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Use of Nuclear and Isotopic Techniques for Addressing Nutritional Problems

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Preface

This *Food and Nutrition Bulletin* supplement represents the proceedings of a symposium on the Use of Nuclear and Isotopic Techniques for Addressing Nutritional Problems. There were five scientific sessions, namely advances in isotopic and nuclear-related methods for nutrition research, isotopic methods for nutrient measurements and energy metabolism, tracer techniques in nutrient metabolism and applications, tracer techniques for evaluating nutritional interventions, and a workshop on stable isotopes in nutrition: opportunities, perspectives, and harmonization. These sessions provide a basic frame of reference for the scope of technical activities carried out by the International Atomic Energy Agency, and formed an integral part of the scientific program at the 17th International Congress of Nutrition held in Vienna, Austria, August 27–31, 2001.

This Congress focused attention on the global social and economic costs of malnutrition and pollution from environmental contaminants, which are enormous. It also helped put into place the commitments to address the issues of health, nutrition, and environment through national and international interventions.

In meeting these challenges, the global nutrition community recognizes the significance of nuclear and isotopic (especially stable isotopic) techniques for accurate and, in some cases, non-invasive measurement of nutrient utilization, nutrient status of the body, and related metabolism. The application of isotopic techniques is well suited for determining the success of food supplementation programs and other interventions aimed at combating many forms of malnutrition. In particular, the doubly labeled water method has been proven to be an accurate tool to study human body composition and energy metabolism while the use of nitrogen-15 has been accepted as an efficient approach to study protein turnover and amino acid metabolism. As analytical tools stable isotopes are now seen to be invaluable, since there is virtually no health risk involved in their use. They are therefore preferred for work in humans, especially in infants and pregnant women. The application of these tools is increasingly recognized as commonplace, since naturally occurring elements exist as a mixture of two or more stable non-radioactive isotopic forms. There are heavy stable isotopes (e.g., ^{54}Fe, ^{56}Fe, ^{57}Fe, ^{58}Fe, ^{64}Zn, ^{66}Zn, ^{68}Zn, ^{70}Zn) and light stable isotopes (e.g., ^{1}H, ^{2}H, ^{13}C, ^{12}C, ^{15}N, ^{14}N, ^{16}O, ^{17}O, ^{18}O). Stable isotopes are thus used in measurements by determining the changes in the ratio of different isotopes. They can be administered either orally (water, food, etc.) or intravenously (in required form) and incorporated into metabolic products, such as body water, urea, or CO_2. Stable isotopes can be sampled in saliva, milk, breath, urine, and stool. The ratio of minor to major isotopes can then be determined by isotope ratio mass spectroscopy, infrared absorption, or emission spectroscopy.

Within the United Nations, several initiatives are being implemented to alleviate micronutrient deficiencies that are of major public health concern in nutrition. The International Atomic Energy Agency (IAEA) is contributing to these efforts by offering technical solutions to improve nutrition monitoring techniques and by identifying effective strategies in nutrition intervention schemes as currently applied in several developing countries. Thus, for many years the IAEA activities in nutrition research have strengthened the use of isotope techniques as tools to evaluate human nutritional status and environmental health.

The IAEA supports activities related to nutrition and environment through research, capacity building, and technical cooperation activities. Coordinated research projects are developed on well-defined research topics for bringing together scientists from developing and industrialized countries to seek solutions to selected problems. Studies supported by the IAEA cover a broad range of problems that include iron-deficiency anemia, protein-energy malnutrition, micronutrient deficiencies with special reference to child health and maternal nutrition and nutrition during pregnancy, osteoporosis, obesity, infection, food composition, and food fortification.

Technical cooperation is aimed at meeting developmental priorities through the application of scientific

and technical capabilities. One such IAEA program of technical activities aligns with the Plan of Action of the World Summit for Children by focusing primarily on improving the heath status of children. The IAEA's program to improve child health includes four principal components: technologies for evaluating child nutrition and dietary status, technologies for control of communicable diseases, technologies for screening childhood diseases, and radiation therapy. These technical packages, along with associated human resource components provide a useful link to national health care, prevention, and disease control programs in member states. Appropriate uses of isotope evaluations result in more children achieving their full potential for growth, performance, and economic contribution to society. At the same time long-term costs to health services are reduced through optimally implemented interventions. In implementing these projects the IAEA works with a network of institutions (spread in many parts of the world) capable of using isotope techniques in analytical studies.

The IAEA operates on the premise that science and technology must serve human development, and the unique and diverse analytical tools referred to in this report are examples of how the Agency and its counterpart institutions contribute to poverty alleviation, food security, and human health priorities. In the interim years until the next Congress, the member states and secretariat of the IAEA will be working to advance the science and technologies that improve human nutrition into new challenges such as the bioavailability of essential micronutrients, such as vitamin A or carotenoids, and quality of life issues and diseases of aging, such as non-insulin dependant diabetes mellitus, coronary heart disease, and obesity. Through its continued collaboration with the international nutrition community the IAEA expects the next Congress to further strengthen the linkage between isotope studies, nutritional status, quality of life, and human development.

Acknowledgments

We wish to acknowledge the invaluable help rendered by Najat Mokhtar and Kerstin Gross in arranging the scientific sessions, Beatriz Miranda da Cruz and Gerd Hinterleitner for shouldering the responsibility of streamlining the symposium structure and proceedings of the meeting, Melinda Fronda and Magali Evrard for their efficient secretarial help and Karen Morrison for the conference organizational support.

We are grateful to Professor Ibrahim Elmadfa, President of ICN-17, 2001, and to the IUNS Council for acommodating the IAEA symposia as part of the ICN congress.

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Considerations on the use of radioisotopes in human nutrition research

Fernando E. Viteri and Rod Warren

Abstract

Tracer methodology using radioisotopes in human nutrition and metabolism research has been very productive but its use has been the object of unjustified limitations because of modeling decisions without adequate consideration of new data. These limitations have encumbered research studies in the metabolism of micro- and macronutrients particularly where nutritional deficiencies are prevalent. Even though stable isotope methodologies in human research are very useful in specific applications they are expensive and are often fraught with serious pitfalls, when compared to studies for the same purposes using radioisotopes. We have been developing safe radioisotopic methods to study the absorption and metabolism of micronutrients, especially iron and zinc, that can be applied to populations in the developing world. These and other applications of radioisotopes should allow scientists to conduct research diminishing the dependence on stable isotopes and on facilities and laboratories existing only in selected sites in the industrial world, and thus should enhance productive local and collaborative research. All radioisotopic research must be subjected to strict safety criteria, based on scientific evidence of risk, but should not be hampered by exaggerated fears of unfounded risk. Examples on the use of radio and stable isotopes to study iron and zinc absorptions are given including the radiation exposure and risk calculations.

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Key words: Radioisotopes, human, use, nutrition, research

Background

Early history of ionizing radiation and research using radio and stable isotopes

By the end of the nineteenth century very exciting research in chemistry and physics extended a conceptual revolution initiated early by Dalton, whose ideas of the atomic nature of matter and classification of elements based on atomic weights were continued by Mendeleev (1860s) who proposed the classification of the elements by their periodic properties. Then came the discovery of x-rays by Roentgen in 1895, the discovery by Becquerel of $\propto$ particles emitted by uranium (radioactivity) in 1896, the isolation of Polonium and Radium by the Curies in 1898, the discoveries of β and γ radiation, and Einstein's theories, all by 1905. Many discoveries followed and the biological effects of ionizing radiation (IR) became clearer, including the discovery of its mutagenic capacity by Mueller in 1927 [1].

The use of radioisotopes in biological research had already been initiated in the 1920s by G. Hevesy who, with rather unique insights and ingenious methods, studied the distribution of naturally occurring radioisotopes of lead, bismuth, and thorium in plants and initiated studies in animals on the distribution and metabolism of ^{32}P. A key step in the use of isotopes in metabolism came from the studies by Schoenheimer who used the stable deuterium (^{2}H) extensively to develop his concept of "the dynamic state of body constituents" [2]. For the purposes of this paper an important step was achieved by Joliot-Curie and Joliot who produced the first artificial radioisotopes in 1934. Their use in biological research, including studies in humans, was significantly increased the world over by the discovery of atomic fission in 1938 and later, particularly after 1945, with the greater availability of radio-

isotopes. Knowledge on metabolic processes and new analytical, diagnostic, and therapeutic methodologies using radioisotopes were greatly expanded. However, early in their use less stringent monitoring of doses was in place compared to that demanded today. This stringent monitoring is prudent because unintentional harm was caused in some instances as in the Vanderbilt iron absorption study in pregnancy by Hahn et al. in 1951 [3], which gave rise to a notorious suit resolved in 1994 by compensating affected families [4].

Essential parts of this paper are some background considerations on the current regulation of the use of ionizing radiation (IR). As early as 1900, evidence was collected which showed that IR could produce damage to biological systems. This promoted the creation of scientific groups such as the American Roentgen Ray Society (ARRS). Later, these groups began taking the role of regulatory agencies. Radiation protection was begun in 1915 by the British Roentgen Society, and by 1928 the International Commission on Radiation Protection (ICRP) was established [1].

The biological effects of high IR doses gave origin to regulations on their use aimed at reducing to a minimum the health risks attributed to IR exposure. A linear model based on the stochastic effects of large IR doses extrapolated health risks to exposure doses thousands of times smaller than those where evidence of biological and health damage existed so that no dose of IR was considered safe. Therefore, the minimal doses currently used in human metabolic research are considered to carry health risks, even though these have never been demonstrated. It is our opinion that an unjustified fear to the prudent use of radioisotopes in human research has permeated society as a consequence of the linear extrapolation models adopted by regulatory agencies.

Up to the 1950s the main concerns were on the detection of genetic effects caused by IR but now other aspects are of greater concern, especially the development of cancer, and the mechanisms involved in cancer initiation and promotion and in the biological reactions secondary to the interactions of IR with biological systems.

In 1945 the Hiroshima and Nagasaki atomic bomb explosions took place. The U. S. Atomic Energy Commission was created in 1946; and in1957 the International Atomic Energy Agency (IAEA) was established by the United Nations "to seek to accelerate and enlarge the contributions of atomic energy to peace, health, and prosperity throughout the world and to guide and promote the peaceful uses of atomic energy." These and other agencies were responsible for the promotion and prudent use of IR including human radiotracer and diagnostic techniques which have resulted in significant advances in many useful applications of IR, including fundamental tracer-based metabolic and nutritional knowledge.

