isotope doses for each individual subject, may not be given clearly. It is accepted that the result is of primary interest but often it is important that the reader should be able to assess the quality of results from published information and to identify possible sources of bias. By on-line publication of supplemental information and primary data, valuable information could be made available.

IAEA's role in strengthening isotopic tools for health and nutrition monitoring

The IAEA through its coordinated research projects (CRP) and technical cooperation projects (TCP) in the areas of health, nutrition, and environment, consistently strives to provide the technical underpinnings to international efforts for improving the quality of life, particularly in the developing world. To date, IAEA's efforts in strengthening applications of isotopic tools have been pivotal for many countries to develop strategies to measure energy metabolism, resistance to insulin, rate of synthesis of fat, changes in protein synthesis, lactation performance, bone mineral density, food composition, efficacy of nutrient fortification, nutrient utilization, and prevalence of infection. Techniques have been applied successfully in more than 50 IAEA member states.

While strengthening the applications of isotopic

techniques for field use, the IAEA recognizes the need for methodological standardization and harmonization of isotopic methods among numerous users to enhance the profile of nutrition metrology, i.e., the science of measurement in nutrition research including all aspects both theoretical and practical with reference to measurements. Thus, the IAEA continues to channel its resources earmarked for current and future projects to improve accuracy and reliability of measurements in food and nutrition research. An example is seen from the results of an IAEA-funded study on energy expenditure of young children in Chile and Cuba based on the doubly-labeled water (DLW) method. These results were used by the joint FAO/WHO/UNU expert committee in 2001 to establish new energy recommendations because of data quality as well as the fact that the measurements represented subjects from developing countries. Prior to this regional project, data on energy expenditures were based on surveys in industrialized countries. Another part of the same study helped to identify several systematic errors in nutritional metrology; physical activity levels (PALs) estimated from questionnaires underestimated total energy expenditure (TEE) compared with PALs measured by the DLW method, and dietary energy intakes generated by conventional methods were underestimated by 11% for women and 55% for men as compared with results obtained by the DLW method [17].

Conclusions

There is much work to be done by the heavy isotope community before meaningful comparisons can be made between different laboratories' work. The light isotope user's group (SIGN) has begun working on standardization and harmonization issues and a similar grouping of heavy isotope users would be beneficial. The workshop discussion can be distilled down into three problem areas: study design, sample analysis, and mathematical methodology. Importantly, each one cannot be tackled in isolation and an interdisciplinary approach is required where nutritionists, clinicians, analytical chemists, mass spectrometrists, and physicists work together as part of the same team. The will to do this is there, as demonstrated by the fruitful discussions contained within this paper. What is needed now is the logistics and organization to realize the full potential of human stable isotope studies in the coming years.

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Total energy expenditure by the doubly-labeled water method in rural preschool children in Cuba

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Abstract

An evaluation of the capacity of the Cuban Nutrition Program for covering the energy requirements of children was carried out in children 5.0 ± 1.0 years of age in a rural mountain community in Cuba. Five males and six females (19 ± 3.05 kg average weight) with a mean BMI 15.4 ± 2.2 kg/m$^{2)}$ were included in the study. Six of the subjects were well-nourished, three were undernourished, and two were overweight. Total energy expenditure was determined by the doubly-labeled water technique. Resting metabolic rate was measured by indirect calorimetry. Energy intake, measured by a three-day weighed dietary record, was 1,527 kcal/day (6.39 MJ). The total energy expenditure of the well-nourished children was 11.8% lower than present energy recommendations (1,773 kcal). This implies that well-nourished children who are moderately to heavily physically active require 82.6 kcal/kg per day. The physical activity level of normal Cuban children is nearly 1.8, which is much higher than that reported in studies of children from industrialized countries using the doubly-labeled water technique. The measured daily energy intake was 1.7 times the resting metabolic rate RMR and 1.04 times the total energy expenditure.

Keywords: energy expenditure, preschool children, doubly-labeled water

Introduction

For more than a decade Cuba has had a nutrition program covering 1,720,359 children under six years of age. A program evaluation using standardized isotopic methods should have great advantages in relation to the costs of the whole program. The main objective of this study was the measurement of the total energy expenditure (TEE) by the doubly-labeled water (DLW) method in rural children under 7 years of age. The results will allow the evaluation of the capacity of the Cuban Nutrition Program to cover their energy requirements.

Subjects and methods

The evaluation was carried out in July 2000 in 4 to 6 year old children who attended a day-care center in the rural mountain community of Las Terrazas at the Sierra del Rosario in western Cuba. The experimental protocol was approved by the Ethics Commission of the Institute of Nutrition and the Ethics Board of the Ministries of Health and Education. Parents provided informed consent after the nature and purpose of the study was explained to them. Eleven children, five males and six females (19.0 ± 3.05 kg average weight) were included in the study taking into account an age proportion similar to that existing in the whole community.

Anthropometry

The children were measured wearing no clothes and shoes. Height was measured with a Holtain stadiometer. Weight was measured on a Soehnle electronic scale (Mod. Galaxy della Portata, DI kg 130-DIV.GR.100, Maurizio Monti, Rome, Italy). Skinfold thickness (triceps, biceps, subscapular, suprailiac) was measured with a Holtain skinfold caliper (Cranlea & Co., Birmingham, England) according to the International Biological Programme [1]. Weight-for-height and height-for-age Z scores were calculated in relation to the median of the NCHS reference population [2] and

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the Cuban reference data [3, 4]. Fat mass and fat-free-mass were estimated using Dugdale and Griffiths´s equation [5].

The doubly-labeled water method

Total energy expenditure (TEE) was measured for 10 days under free-living conditions by using the doubly-labeled water technique. A sample of urine was collected from each subject before isotope administration to determine baseline concentration of ^{18}O and ^{2}H. Each subject was given an oral dose of a mixture containing about 2.0 g $H_2{}^{18}O$ at 10% and 0.12 g $^{2}H_2O$ at 99.9% per kg body weight. The container was then rinsed with 20 to 30 ml of tap water that was also consumed. Urine samples were collected daily in the morning 10 days after the dosing and preserved at 4°C.

Samples were measured for isotope abundance by isotope ratio mass spectrometry (IRMS). The equation for R_{CO2} from Livingstone et al, based on Coward analysis for TEE was used to derive the carbon dioxide production rate. The carbon dioxide production rate was converted to energy expenditure using the equation of Weir [6]. For the calculation of the carbon dioxide production a respiratory quotient of 0.85 was assumed.

Indirect calorimetry

Resting metabolic rate (RMR) was measured during the doubly-labeled water study with the open-circuit GEM calorimeter (Firm NutrEn Technology, Manchester, UK) equipped with a paramagnetic oxygen analyzer (γ = 0–100%, linearity: ± 0.1%, repeatability: ± 0.01% abs) and infrared CO_2 analyzer (γ = 0–2 %, linearity: ± 1%, repeatability: ± 0.02 % abs). The calorimeter was calibrated before each test against standard reference gases. Alcohol burning tests were run five times during the study of the 11 children. The registered respiratory quotient (RQ) values for ethanol were 0.668 (7 July 00), 0.673 (6 July.00), 0.665 (5 July 00), 0.667 (9 July 00), and 0.667 (11 July 00).

The subjects fasted for at least 12 to 14 hours before the test. They were transported to the calorimetry laboratory by car, determined to be free of physiological and psychological stress, and then rested for one hour at a room temperature of 23 to 26°C before the test. The temperature was recorded four times during each test and atmospheric pressure two times daily. An adult-size, transparent, plastic hood was used to collect the expired air for 25 minutes after a five-minute equilibration period.

BMR was also estimated using the equations proposed by the FAO/WHO/UNU Expert Committee [7], the revised Schofield equations [8] and the equations proposed by Henry and Rees for 3 to 10 year-old children in tropical regions [9, 10].

Dietary records

Energy intake was measured by trained dietitians using a 3-day weighed dietary record, which included two week days and one weekend day during the doubly-labeled water study. Energy and macro- and micronutrient intakes were calculated from the Cuban food composition tables included in CERES software developed at the Institute of Nutrition in Havana with the support of FAO [11]

Results

According to NCHS standards 9% of the children were undernourished (–2SD) and 9% were overweight (+2SD) by weight-for-height Z score. Height–for-age Z scores were in the normal range. Girls were heavier and taller than boys, who showed a greater development of fat-free-mass (FFM).

Predicted BMR values using the equations proposed by the 1985 FAO/WHO/UNU report were 4% higher than those obtained by indirect calorimetry. There was a good agreement between the measured resting metabolic rate and the predicted values using the Schofield equations and moreso with the equations proposed by Henry and Rees for 3 to 10 year-old children in tropical regions (table 1). The value of 890 kcal/day (3.73 MJ) for the BMR and the mean variation coefficient lies between previous reported values [10].

The relationship between resting metabolic rate and fat-free mass is estimated to be 56 kcal/kg in 4 to 7 year-old children [12]. The mean resting metabolic rate for children in this study was 57 kcal/kg fat-free mass per day. Values of 53, 56, 51, and 50 kcal/kg/day of fat-free mass (FFM) have been found in white American, Mohawk, Guatemalan, and African American children in this age group [13]. The resting metabolic rate was apparently higher for boys, 899 kcal/day (3.76 MJ) than for girls, 884 kcal/day (3.70 MJ). The percentage of fat-free mass was higher in boys (90%) than in girls (79%). The values expressed per kg FFM were closer (57 vs. 55 kcal/kg FFM/day) and the differences were not significant.

Doubly-labeled water study

The dilution space ratio (table 2) is greater than the value found in adults or in studies conducted in colder climates. The rates of disappearance in these children (kd and ko) are faster than those found in children from industrialized countries, suggesting the influence

TABLE 1. Resting metabolic rate (RMR) of 4 to 6 year-old children from the rural mountain community "Las Terrazas," Sierra del Rosario, Cuba as measured by indirect calorimetry and estimated with different prediction equations for 3 to 10 year-old children

Child	Weight (kg)	Age (yr)	Temperature (°C)	Atmospheric Pressure Mm Hg	Resting metabolic rate (kcal/day)			
					Calculated with equations			Measured by indirect calorimetry
					FAO [7)]	Schofield [8]	Henry-Rees [9]	
G1	15.9	6	24	744	857	809	829	702
G2	25.2	5	23	744	1,066	998	969	962
G3	21.7	5	25	745	988	927	916	1,090
G4	18.5	5	25	745	916	862	868	832
G5	19.2	5	25	744	931	876	879	961
G6	17.7	4	25	745	898	846	856	754
B1	19.9	6	25	745	946	956	941	1027
B2	16.3	4	24	745	864	875	844	916
B3	15.0	5	25	744	835	845	809	785
B4	17.5	4	25	744	892	902	876	956
Mean	19.0	4.91			926	900	890	891
SD	3.05	0.70			69	65	60	122
CV %					7.5	7.2	6.8	13.7

Sources: ref. 7–9.

TABLE 2. Results of the doubly-labeled water study and energy expenditure in 4 to 6 year-old Cuban children

	Age (yr)	Weight (kg)	Rate constant deuterium decay (k_d 1/day)	Rate constant oxygen decay (k_o 1/day)	Dilution space (Nd/No)	TBW (lt)	TBW (%)	Fat by deuterium (%)	CO_2 (mol/day)	Error (%)	TEE (kcal/day)	TEE (kcal/kg/day)
Girls												
G1	5.4	18.2	0.1488	0.1945	1.0480	12.01	66.02	14.37	12.87	4.77	1,481	81.5
G2	5.5	19.0	0.1617	0.2048	1.0620	11.17	58.86	23.66	10.19	5.19	1,151	60.7
G3	4.8	17.7	0.1545	0.2103	1.0450	10.23	57.79	25.24	13.88	4.25	1,616	91.3
G4	6.7	15.7	0.1303	0.1836	1.0710	9.73	61.88	19.95	11.77	5.20	1,374	87.4
G5	5.8	21.8	0.1748	0.2270	1.0470	11.14	51.21	33.58	13.64	4.39	1,567	72.0
G6	5.1	24.8	0.1616	0.2036	1.0400	13.36	54.46	29.36	13.36	5.64	1,520	61.0
Boys												
B1	4.7	17.5	0.1730	0.2160	0.9880	11.00	62.75	14.00	14.24	4.58	1,647	94.1
B2	5.8	15.1	0.2169	0.2748	1.0770	9.01	69.58	21.58	10.14	5.67	1,135	75.2
B3	6.4	19.5	0.1547	0.2203	1.0610	14.55	64.67	14.69	19.61	3.64	2,298	117.7
B4	5.6	21.3	0.1339	0.1754	1.0460	13.14	61.61	15.59	12.92	6.14	1,488	69.8
B5	4.2	16.6	0.1860	0.2318	1.0490	11.54	69.70	8.98	11.77	6.03	1,326	80.1
Mean	5.4	18.8	0.1633	0.2129	1.0485	11.53	61.68	20.09	13.13	5.05	1,509	80.97
SD	0.7	2.9	0.0244	0.0269	0.0233	1.647	5.84	7.43	2.56	0.79	313	16.49

of either a higher environmental temperature and/or physical activity. Observed carbon dioxide production has a mean error of 5.0% in the whole group; this error accounts for the variation of the background isotopic content, lack of covariance of measurement in both ^{18}O and ^{2}H and in general, for analytical and biological contribution to the error.

Total body water decreased in the obese group as compared to the normal and undernourished children. As expected, total body fat increased from 17.1% in the

normal children to 31.5% in the overweight and obese children (table 3).

Total energy expenditures were similar in normal and undernourished children (around 1,550 kcal) and lower in overweight children. The major factors accounting for inter-individual variation in total energy expenditure include body weight, fat-free mass, and resting metabolic rate, which account for 40% to 60% of the variation [14]. TEE was similar between lean (105 kcal/kg FFM/day), obese (97), and normal (100) individuals after taking differences in fat-free mass into account [15–17]. The degree of fatness has negligible effects on TEE, other than a small effect on resting metabolic rate. A combination of the effect of carrying the additional fat mass and the increase in resting metabolic rate as a consequence of the increased fat mass are responsible for that [18].

A gender-related difference in total energy expenditure, in addition to that described for the resting metabolic rate has been reported [13]. In the Cuban study, after taking into account the differences in fat-free mass there were only slight differences in the TEE values per kg FFM for girls and boys (99.4 and 102 kcal/kg FFM/day.

Using the doubly-labeled water technique, the energy requirement of well-nourished children was only 8.6% lower than the 1985 FAO/WHO/UNU recommendations. This implies that well-nourished children who are moderately to intensely physically activity would require 82.6 kcal/kg per day.

Daily energy intakes were 1,313 ± 193 and 1,786 ± 283 kcal for girls and boys, respectively These values were 1.7 times the BMR and were higher than those reported for this age group in other countries [19]. According to the 1985 FAO/WHO/UNU report the recommended dietary allowance for energy was, in general, only 1.051 times higher than the observed intake in these children.

Studies done in other latitudes have shown that TEE in prepubertal children living in Burlington, Vermont, USA [20, 21], Phoenix, Arizona, USA [22], Cambridge, UK [23], and Belfast, Northern Ireland [24] was about 25% lower than that reflected by current recommendations. The TEE for the Cuban children was only 11.8% lower for the total group (1,509 vs. 1,710 kcal/day), 8.6% for the normal weight children (1,560 vs. 1,706 kcal/day), 4.6% for the undernourished children (1,385 vs. 1,452 kcal/day), and 26.9% for the overweight children (1,544 vs. 2,111 kcal/day) as compared to the recommended energy intake of 90 kcal/kg/day. In comparison with the current Cuban recommendations the values were 12% higher for normal children (1,560 vs. 1,773 kcal), 8.7% higher for undernourished children (1,385 vs.1,517 kcal), and 28.4% higher for overweight

TABLE 3. Total energy expenditure by the doubly-labeled water method in 4 to 6 year-old children in the rural mountain community Las Terrazas, Sierra del Rosario, CUBA June–July 2000

	Normal ($n = 6$)	Undernourished ($n = 3$)	Overweight ($n = 2$)	Total group (N = 11)
Age (yr)	5.0 ± 1.0	5.0 ± 1.0	5.0 ± 0	5.0 ± 1.0
Body weight (kg)	19.0 ± 2.0	16.1 ± 1.3	23.5 ± 2.5	19.0 ± 3.0
BMI (kg/m^2)	15.4 ± 1.04	13.1 ± 0.58	18.7 ± 1.81	15.0 ± 2.2
ko (rate constant O_2 decay)	0.206 ± 0.02	0.225 ± 0.05	0.215 ± 0.02	0.213 ± 0.028
Kd (rate constant H_2 decay)	0.157 ± 0.02	0.173 ± 0.04	0.168 ± 0.01	0.162 ± 0.125
Nd/No (dilution space)	1.052 ± 0.01	1.045 ± 0.05	1.044 ± 0.01	1.055 ± 0.012
Body water (%)	63.1 ± 4.5	64.7 ± 4.2	52.8 ± 2.3	61.5 ± 6.1
Body fat (%)	17.1 ± 6.2	18.5 ± 4.0	31.5 ± 3.0	20.7 ± 7.5
RDA (90 kcal/kg/day)	1,706 ± 178.5	1,452 ± 114	2,111 ± 223	1,710 ± 275
Cuban RDA (kcal/day)	1,773 ± 198	1,517 ±128	2,157 ± 228	1,773 ± 278
Energy intake (kcal/day)	1,623 ± 410	1,433 ± 237	1,382 ± 212	1,527 ± 335
RMR (kcal/day)	884 ± 102	814 ± 130	1,026 ± 91	891 ± 122
TEE_{DLW} (kcal/day)	1,560 ± 395	1,385 ± 256	1,544 ± 33	1,509 ± 313
CO_2 (mol/day)	13.54 ± 3.23	12.05 ± 2.06	13.5 ± 0.2	13.13± 2.56
TEE (kcal/kg/day)	82.6 ± 19.6	85.4 ± 9.3	66.3 ± 8.4	80.4 ± 16.3
TEE (kcal/kg FFM_d/day)	100 ± 24	105 ± 7	97 ± 17	101 ± 18
PAL-DLW	1.77 ± 0.4	1.71 ± 0.26	1.51 ± 0.10	1.71 ± 0.32
PAL-time motion	1.3 ± 0.02	1.36 ± 0.13	1.24 ± 0.02	1.31 ± 0.09
RDA-TEE_{DLW} (kcal/day) (%)	13.7	9.1	39.7	14.9
E intake–TEE_{DLW} (%)	4.1	3.5	–10.5	1.1

children (1,544 vs. 2,157 kcal).

In 1995, a panel of the International Dietary Energy Consultative Group agreed that on the basis of recent TEE data, the existing energy recommendations were too high for children less than 7 years of age [10] and that there was a lack of data on the socioeconomic differences and the differences between rural and urban settings. New recommendations should be developed with the measurement of energy requirements based on multiples of measured or predicted REE. The Cuban recommendation for children under 7 years of age should be overestimated by about 15% (1,509 kcal intake vs.1,773 kcal recommended).