Useful terms and units [1, 5 (p. 9–31), 6]

Linear energy transfer

The physical principle behind the effects of IR is the linear energy transfer (LET) of IR-energy to matter, which depends on:

a) (The charge of the incident particles)^2,
b) The electron density of the absorber, and
c) (The velocity of the incident particle)$^{-1}$.

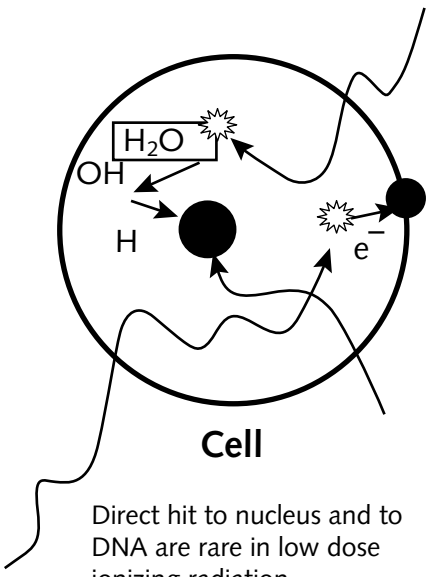
As the electrochemical energy of IR is absorbed, ions are produced mainly in the predominant cellular medium, which is water. These activated chemical ions are "free radicals" capable of changing the structure of cellular molecules, cell function, and life cycle (i.e., arrest or acceleration of cell division and apoptosis), and inducing DNA damage with all its consequences. These can be negative, as in the case of double strand DNA breaks, or even positive by activation of protective reactions against free radicals and reactive oxygen species (ROS), and DNA repair mechanisms. Direct hits to nucleus and DNA and their consequent damage from absorbing all the IR energy are rare in low doses of low LET radiation. X-rays, gamma rays, and β radiation are examples of low LET radiation. Alpha particles, neutrons, and some other uncharged radiation are examples of types of high LET radiation, because they generate high numbers of secondary charged particles in water, becoming very damaging (fig. 1). High LET radioisotopes are not used in metabolic studies, although neutron activation analysis can be used with caution.

Radiation units

The radiation units commonly used for the absorbed dose (or simply the dose) are the traditional rad and the newer SI unit, the Gray (Gy). One Gy = 100 rads. One rad is biologically equivalent to one roentgen (R). The units of risk derived from the absorbed dose, resulting from the dose and the quality factor (Q), itself derived from the LET for different kinds of IR, are the traditional rem (roentgen-equivalent-man) and the newer SI unit, the Sievert (Sv). One Sv = 100 rems.

Relative hazard (H)

The relative hazard (H) of IR delivered by different radioisotopes depends on the amount of radioactive material, A, the toxicity factor of the radioisotope, T, and the use factor, U (H = ATU). Relative toxicity factors (T) have been established by diverse studies and the great majority of radioisotopes employed in human tracer research have a T of 1, compared to 100 for α radiation. ^{3}H has a T of 0.1, and ^{22}Na, ^{47}Ca, ^{125}I, ^{140}Ba have a T of 10. The use factor (U) is the fraction of the IR that is affecting the "target" organ or tissue.



Linear energy transfer (LET) depends on:

a) (The charge of the incident particles)^2,
b) The electron density of the absorber, and
c) (The velocity of the incident particle)$^{-1}$.

Low LET is produced by electromagnetic radiations and by low-energy electrons. X-rays and gamma rays produce a cascade of ions by exciting electrons which damage DNA and membrane structures.

Low energy radiation and electrons lose most of their energy with the first collision with other sub-atomic particles.

Radioisotopes used in research with humans have low LET.

High LET is produced by alpha particles, high-energy X- and gamma rays and high-energy electrons. These are very damaging to cells and their structures and are not used in human research.

FIG. 1. Interactions of ionizing radiation with matter

Equivalent dose

The equivalent dose (H_T) a tissue or organ receives from IR is the product of the average absorbed dose in that tissue or organ and a radiation weighting factor (W_T) for each radiation. This quantity is intended to correlate, independently of each tissue, with the overall detriment to the whole individual. The US National Commission on Radiation Protection (NCRP) specifies a W_T of 1 for X and γ rays, electrons, positrons and muons.

The fact that different tissues respond differently to IR requires that such differences in stochastic responses be weighed in order to calculate total body radiation. This is achieved by considering specific tissue weighting factors which when added sum up to 1. These factors are related to tissue turnover rates and range from 0.2 for gonads to 0.01 for bone surface. The risk derived from considering all stochastic effects is represented by the effective dose (E) and is expressed in rems or Sv: $E = \sum H_T W_T$. When whole body radiation is uniform $E_T = H_T$ because the sum of W_T is 1. However, when a radionuclide enters the body it becomes distributed in various organs and tissues in different concentrations and remains there for different times. The total dose received by an organ or tissue over time is the integrated Committed Equivalent Dose of H_T for that tissue or organ over time. By convention the time estimates are 50 or 70 years, for IR exposed adults and for IR exposed children, respectively. Following this reasoning, the committed effective dose equivalent (CEDE) as recommended by the International Commission on Radiation Protection 30 [7] is the weighted sum of all the committed equivalent doses for all tissues exposed to IR.

Low doses of linear energy transfer ionizing radiation

The evidence or lack of it that low doses of low linear energy transfer (LET) ionizing radiation (IR), similar to those used in tracer radioisotopic studies in nutritional and metabolic research may be damaging, indifferent, or even beneficial is presented below. A consequence of these findings should be a modification of the current linear model for estimating risk for others that include a threshold.

DNA damage, other undesirable cellular effects, and their health and developmental consequences due to high doses of ionizing radiation as well as factors that modify them are well documented. Table 1 summarizes the biochemical effects, the cellular defense or control mechanisms, and the cell to cell communications (clastogenic responses) which amplify these control mechanisms. Table 2 summarizes the end results of these changes due to high doses in terms of cell function and health, as well as the factors that modify the effects of a given dose.

In contrast to the demonstrated effects of high ionizing radiation doses, the effects of very low radiation doses are less well known. Human, animal, and in vitro tissue studies seem to indicate that low LET radiation at low doses, especially those delivered at low dose rates (days rather than minutes or hours), do not cause

TABLE 1. Biochemical basis of radiation damage and repair

Damage	Control mechanisms	Clastogenic responses
Most important damage is to DNA, caused by ionization of water and generation of reactive oxygen species (ROS), producing oxidation of bases (8-OHDG), single strand brakes (SSB) and double strand brakes (DSB), apurinic/apyrimidinic sites (AP sites), chromosomal breakage, and genetic instability. ROS also oxidize proteins and membrane lipids, and affect mitochondrial function.	Activation of DNA damage control system, including complex mechanisms in radical detoxification and DNA repair involving recognition of damaged site, excision of base or nucleotide, repair replication, and rejoining of strand. Stimulation of polymerases, kinases, endonucleases. Arrest cell cycle progression. Removal of damaged cells by apoptosis and globally by immune responses.	Inter-cell communication of adaptive responses to activate DNA damage control systems

TABLE 2. Main undesirable consequences of high radiation doses [above 0.1 Sv (10 rems)] and modifying factors

Undesirable consequences	Modifying factors
Cell death and altered replication Gene and chromosomal damage due to DNA damage Various cancers: skin, lung, mammary gland, gastrointestinal system, leukemia, thyroid Developmental alterations, congenital defects, and mental retardation Genomic transmission and its intergenerational consequences	The type and dose of radiation The rate at which the dose reaches the tissues The stage of the cell cycle when radiation reaches the tissues Sex and age of exposure Exposure to other factors (toxins, chronic inflammation, nutrition) A variety of chemicals can modify the cell killing effect of radiation Modifiers exist which greatly influence the multistep processes of carcinogenesis DNA repair and antioxidant systems The shape of the dose-response relationship: linear, non-linear, linear-quadratic and other complex functions

short- or long-term undesirable effects (table 3) and often perturb, in a favorable way, physiological signaling pathways leading to accelerated DNA repair and increased defenses against free-radical damage [8, 9]. This phenomenon is known as hormesis (table 4).

Our interpretation of current scientific data agree with the conclusions arrived by the Health Physics Society [10] which indicate that health risks due to IR cannot be estimated quantitatively "below an individual dose of 5 rem (0.05 Sv) in one year or a lifetime dose of 10 rem (0.1 Sv) in addition to background radiation," which is estimated to be 200 to 300 mRem/year in the United States and about 250 mRem/year in the United Kingdom [6, 11].

Cancer incidence

No increment in cancer incidence during the remaining years of life among persons exposed to low IR levels has been detected, even when IR sources are high LET. This is in part because "natural" cancer deaths are high (~20% of all deaths in the USA). From linear extrapolations of stochastic incidences of extra cancer deaths from high radiation doses, the Biological Effects of Ionizing Radiation fifth committee report (BEIR-V) [5, p. 9–31] arrives at a figure that for every whole body dose of 100 mGy (10 rads) to all the population the number of cancer cases per 100,000 individuals would increase from about 20,000 to 20,800 over the life-times of all individuals (note that the error in the estimate of "naturally" occurring cancer deaths is close to 4,000

TABLE 3. Human and other studies have failed to demonstrate that low ionizing radiation doses of low LET result in radiation-induced cancers and genetic alterations

Human studies	Other studies
Atomic bomb survivors who received doses < 0.2 Gy Humans exposed to atomic bomb test sites Workers in shipyards with atomic reactors Radiation workers and uranium miners X-ray exposure for TB diagnosis Spondylitis and other therapies High natural background areas	Animal Tissue culture Cellular enzymatic Genomics Intergenerational persistent effects

All strongly suggest a threshold model rather than a linear model.

TABLE 4. Hormesis: "Stimulatory or beneficial effect observed when a biological system is exposed to a low dose of an agent known to be toxic or damaging at a significantly larger dose"

Examples
» In lymphocyte cultures, DNA repair and free radical protecting enzymes are enhanced by exposure to low doses of ionizing radiation (IR). The result is that previous exposure to low IR doses protects from the effects of subsequent exposure to large IR doses not only in the cells receiving a hit (where LET occurs) but also in surrounding cells and in extra-cellular media by clastogenic mechanisms.
» Intestinal cell renewal increases readily when exposed to continuous low IR levels.
» Spleen plaque-forming cells in mice are stimulated by exposure to 25 to 75 mGy.
» Irradiated mice and guinea pigs have a slightly greater life span than controls and develop less tumors at standardized ages.
» Lower lung cancer incidences than expected have been noted in plutonium-plant workers chronically exposed to low IR as well as in individuals exposed to low radon levels.
» High natural background radiation in different parts of the world (Brazil, China, France, India, Iran, Italy, Madagascar, and Nigeria) show lower or the same cancer mortality rates as control populations, even though higher prevalence of apparently innocuous chromosomal aberrations have been found in some areas.
» Strains of non-obese, insulin-dependent diabetic mice subjected to low IR doses are protected from developing the clinical syndrome and exhibit less apoptosis of insulin producing cells as well as stimulated activities of several enzymes in antioxidant defense systems and DNA repair.

Modified from ref. 5 and 9.

per 100,000). The relative risk coefficient derived from estimates of the risk of developing different types of cancer following high IR doses indicates that for every age group the increment in cancer incidence over the natural number of cases is constant but varies with the time of exposure (acute or spread over time), the physiological or developmental stage, and the period elapsed after exposure. For example, exposures of 4.5 to 6 Gy to mammary glands during their development (at age 15 years) increase the risk of cancer by about 25% over the same dose given before their development or after they are fully developed (at ages < 5 or > 25 years) [5 (p. 229–34), 12] (fig. 2).

According to the atomic bomb survivors study, leukemia develops relatively early and solid tumors appear later and cause more deaths. The leukemia incidence, however, appears lower than the "natural" incidence when the IR doses to bone marrow were lower than about 20 rems (0.2 Sv) suggesting a hormetic effect [13, 14] (fig. 3). Other data suggesting a hormetic effect have been obtained in the case of lung cancer incidence and radon exposure in miners as well as in workers in nuclear reactors. Lung cancer incidence among the miners receiving less than 6 Gy/year was not elevated and actually declined as air radon concentration increased within these low radiation exposures. This contrasts with the predicted increases in incidence by the linear regression model [15, 16]. Mortality due to all causes, leukemia, lymphatic, and all blood cancers was lower in nuclear reactor workers than in non-exposed workers* (fig. 4).

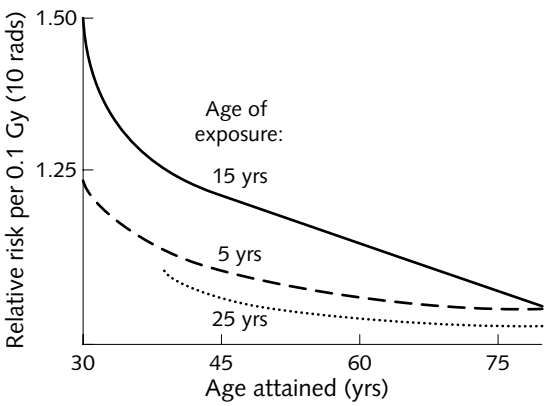


FIG. 2. Relative risk incidence of breast cancer at different attained ages, by age of IR exposure doses between 4.5 and 6 Gy. Source: ref 5

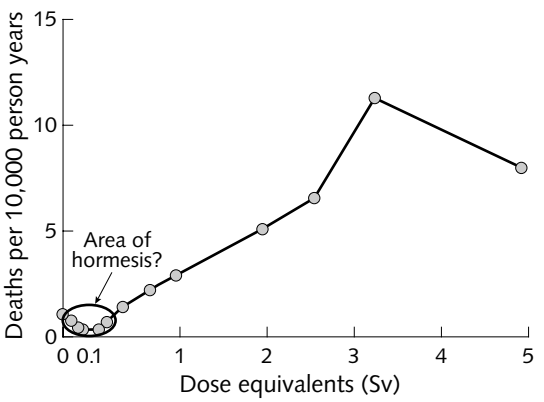


FIG. 3. Leukemia deaths per 10,000 person years consequent to different dose equivalent IR exposures of the bone marrow. Note: by 1985 there were 51 cases in the 0 Sv category and 31 cases in the 0.01–0.1 Sv stratum. Source: ref 5

* Matanoski GM. Health effects of low-level radiation in shipyard workers. Final Report DE-AC-02-79-EV 1000095. Washington, D.C.: Department of Energy, 1991:328–43.

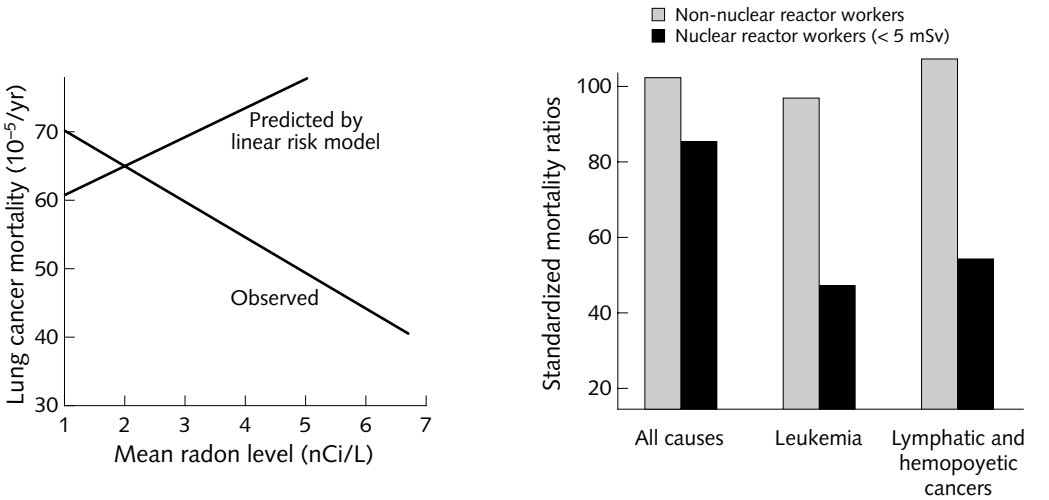


FIG. 4. Cancer mortality rates among miners and nuclear reactor workers

Other effects

The fetus is more susceptible to IR than adults [17] and the moment of exposure to the same high dose of ionizing radiation has different consequences. For example, experimentally, the embryo and the fetus express radiation damage in different forms: death occurs when irradiation exposure takes place in the pre-implantation period; malformations (teratogenesis) occurs during organogenesis; and no immediate damage occurs during the final stages of pregnancy [17–19] but may manifest higher cancer incidence (especially leukemia) early in life [20]. In the human these gestational developmental stages correspond to 9 days, 10 days to 25 weeks, and 26 weeks or later. Fetal exposure to doses (≥ 1Gy) when brain organogenesis is at its peak (gestational weeks 8 to 25) in the human, produces significant mental retardation but has no effect in these functions from week 26 onwards [18] (fig. 5).

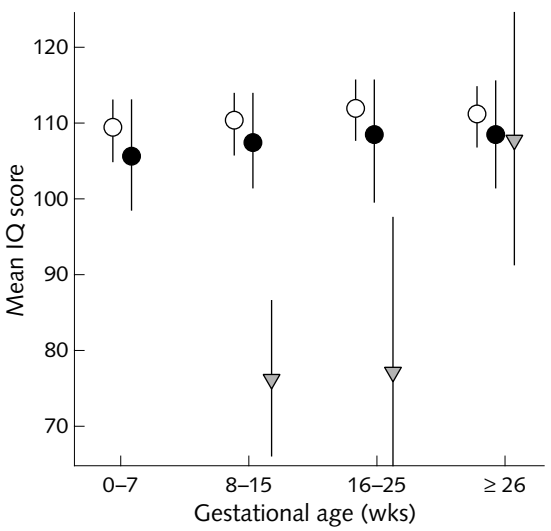


FIG. 5. Effect of different radiation exposures during fetal life

Mutations due to damages to genetic material caused by IR were recognized early in the twentieth century, and experimentally the genetic effects of radiation have been well documented in a large variety of living organisms. However, these damages in different species can not be extrapolated with confidence to the human. For example, data from children exposed to IR from atomic explosions in Japan strongly suggest that humans are less susceptible to radiation damage than mice.

The genetic effects of low doses of low LET IR in the human are still obscure mainly because of several limitations in assessing them. They are not expressed in the person exposed (cancer is possibly an exception). They are expressed in their immediate or remote offspring. When population studies are performed the doses received, the sex, age, and rates of exposure are different, not uniform nor randomly distributed. In addition, all reproductive cells have spontaneously-occurring genetic alterations and small increments in genetic damage due to low IR doses in the human are very difficult to estimate. The BEIR V report arrives at an estimate that the doubling dose for genetic defects in humans is greater than 1Sv (100 rems) and that an equilibrium on clinically mild dominant genetic disorders is reached at 75 cases per million when a population is exposed to 1 rem per generation (the current spontaneous incidence is 7,500/million live-born offspring). All other genetic defects are much lower, including clinically severe, X-linked, recessive, chromosomal translocations and trisomies, congenital abnormalities, and other disorders of complex etiology, such as heart disease, cancer, and "selected others" [5, p. 65–134].

Implications on extrapolation models on the negative effects of low doses of low LET ionizing radiation

Mounting evidence from studies outlined in table 3 and in figures 3 and 4, strongly suggests that there is a threshold for detectable negative effects consequent to low IR doses of low LET and that there can actually be beneficial effects through the phenomenon of hormesis which consists in "a stimulatory or beneficial effect observed when a biological system is exposed to a low dose of an agent known to be toxic or damaging at a significantly larger dose." Examples of hormesis triggered by low IR abound [8, 22–29] and are summarized in table 4.

Based on the above evidence and on the mechanisms involved in response to low IR doses, the development of guidelines for estimating the hazard associated with them based on linear regression models must be reviewed and changed for either a lower risk or a threshold/hormesis model (fig. 6). If these risk models are adopted by regulatory agencies, new guidelines on the permissible use of radiation doses will allow further prudent use of radioisotopes in tracer studies in humans.