The results of our study show that the dietary energy recommendations for 4 to 6 year old children in a rural community in western Cuba are not overestimated as much as those for children in urban areas in industrialized countries.

The physical activity level (PAL) values were greater than those for American children [21], children in the Philippines [25] and than the reported values for Chilean children. This was in agreement with the results of Davies et al. in 5-year-old children [26]. In this study, there was only a 1.1% difference between mean energy intake and expenditure in the entire cohort of children. Values of 3.3, 6.4, 5.2, and –1.0% were reported by Davies et al. in 1994) [27] for preschool children in four age groups. In adults and adolescents, the differences may be higher (20%) [24, 28]. The most frequent source of underestimation of energy intake is due to under recording of the food consumed.

Energy requirements as determined by the doubly-labeled water method and weighed intake were similar in well-nourished and undernourished children (difference of 4.1% and 3.5%), however, dietary intake clearly underestimated the requirements of overweight children by 10.5%. There is no sufficient data on overweight children to make conclusions, but this difference may be related either to efforts made by parents to make their children lose weight or to food consumption unknown to the observers. The body weight was recorded at the beginning and end of the seven day study and there was no change to account for this difference in such a short period.

The mean physical activity level (PAL) of normal children was nearly 1.8, which is much higher than that reported for children from industrialized countries in studies using doubly-labeled water. Using the predicted resting metabolic rates, a sedentary PAL of 1.5 was obtained (for 4 to 6 year old children) in other studies using DLW. Values from Cuban and Chilean children are in the moderate to intense range of physical activity. In overweight children from Cuba, the PAL is 1.51; one factor contributing to the difference between normal and overweight children could be that they were girls.

Recent studies on energy expenditure (by DLW) of children living in industrialized countries, indicate that their energy expenditure was 30% lower than the recommended dietary allowance. The energy expenditure in Cuban children accounted for 90% of the dietary allowance, which is similar to that obtained for Chilean preschool children (86%). The completion of the energy expenditure studies in both countries will contribute to a more valid assessment of the adequacy of present nutrition programs.

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Central obesity and risk for type 2 diabetes in Maori, Pacific, and European young men in New Zealand

Elaine Carolyn Rush, Lindsay Dudley Plank, Elizabeth Mitchelson, and Manaia Sialei Laulu

Abstract

New Zealanders of Polynesian origin have a higher prevalence of obesity and type 2 diabetes mellitus than those of European origin. Risk factors for type 2 diabetes mellitus—decreased energy expenditure, increased body fat mass, and central body fat—in 30 normoglycemic Maori, Pacific, and European men were studied. Biochemical measures of risk for type 2 diabetes mellitus included an oral glucose tolerance test, insulin, lipids, and glycosylated hemoglobin. The groups did not differ significantly in BMI, height, body mass or fat mass (DEXA), or adjusted resting metabolic rate (indirect calorimetry), but the European subjects had significantly lower subscapular to triceps skinfolds and fat-free mass than the Maori and Pacific groups. Central obesity by anthropometry and DEXA showed strong associations with the biochemical measures for type 2 diabetes risk. These findings emphasize the association between body composition and central fat distribution with risk of diabetes independent of ethnicity.

Key words: obesity, central obesity, risk factors, men, insulin resistance, DEXA, ethnicity, resting metabolic rate, blood lipids

Introduction

New Zealand people are getting fatter—on average one gram per day—360g per year and one kilogram every three years [1]. Excess body weight results from a positive energy balance. This is the result of a positive imbalance between the energy absorbed from the macronutrients in food and that used by the body. Maori and Pacific people in New Zealand have a greater prevalence of obesity and type 2 diabetes compared to those of European origin. We hypothesized that this is related to metabolic and fat distribution differences as previously demonstrated in a study of Polynesian and European women [2–7]. Specifically it was hypothesized that

- Maori and Pacific Island men have a lower relative resting metabolic rate than New Zealand men of European origin.
- The commonly accepted body mass index (BMI) ranges for categorizing non-obese and obese New Zealand Europeans are inappropriate for Maori and Pacific Island men.
- Maori and Pacific Island men have relatively more central fat than those of European origin and they have a greater risk of developing type 2 diabetes mellitus.

Experimental methods

Protocol

Male volunteers selected on the basis of their body mass index (BMI) were asked to arrive at the body composition facility in the Department of Surgery in the University of Auckland in the morning after an overnight fast. Informed consent was obtained as prescribed by the Auckland Ethics Committee. Height and weight were measured followed by a dual-energy x-ray absorptiometer (DEXA) scan during which volunteers relaxed in a supine position for approximately 30 minutes. Resting metabolic rate (RMR) was measured by indirect calorimetry for about 30 minutes immediately following this scan. Anthropometric measurements were then made, and an intravenous blood sample taken for fasting glucose concentration as well as insulin concentration, glycosylated hemoglobin, and

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blood lipids. The volunteers then drank a dose of 75g of polycose for the standard glucose tolerance test which was performed over the subsequent two hours during which blood samples were taken at 30, 60, and 120 minutes after drinking the polycose. Water was allowed *ad libitum* during the 3.5 hour period of the study.

Anthropometry

Measurements of weight and height allowed BMI (weight/height squared) to be calculated. Harpender calipers (Mentone Educational Centre, Melbourne, Victoria, Australia) were used to measure skinfold thicknesses at triceps and subscapular sites following standard techniques [8]; an average of three measurements was recorded. The girth of the waist and hips were measured using the guidelines of the Hilary Commission Life in New Zealand Survey [9].

Total body fat and fat distribution

Using whole body dual-energy x-ray absorptiometry (Model DPX+, Lunar Radiation Corporation, Madison, Wisc., USA) fat mass, lean soft tissue mass, bone mineral content, and bone mineral density were determined. While the use of this technique as a "gold-standard" for fat measurement is still under debate [10] it possesses a number of advantages over traditional fat estimation techniques. Principal among these is its high precision (better than 3% for total body fat mass) [11]. Regional analysis was subsequently performed to obtain fat content of peripheral and central regions of the body and abdominal and hip fat. A limitation of the machine is the size of the scanning area. Two volunteers whose body dimensions exceeded those of the scanning area were measured by excluding one arm from the scan and then adding the scanned arm composition to the scan as described by Tataranni and Ravussin [11]. Fat-free mass was calculated as the difference between measured body mass and fat mass.

Resting metabolic rate

Resting metabolic rate was measured by indirect calorimetry (Deltatra MBM-100, Datex /Instrumentarium, Helsinki, Finland). Heart rate was monitored using a Polar Sporstester (Polar Electro, Oulu, Finland) to ensure that the volunteer was in a stable state.

Biochemical measurements

Glucose was measured by the Roche Hitachi glucose oxidase method (Roche Diagnostics, Mannheim, Germany), HbA1c by high performance liquid chromatography (HPLC) affinity chromatography (Primus)(C.V. routinely < 3%). Lipids were by the standard Roche-Hitachi methodology. High density lipoprotein (HDL) was done by direct assay. All assays were within target limits specified by the Royal College of Pathologists of Australasia Quality Assurance Program. The laboratory has continuous IANZ ISO9002 (International Accreditation of New Zealand, International Organization for Standardization) accreditation, both for laboratory and supplier status. Leptin was measured by enyzme-linked immunosorbent assay (ELISA) (American Laboratory Products Company, Ltd., Windham, NH, USA) and insulin by radio immunoassay (IMX Insulin Assay kit, Abbott Diagnostics, Abbott Laboratories (NZ), Ltd., Mount Wellington, Auckland, New Zealand).

The ISI 0–120 index of insulin sensitivity was calculated using the formula of Gutt et al. [12] from the fasting and 120-minute post-oral glucose tolerance test. Homeostasis model assessment (HOMA) indices of insulin sensitivity HOMA S% and beta cell function, HOMA B% were calculated according to Hermans et al. [13, 14].

Data analysis

Analysis of covariance was used to establish differences in RMR between the three ethnic groups by controlling for differences in fat-free and fat mass which bear a strong linear relationship with RMR [15]. Analysis of variance was used to test for differences between ethnic groups for measured and derived variables and, if significant, pairwise *t*-tests were performed. Regression analysis was used to examine the dependence of percentage body fat on BMI. Pearson's correlation coefficient was used to examine correlations between pairs of variables. The 5% level was chosen for statistical significance.

Results

The physical characteristics and anthropometric measurements for the 30 men studied are shown in table 1. The groups did not differ in body mass, body mass index (BMI), or height. Pacific Island men had a significantly higher subscapular to triceps skinfold ratio than the European men ($p = .01$) and the Maori men had an intermediate value that was not significantly different to that of European or Pacific Island men.

Results of the resting metabolic rate measurements are shown in table 1. After adjusting for fat mass and fat-free mass, resting metabolic rate was similar for Maori (1,830 ± 111 (SD) kcal/day) and Pacific Island (1,828 ± 117 kcal/day) groups and slightly lower for the Europeans (1,751 ± 120 kcal/day) although this did not

TABLE 1. Demographic, anthropometric, and resting metabolic rate measurements in Maori, European, and Pacific Island men 18 to 27 years old (mean ± SD)

	Maori (n = 10)		European (n = 10)		Pacific Island (n = 10)		p
Age (yr)	22 ± 3	(18–27)	23 ± 3	(19–27)	24 ± 2	(20–27)	.47
Height (cm)	180.8 ± 6.5	(170.5–189.5)	178.9 ± 4.9	(170.9–187.8)	181.2 ± 9.5	(167.2–196)	.75
Weight (kg)	100.3 ± 29.2	(72.7–147.9)	82.6 ± 11.7	(72.6–109.2)	101.4 ± 14.4	(78.7–125.6)	.08
Waist (cm)	93.6 ± 18.0	(76.2–125.7)	85.9 ± 9.9	(73.8–106.4)	95.2 ± 10.5	(82.6–110.1)	.71
Hip (cm)	111.6 ± 15.9	(92.9–137.6)	101.6 ± 8.0	(93.8–119.3)	111.8 ± 9.2	(97.8–125.8)	.10
Waist:hip ratio	0.83 ± 0.05	(0.76–0.91)	0.84 ± 0.04	(0.79–0.91)	0.85 ± 0.04	(0.80–0.90)	.71
Subscapular (mm)	25.2 ± 18.1	(8.5–57)	14.0 ± 8.9	(7.5–35.2)	24.4 ± 13.1	(5.8–48.2)	.15
Triceps (mm)	18.6 ± 11.7	(8.4–41.5)	13.2 ± 6.9	(6.5–29.5)	14.6 ± 6.2	(4.0–25.4)	.36
Subscapular/ triceps	1.29 ± 0.36^{ab}	(0.90–1.98)	1.05 ± 0.21^{b}	(0.71–1.30)	1.67 ± 0.62^{a}	(1.14–3.13)	.01
BMI (kg.m^{-2})	30.4 ± 7.8	(22.7–43.5)	25.9 ± 4.2	(20.6–34.9)	31.0 ± 4.8	(24.6–38.4)	.12
RMR (kcal.d^{-1})	1,870 ± 255^{a}	(1,657–2,485)	1,620 ± 135	(1,495–1,940)	1,920 ± 225^{a}	(1,565–2,270)	.01

Values *not* sharing a common superscript are significantly different. Values in brackets are ranges.

reach statistical significance. Resting energy expenditure (REE) could be predicted from a linear combination of fat mass (FM) and fat-free mass (FFM):

$$\text{REE (kcal/day)} = 371.146 + (18.344 \times \text{DEXA FFM(kg)}) + (6.651 \times \text{DEXA FM(kg)})$$

The R^2 value was 0.80 ($p < .001$). Ethnicity was not a significant predictor.

Results of the DEXA and biochemical measurements are shown in table 2. The groups were similar for all these measurements except for fat-free mass by DEXA, bone mineral density, and the ISI 0–120 value. Pacific Island men had a higher fat-free mass than European men ($p = .02$) with the Maori men having an intermediate value that was not significantly different from that for either Pacific or European men.

Using multiple linear regression there was no difference in the percentage of body fat from DEXA on BMI between the three ethnic groups. The equation for predicting the percentage of fat from BMI was:

$$\text{DEXA\%fat} = -17.98 - (0.55 \times \text{group}) + (1.53 \times \text{BMI}) \quad (\text{SEE} = 4.7\%, R^2 = 0.79)$$

where the group is coded as 1 for Maori, 2 for European, and 3 for Pacific Island. Hence for a fixed BMI, Maori and Pacific Island men had a lower percentage of fat and therefore more fat-free mass than European men.

There were significant positive relationships between insulin and glycosylated hemoglobin, insulin and the subscapular to triceps ratio, and glycosylated hemoglobin and the subscapular to triceps ratio. Other relationships included significant negative relationships between dietary fiber and total cholesterol, LDL, and triglycerides. Total dietary fiber and the subscapular to triceps ratio were also related ($r = -0.52$, $p = .004$). These significant associations point to the interrelationships of metabolic fuel mix, diet, and fat distribution.

All the men in this study were classified as normoglycemic based on the oral glucose tolerance test. Table 3 demonstrates the associations between central fat and measures of insulin sensitivity. In particular fasting insulin levels, and the areas under the insulin and glucose curves and the ISI 0–120 and HOMA indices showed strong relationships with measures of central fat.

Discussion and conclusion

This report is limited to body composition, resting metabolic rate, and the relationships between central obesity and biochemical measurements of type 2 diabetes risk. The relatively small number of participants in the study limits the number of significant findings that may be reported.

The results of the study show that Pacific Island men had relatively more fat-free mass than Maori men, who had more than European men. The subscapular to triceps skinfold ratio followed the same pattern indicating that Pacific Island men have more subcutaneous central fat than Maori and European men. These observations agree with those made previously reported in Polynesian women and Samoan, European, and Maori men and women [2, 16, 17]. This central fat deposition could be related to the increased prevalence of diabetes and obesity in Maori and Pacific Island people in New Zealand as compared to Europeans. However as the DEXA measurements of the ratio of total fat in the limbs and torso, and between the abdominal and hip regions showed no significant difference between the ethnic groups for these young men, further investigation of the distribution of the subcutaneous to intra-abdominal fat is warranted [18–20].

We did not find a lower adjusted resting meta-

TABLE 2 DEXA and biochemical measurements in Maori, European, and Pacific Island men (mean ± SD)

	Maori ($n = 10$)		European ($n = 10$)		Pacific Island ($n = 10$)		p
DEXA fat mass (kg)	30.4 ± 21.5	(5.5–64.8)	18.9 ± 9.7	(10.0–40.0)	28.4 ± 13.1	(9.8–49.1)	0.22
DEXA fat-free mass (kg)	69.5 ± 7.8^{ab}	(57.7–82.0)	63.4 ± 4.8^{b}	(54.1–71.2)	73.1 ± 8.3^{a}	(57.2–85.6)	0.02
DEXA % fat	27.4 ± 12.8	(7.6–44.1)	22.1 ± 8.2	(13.8–36.9)	27.2 ± 9.9	(10.2–41.3)	0.45
Bone mineral density ($g.cm^{-2}$)	1.32 ± 0.04^{b}	(1.22–1.39)	1.19 ± 0.08^{a}	(1.06–1.33)	1.31 ± 0.08^{b}	(1.18–1.43)	0.01
Central/appendicular fat	1.31 ± 0.29	(0.96–1.92)	1.15 ± 0.30	(0.73–1.67)	1.22 ± 0.21	(1.04–1.70)	0.43
Abdominal/hip fat	0.84 ± 0.25	(0.47–1.17)	0.74 ± 0.23	(0.37–1.15)	0.84 ± 0.15	(0.67–1.16)	0.43
Fasting glucose ($mmol.l^{-1}$)	4.8 ± 0.4	(4.2–5.6)	4.9 ± 0.5	(4.2–5.6)	5.0 ± 0.7	(3.1–5.6)	0.71
Fasting insulin ($pmol.l^{-1}$)	79 ± 58	(21–181)	60 ± 52	(21–181)	83 ± 67	(21–188)	0.66
HbA1c (%)	4.6 ± 0.4	(3.9–5.2)	4.6 ± 0.5	(3.6–5.0)	5.0 ± 0.38	(4.7–5.7)	0.11
AUC glucose ($mmol.l^{-1}.hr$)	13.4 ± 2.1	(10.3–16.7)	11.8 ± 4.0	(8.2–20.6)	10.6 ± 1.3	(8.3–12.4)	0.08
AUC insulin curve ($pmol.l^{-1}.hr$)	1,212 ± 1,096	(323–3,808)	743 ± 631	(342–2,485)	1,318 ± 1,103	(386–3,027)	0.38
ISI 0–120	43.6 ± 18.2^{b}	(24.1–87.0)	89.8 ± 36.5^{a}	(33.2–142.6)	75.6 ± 32.7^{ab}	(38.5–127.0)	0.01
HOMA B%	152.0 ± 76.6	(57.5–262.2)	117.8 ± 66.9	(38.3–236.1)	158.1 ± 95.1	(53.2–316.1)	0.49
HOMA S%	114.3 ± 83.7	(30.0–257.8)	150.2 ± 89.2	(29.9–253.2)	105.7 ± 67.7	(29.1–257.8)	0.43

Values *not* sharing a common superscript are significantly different. Values in brackets are ranges.