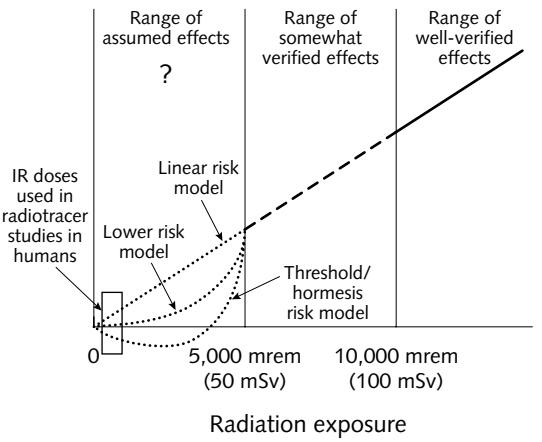


FIG. 6. Models for estimation of risk from ionizing radiation exposure by administered dose. Source: ref 41

Nutritional and metabolic research using radioisotopes has contributed significantly to our knowledge, as exemplified by the number of studies and programs supported, for example by the International Atomic Energy Agency (IAEA) [30–32] and the U.S. Government [33, 34]. Importantly, the majority of studies, even using long-physical-life isotopes have had committed effective dose equivalents (CEDEs) within the low area of undetectable effects (table 5)

In summary, all evidence indicates that when radioisotopes are properly applied, resulting in low CEDES, the health risk is at most, undetectable because it is very low, nil, or may be even protective. In our opinion, the linear no threshold model should be abandoned for a model that accurately depicts the true risks, or lack of them when low dose, low rate, low LET radiation is used.

Comparison of benefits, limitations, availability, and relative costs of tracer studies using radioisotopes and stable isotopes

There is no question that studies in humans using stable isotopes have contributed significantly to our knowledge, and should be promoted. A few exemplary contributions are measuring long-term total energy expenditure with doubly-labeled water, in vivo elucidation of metabolic pathways using ^{13}C and ^{2}H labeled molecules and pool sizes and fluxes by using ^{70}Zn and ^{67}Zn [35–37].

Unfortunately there are serious obstacles to the ample use of stable isotope studies where most nutritional deficiencies exist. These include the often limited isotope availability, their cost, the necessity of expensive equipment affordable only by a few special research settings, the serious technical difficulties in achieving and maintaining standardized and reliable results, and the need of highly trained professionals for maintenance

TABLE 5. Radioisotopic studies supported by the IAEA and the US Government

IAEA	US Government
Coordinated research program on isotopic-aided studies of the bioavailability of iron and zinc from human diets and fortificants (12 countries, 10 of them developing) (1990–1993). Methodology development and its application in nutrition. The majority took place up to 1974,but micro-nutrient bioavailability studies and others (iron, zinc, body composition)are ongoing at present (1999–2001) in selected populations.	Human metabolic research involving the following radioisotopes: ^{131}I, ^{14}C, ^{3}H, ^{24}Na, ^{54}Mn, ^{28}Mg, ^{36}Cl, ^{42}K, ^{60}Co, ^{58}Co, ^{57}Co, ^{47}Ca,^{85}Sr, ^{65}Zn, ^{59}Fe, ^{55}Fe, ^{32}P, ^{51}Cr, ^{111}Ag, ^{75}Se

Importantly, studies can be performed in humans even with very long physical half life, low LET radioisotopes such as ^{14}C and ^{3}H as long as biological half lives are short resulting in low CEDEs. For example, ^{14}C doses administered in several studies were as high as 10 μCi (370 kBq) that in the case of ^{14}C-acetate, with a half-life of 50 days, delivered a very low CEDE of 21 mRems (0.21 mSv). For 46 μCi of $^{3}H2O$ ingested, CEDE was 2.8 mRems (0.028 mSv).

and operation of the sophisticated mass spectrometers required for many specialized metabolic studies.

To these factors must be added the fact that in some micronutrient tracer studies, the need to administer isotope doses large enough to achieve measurable tracer to tracee ratios (relative enrichments) making them not truly tracer studies. For example, the addition of 4 mg of ^{57}Fe to a meal, necessary for reliably detecting ^{57}Fe enrichment of hemoglobin, alters the ratio of iron to phytates and to other minerals in that meal. This is not the case for either ^{55}Fe or ^{59}Fe, where only micrograms of high specific activity tracers are used [38–41].

Table 6 compares the costs of doing metabolic studies in iron and zinc absorption and metabolism. The results of these comparisons are evident. Many other studies using stable isotopes are more expensive even by several orders of magnitude. However, one must profit from advantages in the use of stable and radioisotopic methodologies. For example, we are developing a combination of double stable and single radioisotope methods to validate and standardize measurements of zinc absorption applicable to population studies* [42].

Radioisotopic-aided nutritional and metabolic studies with emphasis on the committed effective dose equivalent (CEDE) administered to humans

We have performed a series of studies on iron and zinc absorption and metabolism using radioisotopes and or combinations of radio and stable isotopes. Table 7 presents the radiation doses administered and their CEDEs, estimated by following the ICRP 30 [7] guidelines.

In terms of excess radiation over the mean yearly natural radiation in the United States and the United Kingdom (200 to 300 mRem/year, or about 2.5 mSv/year), the CEDE amounts to 5.4% for ^{59}Fe, to 0.96% for ^{55}Fe and to 15.8% for ^{65}Zn above the natural yearly background radiation. Expressed in other terms, the excess CEDE amounts to 19.7 days for ^{59}Fe, to 3.5 days for ^{55}Fe and to 57.8 days for ^{65}Zn, above the 365 days of natural background radiation in a year. These data indicate that these CEDEs are near the extreme left of the region where no effects are detected and in the region where hormesis is prone to occur.

Conclusions and recommendations

The dose-risk model being used to establish regulatory IR dose limits assumes a linear relationship with no threshold above zero dose, suggesting that any amount of radiation is damaging. Evidence against this model abounds, indicating a lower risk or a threshold/hormesis model, even resulting in beneficial effects of low LET radiation dose. The bodies recommending appropriate standards for protecting radiation workers and the general public have been aware of the non linear relationship of dose and risk for low total IR dose and dose rates for low LET radiation. In spite of this, the conservative approach taken by these agencies has resulted, over the years, in an unquestioned belief that "there is no safe IR dose," leading to an excessive societal concern regarding the use of minimal amounts of radioactive isotopic material such as those being employed in human tracer research.

The Health Physics Society [10], referring to dose and risk for low dose exposures, "recommends against quantitative estimation of health risks below an individual dose of 5 rem in one year or a lifetime dose of 10 rem in addition to background radiation. Below

* Viteri FE, Mendoza C, Woodhouse LR, King, JC, Brown KH, Mukherjea R, Barnese K. Combination of stable (^{67}Zn and ^{70}Zn) and radioisotopic (^{65}Zn) zinc for measuring zinc absorption. Preliminary report.

TABLE 6. Relative costs of studies using stable or radioactive isotopes

Isotopes	US$ per study	Equipment, US$	Personnel
Stable isotopes			
^{58}Fe ($ 95/mg)	300 PO, 28.5 IV	260,000 to 586,000	Senior experienced scientist (high annual costs)
^{57}Fe ($7.5/mg)	61 PO		
^{54}Fe ($7.0/mg)	120 PO		
^{70}Zn ($100/mg)	160 PO, 40.0 IV		
^{67}Zn ($35/mg)	35 PO		
Radio isotopes			
^{55}Fe ($2.4/μCi)	9.6 PO, < 1.00 IV	20,000 to 70,000	Easily trained technician (moderate annual cost)
^{59}Fe ($1.6/μCi)	3.2 PO		
^{65}Zn ($0.3/μCi)	0.8 PO		

PO, oral dose; IV, intravenous dose.

TABLE 7. Iron and zinc absorption/bioavailability studies using radioisotopes in humans and radiation doses administered

Case A	Oral administration of 4 μCi (148 kBq) of ^{55}Fe and 2 μCi (74 kBq) ^{59}Fe. Half the doses were administered 15 days apart. Total CEDE for 2 μCi of ^{59}Fe: 13.4 mRem. NOTE: Total risk (CEDE) is condensed to the year of dosing. Total CEDE for 4 μCi of ^{55}Fe: 2.4 mRem;
Case B	IV administration of 0.25 μCi ^{65}Zn (9.25 kBq), and 6 and 12 days later, 1.25 μCi ^{65}Zn (46.25kBq) orally. Total CEDE for 2.75 μCi of ^{65}Zn: 39.6 mRem
Permissible doses	Nuclear Regulatory Commission: 100 mRem/year (1mSv/yr) for the general population. Pregnancy (after 26 weeks): maximum total 50 mRem (500 μSv). In vivo neutron activation analysis (IVNNA): 20–30 mRem (200–400 μSv)
Therefore, in all cases the CEDE is less than 40% of the yearly permissible dose.	

these dose levels risk estimates should not be used." More research needs to be done in order to reduce the uncertainties in risk assessment models of low LET IR [8]. (This is ongoing from genomic expressions to long-lasting epidemiological research). Important research in nutrition and metabolism has been hampered because of an overestimated fear of radioisotopic tracer studies. Unfortunately, most stable-isotopic-based research is extremely expensive and often less reliable than radiotracer studies, limiting their application. Combined use of stable and radio isotopes should be explored further.

The IAEA and affiliated centers should make better known their health physics expertise and their capability to advise, support, and monitor researchers on the safe use of radioisotopes in human and animal nutritional and metabolic research. They should take advantage of all communication means available now to produce a template to be filled by all researchers using radioactive tracers. Additional advice and support, based on this template, should be provided by the Agency and its affiliated centers.

Considering the accumulated evidence that low IR doses of low LET have no harmful effects and may be even beneficial, the regulatory agencies must conduct a public information campaign following and expanding the Health Physics Society statements. This campaign should be addressed to eradicate the unfounded fear of using prudent doses in tracer radioisotope-based research.

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Uses of stable isotopes in the assessment of nutrient status and metabolism

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Abstract

Stable isotopes are nonradioactive and can be safely administered to humans; yet, because of the isotopic difference, can be distinguished from the unlabeled moiety and thus trace the nutrient uptake and elimination. Stable isotope applications include measurement of nutrient absorption, determination of nutrient body stores, tracing routes of nutrient metabolism, measuring nutrient fluxes through specific pathways, and measuring nutrient elimination. The ability to assess the dynamics of nutrient metabolism in vivo has been vital in the study of nutrient requirements, nutrient metabolism, mechanisms of nutrient homeostasis, and nutrient toxicity. Stable isotopes provide a window into human metabolism that is particularly valuable to the quantitative study of human nutrition.

Key words: Mass spectrometry, doubly-labeled water, breath test

Nutrition differs in principle from many other sciences because of its essential quantitative nature. Research directed toward an understanding of cause and effect, biochemical mechanisms, and basic nutritional principles form the basis of nutritional science, the ultimate nutrition research question is how much of a nutrient is required to maintain health and optimize well-being in human nutrition. Unfortunately, humans generally do not make good subjects for quantitative research. We have a diet that is highly varied with respect to food items and meal patterns; and we do not like to participate in scientific studies that restrict our ability to self-select a diet, limits our daily schedule, or requires invasive sampling. Finally, responses to nutritional perturbations are often slow play out and difficult to measure.

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A tool available to nutritionists that can improve the ability to accurately and precisely measure nutrient requirements is stable isotope tracer technology. Because stable isotopes do not present a radiation hazard, they can be safely utilized in human research as long as requirements for chemical purity are met [1]. The only stable isotope known to have any toxic isotope effects is deuterium and even this requires large doses that would comprise more than 15% of body pool [1]. Deuterium labeling, however, can alter the metabolism of the labeled compound if the isotopic label is inserted at a key reaction site, an issue that should be considered during the design of the study [2].

The advantages and some of the limitations of stable isotope methods can be illustrated with a discussion of the doubly-labeled water method for the assessment of energy requirements [3, 4]. The doubly-labeled water method, which was developed by Lifson, is a stable isotope method for measuring total daily energy expenditure for a 4 to 20 day period [5]. A loading dose of water labeled with both deuterium and oxygen-18 is administered by mouth. The two tracers distribute in body water and the deuterium is eliminated as water. The oxygen-18 is eliminated as water and carbon dioxide. The difference between the two eliminate rates is therefore a measure of carbon dioxide production. Energy expenditure can be calculated from carbon dioxide production; but this does require an estimate of the respiratory quotient, illustrating that stable isotope techniques cannot totally replace traditional research techniques. The isotope elimination rates are determined from the isotope concentrations in spot urine samples collected shortly after the loading dose and at the end of the metabolic period. The advantage of the method is that the subject is free to engage in his/her normal activities during the period and that the period can be long enough to average much of the day-to-day variation in physical activity.

Prior to the use of the doubly-labeled water method for measurement of energy expenditure, energy requirements were estimated from dietary energy intake during weight maintenance or a factorial calculation of

energy expenditure. These estimates formed the basis for establishing energy requirement recommendations. In so doing, large differences in the requirements of adults were frequently observed and much research was directed towards identifying the mechanisms through which many individuals lead healthy, productive lifestyles on apparently small energy intakes [6]. With the advent of the doubly-labeled water technique, direct objective measurement of energy expenditure under free-living conditions became possible. Carefully prepared, self-reported energy intakes using a weighed dietary record were utilized by Livingstone et al. [7] to identify groups of individuals who were classified as small or large eaters. These reported consuming about 1.3 or 1.8 times their calculated basal metabolic rate, and thus indicated unusual energy requirements. Measurement of energy expenditure by doubly-labeled water, however, contradicted this dietary data. The small eaters were found to have energy expenditures that were about 1.8 times their basal metabolic rate and did not differ from that of the large eaters. These data indicated that the self-recorded energy intakes were not accurate and that energy requirements for individuals of similar body size and lifestyle were not as variable as had been previously thought [7]. Similarly, in a study of women living in an agrarian society in the Gambia energy expenditures were twice as great as had been indicated by previous dietary intake data [8]. Results from both of these studies have been instrumental in strengthening the basis for establishing energy requirements and, more importantly, redirecting research efforts. Before the doubly-labeled water data on energy expenditure became available, the dietary energy intake data suggested that human energy requirements were very flexible in the face of changes in energy intake. This suggestion led to a considerable research effort directed towards identifying those mechanisms. The results of doubly-labeled water studies, however, now indicate that the dietary data was biased and that research needs to be redirected.

In a similar vein, the doubly-labeled water has been used in two milestone efforts to refine the energy requirements for infants and children. Prentice et al. [9] demonstrated that previous energy requirements for infants and children were as much as 20% too high. To better understand how these older estimates could have been so high, Lucas et al. [10] measured the energy density of human breastmilk. It was found that the density was 0.6kcal/ml (0.25 MJ/dl), which is substantially less than had been previously estimated on the basis of analysis of expressed breastmilk.

A second area in which stable isotope techniques have advanced nutritional science during the past decade is the carbon-13 breath test. The stable isotope breath test methodology was of special interest of Dr. Peter Klein, who championed much of the early methodology development [11]. The concept of the breath test contrasts that of the doubly-labeled water technique in that the tests are generally semi-quantitative; yet like the doubly-labeled water technique the carbon-13 breath test opens a noninvasive, nonhazardous window into the workings of human metabolism. The breath test is designed to monitor the activity of a specific enzyme or metabolic system in vivo. A substrate is labeled with carbon-13 in a position targeted by the enzyme of interest. Cleavage of the substrate releases the carbon labeled, which is then oxidized to carbon dioxide and excreted in breath, where it can be measured by isotope ratio mass spectrometry. The ideal substrate is designed such that the target bond cleavage is the rate-limiting step in labeled carbon dioxide production and that production rate becomes a measure of the activity of that enzyme or system [11]. An early example of the breath tests used for the study of nutrition was the glycocolate breath test for bacterial overgrowth in the small intestine [12]. More recently, fat breath tests have been compared to investigate the relationship between fatty acid chain length and saturation on oxidation [13]. The breath test concept, like the doubly-labeled water method did not immediately take hold. Like the doubly-labeled water technique the information provided by the test did not at first justify the expense. Two things, however, changed that in the past decade. The first was the development of a breath test that provided vital and virtually unique information to the investigator and the second is technological development. The urea breath test measures gastric infection by *helicobactor pylori*, the cause of most gastric ulcers, which previously was only possible by a biopsy of the stomach [14]. The ability to monitor the presence of helicobactor pylori with a noninvasisve breath test has made it possible to improve treatment and perform epidemiologic studies in field situations. This development, like the development of the doubly-labeled water method, has redirected research in the field. The second factor that is just now beginning to impact carbon-13 breath tests is the development of a lower cost, easier to use, infrared spectrometer for the measurement of carbon-13 in carbon dioxide [15]. The product of over 30 years of development, the optical technique has achieved a level of precision that can detect less than 1 part of excess carbon-13 in 100,000 parts of unlabeled carbon dioxide, which is the sensitivity necessary to compete with isotope ratio mass spectrometry and keep the isotope costs of the breath tests low.

Breath test approaches have also been applied to the study of macronutrient metabolism. Vernon Young has been a leader in the field of stable studies of amino requirements and one of several that have been involved in the development of amino acid oxidation approach to measurement of indispensable amino acid requirements [16, 17]. The concept of this technique was that the body would conserve the labeled amino

acid when it is supplied at levels below the minimum requirement, but oxidize increasing amounts as the amino acid intake is increased above the minimum requirement. This approach in combination with more invasive constant infusion approaches has been instrumental in understanding amino acid requirements and particularly the changes that occur during trauma and other metabolic stresses [18]. The stable isotope technology for the study of amino acid requirements, however, has not been extensively applied under field conditions in developing countries, which may be a product of the often complex and invasive infusion protocols that have proven so useful in developed countries. To counter this limitation, the International Atomic Energy Agency developed a simplified protocol that eliminated blood sampling and used oral tracer administration [19]. Although the protocol may prove to be somewhat less quantitative than the more complex infusion protocols, the simplified protocol lends itself to use under field conditions and promises to provide insights into amino acid requirements in situations that could never before be studied except with more traditional anthropometric techniques.

Isotopic techniques have been developed for a wide range of nutritional studies that go beyond energy expenditure and macronutrient metabolism. Stable isotope dilution techniques are making it possible to measure nutrient stores such as vitamin A, which previously could not be assessed without the use of invasive liver biopsies [20]. Even questions of molecular and cellular turnover are now being addressed with stable isotope techniques and are providing answers to human questions that are beginning to arise from molecular biological investigations [21].

Stable isotope techniques, like any technique, are not a panacea for all research problems. There are barriers to their use and limitations to their precision and accuracy. For example, the doubly-labeled water technique requires large amounts of oxygen-18 water. Twice in the past 20 years, world production has failed to keep pace with demand and many investigators were forced to halt doubly-labeled water-based research. Even when it was available, the cost of the oxygen-18 alone for a single adult study exceeded $500 (US). Furthermore, the analysis of the isotope concentrations requires expensive mass spectrometry and analytical expertise that limits the use of the method to those investigators that are working in or collaborating with the limited number of centers across the world that have both. Continued centralized efforts by major research agencies are needed to address these limitations so that more investigators can begin to realize the advantages of stable isotopes in their nutritional research.

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^{13}C breath tests in infections and beyond

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Abstract

Stable isotope labeled compounds are widely used as diagnostic probes in medicine. These diagnostic stable isotope probes are now being expanded in their scope, to provide precise indications of the presence or absence of etiologically significant change in metabolism due to a specific disease. This concept exploits a labeled tracer probe that is a specifically designed substrate of a "gateway" enzyme in a discrete metabolic pathway, whose turnover can be measured by monitoring unidirectional precursor product mass flow. An example of such a probe is the ^{13}C-urea breath test, where labeled urea is given to patients with H. pylori infection. Another example of this kind of probe is used to study the tripeptide glutathione (glu-cys-gly, GSH), which is the most abundant cellular thiol, and protects cells from the toxic effects of reactive oxygen species. Within the gamma glutamyl cycle, 5-oxoproline (L-pyroglutamic acid) is a metabolite generated during GSH catabolism, and is metabolized to glutamic acid by 5-oxoprolinase. This enzyme can also utilize the substrate L-2-oxothiazolidone-4-carboxylate (OTC), to generate intracellular cysteine, which is beneficial to the cell. Thus, labeled (^{13}C) OTC would, under enzymatic attack yield cysteine and $^{13}CO_2$, and can thus track the state and capacity of glutathione metabolism. Similarly, stable isotope labeled probes can be used to track the activity of the rate of homocysteine clearance, lymphocyte CD26, and liver CYP (cytochrome P450) enzyme activity. In the future, these applications should be able to titrate, in vivo, the characteristics of various specific enzyme systems in the body and their response to stress or infection as well as to treatment regimes.