TABLE 3. Relationships (correlation coefficients) between indices of central fat and Insulin sensitivity

	Waist	Waist:hip ratio	Subscapular skinfold	Subscapular/triceps	% Fat	Fat-free mass	Fat mass	Central/appendicular	Abdominal/gluteal
Fasting glucose	–0.120 (0.529)	0.273 (0.145)	–0.080 (0.673)	–0.060 (0.752)	–0.000 (0.999)	–0.054 (0.776)	–0.050 (0.792)	0.245 (0.192)	0.133 (0.485)
Fasting insulin	0.765 (0.000)	0.530 (0.003)	0.786 (0.000)	0.549 (0.002)	0.784 (0.000)	0.280 (0.134)	0.795 (0.000)	0.130 (0.492)	0.577 (0.001)
HbA1c	0.130 (0.494)	0.0451 (0.813)	0.195 (0.301)	0.310 (0.096)	0.173 (0.361)	0.148 (0.436)	0.148 (0.436)	0.030 (0.876)	0.252 (0.178)
AUC glucose	0.377 (0.040)	0.366 (0.047)	0.335 (0.070)	0.071 (0.709)	0.448 (0.013)	–0.145 (0.443)	0.382 (0.037)	0.475 (0.008)	0.503 (0.005)
AUC insulin	0.701 (0.000)	0.455 (0.012)	0.729 (0.000)	0.623 (0.000)	0.720 (0.000)	0.237 (0.208)	0.723 (0.000)	0.083 (0.663)	0.471 (0.009)
ISI 0–120	–0.456 (0.011)	–0.193 (0.307)	–0.442 (0.015)	–0.116 (0.541)	–0.566 (0.001)	0.031 (0.871)	–0.518 (0.003)	–0.224 (0.234)	–0.397 (0.009)
HOMA B%	0.777 (0.000)	0.583 (0.001)	0.781 (0.000)	0.589 (0.001)	0.764 (0.000)	0.290 (0.120)	0.781 (0.000)	0.071 (0.771)	0.537 (0.002)
HOMA S%	–0.654 (0.000)	–0.375 (0.041)	–0.683 (0.000)	–0.469 (0.009)	–0.737 (0.000)	–0.223 (0.236)	–0.702 (0.000)	–0.136 (0.474)	–0.548 (0.002)

p values in parentheses.

bolic rate in the Polynesian men as compared to the European men. In contrast, similar measurements performed on women yielded a lower relative resting metabolic rate in the Polynesian group [3]. It has been shown [21] that as the risk for diabetes increases so does the resting metabolic rate. This effect may be a confounding factor in the interplay between central obesity, ethnicity, and metabolic rate. The indices of insulin resistance and diabetes risk were higher in the normoglycemic men with more central fat. Finally, there were strong associations between the total fatness and central obesity measurements and risk of type 2 diabetes. These findings emphasize the relationships between body composition and fat distribution with the risk of diabetes independent of ethnicity.

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New iodine models family for simulation of short-term biokinetics processes, pregnancy and lactation

Vladimir Berkovski

Abstract

Research on iodine metabolism was reviewed with special reference to short-term processes, pregnancy, and lactation. A detailed discussion of the new physiologically-oriented biokinetic model and a model parameterization procedure are given. Predictions of the new model are applicable for the analysis of in vivo and in vitro clinical data gained using iodine or radioactive tracers, as well as for the assessment of radiation exposure doses to mothers and offspring. The model can be used to simulate biokinetic processes in the human body, for prediction of biokinetic parameters such as the time course of thyroid uptake (both for mother and fetus), the rate of exertion with urine, and the level of iodine secretion with saliva and breastmilk. The structure of the model is applicable in a wide range of stable iodine in the diet, both for pregnant and non-pregnant persons.

Key words: iodine, short-term biokinetic processes, pregnancy, fetus, lactation, thyroid, radiation, dosimetry, euthyroidal person

Introduction

This paper continues the discussion of the model for the transfer of iodine to the fetus, which has been developed by the author [1, 2] for new publication of the International Commission on Radiological Protection (ICRP) [3]. The model includes a more complex representation of iodine biokinetics in the mother than used previously by ICRP [4] and others [5–7]. Changes to the adult model were considered necessary to provide adequate estimates of tissue exposure from short half-life iodine isotopes and radiation doses to the embryo from iodine in non-thyroidal maternal tissues. The model takes account of available data on changes in iodine metabolism during pregnancy, uptake of iodine by the placenta, amniotic fluid and the fetus, and changes in fetal thyroid mass and iodine concentration. Due to the radiological destination of the discussed model the scope of this paper is restricted by deliberate neglect of the mechanism of hormone metabolism. The metabolism of synthetic iodine-containing compounds which are foreign to the body is also out of the scope of this paper.

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Development of the models

Model of short-term biokinetic processes

The structure of the model is presented in figure 1 and parameter values estimated for the euthyroid adult are discussed below and summarized in table 1. The notation of model compartments is explained in figures 1 and 2.

Alimentary tract

Absorption

The absorption rate of ingested iodine from the gastrointestinal tract as a whole was estimated to be in the range 0.01 to 0.05 min^{-1} [8]. Ingested iodide (I^-) is absorbed rapidly from the small intestine (compartment (**SI**) and more slowly from the stomach (**St**) [9].

Gastroenteric cycle of iodide

The stomach and a portion of the small intestine have an active (against the gradient of concentrations) iodide transport from the serosal to the mucosal surface. The iodide appears in the stomach contents within 0.5 to 1 hour after the intramuscular injection of $Na^{131}I$ [8]. The concentration ratios of iodide in saliva and gastric juice relative to plasma are 30 to 50 [8–10]. In one ICRP publication 23 [11] concentrations of iodide in saliva and gastric juice are given as 3.5 to 24 μg dl^{-1}.

The secretion of iodide into saliva (**S**) and gastric

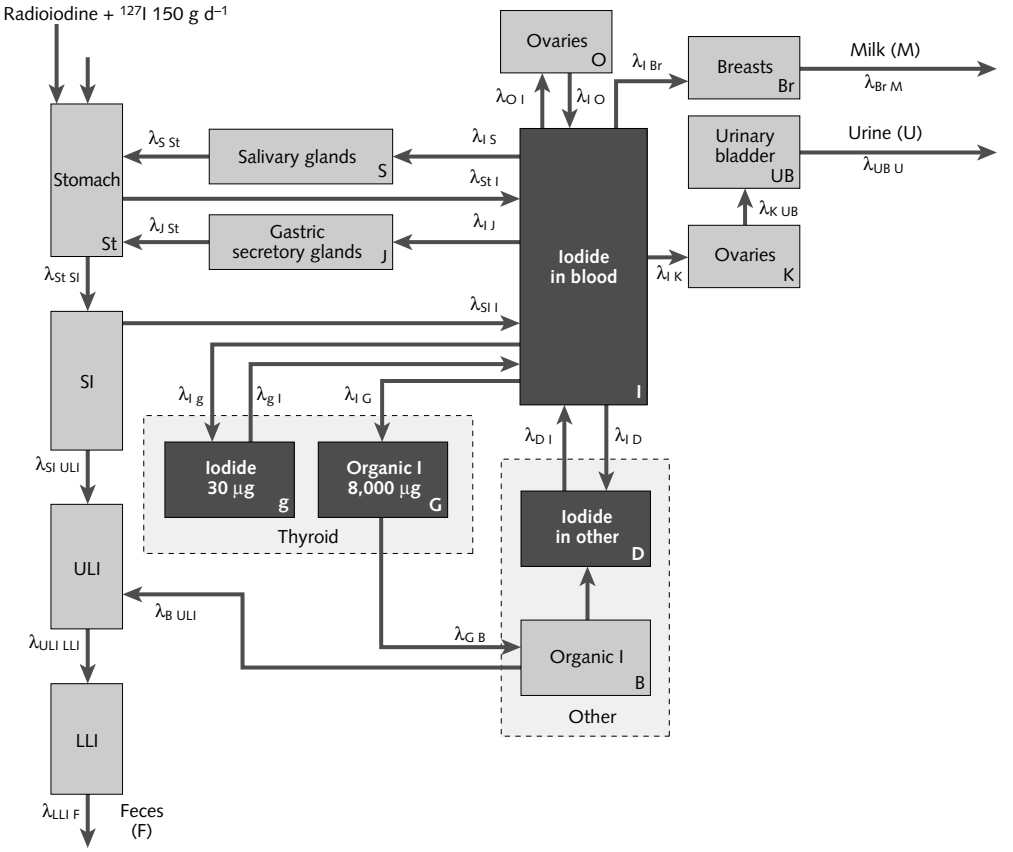

FIG 1. Iodine biokinetic model for short-term processes and lactation. SI, ULI, LLI are Small Intestine, Upper Large Intestine and Lower Large Intestine, respectively.

juice (**J**) and its subsequent re-absorption represents the gastroenteric iodide cycle. Within three hours after ingestion, this cycle has been estimated to account for up to 23% of intake in humans; in dogs this value is about 20% to 30% [9, 12]. The daily secretions of saliva and gastric juice are 0.5 to 2 L and 2 to 2.5 L, respectively [11, 12].

TABLE 1. Transfer rates (d^{-1}) [a]

St→I	40	**I→K**	6.4	**B→D**	0.03
St→SI	24	**D→I**	19	**B→ULI**	0.0074
SI→I	300	**g→I**	1.6	**G→B**	0.0063
SI→ULI	6	**S→St**	10	**I→Br**[b]	4.3
I→D	48	**J→St**	20	**Br→M**[b]	2.0
I→g	2.3	**K→UB**	10	**I→O**	1.7E+1
I→G	2.3	**ULI→LLI**	1.8	**O→I**	5.9E+2
I→S	3.0	**LLI→F**	1.0		
I→J	8.6	**UB→U**	12		

a. The 1.7E+1 denotes 1.7×10^1

b. Non-zero for post-partum. Parameters are discussed in the section "Model for lactation"

St, stomach; SI, small intestine; ULI, upper large intestine, I, iodine; D, iodide in other; g, iodide; G, organic iodide; S, salivary glands; J, gastric secretory cells; K, kidneys; UB, urinary bladder; LLI, lower large intestine; F, feces; U, urine; B, organic iodine; Br, breasts; M, milk; O, ovaries.

In the classical Riggs's review [6] both the delay in absorption and the delay in distribution are disregarded. Riggs assumed that the radioactive iodine is instantaneously and evenly distributed throughout the iodine compartment. At the same time, Riggs indicated that "This assumption is obviously not legitimate if much importance is to be attached to the fate of radioactive iodine during the first hour or two after administration." The model developed in this study has demonstrated that the iodine early kinetics is a most important process, which governs the dose estimation for short-lived radioiodines, such as ^{132}I, ^{133}I, and ^{135}I.

Waune [13] stated that iodine in saliva is entirely inorganic, even in pathological cases, when significant amounts of the organic iodine are present in the urine. Putz et al. [14] reported a value 1 nmol l^{-1} as a mean level of thyroxine (T_4) in saliva. There was a good correlation between the saliva and serum T_4 concentrations (r = 0.74) and between saliva T_4 values and the T_4/(thyroxine-binding globulin) ratio (r = 0.83).

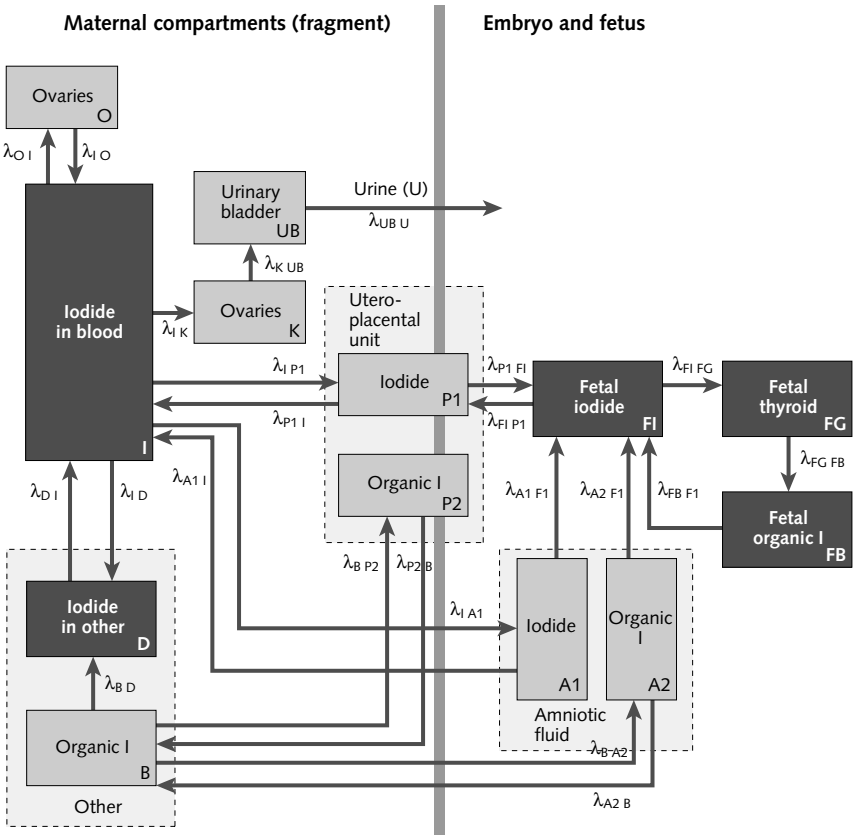

FIG 2. Iodine biokinetic model of the transfer of iodine to the fetus

Model parameterization

Values of 40 d^{-1} (about 3% $min.^{-1}$) and 300 d^{-1} were adopted in the model for iodine absorption from **St** and **SI**, respectively. The value for absorption from the stomach was based on the results of Egoroff et al. [8], and the value for intestinal absorption corresponds to the gut absorption coefficient $f_1 = 0.98$. The iodide flux with saliva and gastric juice can be estimated by equations $\varphi_{S\to St} = C_{SJ}K_S = 63$ μg d^{-1}, and $\varphi_{J\to St} = C_{SJ}K_J = 180$ μg d^{-1} where $C_{SJ} = 9$ μg d^{-1} is the iodide concentration in saliva or gastric juice, $K_S = 0.7$ *l* d^{-1} is the salivary gland clearance rate and $K_J = 2$ l d^{-1} is the gastric secretory cells clearance rate. The (saliva or gastric juice iodide)/(plasma iodide) concentration ratio of $N = 30$ has been adopted for setting iodide transfer rates: $\lambda_{I\to S} = K_S N / V_I = 3$ d^{-1}, and $\lambda_{I\to J} = K_J N / V_I \approx 8.6$ d^{-1}, where $V_I = 7$ l is the volume of the compartment **I** (the discussion see below). Values $\lambda_{S\to St} = K_S / V_S = 10$ d^{-1} and $\lambda_{J\to St} = K_J / V_J = 20$ d^{-1} have been used in the model ($V_S = 70$ ml and $V_J = 100$ ml are adopted in the model volumes of the iodide pool in secretory glands). In equilibrium the compartment **I** for non-pregnant women with a daily iodine intake of 150 μg d^{-1} has 21 μg of iodide (see below). Calculated in such manner transfer rates provide the required iodide flux with saliva and gastric juice.

Urinary excretion of iodide

The most important route of iodide excretion is by filtration through the kidney into the urine. Renal iodide clearance is remarkably constant for non-pregnant euthyroidal persons over all ranges of plasma iodine considered. In the euthyroidal state it is mostly the inorganic iodine component that is excreted in urine. Organic compounds are present in the urine in thyrotoxicosis and dyshormonogenesis (dehalogenase deficiency) only [13]. Moskalev [12] proposed a standard daily excretion rate of 135 μg d^{-1} for reference adults with a body weight of 70 kg.

Model parameterization

Renal clearance of iodide was assumed to be 45 ml min^{-1}, resulting in transfer rates from blood to kidneys (**K**) 6.4 d^{-1} ($\lambda_{I\to K} = K_U / V_I$). The rate of transfer from K to urinary bladder (UB) was set to 10 d^{-1} in order to maintain a kidney iodine concentration of 5 μg dl^{-1}. The rate of transfer from UB to urine (U) was taken to be 12 d^{-1}, corresponding to six voids per day [15].

Uptake of iodide by non-thyroidal tissues

Model parameterization

In the model the total maternal iodide distribution space was assumed to be 25 L [7, 16, 17], with 7 L cor-

responding to iodide in blood and 18 L corresponding to tissues other than the thyroid. The homogenization in blood (compartment **I**) is taken to be more rapid than diffusion towards the tissue compartment (**D**). Brownell [18] estimated a rate of exchange between **I** and **D** of 14 l h^{-1} and, accordingly, using the volumes of compartments **I** and **D**, transfer rates were calculated as 48 d^{-1} for **I** to **D** and 19 d^{-1} for **D** to **I**. For an iodine intake of 150 μg d^{-1}, the model predicts the contents of compartment **I** and **D** at equilibrium as $Q_I = 21$ μg and $Q_D = 57$ μg of iodide, corresponding to reported concentrations of about 0.3 μgdl^{-1} [8, 10, 13].

Ovaries

In the model, uptake of iodide by the ovaries is treated separately from uptake in other non-thyroidal tissues. There is some evidence that iodine is concentrated in the ovary, particularly during the phase of follicular development [9]. Laginskaya et al. [19] reported peak radioiodine uptake by ovaries of rats in the range 0.3% to 5%. Iodide uptake of 1% and clearance with a half-time of 1 hour have been adopted (uptake rate of 17 d^{-1}) for ovaries compartment.

Uptake of iodide by the thyroid and hormonal circulation

Thyroid

In contrast to renal clearance, thyroid clearance is sensitive to changes in the plasma concentration of iodide and varies greatly with the functional activity of the gland. In normal individuals, thyroid clearance averages 10 to 20 ml min^{-1}, while in cases of exophthalmic goiter, a clearance of over 100 ml min^{-1} and even over 1,000 ml min^{-1} is possible [8]. According to Aboul-Khair et al. [20], iodide uptake by the gland in the control group (non-pregnant women) is 42 μg d^{-1}. The organic iodine space in compartment **G** contains 8,000 μg of iodine [17].

Iodide is accumulated rapidly by the thyroid gland. Oxidation of iodide is a highly efficient process such that the concentration of I^- in the gland is about 0.3% to 0.4% of the concentration of organically-bound iodine [12]. The model includes an iodide compartment in the thyroid (**g**) with 30 μg of stable iodine, that exchange with the organic iodine compartment (**G**) through the blood (**I**) and is included as a means of fitting data on the short-term uptake of I^- by the thyroid. The model gives total uptake of 9% of iodine reaching blood after two hours and 25% after 24 hours, consistent with published data [21, 22]. The data of Mirkhodzhaev [22] are summarized in the table 2. The rates of transfer from **I** to **G** and **I** to **g** were taken to be equal and the rate from **g** to **I** fitted to correspond to the accumulation of 30 μg of stable iodide in **g**.

Model parameterization

The initial estimation of the thyroidal uptake rate was calculated by the equation $\lambda_{I\to G} = K_U S_I / InV_I$, where

TABLE 2. Average values of ^{131}I thyroidal uptake (ingestion) reported for persons living in iodine replete areas [22]

	Time after intake	
Thyroid status	2 hours	24 hours
Hypothyroidism	3%	5%
Euthyroidal	10%	25%
Diffuse goiter	41%	64%

K_U is renal clearance. S_I is the absolute iodine uptake by the gland (42 μg d^{-1}). V_I is the volume of the compartment **I** (7 l). *In* is the daily intake of stable iodine (150 μg d^{-1}). Further adjustments of $\lambda_{I\to G}$ were then performed to take into account the loss of iodine in feces.

Analysis demonstrates that the widely used one-compartmental presentation of the thyroid gland gives a substantial error (2 to 3 times) for the early thyroid uptake. The introduction into the model of a compartment **g** with 30 μg [22] of stable iodide adjusts the early thyroid uptake to values observed for euthyroidal persons. Fast exchange of iodide in the compartment **g** with blood acts simultaneously with processes of the thyroid hormones (THs) synthesis (compartment **G**). Both thyroid compartments (**g** and **G**) have the direct communication with 'iodide in blood' compartment **I**. It is assumed $\lambda_{I\to g} = \lambda_{I\to G}$; to provide the accumulation of the 30 μg of stable iodide in the thyroid of non-pregnant persons the value $\lambda_{g\to I}$ has been estimated as $\lambda_{g\to I} = Q_I \lambda_{I\to g} / 30$ μg.

Protein-bound iodine

Protein-bound iodine (PBI) in extrathyroidal tissues is represented in the model by compartment **B**. A volume of 25 L was adopted for **B** [17, 23]. For an iodine intake of 150 μg d^{-1} by a euthyroidal person the model predicts the content of iodine in circulating PBI in compartment **B** as 1,350 μg, corresponding to a concentration of 5.4 μg dl^{-1}, consistent with reported concentrations of 5 to 6 μg dl^{-1} [24]. The transfer rate from the thyroid was chosen to give hormonal iodine secretion of 50 μg d^{-1} [20].

Deiodination of thyroid hormone and return to the iodide pool is represented by the transfer **B** to **D** while fecal excretion of iodine via bile is represented by **B** to **ULI** (upper large intestine). Together these transfer rates govern the concentration of hormonal iodine in plasma during pregnancy. A value of 10 μg d^{-1} was used for transfer to ULI [17] and the transfer from **B** to **D** was chosen in order to ensure agreement with the clinical data [24].

Model for pregnancy

Pregnancy has been shown to be associated with increases in iodine uptake by the maternal thyroid gland and increases in concentrations of PBI in

maternal blood. The parameters of iodine turnover (renal and thyroidal clearance, plasma inorganic iodine concentration and absolute thyroid uptake) during the course of pregnancy have been reported elsewhere [20]. The daily stable iodine uptake by the thyroid during pregnancy was shown to vary from values similar to those in non-pregnant controls to increased values by about a factor of two (table 3). Berghout et al. [24] determined the levels of thyroid hormones in the blood at different gestation stages (table 4).

The structure of the model is presented in figure 2 and parameter values estimated for the euthyroid woman, under conditions of optimal intake of stable iodine (150 μg d^{-1}), are given in tables 5 and 6. The behavior of iodine in the embryo and fetus is then addressed, considering the fetal hormonal cycle, bidirectional transfer and accumulation by the placenta, and iodine transfer via the amniotic fluid.

Renal clearance during pregnancy is increased from 31 to 64 ml min^{-1}. Based on the data of Aboul-Khair et al. [20] on renal clearance $K_U(\tau)$ of iodide (table 1) the transfer rates from blood to kidneys (**K**) for different gestation stages were estimated using the equations described earlier. The time course of maternal thyroid clearance during pregnancy was chosen so that model predictions are consistent with the data of Aboul-Khair et al. [20]. The dynamics of ^{131}I in the placenta, amniotic fluid, fetal extrathyroidal tissues, and thyroid also have been studied by Aboul-Khair et al. [25]. Data are also available from other studies [26–30]. Radioactive iodide accumulates in the fetus before the thyroid starts functioning and is mainly concentrated in the liver and the intestine. The concentration of radioiodine in the fetal thyroid is always higher than in the mother's thyroid [25, 29]. During gestation the concentration of radioactive iodide in the fetal thyroid (**FG**) increases and towards the end of gestation it can exceed by 3- to 10-fold that in the maternal thyroid [31]. On the basis of human and animal data that fetal production of thyroxine (per kg body mass) during the last trimester will exceed that in the mother by 6 to 8 times [32–35], a range of 4 to 8 μg d^{-1} per kg body mass was assumed for the third trimester. The fetal thyroid at term was taken to have an iodine concentration of 450 μg g^{-1}, corresponding to 580 μg of iodine in a total mass of tissue of 1.3 g (fig. 3). Hormonal iodine production at term of about 6 μg per kilogram of fetal mass (3.5 kg total) was achieved by choosing a constant half-time of clearance from the gland of 20 days, i.e., a transfer rate of 0.035 d^{-1}. Hormonal iodine production throughout fetal development was calculated using data for the increase in fetal mass and uptake of ^{131}I by the fetal thyroid; values obtained were 0.7, 1.5, and 2.4 μg kg^{-1} fetal mass for gestational ages of 15, 20. and 25 weeks, respectively.

Iodine and circulating thyroid hormone within the fetus were modeled by compartments **FI** and **FB**, respectively. It was assumed that both compartments occupy 50% of the total fetal volume. At term, the reported concentration of PBI in the fetus of 7.2 μg dl^{-1} [34] corresponds to a total content of 130 μg. In determining the rate of iodine uptake by the gland, a concentration of 0.3 μg dl^{-1} of iodide at term in blood was assumed.

Placenta and amniotic fluid estimates of the total transfer of I^- from maternal to fetal circulations were based on experimental observations of fetal uptake of ^{131}I [25–30, 36]. Placental uptake peaks within 0.5 to 3 hours of the ^{131}I injection [25, 30]. Palmer and Preece [30] also reported a value of 1% for ^{131}I placenta

TABLE 3. Time course of iodine biokinetic parameters during pregnancy [20]

Measurement	Non-pregnant control	Weeks of pregnancy					Weeks post-partum		
		Up to 12	16	24	32	36	2	6	12
Renal clearance l $hr.^{-1}$ ± SEM	1.9 ± 0.22	2.9 ± 0.26	3.5 ± 0.14	3.7 ± 0.25	3.8 ± 0.19	3.4 ± 0.20	2.7 ± 0.35	2.0 ± 0.16	2.1 ± 0.08
Plasma inorganic iodide μg dl^{-1} ± SEM	0.2 ± 0.02	0.13 ± 0.02	0.12 ± 0.02	0.06 ± 0.02	0.08 ± 0.01	0.12 ± 0.03	0.11 ± 0.03	0.18 ± 0.04	0.15 ± 0.07
Absolute iodine uptake by gland μg d^{-1} ± SEM	42 ± 5	82 ± 14	82 ± 12	39 ± 5	50 ± 5	92 ± 22	61 ± 10	50 ± 10	40 ± 7

TABLE 4. Thyroid hormone (THs) during pregnancy in 10 healthy women in iodine replete area [24]

	Before	1st trimester	2nd trimester	3rd trimester
T4 (nmol/L)	105 ± 11	165 ± 16	153 ± 19	158 ± 26
T3 (nmol/L)	1.94 ± 0.3	2.88 ± 0.5	2.83 ± 0.5	2.88 ± 0.5
rT3 (nmol/L)	0.21 ± 0.06	0.31 ± 0.08	0.26 ± 0.05	0.32 ± 0.08
Iodine in THs (μg/dl)	5.4 ± 0.6	8.5 ± 0.8	7.9 ± 1.0	8.1 ± 1.3

TABLE 5. Time-dependent parameters of the model for pregnancy (d^{-1}) [a]

	Time (wk)[b]								
	0	3	11	12	16	24	32	36	38
I→G	2.3E+0	5.1E+0	6.5E+0	7.5E+0	8.4E+0	9.3E+0	9.1E+0	8.8E+0	8.7E+0
I→K	6.4E+0	7.0E+0	8.9E+0	1.0E+1	1.2E+1	1.3E+1	1.2E+1	1.2E+1	1.2E+1
I→P1	0.0E+0	0.0E+0	3.3E+1	3.3E+1	3.3E+1	3.3E+1	3.3E+1	3.3E+1	3.3E+1
I→A1	0.0E+0	7.5E–1	7.5E–1	7.5E–1	7.5E–1	7.5E–1	7.5E–1	7.5E–1	7.5E–1
B→D	3.0E–2	3.8E–2	3.8E–2	3.8E–2	3.8E–2	3.8E–2	3.8E–2	3.8E–2	3.8E–2
B→ULI	7.4E–3	9.4E–3	9.4E–3	9.4E–3	9.4E–3	9.4E–3	9.4E–3	9.4E–3	9.4E–3
B→P2	0.0E+0	0.0E+0	1.8E+1	2.0E+1	2.4E+1	3.1E+1	4.0E+1	4.6E+1	5.1E+1
B→A2	0.0E+0	5.5E+0	5.5E+0	5.5E+0	5.5E+0	5.5E+0	5.5E+0	5.5E+0	5.5E+0
P1→I	1.1E+4	1.1E+4	1.1E+4	3.6E+3	1.4E+3	5.1E+2	2.7E+2	1.9E+2	1.6E+2
P1→FI	0.0E+0	0.0E+0	0.0E+0	3.3E+1	3.3E+1	3.3E+1	3.3E+1	3.3E+1	3.3E+1
P2→B	1.8E+4	1.8E+4	1.8E+4	7.6E+3	3.9E+3	1.9E+3	1.5E+3	1.5E+3	1.6E+3
FI→P1	0.0E+0	0.0E+0	0.0E+0	1.0E+2	6.0E+1	3.5E+1	2.0E+1	1.5E+1	1.2E+1
FI→FG[c]	0.0E+0	0.0E+0	0.0E+0	1.8E+0	3.5E+0	5.0E+0	6.0E+0	6.0E+0	6.0E+0
G→B	6.3E–3	1.3E–2	1.3E–2	1.3E–2	1.3E–2	1.3E–2	1.3E–2	1.3E–2	1.3E–2
A1→I	1.3E+2	1.3E+2	1.3E+2	4.3E+1	1.7E+1	6.0E+0	3.2E+0	2.2E+0	1.9E+0
A1→FI	0.0E+0	0.0E+0	0.0E+0	2.5E–1	2.5E–1	2.5E–1	2.5E–1	2.5E–1	2.5E–1
A2→B	6.4E+3	6.4E+3	6.4E+3	2.3E+3	9.9E+2	3.8E+2	2.3E+2	2.0E+2	1.9E+2
A2→FI	0.0E+0	0.0E+0	0.0E+0	2.5E–1	2.5E–1	2.5E–1	2.5E–1	2.5E–1	2.5E–1

a. The 1.7E+1 denotes 1.7×10^1

b. Time after conception.

c. This rate is zero until 11 weeks. It is then linearly interpolated between zero and the value at 12 weeks. All other rates are linearly interpolated throughout.

TABLE 6. Time-independent transfer rates (d^{-1})

St→I	4.0E+1	**I→O**	1.7E+1	**FB→FI**	1.3E–1
St→SI	2.4E+1	**D→I**	1.9E+1	**LLI→F**	1.0E+0
SI→I	3.0E+2	**g→I**	1.6E+0	**UB→U**	1.2E+1
SI→ULI	6.0E+0	**S→St**	1.0E+1	**ULI→LLI**	1.8E+0
I→D	4.8E+1	**J→St**	2.0E+1	**I→g**	2.3E+0
I→S	3.0E+0	**K→UB**	1.0E+1	**O→I**	5.9E+2
I→J	8.6E+0	**FG→FB**	3.5E–2		

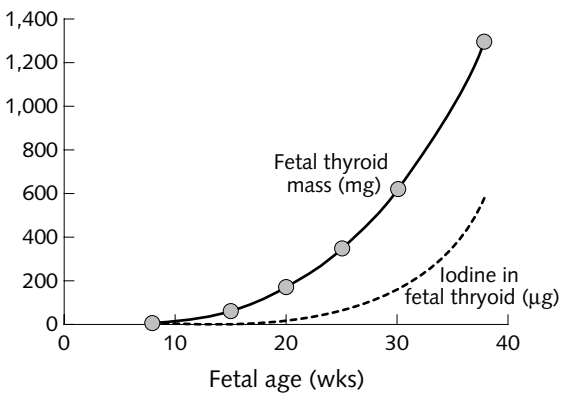

FIG. 3. Time course of the fetal thyroid mass and the assumed iodine accumulation in the thyroid gland.

uptake in guinea pigs at the third stage of gestation. To reproduce the clinically observed rapid accumulation of radioiodine in the placenta and fetus an average half-time of 0.5 hours was assumed for transfer from compartment **I** to **P1** and from **P1** to **FI** (fig. 2). The rate of the recycled flow **P1** to **I** was set to fit the available data for short-term retention in the placenta.

The transfer rate **B** to **P2** was calculated as: $\lambda_{B\to P2} = K_P / V_B$, where K_P is the uteroplacental blood flow. K_P reaches the value 900 ml min^{-1} at term, so $\lambda_{B\to P2} \approx 51\ d^{-1}$ (K. Eckerman, Oak Ridge National Laboratory, Tenn., USA, personal communication, 1998). The rate of the recycled flow **P2** to **B** was set to fit data for the longer-term retention of iodine in the placenta. Data for the mass and blood content of the placenta at different stages [12] have been used to provide additional verification of estimated transfer rates.

Tomoda [37] determined the dynamics of amniotic fluid and fetal swallowing rate in sheep; clearance rates averaged 5% h^{-1} and ingestion of fluid by the fetus was about 1000 ml d^{-1}. On the basis of these data, values $\lambda_{I\to A1} = 0.75\ d^{-1}$ and $\lambda_{A2\to F1} = 0.25\ d^{-1}$ were adopted. A half-time of 3 hours was assumed for transfer rate B to A2; 5.5 d^{-1}. Rates $\lambda_{A1\to I}$ and $\lambda_{A2\to B}$ were derived to fit the available data on retention of iodine in amniotic fluid.

Model for lactation

The protection of the fetus and the embryo with respect to its iodine requirements is carried over into the immediate postnatal period with the marked excretion of iodine in the milk of the lactating mother, thereby constituting a major pathway for the elimination of iodine. In the WHO/IAEA report [38] the large statistical data about the concentration of stable iodine in breastmilk is reported. For most countries the observed values were in the range of 1 to 100 μg dl^{-1}. ICRP [11] gives the range of 2 to 15 μg dl^{-1} with an average of 7 μg dl^{-1}. Up to 90% of injected radioiodine can be lost by this route within 48 hours after administration. Milk to plasma iodide ratios can reach 40 and this concentration gradient can be abolished by treatment with thiocyanate, stable iodine, or perchlorate. Although a high iodine level serves to provide the newborn with an adequate iodine intake, maternal iodide stores may become depleted to a point where the mother exhibits deficiency symptoms. Enlargement of the thyroid gland has been observed in lactating women [9].

Model parameterization

Transfer rates have been estimated in the model in the assumption of the milk/plasma concentration ratio = 35, milk clearance $K_{milk} = 0.85\ l\ d^{-1}$ [11], and the effective volume of mammalian glands $V_m = 0.42$ l: $\lambda_{I \to Br} = 35K_{milk} / V_I \approx 4.3\ d^{-1}$, $\lambda_{Br \to Milk} = K_{milk} / V_m \approx 2\ d^{-1}$.

Model predictions

There is good agreement between model predictions and clinical data for fetal thyroid uptake (fig. 4). A 'conservative' data fit was applied at the early stages of gestation in order to prevent underestimation of radiation doses to the fetus. The model predicts fetal thyroid uptake at term of about 8%, which is in good agreement with that reported elsewhere [30]. For humans, this value corresponds to "fetal to mother concentration ratio" CF:CM ratios of about 3 to 4; Book and Goldman [39] reported such values as typical for the last trimester.

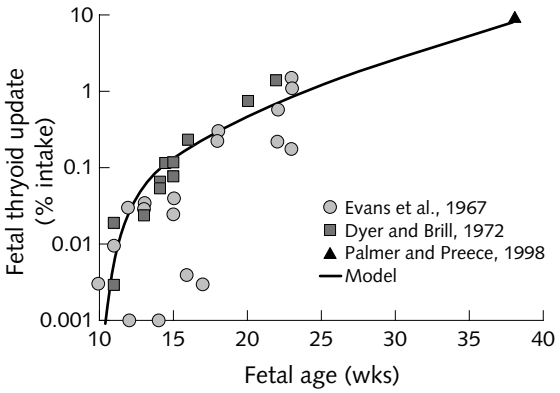

FIG. 4. Uptake of ^{131}I by the fetal thyroid and model predictions. Data are for humans except for the study of Palmer and Preece [30] using guinea pigs.

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Energy requirements and body composition in stable pediatric intensive care patients receiving ventilatory support

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Abstract

Energy requirements of pediatric intensive-care patients are unknown due to the difficulty of measuring total energy expenditure in free-living conditions. We investigated energy expenditure and body composition in stable pediatric intensive-care patients receiving long-term ventilatory support. Total energy expenditure and total body water were measured in 10 such patients using the doubly-labeled water method. The patients had significantly lower energy expenditure than healthy children of the same age. Relative to length, fat-free mass deposition was significantly lower, and fat deposition was significantly greater, than in healthy subjects. In general, total energy requirements of these patients are significantly reduced as compared to healthy children, which can be attributed to their lower activity levels and their reduced muscle mass. Although they gain weight similar to healthy children, their weight gain is disproportionately higher in fat. However, one patient with myofibromatosis contradicted this general pattern.

Key words: fat-free mass, body fat, total energy expenditure, doubly-labeled water, stable isotopes

Introduction

Energy intake is a vital component of patient management in pediatric intensive care. Energy is required for basal metabolism, growth, and the level of physical activity allowed or imposed by the disease state. In contrast to most nutrients, there is only one level of energy intake that is appropriate for each individual given their body composition, health status, and activity level [1], and inappropriate provision of energy may lead to excess weight gain or to growth being constrained.

Energy requirements are primarily determined by absolute body size, body composition, and activity level, with a disease state also potentially exerting additional effects. Of these factors, only the first is readily measured in typical intensive-care unit (ICU) conditions. The ability of body weight to predict energy requirements is poor even in healthy infants [2], and is further reduced if underlying body composition is significantly different from normal. For example, if body fatness is low, a larger than expected proportion of body weight may require energy. Conversely, if fatness is high, the relative proportion of fat-free mass (FFM) and hence energy requirements will be reduced. Information on body composition would clearly help clinicians to estimate energy requirements, however few methods are suitable for young patients, and those that are simple to perform have poor accuracy and precision.

Activity level also poses a significant problem. Many stable ICU patients are not bed-bound, and so measurement of resting metabolic rate by classical indirect calorimetry is neither an appropriate nor a feasible source of information on total daily energy requirements. At the same time, their activity level may be reduced as compared to that of healthy children of the same age, due to the constraints of the hospital environment and the disease-state. For example, ventilated patients with chronic lung disease are not bed-bound, but are constrained physically by the artificial ventilator to which they are attached, and physiologically by the condition of their lungs.

Estimation of energy requirements in such patients is therefore extremely difficult. Dietitians attempt to provide intakes consistent with normal weight gain, but are unable to confirm that this weight gain reflects the composition found in healthy subjects. Management is obliged to proceed on the basis of trial and error, using indications of excess or inadequate weight gain to mold

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intake to perceived requirements.

We measured total energy expenditure (TEE) and body composition, using the doubly-labeled water method, in pediatric patients receiving long-term artificial ventilation. The study tested two hypotheses—that body composition in these patients is significantly different from that of healthy subjects and that energy requirements are reduced compared to those of healthy subjects.