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Key words: metabolic probes, gateway enzymes, glutathione

Introduction

Isotopic tracers have been used to determine dynamic aspects of metabolism in both the intact animal as well as in isolated tissues. These tracers can be radioactive or stable, and the latter allows the use of tracers in vulnerable groups, such as pregnant women and children where radioactive tracers may pose an unacceptable medical risk. Stable isotopes of an element differ in their neutron numbers, and are, as their name suggests, non-radioactive. Thus, stable isotope tracers have been used to determine quantitative aspects of substrate metabolism, such as rates of synthesis, transformation, or degradation.

The method followed in human stable isotope tracer-based studies is to administer the labeled compound by mouth or intravenously, such that the labeled compound (tracer) mixes within the compound that is being traced (tracee) within the body. Analysis of blood samples to assess the tracee/tracer rates of appearance and disappearance over different time periods, or physiological states, as well as analysis of excreted tracer in order to quantify oxidative processes is possible with a variety of compounds [1]. However, there are several conditions that need to be met in human studies, in order to use kinetic data effectively. These include conditions like the physiological state, the steady state, as well as other technical issues such as tracer recycling. In addition, these studies are usually difficult to perform, and technically complex. Hence there is a need for simpler techniques to use, especially in clinical or field situations, where demanding protocols may not be possible to implement. Such a technique would ideally involve the non-invasive (oral) administration of tracer, and collection of labeled end products in the breath, but without losing diagnostic value in terms of sensitivity and specificity. This easily implemented design of study is now available through

the use of metabolic probes (or metaprobes), which assess the activity of specific enzymes in the body [2] and since breath sampling is being discussed here, ^{13}C, which is stable and safe for general usage, would be the label of choice.

The ^{13}C breath test

This test is based on the delivery of a ^{13}C-labeled substrate into the body by oral ingestion or by injection. A specific enzyme in the target tissue then selectively metabolizes the substrate such that the tracer is irreversibly released as $^{13}CO_2$ into the body CO_2 pool [2]. The tracer is then transported to the circulation and excreted in the breath, such that a pattern of breath enrichment over time can be obtained. The enrichment of $^{13}CO_2$ in the breath can be measured by isotope ratio mass spectrometry (IRMS), which typically would require about 10 ml of breath, which can be easily collected in a vacutainer or sealed glass tube. The enrichments that can be measured by this system are low (in a range of 0.0001 atom percent excess) with a high precision, meaning that less tracer can be administered (thus minimizing costs). From this pattern and extent of enrichment, qualitative conclusions can be reached about the presence or absence of specific enzyme activity. If the breath enrichment data is subjected to pharmacokinetic modeling which correlates the input dose to the output of tracer, a numeric index of the metabolism of the labeled substrate can be obtained [2]. This metabolic index provides some quantitative information about the handling of the labeled substrate by the body, but is not able to provide more detailed metabolic information about the pathways of metabolism of the substrate prior to oxidation. Thus, detailed information about pool sizes of the substrate, or of its fluxes into and out of different pools cannot be obtained. Nevertheless, the requirement of a complex methodology and design to obtain the latter information should not offset the potentially valuable information that the breath test can provide in studies on nutrition, body function and disease.

Commonly used breath tests

There are a number of ^{13}C based breath tests that are currently available to determine the metabolic status of various organ systems in the body. A classic application, in terms of the informatics that can be generated on individuals, is the use of ^{13}C-aminopyrine to distinguish among different degrees of liver disease affecting the hepatic P-450 demethylation of ^{13}C-aminopyrine and subsequent production of $^{13}CO_2$ [3, 4], or by the use of ^{13}C-methacetin [4–6]. Investigators have recently used labeled α-keto analogs of the branched-chain amino acids to probe hepatic mitochondrial function by measuring the exhalation of labeled CO_2 [7]. Other breath tests include gastric emptying using ^{13}C-octanoic acid [8, 9], maldigestion using a variety of tracers such as ^{13}C-trioctanoin, ^{13}C-triolein etc [10–13], chylomicron remnant catabolism in obesity [14] and familial hypercholesterolemia [15], fat digestion at a very young age, by the use of ^{13}C labeled mixed triglycerides [16, 17] and the development of specific P450 dependent enzyme systems in infants [18].

In terms of infection, the most common breath test used is the ^{13}C-urea breath test, which is used to diagnose the presence of *Helicobacter pylori* infection in the stomach. *H. pylori* is a common infection in children studied in communities in Bangladesh [19], India [20], and Turkey [21], for instance. High infection rates of *H. pylori* among newborns and young children in developing countries appear to be a major cause for chronic under-nutrition and diarrhea syndrome with failure to thrive. It has been shown the height-for-age is lower in *H. pylori* infected children than in uninfected children [22]. In terms of causative links, an increased incidence of gastrointestinal infections has been hypothesized as a cause of growth faltering.

In this test [23, 24], ^{13}C-urea is administered orally, as a solution to drink, and the urease that is secreted by the *H. pylori* in the stomach acts on the labeled urea to release the label as $^{13}CO_2$. The $^{13}CO_2$ then enters the body pool to be excreted in the breath immediately. Therefore, an increase in breath enrichment of $^{13}CO_2$ immediately after ingestion of the ^{13}C-urea is indicative of an infection with this organism. Typically, breath samples are taken about 30 and 60 minutes after the dose, and are sufficient for a reliable diagnosis of the presence of *H. pylori*. Several studies are now available on the prevalence of this infection in developing countries, as well as its role in growth faltering in children. In India, based on this test, we have recently measured the prevalence of *H. pylori* in different age groups of children from rural and urban India, and found it to be of the order of about 50% to 85% in urban, low socioeconomic children between the ages of 6 to 16 years from North and South India (Raj & Kurpad, 2001, unpublished data, Mahalanabis & Kurpad, 2001, unpublished data).

The concept of a metabolic probe

In this paper, we describe the possibility of using stable isotope labeled compounds to explore specific enzymatic activity, through the use of metabolic probes, which we call 'metaprobes' [2]. Within the framework of this concept, intact organisms or humans can be studied through the oral administration of specific labeled molecules in order to characterize specific enzymatic activity through the measurement of the excre-

tion pattern of the released label in breath or urine. The concept exploits the possibility of studying specifically designed substrates of specific enzymes in a metabolic pathway. The labeled substrate (input) is administered to a subject, followed by the characterization of the labeled product (output, $^{13}CO_2$) of the enzyme activity in the breath, and the kinetics of the appearance of the product are calculated. A diagnostic determination of the metabolic activity of interest can be made based on the substrate-product conversion rate (input vs. output as a function of time) by the specific enzyme, by correlating to the metabolic condition of interest. The method can also allow for a non-invasive assessment of the effectiveness of a therapeutic intervention.

The term 'gateway' enzyme has been coined to mean those components of dynamic processes in the living organism whose turnover can be monitored by measuring unidirectional precursor-product mass flow [2]. In addition, for an enzyme system to qualify as a gateway that can be assessed practically, its metabolic products must be amenable to capture in a readily accessible metabolic compartment such as the breath, and be easily analyzed. The gateway enzyme should also be an indispensable part of a pathway, at which point a quantitative determination of the throughput (movement) of metabolites can be monitored. This is bound to be variable in populations, and therefore it is necessary to establish normative values of the labeled substrate-product conversion rate that is measured with the administration of the labeled substrate and measurement of the appearance of the labeled product. These normative data should also include conditions under which the enzyme system adapts or is regulated in health and disease, since the gateway enzyme would be chosen for study precisely because they straddle the boundaries of the divergent pathways of health and disease.

The term coined for the labeled substrate that measures gateway enzyme activity is 'metaprobe,' or a short form of metabolic probe [2]. This is a substrate that is acted upon by the gateway enzyme, and releases a labeled product that is immediately excreted in the breath (only breath tests are being referred to here; the product can also be excreted in the urine or be sampled in the blood). It is important that after the enzyme acts upon the labeled substrate, the labeled portion of the product ($^{13}CO_2$) is immediately excreted. Not all the labeled product is usually excreted, since there is retention of the $^{13}CO_2$ in various body CO_2 pools, and this incomplete recovery is usually corrected for in classical ^{13}C-substrate kinetic studies. However, from the viewpoint of a diagnostic test, the recovery of the labeled product can be corrected for, by some assumptions of the rate of CO_2 production, and meaningful indexes of the metabolism of the labeled substrate obtained.

The structure of the ^{13}C labeled metaprobe would depend on the particular gateway enzyme structure and substrate biochemistry and the metabolic pathway of interest. Ideally, the metaprobe would contain what is called a 'release tag,' which is CO_2 or bicarbonate in this case, and a core component, which is that portion of the molecule that is acted on to trace the biochemical process being investigated.

A specific metabolic probe: L-2-Oxothiazolidine-4-carboxylic acid (^{13}C-OTC)

Glutathione (GSH), which is present in high concentrations in mammalian tissues, has many diverse functions related to the protection of cells [26, 27]. Low GSH levels are associated with increased oxidative damage in mitochondria [27], and it has been suggested that aging is due to increased free radicals in the presence of low GSH levels [28, 29]. It is of interest, therefore, to study the modulation of GSH levels in health and disease.