Methods

Patients were recruited into the study from the ICU and transitional care wards at Great Ormond Street Hospital, London. Those eligible for participation were patients whose condition was stable, but who required long-term ventilatory support. Ethical permission was granted by the research ethics committee of Great Ormond Street hospital. The study was initially intended to measure 12 patients, but will be continued in the future in order to obtain further information about specific disease states. This paper refers to the first 10 patients measured.

Weight was measured where possible with Seca 727 electronic scales (CMS Weighing Ltd., London, UK), or, where this was beyond patient capability, using a weighing chair. Length was measured with a Rollametre (Raven Equipment Limited, Essex, UK). Skinfold thickness was measured at the triceps and subscapular sites using Holtain calipers. Weight, height, body mass index (BMI), and skinfold standard deviation (SD) scores were calculated using current UK reference data [3–5].

TEE and total body water (TBW) were measured using the doubly-labeled water method, described in detail elsewhere [6]. Briefly, a dose of $^{2}H_{2}{}^{18}O$ was given by nasogastric or gastrostomy tube, and flushed with sterile water. Urine samples were collected predose and on days 1, 2, 3, 5, 6, and 7 post-dose, with the time of the post-dose samples known to within 30 minutes. Urine samples were analyzed for ^{2}H and ^{18}O enrichment using isotope-ratio mass spectrometry. All samples were analyzed in duplicate.

Isotope dilution spaces and rate constants were calculated, allowing calculation of total energy expenditure using established equations [6]. Fat-free mass (FFM) was calculated by adjusting total body water (TBW) for the water content of fat-free tissue [7]. Fat mass (FM) was calculated as weight minus FFM. Both FFM and FM were adjusted for height by dividing by height squared to give the fat-free mass index (FFMI) and fat mass index (FMI) [8, 9].

Reference data for TEE, FFMI and FMI were derived from previously published data [10–12]. Smoothed curves were obtained for mean ±1SD. Individual data points for patients were plotted on graphs of the reference data. Difference from the age-specific mean was calculated for all variables. Compared to hypothesized differences between patients and controls, sex-differences in body composition and energy expenditure were relatively minor [11, 12], and the reference data was therefore not split by sex.

Results

All patients invited to take part in the study agreed to participate, and all successfully completed the protocol. Age, anthropometric SD scores, and diagnosis of the patients are given in table 1. Mean (SD) standard deviation scores were –0.33 (2.00), 0.15 (3.08), and –0.53 (1.68) for weight, height, and BMI, respectively. These mean values were not significantly different from zero.

Total energy expenditure was lower than the average for healthy children of the same age (mean (SD) deficit 122 (200) kcal/day; $p = .086$). This difference was significant when expressed per kg body weight (deficit 12.8 (18.5) kcal/kg/day; $p = .020$) (fig. 1), but not when expressed per kg FFM (deficit 1.66 (2.49) kcal/kg/day; $p = .81$) (fig. 2).

FFMI was likewise reduced (deficit 1.66 (2.49) kg/m^2; p = 0.064) (fig. 3). Mean FMI was higher than

TABLE 1. Disease state, age, sex and anthropometric status of the 10 patients

Disease state Skinfold SD score[a]	Age (yr)	Sex	Weight SD score	Height SD score
Tracheobroncho-malacia 1.06	1.25	M	–2.23	–3.32
Tracheobroncho-malacia 1.28	1.25	M	0.16	–0.02
Tracheobroncho-malacia 2.12	1.25	M	–2.55	–3.19
Tracheobroncho-malacia 2.09	2.00	M	1.08	0.68
Disseminated myofibromatosis –0.50	0.50	M	–3.81	–6.32
Congenital myasthenia 2.21	0.35	F	–0.16	4.01
Congenital myasthenia 2.56	0.90	F	–1.14	1.07
Neuropathy 2.12	1.15	M	0.14	0.74
Neurometabolic problem 1.62	0.50	F	–1.56	–0.41
Spinal cord injury –	3.25	F	1.54	0.87

a. Mean of triceps and subscapular values.

in healthy subjects (excess 1.21 (1.93) kg/m^2; $p = .08$) (fig. 4). The majority of the patients conformed to this pattern, however, the patient with myofibromatosis contrasted markedly and had significantly higher FFMI (> 2SD scores) and significantly lower FMI (< –2SD scores). When this patient was excluded, the results for body composition achieved statistical significance (FFMI deficit 2.26 (1.68) kg/m^2; $p < .004$; FMI excess 1.66 (1.40) kg/m^2; $p < .008$).

Discussion

On average, stable patients receiving long-term artificial ventilation in the ICU require less energy than healthy subjects. Some of this deficit can be attributed to differences in body composition and some to reduced activity levels. However, within this general pattern it is apparent that not every disease state has the same effect. Thus, further research is required to provide more detailed data for specific disease states.

As a group, the patients had low levels of FFM deposition relative to height. This may be due to the lack of stimulation of muscle growth due to physical exercise, as well as possible effects of disease states themselves on growth. However, the patient with disseminated myofibromatosis contradicted this general pattern, having significantly higher FFMI than healthy subjects. This enhanced FFM deposition may be attributed to tumor growth in his muscles.

The dietetic management of these ICU patients aimed to achieve normal rates of weight gain. This is reflected in their mean Z score for weight of near zero, although the range in individuals was from –3.8 to 2.9. However, given the low FFM deposition, the normal weight is associated with high levels of fat. We have

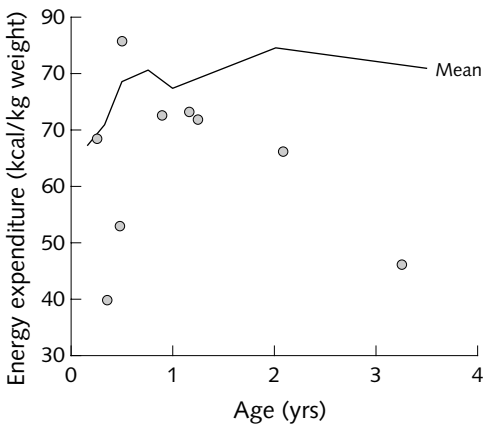

FIG. 1. Total daily energy expenditure expressed per kg body weight in patients (filled circles) as compared with mean of reference data (line)

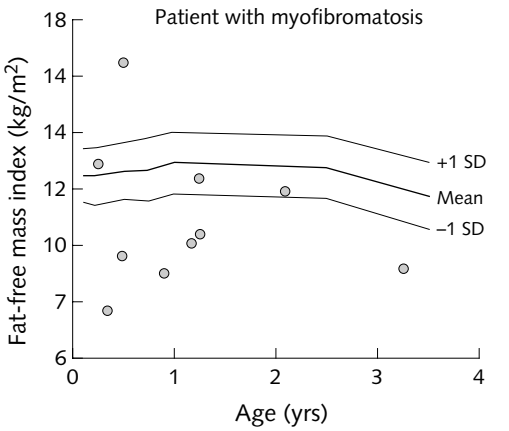

FIG. 3. Fat-free mass index (fat-free mass adjusted for body length) in patients (filled circles) as compared with mean ± SD of reference data (lines)

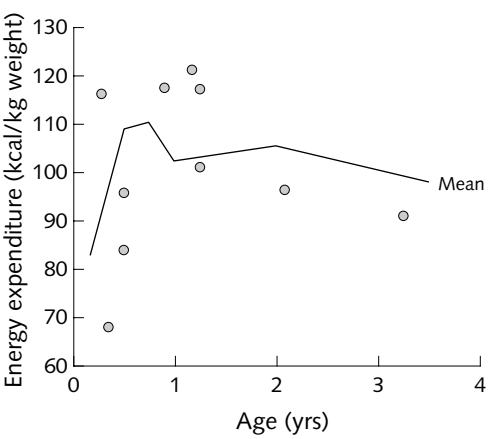

FIG. 2. Total daily energy expenditure expressed per kg fat-free mass in patients (filled circles) as compared with mean of reference data (line)

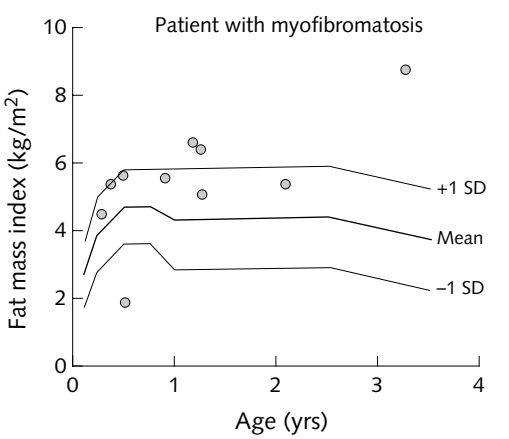

FIG. 4. Fat mass index (fat mass adjusted for body length) in patients (filled circles) as compared with mean ± SD of reference data (lines)

expressed data relative to length rather than as percentage of fat because the low FFM deposition would artificially inflate the percentage of fat [9]. However, even avoiding this artefact, fatness was significantly higher than in healthy children. The most extreme results were found in the three least active patients with congenital myasthenia ($n = 2$) and spinal cord injury, who had the lowest FFM deposition and the highest FM deposition. Excess body fat despite normal weight is likely to be found in many hospital patients, but will not be detected unless body composition, rather than anthropometry, is measured. Again, the patient with myofibromatosis conflicted with this pattern, having significantly depleted levels of fat.

The low levels of FFM in these patients, along with the reduced activity levels, are likely to explain the reduced TEE and TEE per kg of body weight. The fact that TEE per kg of FFM is similar to that of healthy children, despite the difference in activity level, is probably due to a shift in the ratio of organ tissues to muscle tissues within the FFM. Organ tissues have higher mass-specific metabolic rate [13], so if limb muscle mass is constrained compared to organ mass, energy expenditure per unit of FFM is increased.

Measurement of body composition by isotope dilution requires adjustment of total body water for the hydration of FFM. In our study, we assumed FFM hydration to be the same as in healthy subjects, however, this may not be true. For example, a change in the organ: muscle tissue ratio of FFM is predicted to alter FFM hydration, as organs have a higher water content. Thus, our values for FFM and FM may be biased. Nevertheless, our findings of enhanced FMI are supported by the skinfold SD scores, which are on average significantly greater than zero, but depleted in the subject with myofibromatosis.

Our study highlights the limitations of basing energy requirements of patients on body weight alone, and demonstrates important associations between body composition, energy utilization, and disease state.

Acknowledgements

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Body composition in Mexican adults by air displacement pletismography (ADP) with the Bod-Pod and deuterium oxide dilution using infrared spectroscopy (IRS-DOD)

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Abstract

Thirty four subjects (13 men and 21 women), 24 to 70 years old from northern Mexico, were measured for body density by air displacement plethysmography (ADP) with the BOD-POD, and for total body water by deuterium oxide dilution and infrared spectroscopy (IRS-DOD). Subjects were given a 30 g dose of deuterium oxide. Saliva samples were filtered, sublimated, and deuterium was measured using a Miran 1 FF, IRS. Linear regression of the fat mass (FM) derived from both methods showed that the intercept (0.071) was not different from zero ($p = .96$) *and the slope was 0.96* ($p < .0001$) *demonstrating the techniques to be equivalent. Further, mean FM was 26.7 ± 12.4 and 25.6 ± 12.4 kg, for IRS-DOD and ADP techniques, respectively* ($p = .08$). *Precision analysis by the model R^2 showed that 92.3% of the variability was explained (SEM = 3.4 kg). Bland-Altman analysis showed no significant bias* ($r = 0.017$; $p = .93$). *Mean difference between methods was –1.08 (CI: –2.3 to + 0.13) kg FM.*

Key words: body composition, density, deuterium dilution, infrared

Introduction

Until recently, most body composition studies in developing regions had been limited to the use of anthropometric techniques or unvalidated bioimpedance analysis. The use of dilution principles to measure total body water using deuterium oxide has also been restricted due to elevated costs of mass spectrometry equipment and its operation. Deuterium quantification by infrared spectroscopy (IRS) for both body composition and lactation studies has been shown to be an alternative procedure [1–3]. Laboratories in developing regions could consider the use of this methodology for research and the evaluation of nutrition intervention programs. Air displacement plethysmography with the BOD-POD is a new densitometric technique that has been found to be accurate to measure body composition in healthy adults under the two compartment model [4–8]. Measurements in the BOD-POD are practical and the subject does not have to be immersed in water or go through complicated residual lung volume determinations [9].

The purpose of this study was to examine the accuracy, precision, and bias of body fat estimation using deuterium oxide dilution measured by infrared spectroscopy, relative to body density by air displacement plethysmography.

Methods

Thirty-four healthy subjects, 21 women and 13, men consented to participate in this study. The research protocol was approved by the internal-external Ethical Committee of Centro de Investigación en Alimentación y Desarrollo, where the study took place. Subjects were dosed with 30g deuterium oxide. Saliva samples were collected before dosing and after a four hour equilibration period.

Anthropometry

Weight was measured on an electronic scale of 150 kg ± 0.01 kg capacity, attached to the air displacement plethysmograph BOD-POD (BOD-POD Body Composition System, Life Measurement Instruments, Concord, Calif., USA). Height (cm) was measured using a Holtain stadiometer (Holtain Limited, Dyfed, UK). Body mass index (BMI, kg/m^2) was calculated from these measurements. Waist circumference was measured in the supine position at the umbilicus level

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using a fiber glass measuring tape (Lafayette Instruments Company Inc., Ind, USA) and hip circumference was measured at the level of the most prominent part of the gluteus. The waist/hip ratio was determined from these measurements [10].

Body composition by infrared spectroscopy and deuterium oxide dilution (IRS-DOD)

Sample sublimation

A simple sublimation system connected to a freeze drier was used to obtain water from saliva [3]. Each frozen saliva sample was placed inside an ice bath. Vacuum was applied and sublimated water was condensed in a trap to constant temperature (–30°C). Deuterium in different concentrations was completely recovered in condensation flasks to avoid fractionation. Rate of sublimation for saliva was 1 ml/hour.

Deuterium oxide measurements by IRS

Deuterium abundance was measured by IRS with a calcium fluoride (CaF_2) cell (Miran 1FF, Foxboro Co., USA). Measurements were taken after filtration (0.22μm) at 2513 cm^{-1} under controlled temperature (15°C). A programmable voltmeter (Fluke Corporation, Mod. 83. Ill., USA) was installed to the detector signal for digitizing and averaging absorbance measurements. A calibration curve was checked every day using a deuterium stock solution (2000 ppm). Each dilution or sample was loaded into the cell using a syringe. All standards and sublimated samples were measured in duplicate after adjusting the absorbance to 0 with water or basal saliva. Fat free mass was calculated from total body water using a hydration constant of 0.73 [11].

Densitometry by air displacement plethysmography

Body density was measured using air displacement plethysmography (BOD-POD Body Composition System, Life Measurement Instruments, Concord, Calif., USA). The BOD-POD has a single structure containing two chambers. A volume perturbation device situated between the two chambers, electronically controlled by a servo system, produces pressure fluctuations in both chambers which are used to assess the total body volume. The system has been described in detail elsewhere [12]. Each subject was weighed on a calibrated scale to a resolution of 0.01 kg. A two-point calibration was then performed at baseline, with the chamber empty and using 50 liters as the calibration for the cylinder. With this data a regression equation that relates volume to ratio of the pressure in the two chambers was developed. The subject entered the chamber in a tight swimming suit and cap and sat down for the volume measurements. Thoracic gas volume (TGV) was also measured as part of the test process [9]. The TGV includes the lung air volume and any air that can be trapped in the thorax. This evaluation is very similar to the plethysmographic measurement of TGV used in pulmonary function testing [12]. Siri's equation was used for calculation of body density [13].

Statistical methods

Data were analyzed using the statistical program NCSS 1997 (Number Cruncher Statistical System for Windows, Kaysville, Utah, USA). All results are expressed as mean ± standard deviation (SD). The accuracy of the IRS-DOD technique was examined by regression procedures. The comparison was considered accurate if the regression between fat mass by ADP with the BOD-POD and the IRS-DOD had a slope not significantly different from 1.0, and an intercept not significantly different from zero. Precision was assessed by the model R^2 and the standard error of the estimate from the regression procedures described above. Bias was examined using Bland and Altman's procedure [14]. This procedure tests the hypothesis that methodological error is randomly distributed across the spectrum of body fat content, as indicated by a non-significant regression between technique error and body fat content.

Reproducibility of body density measurements and validation of TGV has been reported elsewhere [9]. The reproducibility of our BOD-POD was tested in 91 duplicate measurements in the same subject during the same session. Mean body densities of groups 1 and 2, were 1.0292 ± 0.019 and 1.0296 ± 0.019, respectively. Both sets of measurements had a coefficient of variation of 1.89%.

Results

The characteristics of subjects are presented in table 1. Considering both men and women together there was a wide range of body size and composition. Body weight ranged from 45 to 114 kg and BMI from 17 to 47. Mean and range of body mass index and percent of body fat by IRS-DOD were 26.5 (17.3 to 47.1) and 35.5 (8.8 to 52.8), respectively. The percent of body fat was calculated by each method based on a two-compartment model in each case

Linear regression of the fat mass (FM) derived from IRS-DOD and ADP methods showed that the intercept (0.071) was not different from zero ($p = .96$) and the slope was 0.96 ($p < .0001$) demonstrating the tech-

TABLE 1. Characteristics of study group

	Females (n = 21)			Males (n = 13)		
	Mean	SD	Range	Mean	SD	Range
Age (yr)	37.1	10.8	24–57	39.2	13.8	27–70
Weight (kg)	70.2	17.4	45.3–114.7	75.1	19.5	47.9–108.6
Height (m)	1.6	0.06	1.5–1.71	1.72	0.05	1.66–1.80
BMI (kg/m^2)	27.2	6.6	18.3–47.3	25.1	6.28	15.5–34.0
Waist/hip	0.77	0.05	0.7–09	0.91	0.06	0.83–1.03
Deuterium oxide						
Total body water(kg)	29.7	4.6	23.8–42.1	38.6	7.4	28.1–53.1
Fat-free mass (kg)	40.7	6.4	32.7–57.7	52.9	10.1	38.4–72.8
Fat mass (kg)	30.2	13.2	4.0–56.9	22.1	10.1	8.9–35.8
BOD POD						
Density	1.013	0.018	0.988–1.056	1.040	0.020	1.009–1.066
Fat mass (kg)	28.4	12.3	8.6–58.7	21.2	11.8	6.9–44.1

niques to be equivalent (fig. 1). Further, the mean FM was 26.7 ±12.4 and 25.6 ± 12.4 kg, for IRS-DOD and ADP techniques, respectively (p = .08). Precision was examined by the model R^2 and the standard error of the estimate, regressing FM by IRS-DOD and FM by ADP. This analysis showed that 92.3% of the variability was explained, however the standard error (SEE) of the estimate was 3.4 kg. Using Bland and Altman's analysis we examined the discrepancy between the techniques as a function of FM and there was no significant bias as determined by the two methods (r = 0.017; p = .93) (fig. 2). Mean difference between methods was –1.08 (CI: –2.3 to +0.13) kg FM.