GSH is synthesized through the γ-glutamyl cycle [30]. In this cycle (fig. 1), 5-oxoprolinase is the enzyme that catalyzes the reaction converting 5-oxoproline to

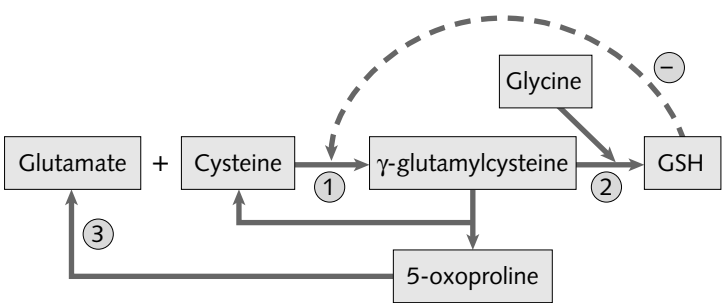

FIG. 1 GSH is synthesized in two steps from glutamate, cysteine, and glycine by 2 enzymes: 1) γ-glutamylcysteine synthetase and 2) GSH synthetase. GSH controls its production by negative feedback on reaction 1 (−). If γ-glutamylcysteine is not converted to GSH, it follows an alternate pathway to produce cysteine and 5-oxoproline, which is converted to glutamate via 5-oxoprolinase (reaction *3*). Source: ref. 42

glutamate, which is required for the first step for GSH synthesis, and a potential site for modulation of GSH synthesis [31, 32]. 5-oxoprolinase can also utilize the analogue, L-2-oxothiazolidine-4-carboxylate (OTC) to generate intracellular L-cysteine with the release of CO_2, and the administration of OTC can prevent hepatic toxicity by exogenous or endogenous agents, by the stimulation of GSH formation [33, 34]. OTC can also be called a 'prodrug' of cysteine, since it is a cysteine precursor [35, 36] and the latter is thought to be the amino acid that is rate limiting for oxoproline and GSH synthesis [37], and OTC has been established as an effective and relatively non-toxic cysteine delivery agent that is potentially useful in increasing intracellular GSH levels [38, 39]. Because of the efficient release of CO_2 from OTC, it is possible to measure the activity of 5-oxoprolinase indirectly, using a 5-^{13}C-oxoproline analog such as ^{13}C-OTC and then quantitating the $^{13}CO_2$ accumulation in expired CO_2 [2]. The metabolism of OTC by 5-oxoprolinase is dependent on the availability of the natural substrate, 5-oxoproline, for this enzyme. Under conditions of adequate GSH (or basal) availability, the GSH titer is high and will inhibit the synthesis of γ-glutamylcysteine, which is formed by the action of γ-glutamylcysteine synthetase on cysteine and glutamate as well as GSH. The flux of the glutamyl precursors can shunt to form 5-oxoproline, which will be acted upon by the 5-oxoprolinase, such that an analog of OTC administered under these conditions would be metabolized to a lesser extent, resulting in a lower rate of ^{13}C-OTC conversion and a lower cumulative appearance of $^{13}CO_2$ in the breath. Conversely, when GSH demand is high, γ-glutamylcysteine moves into GSH synthesis, leaving the 5-oxoprolinase pathway open for OTC metabolism and conversion to $^{13}CO_2$ [2] and resulting in the appearance of higher amounts of $^{13}CO_2$ in the breath. Therefore, this paradigm can delineate two conditions of adequate, and low GSH stores, and is the basis for the use of ^{13}C-OTC as a probe for precursor mobilization for GSH synthesis.

The quantitation of $^{13}CO_2$ in the breath can be used to generate some numerical evaluation of the metabolism of ^{13}C-OTC [2]. Therefore, by quantitating the appearance of the label in the breath over five hours after the dose, the mean residence time (MRT) can be calculated. The inverse of the MRT, which is equivalent to the fractional catabolic rate outside of the central compartment can then be multiplied by the dose of OTC per kilogram body weight, adjusted for insensible losses, to yield the 'metabolic rate index' (MRI, nmol.kg^{-1}. min^{-1}) for OTC. The information that can be gleaned by the use of this test can be improved by creating a stimulation of GSH synthesis by first depleting GSH stores. It is known that therapeutic doses of acetaminophen can stimulate the turnover of the pool of cysteine available for the synthesis of GSH. This is most likely due to an increased rate of synthesis of GSH which is required to detoxify the toxic metabolite of acetaminophen [40, 41]. Thus Fukagawa et al. [42] have used the paradigm of the MRI of ^{13}C-OTC to indirectly assess precursor (cysteine) mobilization for GSH synthesis when GSH stores are reduced by an acute ingestion of acetaminophen. In their study, the MRI of the ^{13}C-OTC probe was found to be significantly higher after reduction of GSH content (or the stimulation of GSH synthesis) by acetaminophen. They also used this test to assess the difference in adaptive capacity (difference between basal and post-acetaminophen MRIs) between young and old individuals, and showed that this capacity was two times as large in the young subjects when compared to the old subjects (table 1) [42]. These data support the use of ^{13}C-OTC as a probe to characterize individuals who may be at risk for low GSH stores or who have an impaired capacity to synthesize GSH, demonstrating the possibility of monitoring the metabolism of an oral tracer as an index for precursor mobilization in an ubiquitous metabolic pathway. In the post-genomic era, this also assumes importance in terms of phenotyping individuals with genetic defects of differences in the enzymes of the gamma glutamyl cycle.

GSH is important in clinical conditions, since

TABLE 1. Metabolic OTC index after an oral dose of ^{13}C-OTC without or with acetaminophen, in young and old individuals

Metabolic rate index (MRI)	Subjects		
	Young (n = 7)	Old (n = 5)	HIV (n = 5)[a]
Basal	286 ± 111	335 ± 110	375 ± 258
After acetaminophen	547 ± 269	452 ± 161	342 ± 243
Stimulated MRI	261 ± 115*	117 ± 40*	–33 ± 81

Data on young and old subjects from ref. 42.
Values are means ± SD; units of MRI are nmol. Kg^{-1}. min^{-1}.
OTC: L-2-oxothiazolidine-4-carboxylic acid
Stimulated MRI: difference between 'basal' and 'after acetaminophen'.
* $p < .05$, young vs. old.
a. Authors' unpublished data on HIV subjects (2001).

patients who are not able to respond to a demand on their stores of GSH by increasing the synthesis of GSH may be at higher risk of developing hepatic injury from drugs that require GSH for their detoxification [43]. The OTC paradigm can be extended to evaluating the need for specific therapies in patients, by characterizing these patients in terms of their basal MRI and their adaptive capacity in response to a controlled insult such as that provided by acetaminophen. Thus, patients with a high basal MRI or a low stimulation score might be less likely to benefit from antioxidant and related therapies aimed at boosting the GSH pool and might be the most susceptible to harm from treatments such as chemotherapy.

GSH levels are known to be decreased in several clinical conditions ranging from inflammatory bowel disease to HIV infection [44–47]. GSH levels are low in plasma and other body fluids [46, 47] in HIV patients, and this is clinically significant, since there is a strong association between survival and GSH deficiency in CD4 cells in these patients [48]. It is thought that the oxidative stress induced by reactive oxygen species may contribute to the immunodeficiency in HIV infection [49, 50], and studies have shown that there is an increased oxidative stress in HIV infection by an increase in lipid peroxidation [51]. Jahoor et al. have shown that the absolute synthesis rate and concentration of erythrocyte GSH are reduced in HIV subjects, and at least in part, this is cause by the availability of cysteine [52]. Other studies have also shown cysteine to be rate limiting for the synthesis rate and turnover of GSH in blood [53]. Therefore it is of interest to determine if the OTC metaprobe could evaluate this decrease in GSH synthesis rates, more particularly within the paradigm of an evaluation of the adaptive capacity of this process when a controlled insult by acetaminophen is administered. We evaluated the MRI of OTC under basal and stimulated conditions in 6 HIV infected patients, who were otherwise asymptomatic, and showed that the MRI (mean basal: 374.6, and mean post acetaminophen: 342.2 nmol.kg^{-1}.min^{-1}, table 1) did not change after the administration of acetaminophen. This suggests that the OTC probe can be used to evaluate the GSH synthesis capability in patients.

Other possible metaprobes

Homocystinuric patients have a propensity for arteriosclerosis, leading to myocardial infarction and stroke [54]. While transitions in lifestyle and nutrition in developing countries have led to an epidemic of cardiovascular disease in these populations [55], epidemiological studies also suggest that an elevated plasma homocysteine is a risk factor for cardiovascular disease. Therefore, it is worthwhile to measure the homocysteine status in the body. Homocysteine is cleared from body pools due to the activity of two enzyme systems: the remethylating and the transulfurating enzymes (fig. 2). The former are vitamin (B_{12} and folate) dependent, while the latter is pyridoxine dependent. Intervention trials with these vitamins have shown that homocysteinemia can be reduced by up to 25%, suggesting that a vitamin deficiency may be partly responsible for the elevated plasma homocysteine levels (reviewed in 55).

However, the genes coding for the enzymes that indirectly help in clearing homocysteine, may also be subject to mutations [56, 57]. In addition, the enzyme 5,10-methylene tetrahydrofolate reductase (MTHFR) that is involved in folate metabolism and in homocysteine clearance through its remethylation, is coded for by the MTHFR gene [58]. The gene for this enzyme is located on chromosome 1, and two common alleles, the C677T (thermolabile) allele, and the A1298C allele, have been described elsewhere [59]. The thermolabile variant of the MTHFR gene (C677T) has been identified as a determinant of elevated homocysteine levels which are known to be a risk factor for arterial and thrombotic vascular disease [60]. Therefore, the ultimate phenotypic expression of hyperhomocystenemia may be due to a combination of environmental and genomic effects. It is also likely that this phenotype of a high homocysteine level is a late manifestation of variations in the clearance kinetics of homocysteine. It would therefore be useful to have a dynamic assessment of the homocysteine clearance system so that earlier diagnosis and risk assessment is possible, and the effect of interventions such as vitamin supplementation can be studied effectively. In humans the measurement of remethylation and transulfuration rates of homo-

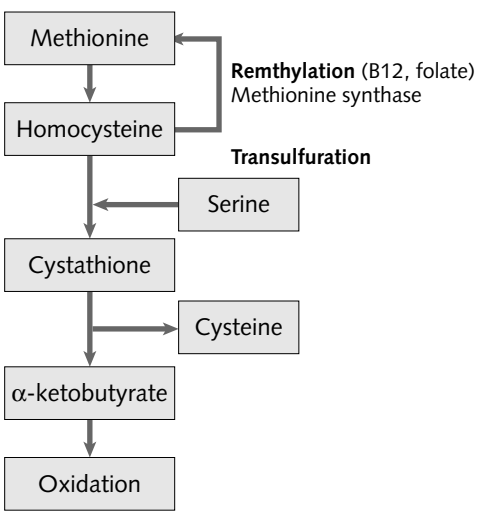

FIG 2. The metabolic fate of methionine, via remethylation and transulfuration reactions.

cysteine is possible through the use of a double-labeled methionine tracer infusion method [61], but this is a technically demanding protocol to use.