Discussion

The wide range of body size and composition of the subjects were within the expected values for the population in this region of Mexico. The procedure used for the dosing and time of equilibrium is similar to that reported by others [1]. The estimation of total body water and of fat-free mass with a standard hydration constant (0.73) allowed for the calculation of the fat mass using a two compartment model approach. In this study we used densitometry with the BOD-POD as the reference procedure. The comparison of deuterium oxide dilution procedure with BOD-POD, showed a 1.6% of difference in the percent of body fat. The methods were not statistically different. This was tested directly using the fat mass compartment. The relationship of the two methods did not differ from the line of identity since the intercept was not significantly different from zero or the slope different from 1.0, showing methods to be equivalent. Precision analysis determined from the model R^2 explained 92.3% of the variability. However, the SEE was 3.49 kg, which is somewhat higher than similar studies have reported at

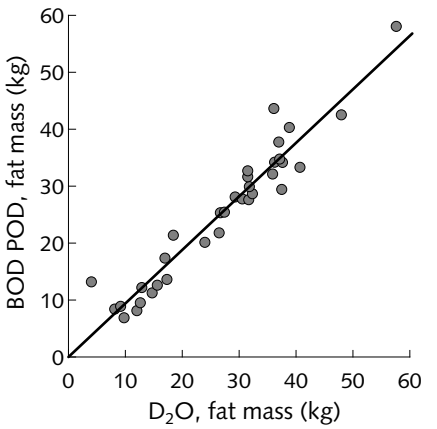

FIG 1. Comparison of fat mass (kg) by BOD POD and D_2O. The solid line is the line of identity

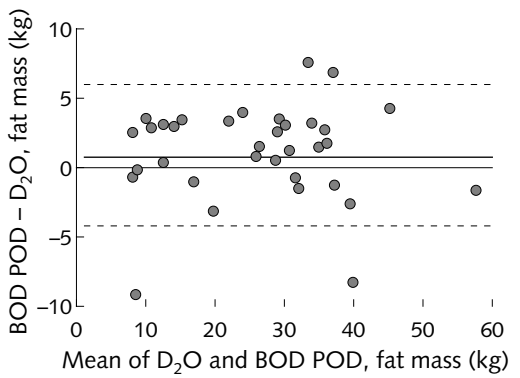

FIG. 2 . Plot of intermethod differences between fat mass (kg) values versus their mean values in healthy men and women (n = 34, age = 38.2 ± 11.78683)

this level of R^2 with values of 2.4 kg of fat mass [15].

Using Bland-Altman's analysis [14] we examined the discrepancy between the techniques as a function of FM and found no significant bias. Still, this is a preliminary study with a small number of subjects and further validation of our IRS-DOD method is required by comparison with mass spectrometry quantification of deuterium oxide.

Alternative methods for measuring body composition under field conditions in developing regions without access to gold standard techniques such as deuterium dilution by isotope ratio mass spectroscopy, are very important. They are required to evaluate the impact of nutrition intervention programs and to assess risk in nutrition-related diseases. Infrared spectroscopy aided by a simple sublimation system is a plausible analytical procedure.

Aknowledgements

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Application of isotope dilution technique in vitamin A nutrition

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Abstract

The isotope dilution technique involving deuterated retinol has been developed to quantitatively estimate total body reserves of vitamin A in humans. The technique provided good estimates in comparison to hepatic vitamin A concentrations in Bangladeshi surgical patients. Kinetic studies in the United States, Bangladesh, and Guatemala indicated the mean equilibration time of 17 to 20 days irrespective of the size of hepatic reserves. Due to the controversy surrounding the efficacy of a carotene-rich diet on improvement of vitamin A status, the isotope dilution technique was proposed to pursue this research question further (IAEA's coordinated research program). In the Philippines, schoolchildren with low serum retinol concentrations showed significant improvement in total body vitamin A stores following intake of carotene-rich foods (orange fruits and vegetables), using a three-day deuterated-retinol-dilution procedure. When Chinese kindergarten children were fed green and yellow vegetables during the winter, their total body vitamin A stores were sustained as compared to a steady decline of vitamin A stores in the control children. Likewise, daily consumption of purified beta-carotene or a diet rich in provitamin A carotenoids were shown to prevent a loss in total body vitamin A stores among Thai lactating women during the rice-planting season. These studies demonstrate potentials of the isotope dilution technique to evaluate the impact of provitamin A carotenoid intervention programs.

Key words: isotope dilution, vitamin A, body stores, provitamin A carotenoids, dietary intervention

Introduction

Vitamin A deficiency represents a continuum of stages beginning from poor dietary intake to depletion of body stores followed by reduction in circulating and tissue levels which later result in functional impairment (vision, epithelial differentiation, immune system, etc.) and finally, the eruption of clinical signs or xerophthalmia [1]. Assessment of vitamin A status is critical to program design, implementation, monitoring, and evaluation. Biochemical methods to assess vitamin A status depend on the metabolic pattern of vitamin A. Under normal physiological conditions, dietary vitamin A in the form of retinyl esters is hydrolyzed, in the intestinal lumen, to retinol which is well absorbed in the presence of fat. Retinol is re-esterified in the intestinal mucosa and incorporated into chylomicra prior to release into the circulation. Through the degradation by lipoprotein lipase, retinol is released, taken up, and stored in the stellate cells of the liver as retinyl esters which accounts for about 80% of total vitamin A reserves in the body. Hepatic vitamin A stores in adult men turn over slowly with a half-life of 126 to 140 days. To release the stores, retinol combines with retinol-binding protein (RBP) in the parenchymal cells of the liver and is released into the plasma as holo-RBP. As vitamin A stores decrease, the body turns to conservation of the vitamin A by reducing the catabolism of vitamin A, resulting in less excretion of vitamin A metabolites in the feces and urine [2, 3]. Such homeostatic regulations can be disrupted by the presence of infections, primarily through the reduction of plasma retinol concentrations, enhancement of catabolic rate, and increased urinary excretion.

Isotope dilution technique

Vitamin A concentration in the plasma is widely used to assess vitamin A status. However, the level of circulating vitamin A is under a homeostatic control over a broad range of body stores and only reflects status when the stores are very low or very high. Although hepatic concentration of vitamin A is considered the best indicator of vitamin A status, direct biopsy of the

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liver is not feasible for a population study. Indirect biochemical assessments, such as the dose response test, are useful to identify the at-risk groups in need of intervention but they cannot provide quantitative estimates of vitamin A stores [4]. The isotope dilution technique has been developed to determine body stores of vitamin A [5]. The method is based on the equilibration of newly absorbed vitamin A, labeled with isotopic tracers with the existing body pool of vitamin A after which the body pool can be calculated by a dilution equation. A known oral dose of vitamin A in oil will equilibrate well with total body reserves of vitamin A after 25 days or so whereas the ratio of deuterated to non-deuterated retinol in the plasma reflects total body stores. The isotopic dilution will be great if the initial reserves are large while a smaller isotopic dilution reflects lower body stores. The technique has been shown to provide a good estimate of liver reserves in Bangladeshi surgical patients despite a wide prediction interval for individual subjects [6]. A study on the kinetics of an oral dose of deuterated retinyl acetate in healthy subjects in the United States as well as in Bangladeshi women (24 to 36 years old) indicated a mean equilibration time of 16 to 17 days [7] which was not affected by the size of hepatic reserves. A recent study in Guatemalan elderly [8] showed that equilibration occurred by 20 day post-dosing which is in agreement with the former report. Based on this evidence, total body vitamin A stores can be estimated according to the principles of isotope dilution, a set of assumptions regarding retention of the labeled dose, the ratio of specific activities of plasma to liver vitamin A, and the irreversible loss of vitamin A over time. Total body reserves can be calculated using the Furr equation [5].

The challenges concerning bioavailability of provitamin A

Rural communities depend primarily on food rich in provitamin A carotenoids, such as dark green leafy vegetables and yellow and orange vegetables and fruits for their vitamin A supply. However, the bioavailability and bioconversion of provitamin A carotenoids are influenced by a number of factors, i.e., chemical structure, food matrix in which a carotenoid is incorporated, cooking methods, dietary fat, nutrient status (vitamin A, protein, zinc) of the host, genetic factors, and interactions among these factors [9]. In 16 studies conducted from 1958 to 1994 preschool children were fed dark green leafy vegetables, carrots, or purified beta-carotene [9]. Of these, 13 reported an elevation of serum retinol from 3 to 24 μg/dl, while three others found no improvement (table 1). Nevertheless, these studies were consistent in demonstrating a higher serum response with the administration of purified beta-carotene or retinol supplements. The conflicting evidence led to a controversial issue since these studies had certain limitations in research design and methods. In a study in Indonesian lactating women [10] there was no change in serum and breastmilk vitamin A to a routine diet of dark green leafy vegetables (3.5 mg beta-carotene daily for 12 weeks), while there was a strong improvement to a similar amount of beta-carotene in an enriched wafer. The evidence from this study created doubts as to the efficacy of provitamin A-rich foods in the prevention of vitamin A deficiency [10]. The International Atomic Energy Agency (IAEA) set up a coordinated research program (CRP) on vitamin A nutrition using the isotope dilution technique before and after dietary interventions in China, the Philippines, and Thailand [11].

TABLE 1. Feeding trials (dark-green leafy vegetables, carrots, purified beta-carotene) in preschool children during 1958–1994

Country, no. of studies	Age (yr)	No. of subjects per group	β-carotene per day	Duration	Response
13 studies Rwanda, 1 India, 7 Thailand, 1 Brazil, 1 Egypt, 2 Indonesia, 1	2–16	15–60	1.2–12.7 mg	2 wk–3 mo	Serum retinol elevation (3–24 mg/dl) Serum carotene variable
3 studies Indonesia, 2 Guatemala, 1	3–13	17–43	1.8–3.0 mg	20 days–3 mo	No elevation in serum retinol

Source: adapted from ref. 9

Impact of dietary intervention by isotope dilution

China

The study in China aimed to determine whether plant foods rich in provitamin A carotenoids can sustain or improve vitamin A status in kindergarten children during the fall season [12]. Forty-six children aged 5 to 6.5 years received either green and yellow vegetables (4.7mg beta-carotene/day) or light-colored vegetables (0.7mg beta-carotene/day) every weekday for 10 consecutive weeks. Octadeuterated and tetradeuterated vitamin A were given before and after the dietary interventions, respectively. Serum retinol concentrations were sustained in the green and yellow vegetable group but they decreased significantly in the light-colored vegetable group. Estimation of body stores using isotope dilution confirmed that children fed light-colored vegetables showed a decrease, on average, of 27 μmol or 7,700 μg retinol per child. On the other hand, the total body retinol stores of children in the green and yellow vegetable group were sustained. Therefore, the study indicated that carotenoid-rich vegetables were effective in maintaining adequate vitamin A nutrition and in protecting the children from becoming vitamin A deficient during the season when such food sources are scarce.

Philippines

In the Philippines [13], 7 to 13 year old schoolchildren with marginal serum retinol concentrations (0.32–0.93 μmol/L) received lunch and snacks rich in provitamin A carotenoids on weekdays for 12 consecutive weeks. The foods provided on average, 13 mg beta-carotene per child per day. The study also tested the ability of the three-day deuterated-retinol-dilution (DRD) procedure to detect changes in the pool size of body vitamin A. Following the intervention period, both serum retinol and beta-carotene concentrations significantly increased. In addition, the three-day DRD showed an improvement in body vitamin A stores, especially among those whose status was low at baseline. In summary, the study suggested that bioconversion of provitamin A carotenoids in plants to vitamin A in the body varies inversely with vitamin A status. In addition, the three-day DRD procedure can be used to detect changes of body vitamin A stores and a predictive equation should be developed further.

Thailand

The study on lactating women in Northeast Thailand evaluated the efficacy of a provitamin A mid-day meal given five days per week for 12 weeks on total body retinol stores by isotope dilution and other vitamin A status indices [14]. Eighty-five women with low serum retinol concentrations (< 0.87 μmol/L) were randomly given a meal with either dark green leaves and yellow or orange vegetables and fruits (4.7 mg beta-carotene), purified beta-carotene (3.6 mg), or low-carotenoid foods (< 0.5 mg beta-carotene). Following the intervention, serum retinol increased similarly in all groups, which is likely to reflect seasonal influences on habitual diet. On the other hand, breastmilk retinol increased more in women receiving the provitamin A diet. Total body retinol stores tended to decline more in the control group. In summary, a short-term (3 months) increase in dietary intake of beta-carotene-rich foods had no effect on serum retinol and modestly increased breastmilk retinol concentration but may have prevented a slight decline in total body retinol stores, that could result from breastfeeding. Further study should extend dietary intervention through a full year to avoid seasonal influences and to properly evaluate long-term effects of diet on vitamin A status and body stores of vitamin A.

Summary and future needs

The isotope dilution technique has been developed to assess body stores of vitamin A. The method involved a dilution of an oral dose of isotopic vitamin A which equilibrates with total body reserves after 25 days. A larger isotope dilution indicates a large initial pool size and vice versa. The total body stores can be calculated by a dilution equation. The estimated vitamin A reserves were validated through liver biopsy in tested subjects and showed a good reliability. Studies in the United States, Bangladesh, Guatemala, China, Philippines, and Thailand demonstrated the potential of using the isotope dilution technique to determine total body reserves in an individual and to assess the impact of dietary interventions on changes in body stores of vitamin A. Further application of isotope dilution in program evaluation should be examined. In addition, future research should explore the use of isotopic ratio at day three post-dose to estimate body vitamin A stores as well as the refinement of the isotope dilution technique for application in different age groups.

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Validity of dual-label stable isotopic protocols and urinary excretion ratios to determine folate bioavailability from food

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Abstract

We tested the hypothesis that some cereal-product vehicles may reduce fortificant bioavailability below 85% and examined the feasibility of using single dose, dual-label, non-saturation protocols for studying bioavailability based on urinary excretion ratios (UER; % oral $^{13}C_6$ isotope dose excreted in intact folate/% IV $^{2}H_4$ isotope dose excreted). Fifteen females received 225 μg oral folate (capsules, fortified bran flakes, and fortified white bread), mainly as $^{13}C_6$-PteGlu, followed by IV injection of 100 μg $^{2}H_4$-PteGlu. UERs were used as the primary index of absorption. Urinary folate was cleaved to p-aminobenzoylglutamic acid, dervivatized and determined by gas chromatography/mass spectroscopy (GC/MS). The UER mean (95%CI) for folic acid was 2.18 (1.2–3.8) at 48 hours and as these were greater than 1.0, it was concluded that oral and IV isotopes of folic acid are handled differently by the body. Compared to the 48 hour UER for folic acid, UERs for white bread and bran flakes were 0.71 and 0.37, respectively, thus indicating some matrix inhibition of absorption. Consideration should be given to the choice of cereal-based fortification vehicles in order to maximize bioavailability. Plasma enrichment of folate can be measured using LC/MSMS (liquid chromatography/mass spectroscopy-mass spectroscopy) but seems unfeasible with the GC/MS method.

Key words: folic acid, stable-isotope, bioavailability, human food

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Introduction

Folates are crucial for nucleotide synthesis, cell division, and gene expression [1]. Periconceptual supplementation of women with folic acid has been shown to significantly reduce the incidence and reoccurrence of neural tube defects, such as spina bifida [2, 3]. Marginal folate deficiency is also associated with elevated plasma homocysteine, an emerging risk factor for vascular diseases and stroke [4, 5], linked to some cancers [6, 7] and age-related cognitive decline [8]. Folate fortification of cereal-based foods with folic acid at a concentration of 140 μg/100g has been mandatory in the United States since 1998 [9], and it is currently under review in the United Kingdom and other European countries.

Although humans absorb folic acid well in the absence of food, little is known about its absorption from fortified foods. Published studies have provided conflicting results, with absorption values of PteGlu (pterolymonoglutamic acid) from fortified foods ranging from 30% to 90%, and the food matrix having considerable, small, or no effect on the amount absorbed. This variation may be due to methodological factors, such as the very limited short-term plasma response which is generally thought to make plasma kinetics of limited usefulness in assessing the relative bioavailability of nutritionally relevant oral doses of folate, the influence of pre-study folate-saturation, the use of large, less nutritionally relevant, oral test doses, and the potential for incomplete retention of folate during preparation of food test products.

Isotopic methods permit the examination of folate absorption and metabolism with a high degree of specificity and sensitivity [10]. These methods are based on a single-dose dual-label approach in which two isotopically labeled forms of folic acid [^{13}C and ^{2}H] are administered; one as an oral [^{13}C] and one as an intravenous [^{2}H] dose. Urinary excretion (24 hours) of both isotopes, in isolated intact folate, are measured and the percentage of the oral and IV doses excreted calculated, and then expressed as the urinary excretion ratio (UER).

The purpose of this study was threefold. First, to critically evaluate the dual-label method in a human study, with no folate pre-saturation, and where post-dose urine was collected long enough (48 hours) to encompass two potentially differing pulses in excretion of isotope-labeled folate. Second, the dual oral/IV method was evaluated in the context of studying potential matrix effects on the bioavailability of folic acid from two fortified cereal-based foods.Third, the advantages of using a direct LC-MS/MS procedure for the determination of isotopically labeled folates in biological samples were compared to the current gas chromatography/mass spectroscopy (GC/MS) procedure.

Methods and materials

Materials

$^{13}C_6$PteGlu (labeled with [$^{13}C_6$] in the *p*-aminobenzoate moiety; > 95% isotopic enrichment), and $^{2}H_4$-PteGlu (labeled with [$^{2}H_4$] in the glutamate moiety; >93% isotopic enrichment) were prepared from [$^{13}C_6$]-aniline and 3,3,4,4-[$^{2}H_4$]-L-glutamic acid using a previously developed method [11]. Singly-labeled [$^{13}C_6$]- and [$^{2}H_4$]-*p*-aminobenzoyl-L-glutamate (PABG) was prepared for use as calibrants, and doubly-labeled [$^{13}C_6$, $^{2}H_4$]-PABG was prepared as an internal standard (isotopic enrichments were 96%, 90%, and 82%, respectively).

Gelatin capsules, containing $^{13}C_6$-PteGlu in a stable formulation, were manufactured to GMP standards by RP Scherer Ltd, Swindon, UK. The contents of 20 capsules were assayed for PteGlu, using a high performance liquid chromatography (HPLC) procedure with ultra violet detection [12], and found to contain 56.0 μg per capsule. Capsules were stored in the dark at +4°C. The folate content was checked every six months and found to be stable for the period of study. Solutions of $^{2}H_4$-PteGlu for intravenous (IV) injection were prepared using a previously published procedure [13] by the Pharmacy Manufacturing Unit, Ipswich NHS Hospital Trust, Ipswich, UK. Ten percent of ampoules were selected for virulence testing, and the remainder stored frozen at –18°C. The mean concentration of PteGlu in 5 vials, as determined by HPLC [12], was 87.2 μg/ml.

Two fortified foods were prepared commercially containing $^{13}C_6$PteGlu: a breakfast cereal (bran flakes) and a white sliced bread as reported elsewhere [12]. Homogeneity of added folic acid to the bran flakes and white bread was checked by measuring total folate by microbiological assay using hog kidney deconjugase and α-amylase [14]. The mean (±SEM) concentration of folate in fortified and unfortified bran flakes was 248 (10.4 μg/100g) and 33 μg/100g, respectively, indicating that 13% of the total folate present in the fortified bran flakes was of natural origin. The mean (±SEM) concentration of folate in fortified and unfortified white bread was 191 (6.6 μg/100g) and 21 μg/100g, respectively, indicating that 11% of the total folate present in the fortified bran flakes was of natural origin.

Experimental design and study volunteers

The study was approved by the Norwich and District Ethics Committee (Norfolk and Norwich Area Health Care Trust). Fifteen healthy women volunteers (aged 19 to 43 years) were recruited from the staff and the local population. An initial fasting blood sample was taken from all volunteers to check for normal blood biochemistry, serum B_{12}, plasma and erythrocyte folate, and total homocysteine concentrations. After giving written consent, each volunteer participated for three test days (folic acid capsules, fortified white bread, and fortified bran flakes), which were scheduled at least three weeks apart to minimize the risk of isotope carry-over between doses. Each volunteer received the treatments in a random cross-over fashion. The volunteers were asked to avoid excessive consumption of folate-rich or fortified foods (a list was provided) for three days prior to and subsequent to each of the test days. For the remainder of the study they ate and recorded (weighed-record) their usual diet. For each test day, following an overnight fast, volunteers received an oral dose of folate in the form of either four gelatin capsules containing $^{13}C_6$-PteGlu (224 μg total), 91g fortified bran flakes containing a total of 225 μg folate (196 μg $^{13}C_6$-PteGlu and 29 μg folate of natural origin) or 118g white sliced bread containing a total of 225 μg folate (200 μg $^{13}C_6$-PteGlu and 25 μg folate of natural origin). All doses were digested within 15 minutes, with the aid of water only, and an intravenous injection of 100 μg $^{2}H_4$-PteGlu in saline was administered 15 minutes after the start of consumption. Volunteers were given a light, low-folate, lunch four hours after the oral dose. Three 24-hour urine samples were collected from each volunteer (24 hour period prior to treatment, 0 to 24 hour period post-treatment and 24–48 hours period post-treatment).

Habitual dietary intake data was obtained using written, weighed, records completed every sixth day for seven weeks during the study period, excluding test days and the three days before and after, when volunteers were asked to avoid excessive consumption of folate-rich or fortified foods. Dietary records were coded [15] and mean daily nutrient intakes calculated.

Methods

Red-cell folate and serum B_{12} were measured by chemiluminescent immunoassay (Beckman Access,

Beckman-Coulter, Ltd. High Wycombe, UK) and enzyme immunoassay (Abbott Laboratories, Maidenhead, UK), respectively, at a local hospital, and total plasma L-homocysteine by fluorescence polarization immunoassay (IMx System, Abbott Laboratories; [16]. A modification of a previously published GC/MS procedure was used to determine total labeled urinary folate [17]. Full details for sample preparation and determination are given elsewhere [12]. The method comprises of extraction and isolation of intact folate from urine, chemical cleavage of the C-9/N-10 bond to PABG, isolation of para-amino benzoyl glutamate (PABG) by HPLC, derivatization of PABG and GC/MS of the resulting lactam (fig. 1). The excretion of each isotopomer (M0, M4, and M6 derivatized PABG fractions) in intact urinary folate was calculated from the total daily urine volume and the ratios M0/M4 and M0/M6. To facilitate comparisons between test-days, excretion data were calculated as urinary excretion ratios (UERs).

Volunteers acted as their own controls. Data for the percentage dose of each isotopomer excreted into the urine in intact folate, and urinary excretion ratios (UER), were mathematically-transformed ($\log_{10}$) to normalize distributions. The results were then examined 'within-subject' using 1-way analysis of variance (1-AOV) and least significant difference (LSD) tests for planned comparisons.

Results and discussion

All volunteers had normal hematological and plasma glucose, and lipid status (table 1). Folate status (plasma and erythrocyte folate concentrations), total plasma homocysteine and average folate intakes were well within accepted normal ranges. Total urinary excretion of both isotopes in intact folate by 48 hours post-dose (expressed as % of initial oral or IV dose), for the three treatments are given in table 2, together with calculated urinary excretion ratios (UERs).

Pre-saturation versus non-saturation protocols

The mean urinary recovery of total labeled folates was found to be 0.3% to 1.3% of the administered dose during the 48-hour post-dose period. This excretion pattern corresponds favorably to other published data using a dual-label stable isotope protocol in which 0.1% to 0.5% of the administered dose was found during a 36-hour post-dose period [18]. The recovery of intact labeled folate in urine is much higher (typically 10% to 30% of the dose) when single dose administration of stable isotope labeled folates follows pre-dose satu-

TABLE 1. Female volunteer characterization for the study ($n = 15$)

Biochemical index	Mean (SEM)
General	
Age (yr)	32.4 (1.9)
BMI	23.3 (0.8)
Nutrient intakes	
Folate (μg/day)	288 (26)
Vitamin B_6 (mg/day)	2.2 (0.8)
Vitamin B_{12}(μg/day)	4.3 (0.3)
Status	
Hemoglobin (g/L)	13.1 (0.2)
Glucose (mmol/L)	4.0 (0.1)
Cholesterol	4.7 (0.3)
Plasma folate (μg/L)	10.9 (1.6)
Erythrocye folate (μg/L)	474.1 (54.6)
Serum B_{12} (μg/L)	0.463 (0.043)
Total plasma homocysteine (μmol/L)	7.5 (0.4)

TABLE 2. Cumulative doses of each isotopomer excreted by 48 hours post-dose and urinary excretion ratios (UERs) in 15 adult females

	Oral dose[a]	% Dose of each isotopomer excreted[b]		
Treatment group	Total folate [as $^{13}C_6$-PteGlu]	$^{13}C_6$-folate	$^{2}H_4$-folate	Urinary excretion ratio[c]
Capsules	224 μg [224 μg]	1.259* (0.655–2.421)	0.578† (0.320–1.045)	2.18* (1.2–3.8)
White bread	225 μg [200 μg]	0.614* (0.277–1.358)	0.397† (0.201–0.785)	1.55* (1.0–2.5)
Bran flakes (cereal)	225 μg [196 μg]	0.260 (0.135–0.5)	0.324 (0.203–0.519)	0.8† (0.5–1.4)

a. The IV dose was 100 μg $^{2}H_4$-PteGlu in all cases.

b. Values are geometric means (95% confidence interval) of $\log_{10}$-transformed data; means, within a row, not sharing the same superscript are significantly different ($p < .05$); 1-AOV 'within-subject,' and least significant difference (LSD)-test for planned comparisons.

c. Values are geometric means (95% confidence interval) of $\log_{10}$-transformed data; means, within a column, not sharing the same superscript are significantly different ($p < .05$); 1-AOV 'within-subject', and LSD-test for planned comparisons.

ration with folic acid [18]. However, the use of folate saturation is considered to be non-physiologic with different *in vivo* folate kinetics compared to the non-saturation protocols used here. Complete recovery of the isotopically labeled dose as intact urinary folate is not expected because of *in vivo* retention, catabolism, and fecal excretion. The low enrichments in urine, along with low rates of folate excretion, contribute to large between-subject variations but this variability is offset by the use of the urinary excretion ratio as the primary indicator of bioavailability. Increasing the urinary excretion of the label might be possible by using larger oral doses, or by multiple-doses over several days, and this could be used to improve between-subject variability. However, once doses exceed 400μg, the possibility that unmetabolized folic acid appearing in the plasma must be taken into account.

Handling of oral and IV isotopes by the body

Provided doses are not excessive (< 400μg/dose), oral folic acid absorbed by enterocytes will be reduced and methylated prior to transfer into the body proper where it will appear in the circulation as [$^{13}C_6$]-5-CH_3H_4PteGlu. In the dual-label stable isotope protocol it is assumed that the *in vivo* kinetics, distribution, and excretion of both the oral [^{13}C]folate, and IV [^{2}H]folate are equivalent and independent of the mode of administration. If absorption of the oral dose was100%, then a UER which does not depart significantly from unity would be expected.

Of the total dose of isotope excreted over the 48-hour period, the proportion of the oral $^{13}C_6$ dose excreted in the first 24 hours for capsules, white bread, and bran flakes was 0.90, 0.95, and 0.77, respectively. These were significantly greater than the comparative proportions of IV $^{2}H_4$ dose excreted in the first 24 hours, which were 0.53, 0.69, and 0.62, respectively. As indicated by 1-AOV 'within-subject,' there was no significant difference in the proportion of the IV $^{2}H_4$ dose excreted by 48 hours for capsules, white bread, and bran flakes ($p = .328$).

Geometric mean of urinary excretion ratios for folic acid capsules, 3.68 at 24 hours and 2.18 at 48 hours, resulting from what appears to be two distinct clearance patterns for $^{13}C_6$ and $^{2}H_4$, leave no doubt whatsoever that oral and IV isotopes of folic acid are handled differently, since ratios significantly greater that 1.0 are theoretically impossible if the isotopes were handled similarly by the body. This undermines the premise upon which the dual-label protocols are based. The recent commercial availability of an isotopically-labeled version of the [6S]-isomer of 5CH_3H_4PteGlu (natural isomer) should permit further dual-label studies to be undertaken with greater confidence.

Folic acid absorption from fortified foods

Although, in the current study, it can be concluded that 'absolute' absorption of oral dose cannot be calculated using urinary excretion ratios, the 'relative ratio' of test food UERs compared to folic acid capsule (control) UER may still be of some use. The 'relative ratio' approach would still retain the advantages of an increase in the precision of analysis while avoiding multi-blood sampling from volunteers. The 48 hour $^{13}C_6/^{2}H_4$ urinary excretion ratio for white bread and bran flakes, when compared 'within-subject' to that for folic acid capsules, results in a geometric mean 'relative ratio' of 0.71 and 0.37, respectively, which suggests that the matrix of these cereal-foods has some inhibitory effect on intestinal absorption of folic acid added to the food as a fortificant. This supports earlier findings of relatively low folic acid absorption from fortified foods, as compared to folic acid alone, based on either short-term changes in plasma folate [19], or longer-term studies measuring changes in erythrocyte folate concentrations [20].

Relationship between urinary excretion of labeled folate and initial red blood cell folate (RBC) concentrations

Urinary excretion of $^{13}C_6$ from folic acid capsules, but not white bread or bran flakes, was significantly correlated with the erythrocyte folate concentration of volunteers ($r = 0.70$, $p = .004$). RBC are known to correlate with liver folate stores, the major organ store in the body. Liver folate also correlates with whole body folate stores. It is suggested that the higher liver folate concentration, the lower the first pass absorption by the liver, and the higher urinary excretion of the dose. This is an area that needs further investigation.

Comparison of GC/MS and LC/MS procedures for determining labeled folate species

The GC/MS method used in this work is outlined in figure 1. The procedure is very specific for folate and has a limit of detection of 0.25 ng/ml (0.11μM) of 5-MTHF in plasma. However, the method is tedious and is prone to both chemical and physical losses during sample preparation and derivatization. In particular, the cleavage reaction involving the conversion of 5-methyltetrahydrofolate to PABG using hydrogen peroxide and catalase is technically difficult to perform with low conversations often found (0 to 20%). In addition, the procedure requires a high resolution mass spectrometer, or a tandem instrument.

Alternate LC/MS and LC/MS/MS procedures have

been developed [21] which require much simpler and rapid extraction and clean-up steps. A folate binding protein affinity column is used for sample clean-up, prior to determination by LC/MS or LC/MS/MS. The uptake of newly absorbed folate by the liver, resulting in the apparent limited short-term appearance in plasma of folate of oral origin, until recently has been seen as a limiting factor in the use of plasma kinetics in assessing absolute absorption [10]. However, the combination of this approach of using dual-label isotope protocols with 6S-5MTHF as the IV dose, with compartmental modeling techniques offer the potential for measuring absolute absorption of folates, and partitioning kinetics into other body pools.

Conclusions

Folic acid cannot be used as the IV dose in dual-label isotopic experiments. Where it has been used, even 'relative ratios' of test material UERs to a folic acid control UER should be viewed cautiously. In view of these findings, great care needs to be taken in the design of dual-label stable isotope protocols. We suggest that the only folate theoretically appropriate for IV use would be the natural isomer of 5-methyl-tetrahydrofolic acid ([6S]-5CH_3H_4PteGlu).

The matrix of some cereal-based foods may have an inhibitory effect on intestinal absorption of folic acid from some fortified foods, resulting in reduced bioavailability to levels that are similar to natural food sources. Recommendations to fortify all types of flour with folic acid may be inappropriate for wholemeal flours because of lower folate bioavailability from products made from these flours. Further consideration should be given to the type and formulation of breakfast cereals, used as vehicles for folic acid fortification, in order to maximize folate bioavailability.

The use of plasma responses suffers from the extensive first-pass hepatic uptake and enterohepatic recycling of newly absorbed folates from the oral dose but it is possible that the development and application of compartmental modeling techniques may be possible for determining absolute absorption and *in vivo* kinetics. The development of LC/MS and LC/MS/MS techniques offer advantages over the GC/MS approach both in terms of sample throughput (handling capacity) and improved precision. In addition, LC/MS offers the potential to quantify other reduced folate forms, that are important in metabolism, something that is difficult using the GC/MS approach because of the varying cleavage conditions for each folate.

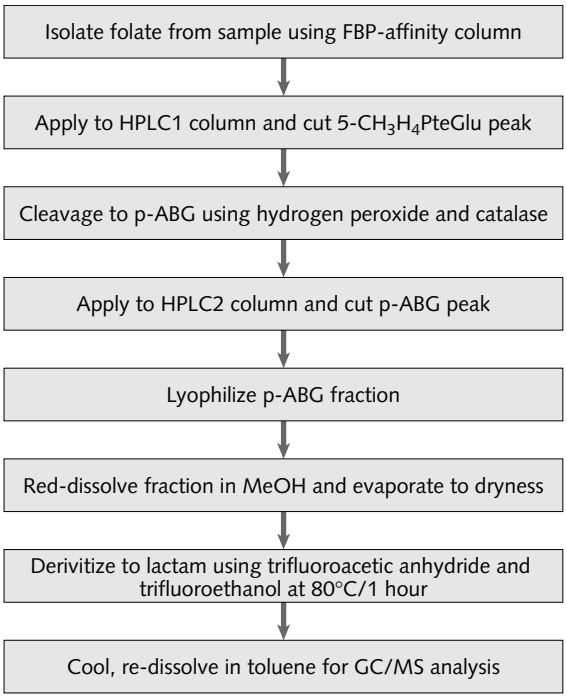

FIG 1. GC/MS procedure for folates

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In vivo stable isotope measurements of methyl metabolism: Applications in pathophysiology and interventions

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Abstract

With intravenous infusion of doubly-labeled [$^{2}H_{3}$C-1-^{13}C-] methionine and stable isotope enrichments in plasma free methionine and carbon dioxide in breath air, whole body transmethylation, transsulfuration, and remethylation rates can be calculated. This technique demonstrated impaired recycling as the major disturbance to explain hyperhomocysteinemia in patients with end-stage renal failure, and can be used to optimize interventions with folate, B_6, and B_{12} supplementation in this patient group. Intravenous infusion of [2,3,3-$^{2}H_{3}$] serine has also been applied to demonstrate the appearance of [$^{2}H_{2}$]- as well as [$^{2}H_{1}$]-methionine in plasma and protein, suggesting transfer of a one-carbon group from serine via 5,10-methylenetetrahydrofolate in human hepatocyte cytosol and mitochondria, respectively. In sheep, tissue free methionine enrichments after infusion of universally labeled [U-^{13}C] methionine showed the highest remethylation activity in postmortem investigation of jejunum, liver, and kidney tissue samples, but no such activity in muscle and brain samples. Methods to quantitate one-carbon acceptor metabolism pathways and folate metabolism have recently become available.

Key words: folate, homocysteine, metabolism, methionine, methylation cycle, stable isotope

Introduction

Methionine is an essential sulfer-containing amino acid which is derived from ingested protein. It can also be formed by recycling its metabolic product, homocysteine, in a metabolic cycle which involves the transmethylation pathway (three enzymatic steps via S-adenosyl methionine) and the remethylation of homocysteine (two enzymatic steps). Together with serine, methionine is a major donor of one-carbon (methyl) units for methyl acceptor reactions which involve, among others, arginine, phospholipid, glutathione synthesis, and biosynthesis (nucleotide formation and DNA methylation).

In the breakdown of homocysteine in the transsulfuration pathway, the carbon skeleton of methionine can be degraded into, ultimately, α-ketobutyrate which is a fuel for the tricarbocyl cycle. Serine is required in this reaction which results in the incorporation of the sulfer atom of homocysteine into a newly formed cysteine molecule.

The B vitamins folate, pyridoxin (B_6), and cobalamin (B_{12}) are closely connected with the metabolism of methionine and serine in a number of ways (fig. 1). Vitamin B_6 is a cofactor in the transsulfuration pathway for the enzyme cystathionine β synthase and is also a cofactor for serine hydroxymethyltransferase, which generates 5,10-methylene tetrahydrofolate from tetrahydrofolate. Folate coenzymes serve as acceptors or donors of one-carbon units in a variety of reactions. The enzyme 5,10-methylene tetrahydrofolate reductase is the regulating enzyme in the remethylation pathway, where vitamin B_{12} is a cofactor for the enzyme methionine synthase. The tetrahydro derivatives of folate are cofactors for several (acceptor) methyl transferase reactions.

One carbon metabolism in humans, and in particular the interrelationships among folate and vitamin $B_{12,}$ as well as those of methionine, homocysteine, and vitamin B_6 have gained the interest of scientists since the discoveries of their roles in macrocytic megaloblastic anemia and the vascular complications in patients with cystathionine β synthase deficiency, respectively. In recent years, folate supplementation has received attention because of its preventive effect in relation to neural tube defects and other congenital malforma-

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tions. Reduced vitamin B_{12} status and elevated blood homocysteine levels (denoted hyperhomocysteinemia) have been associated with birth defects as well, and also with adverse pregnancy outcome. All women in their reproductive years should increase their folate intake above their regular intake with at least 400 μg per day [1]. In the United States, folic acid has been added to grain products since January 1, 1998. It has been calculated that this raises the adult intake by about 100 μg per day, and reduced the incidence of neural tube defects by 19% [2]. Hyperhomocysteinemia has emerged as a prevalent risk factor for atherosclerotic vascular disease in the coronary, cerebral and peripheral blood vessels and thrombotic events [3]. Genetic polymorphisms in methylenetetrahydrofolate reductase constitute a risk factor for hyperhomocysteinemia and pregnancy complicated by neural tube defects. Apart from mortality due to cardiovascular events, hyperhomocystenemia has also been associated with elevated total mortality in patients with diabetes mellitus and in the general population [4]. Supplementation with B vitamins, in particular with folate, is efficient and effective in reducing an elevated homocysteine level in the blood [5]. The clinical and epidemiological endpoints of B vitamin supplementation are the subject of ongoing trials.

Genetic studies and assessment of vitamin status have, in addition to measurements of the blood levels of sulfur-containing amino acids, revealed many key aspects of the role of methionine and its metabolic associates in humans. Clinical studies in patients with cystathionine β synthase deficiency and in patients with end-stage renal disease, in whom hyperhomocysteinemia has a high prevalence, have also provided insights into the pathophysiology of sulfer-containing amino acids and the efficacy of interventions. However, although many associations have been demonstrated and single enzyme characteristics and mutations are well studied, their impact on elucidating the biology and physiology of one-carbon unit metabolism remains largely unknown. This hampers the possibility of educating the population with respect to B vitamin intake and bodily functions and also requires large and costly intervention studies to determine the optimal dosage for vitamin combinations with respect to interindividual phenotypical and genotypical variations.

Stable isotope tracer studies are safe and provide information of the flux rates in metabolic pathways. Many applications in nutritional and micronutrient studies have been developed.

Tracer methodology: methionine and one-carbon metabolism

In the mammalian body, the complexity of methionine metabolism (depicted in fig. 1) would require the study of tissues and organs as well as their contributions to the condition of the whole organism. In humans, the limitations with regard to obtaining tissue samples are obvious. Apart from minimal invasive methods applicable to humans, an invasive procedure will also be discussed which can be used in animal studies. Alternatively, methionine tracers can be used for noninvasive measurement of tissue protein synthesis and the use of metaprobes in homocysteine metabolism will also be discussed. The overview is summarized in table 1.

Whole body methionine fluxes

[$^2H_3$C-1-^{13}C] methionine has stable isotope tracers on its methyl group (m+3) and on the carbon skeleton (m+1). This tracer allows the measurement of methionine transmethylation, remethylation, and transulfuration and corrects for exchange with body protein pools [6]. The method has a solid background because the study protocol resembles that of other essential amino acid tracers such as [^{13}C] leucine and [^{13}C] valine. In the fasting condition, a primed, continuous intravenous infusion of doubly-labeled [$^2H_3$C-1-^{13}C-] methionine is given, and plateaus in stable isotope enrichments in plasma-free methionine and carbon dioxide in breath air are obtained after 4 to 6 hours. Validity with respect to the use of methionine as a precursor of homocysteine has been addressed [9]. The method can be applied to study (patho)physiology and interventions on homocysteine metabolism, as shown below.

Whole body serine fluxes

[2,3,3-$^2H_3$] serine (m+3) donates one deuterium to glycine in its conversion by both cytosolic and mitochondrial serine hydroxymethyltransferases, and the resulting [5,10-C$^2H_{2]}$] tetrahydrofolate molecule loses another deuterium atom by the action of methyltetrahydrofolate dehydrogenase which is present in mitochondria but not in the cytosol. Gregory et al. discovered that these two subcellular pathways are present in a primed, continuous infusion protocol in one fasting adult [7]. This conclusion became apparent when they found $^2H_1$ and $^2H_2$ methionine isotopomers in the blood. $^2H_1$ serine was the predominant form of labeled serine (m+1) in apolipoprotein-B100 (a liver derived protein), which shows the highly active state of hepatic serine undergoing catalytic cleavage into glycine. However, no detectable labeling in glycine was found. The presence of m+3 cystathionine resulted from the reaction of homocysteine with serine m+3 catalyzed by cystathionine β-synthase, the rate limiting step in the transulfuration pathway. This stable isotope approach demonstrates the usefulness of tracers in the study of cellular metabolism.

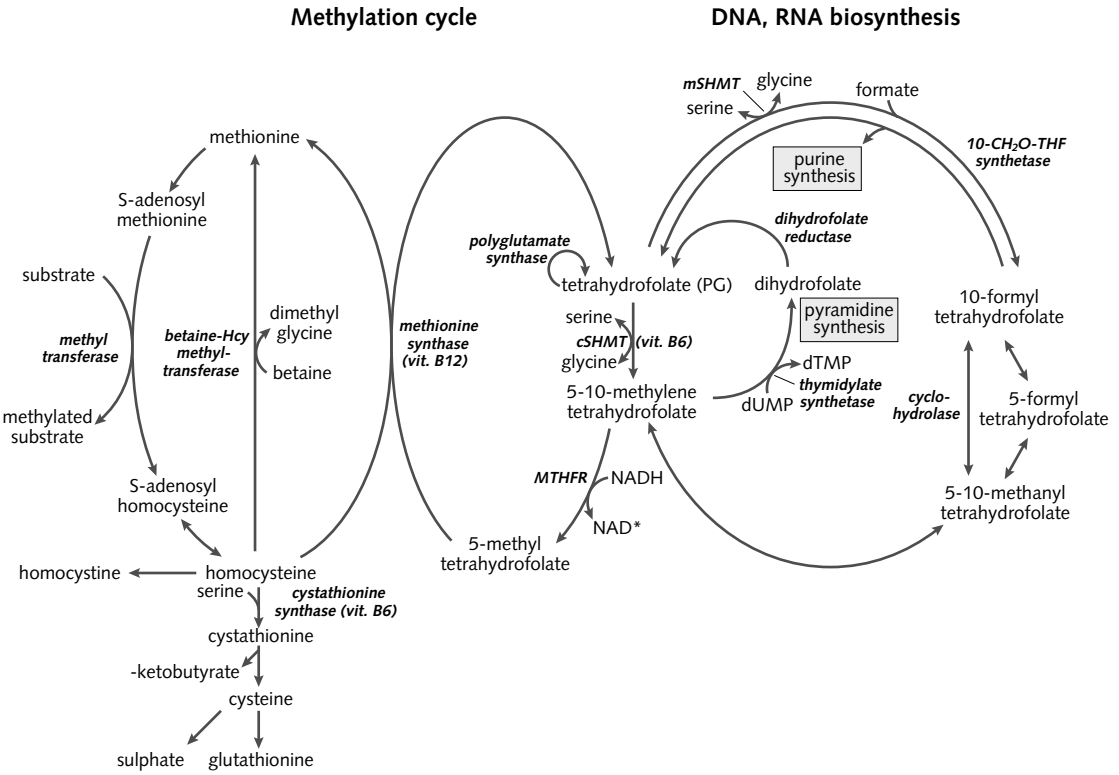

FIG. 1. Schematic presentation of one carbon metabolism. MTHFR: methylenetetrahydrofolate reductase; cSHMT: cytosolic serinehydroxymethylytransferase; mSHMT: mitochondrial serinehydroxymethylytransferase; THF: tetrahydrofolate; PG: polyglutamate.

TABLE 1. Stable isotope methods for in vivo studies of methionine and one-carbon metabolism

Tracer	Process	Metabolite of interest	Measured by
[$^2H_3$C-1-^{13}C] methionine [6]	Transmethylation/ remethylation	Methionine/homocysteine	MS; plasma m+3/m+4/m+1
	Oxidation/transsulfuration	Methionine/homocysteine	MS; $^{13}CO_2$ in breath air
	Protein synthesis	Methionine	MS; plasma m+4
[$^2H_3$] serine [7]	Serine metabolism/ remethylation	Methionine/homocysteine	MS; plasma [$^2H_2$] and [$^2H_1$] methionine
[U-^{13}C] methionine and [$^2H_3$C] and [1-^{13}C] methionine [8]	Tissue methionine/ remethylation	Methionine/homocysteine	MS; plasma and tissue m+5/m+4/m+1
1-^{13}C] homocysteine [9]	Remethylation	Homocysteine	MS; plasma m+1
	Transsulfuration	Homocysteine	MS;$^{13}CO_2$ in breath air
2-oxo-thiazine 4-carboxylic [1-^{13}C] acid ethyl ester [10]	Transsulfuration	Homocysteine	MS; $^{13}CO_2$ in breath air
H_3C-[1-^{11}C] methionine [11]	Protein synthesis	Mixed muscle protein	PET scanning

MS, mass spectrometry; PET, positon emission tomography; m, denotes the mass of tracee methionine.

Tissue methionine metabolism

Universally labeled [U-^{13}C] methionine (m+5) can be obtained from an amino acid mixture produced from hydrolyzed algae protein. This tracer has been applied in a study in sheep, who were randomized to study the effect of choline plus creatine on methionine metabolism [8]. The protocol involved infusions on days 5 and 8 with either the aforementioned tracer or a combination of [$^{2}H_3$C] methionine (m+3) and [1-^{13}C] methionine (m+1), and sampling of blood and tissues after termination of the animal. Because methionine has five carbons, the enrichment of the [U-^{13}C] labeled molecule represents the total methionine turnover. The m+4 enrichment represents the remethylation rate, because the transmethylation reaction converts methionine m+5 into homocysteine m +4, which is reconverted into methionine m+4 by the enzyme methionine synthase which catalyzes the replacement of an unlabeled methyl carbon moiety. In this study procol, remethylation cannot be measured with the m+3 and m+1 tracers because the remethylated tracer molecules (with mass m and m+1, respectively) enter the methionine pools which are diluted by methionine from protein breakdown. From comparisons of methionine enrichment in plasma obtained from the aorta, and portal and hepatic veins, Lobley et al. concluded that the viscera and liver were the major sites of remethylation [8]. This was confirmed by enrichment analyses in tissue free methionine, which showed the highest remethylation rates in rumen, jejunum, and liver and a lower contribution in kidney but no substantial remethylation in skeletal and heart muscle, skin, and lung. Remethylation calculated from plasma enrichement was not significantly changed during choline plus creatinine supplementation in this ruminant model.

Tracing homocysteine metabolism—^{13}C-homocysteine

[1-^{13}C] homocysteine would appear to be an attractive candidate tracer for homocysteine clearance studies. Its derivatization and mass spectrometry procedures for measuring enrichment in plasma are feasible [8]. In theory, a ^{13}C stable isotope tracer introduced in the transsulfuration pathway may be used to assess its activity in a noninvasive way by measuring $^{13}CO_2$ enrichments in exhaled breath samples. This would seem to provide more accurate pathophysiological data in subjects with elevated homocysteine levels than the methionine loading test which is presently used but which involves superphysiological doses of methionine in order to be able to raise the homocysteine concentration in susceptible patients. Use of ^{13}C-homocysteine as a tracer is hampered by its availability and (as yet unsubstantiated) concerns that its infusion may cause local reactions in the canulated effluent peripheral blood vessels.

Tracing homocysteine metabolism: homocysteine metaprobe

Use of a metabolic probe (an isotopically labeled precursor probe that is converted to a labeled product by the action of the enzyme or process of interest, also coined as metaprobes) which is metabolized to homocysteine would provide an alternative. Such a probe, using oxoprolinase to convert the metaprobe (labeled 2-oxo-thiazine-4-carboxylic acid) into a labeled homocysteine homologue, has been developed and studied in rats [10]. Studies in humans would require safety tests of the metaprobe.

Tracing protein synthesis: positron emission tomography (PET) scanning

A radioactive [$H_3{}^{11}$C-] methionine tracer has been used for the noninvasive measurement with PET scanning of mixed protein synthesis in skeletal muscle in adults [11]. It has the advantage of not requiring muscle or blood samples. This method can theoretically also be used to follow protein catabolism, by continuous measuring after stopping the label infusion. Its disadvantages are radiation dose, and costs of the label and PET scanning. Any essential amino acid labeled with ^{11}C could be used for the same purpose, and this particular method thus is not confined to the use of methioinine. The use of PET scanning using labeled methionine or serine tracers in the elucidation of regional organ metabolism of one-carbon donors, e.g., in the central nervous system, awaits further exploration.

Tracer methodology: one-carbon acceptor metabolism

Interrelations between the one-carbon donor pathways and methyl transferase acceptor pathways are pivotal for the understanding of one-carbon metabolism. Methionine transmethylation is a primary contributor to the acceptor reactions, and 5-methyltetrahydrofolate is another important donor. Serine conversion into glycine is another quantitatively important route, resulting in the formation of 5,10-methylene tetrahydrofolate and, via methylenetetrahydrofolate reductase, in 5-methyltetrahydrofolate. The relative contributions of carbon precursors from food, and the buffering effect of the intracellular folate co-enzyme pools have not yet been studied in vivo. Tracer methods for methionine and serine, such as those discussed above, could be applied for that purpose. Development of methods to measure and trace enrichments in the methyl and formyl moiety of folate is pending.

Interests can be focused on the quantification and regulation of methyl acceptor pathways, including those involved in arginine and nitrous oxide production, glutathion and creatine synthesis, phospholipid synthesis and cell replication (nucleotide synthesis and DNA-methylation). Minimal- and noninvasive stable isotope methods are available for the quantification of these processes and include tracer technology and, in the case of creatine metabolism, ^{1}H and ^{31}P magnetic resonance spectroscopy. These methods are summarized in table 2.

B-group vitamin interactions: labeled folates

Deuterium and carbon labeled folic acid have been used to study folate absorption and cellular retention in a variety of clinical protocols, using 24 or 48 hour urinary excretion ratios of oral and intravenous doses, or short-term enrichment in plasma folate as measurement [19, 20]. Isotopically labeled folates, especially [6S]-5CH_3H_4PteGlu (natural l-isomer), have been commercially available since 2000 (Eprova AG, Schaffhausen, Switzerland). Rapid measurement will be facilitated with the combined development of liquid chromatography-tandem mass spectrometry to measure enrichments in 5-methyltetrahydrofolate and other folates involved in 1-carbon metabolism in small plasma samples, as shown elsewhere in this supplement [21], and kinetic modeling techniques.

TABLE 2. Stable isotope methods for in vivo studies of methyltransferase reactions in one-carbon donor metabolism

Process of interest	Measurement available	Tracer method
Arginine synthesis [12]	Yes	Labeled arginine
Nitrous oxide synthesis [13]	Yes	[^{15}N] guanidino arginine
	No	Metaprobe (homoarginine)
Phospholipid synthesis [14]	Yes	[U-^{13}C] glucose -> palmitate
Glutathion synthesis [15]	Yes	[2-^{13}C] oxothiazolidine -4-carboxylic acid
Creatine synthesis [16]	Yes	(Phospho-)creatine with MRS
Nucleotide synthesis [17]	Yes	[U-^{13}C] glucose; MIDA
DNA methylation [18]	No	$^{15}NH_2$-deoxycytidine[a]

a. In vitro study only.
MRS, magnetic resonance spectroscopy; MIDA, mass isotopomer distribution analysis.

Pathophysiology of hyperhomocysteinemia in patients with end-stage renal disease

Hyperhomocysteinemia is reported in 85% to 100% of patients with end-stage renal disease (ESRD), and elevated plasma homocysteine levels have been shown to be an independent risk factor for cardiovascular disease not only in the general population but also in ESRD patients [22, 23]. It has been indicated that the functional loss of the kidney to clear homocysteine is the putative pathophysiological factor to explain hyperhomocysteinemia in ESRD patients [24], but this explanation has been questioned [25]. Studies in rodents showed that normal rats and those with hyperhomocysteinemia have a significantly lower concentration of homocysteine in the renal vein than in the artery, and transsulfuration activity in the rat renal tubule has been held responsible for this finding [24, 26]. These experiments support the hypothesis that loss of kidney function results directly in inability to clear homocysteine. However, in fasting humans with normal renal function who underwent catheterization no significant net renal uptake of homocysteine was found, suggesting that, contrary to the findings in rodents, loss of such uptake cannot cause hyperhomocysteinemia in humans [27]. Studies with [$^{2}H_3C$-1-^{13}C] methionine showed that in patients with ESRD as compared with healthy humans remethylation and transmethylation rates were markedly (up to 27%) and significantly decreased [28]. Although transsulfuration was 17% lower in the patients with ESRD in that study, the difference was not statistically significant. This may have been due to an insufficient statistical power in that study.* Preliminary findings, published in abstract form, suggested that supplementation with folic acid (5 mg/day during three weeks) significantly improves the remethylation and transmethylation rates in the ESRD patients, but does not result in mean rates comparable to those of healthy controls.** We are presently using this stable isotope method for studying the effects of

* Stam F, de Meer K, Kulik W, van Guldener C, ter Wee PM, Jakobs CAJM, Smith D, Donker AJM, Stehouwer CDA. Pathophysiology of hyperhomocysteinemia in end stage renal disease: In vivo stable isotope observations. Presented at the 3rd International Conference on Homocysteine Metabolism in Sorrento, Italy, July 1–5, 2001.

** Stam F, de Meer K, Kulik W, van Guldener C, ter Wee PM, Jakobs CAJM, Smith D, Stehouwer CDA. Effects of vitamin B_{12} and B_6 supplementation on homocysteine metabolism in end stage renal disease: Paradoxical interactions with folic acid? Presented at the 3rd International Conference on Homocysteine Metabolism in Sorrento, Italy, July 1–5, 2001.

vitamins B_6 and B_{12} in relation to transulphuration and folate and B_{12} in relation to remethylation. It can also be used to evaluate the effects of pharmacological interventions affecting homocysteine metabolism. As a result of the lower coefficients of variation for the methylation flux rates than for plasma homocysteine, and because the effects on specific pathways can be distinguished, such intervention studies can be done with a much smaller group size without loss of power when stable isotope methods are used than when homocysteine blood levels are used as the end-point.

Conclusions

Methionine and one-carbon metabolism, and their interactions with B vitamins, take place at different rates in the tissues and contribute to the effects observed at whole body levels, e.g. in patients with elevated blood homocysteine levels. The physiology and pathophysiology in humans can be further studied using several stable isotope methods. Methods have been developed, many of these recently, to study transmethylation, remethylation, and transsulfuration fluxes, folate absorption and retention, and one-carbon acceptor reactions in a quantitative way. There are emerging possibilities to study interactions between vitamins and these metabolic pathways. These in vivo isotope techniques are available for various studies of pathophysiology and interventions in methyl group transfer involving folate, vitamin B_6, vitamin B_{12}, and pharmacological and food derived substances.

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