At present, the measurement of homocysteine clearance by the isotope labelled metaprobe method has only been performed in animals [2] and is not available for use in humans, although this is anticipated. The metaprobe used is L-2-oxo-tetrahydro,1,3 thiazine-4-carboxilic (1-^{13}C) acid, which releases labelled homocysteine intracellularly, under the influence of oxoprolinase. The labelled homocysteine is then transulfurated to cysteine and ^{13}C-aminobutyrate. The latter, on oxidation, would release the label into the CO_2 pool, and this would then be detected in the breath. Therefore, a breath pattern of isotopic label release can be used to assess homocysteine dynamics, under different metabolic conditions, such as fasting or after ingesting a methionine load. Preliminary studies in rabbits support these projections [2] where the MRI under basal conditions was 85 ± 14 μmol.kg^{-1}.h^{-1}, which reduced to 28 ±16 μmol.kg^{-1}.h^{-1} with a methionine load. The difference between these two MRIs is a measure of the body's capacity to remove homocysteine from the intracellular pool, and therefore a measure of homocysteine clearance. The basal MRI would need to be characterized in healthy normal individuals and this database could be used as normative data for characterizing those individuals with low basal MRIs who would be at risk of developing homocysteinemia. However, at the present time, it cannot be assumed that this technique is easy to perform on an epidemiological basis.

Another possible metaprobe is based on an endopeptidase present on the lymphocyte surface, called dipeptidyl peptidase IV [61], or the differentiation antigen CD26, which is important in lymphocyte activation and recognition [62–64]. The metaprobe gly-pro-2-aminobutyric-1-^{13}C (2) can evaluate the activity of this enzyme in vivo, since it would cleave off the L-2-aminobutyrate, which would transaminate [65] and then yield $^{13}CO_2$ on subsequent decarboxylation of its ketoacid [66]. In this case, the metabolism of L-2-aminobutyrate by itself would need assessing as well, since there could be differences in the way in which this molecule is processed by different groups of individuals. This metaprobe has been assessed in a septic rat model [2], and it has been shown that in rats with sepsis, the release of $^{13}CO_2$ during sepsis was significantly higher. This kind of test is a surrogate for the efficiency and activation of the immune system and it is also possible to think of exploiting the same strategy for assessing the efficacy of therapeutic modalities that depend on the modulation of CD26.

The future

Breath tests have been in diagnostic medicine for a long time, and coupled with stable isotopes, have a promising future. Nonetheless, even though the methodology is available, and a number of breath tests have recently been proposed and evaluated for the measurement of intestinal function [67–71], their use as a routine clinical test is still limited. These are possibly because of the relative lack of expensive mass spectrometers required to measure stable isotopic enrichments in breath as well as the technical knowledge that is necessary for their operation and the lack of standardized protocols for running these tests [72]. In addition, there is a need for appropriate cutoffs for the diagnosis of disease in specific populations [73]. There is also a lack of a normative database of normal responses to these tests. As new substrates and cheaper instrumentation for analysis are developed, it is possible that the usage of these tests will grow in diagnostic medicine, and contribute to the generation of normative data. Further, breath tests can be used for evaluating liver CYP (cytochrome P450) function, as for example, the ^{14}C labeled erythromycin breath test in the evaluation of liver CYP3A function [74, 75], can offer a way to evaluate the hepatic response of individuals to some drugs. It is known that genotypic variants of liver CYP enzymes can affect cure rates of *H. pylori* with different drug doses [76], and therefore, the rationale for drug dosing will also change depending on the development of easily available, quick, and non-invasive breath tests for this purpose.

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Assessing physical activity of women of childbearing age. Ongoing work to develop and evaluate simple methods

Marie Löf, Ulf Hannestad, and Elisabet Forsum

Abstract

Simple methods were developed and evaluated to assess total energy expenditure in 24 healthy, Swedish women planning pregnancy. Total energy expenditure was measured by the doubly-labeled water method (reference method) and three simple methods: heart rate recording, movement registration by an accelerometer, and a questionnaire. Mean total energy expenditure obtained by the four methods varied between 2,530 kcal per 24 hours (10,570 kJ/24 hours) and 2,730 kcal per 24 hours (11,420 kJ/24 hours). No significant difference between the results obtained by the different methods was found. The mean difference between the simple method and the reference method was for the questionnaire 204 ± 508 kcal per 24 hours (853 ± 2,124 kJ/24 hours), for the heart rate recorder 58 ± 338 kcal per 24 hours (241 ± 1,416 kJ/24 hours) and for the accelerometer 6 ± 325 kcal per 24 hours (25 ± 1,360 kJ/24 hours). The heart rate recorder and the questionnaire overestimated high and underestimated low energy expenditures. The accelerometer and the heart rate recorder were able to assess mean total energy expenditure of groups. No systematic bias was found when the accelerometer was used.

Key words: accelerometer, adult women, basal metabolic rate, doubly-labeled water method, heart rate recording, physical activity level, total energy expenditure, questionnaire

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Introduction

The fact that early nutrition may program health later in life has recently attracted interest [1]. As a consequence, the nutritional situation of women of childbearing age comes into focus. Nutritional assessments, including estimates of dietary intake, are thus of interest. However, estimates of energy intake are often underestimates of the true intake [2]. Therefore, ideally, studies of dietary intakes should include an independent assessment of the total energy expenditure (TEE) of the subjects investigated. Thus there is a need for simple and inexpensive methods for such measurements.

The objective of this study was to develop and evaluate simple methods to assess TEE using heart rate recording, movement registration (measured by an accelerometer), and a questionnaire in healthy, Swedish women of childbearing age.

Material and methods

Information about the 24 women participating in the study is given in table 1. These women, who were all planning pregnancy, showed a wide range of body mass indexes. Half of the subjects were employed in office work and the other half in childcare or nursing jobs.

The doubly-labeled water (DLW) method was used as the reference method. The subjects were given a dose of doubly-labeled water (0.05 g deuterium and 0.15 g oxygen-18 per kg body weight) after collecting baseline samples of urine. The subjects collected another five urine samples during the next 14 days. Isotopic

TABLE 1. Characteristics of the subjects (N = 24)

	Mean ± SD	Range
Age (yr)	30 ± 4	21–36
Height (cm)	167 ± 7	154–178
Weight (kg)	67 ± 10	50–95
BMI (kg/m2)	24 ± 4	20–39

enrichment of the dose and urine samples was analyzed using an isotopic ratio mass spectrometer (Deltaplus XL, Thermoquest). Carbon dioxide production and TEE were calculated as described earlier [3, 4] assuming 30% of water losses to be fractionated. The subjects wore an accelerometer (Computer Science and Applications, Shalimar, Fla., USA) and a heart rate monitor (Polar, Stockholm, Sweden) during the same two weeks as TEE was measured by the DLW method. On the day of dosing each subject performed six standardized activities while wearing the heart rate monitor and the accelerometer. Individual calibration curves were established by assigning different conversion factors [5] to the six activities. After the measurement period, the recorded heart rate and accelerometer outputs were converted to TEE by using the individual calibration curves and basal metabolic rate (BMR) calculated using prediction equations [6]. Furthermore, after the experimental period, using a questionnaire, each subject estimated how much time she had spent in six different activity categories during the last two weeks. Appropriate conversion factors [5] assigned to the different activity categories and predicted BMR were used to calculate TEE.

Results

Figure 1 shows mean TEE of the subjects as obtained by the questionnaire, the accelerometer, the heart rate recorder, and the doubly-labeled water technique. This figure varied between 2,530 kcal per 24 hours (10,570 kJ/24 hours) and 2,730 kcal per 24 hours (11,420 kJ/24 hours). No significant difference was found between results obtained by the different methods.

Agreement between each of the three simple methods and the doubly-labeled water method was assessed according to Bland and Altman [7]. Thus for each subject, the difference between the simple and the reference method was plotted against the average of these two estimates. The mean of these differences gives an estimate of the bias of the simple method when compared to the reference method. The standard deviation of the differences gives an estimate of the precision of the simple method.

Figure 2 shows the Bland and Altman plot for the questionnaire. The mean difference was 204 kcal per 24 hours (853 kJ/24 hours), two standard deviations being 1,015 kcal per 24 hours (4,248 kJ/24 hours). This indicates that the questionnaire was both inaccurate and imprecise. Also, there was a significant ($r = 0.56$, $p < .05$) linear relationship between the average of the TEE obtained by the questionnaire and the DLW method and the difference between these two methods indicating a systematic bias. The questionnaire thus overestimated high and underestimated low energy expenditures.

Figure 3 shows the Bland and Altman plot for heart rate recordings. Here the mean difference was 58 kcal per 24 hours (241 kJ/24 hours) while two standard deviations were 677 kcal per 24 hours (2,832 kJ/24 hours). This method was apparently more accurate than the questionnaire but still fairly imprecise. Also, the heart rate recorder showed a systematic bias ($r = 0.51$, $p < .05$) overestimating high and underestimating low energy expenditures.

Finally, figure 4 shows the Bland and Altman plot for the accelerometer. In this case the mean difference was very small, only 6 kcal per 24 hours (25 kJ/24 hours). However, two standard deviations were still as large as 650 kcal per 24 hours (2,720 kJ/24 hours). The accelerometer was thus more accurate than the other two methods but its precision was still low. No systematic bias was found for this method.

To illustrate the potential of the three methods to predict TEE of individuals we calculated the physical activity level (PAL) of our subjects. The PAL was the

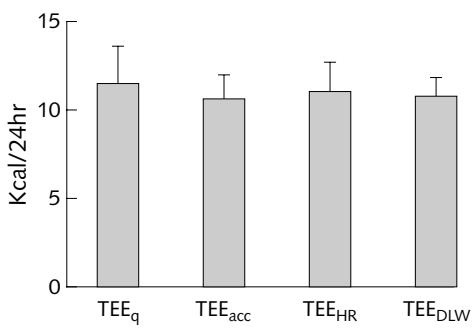

FIG. 1. Total energy expenditure (TEE) measured by a questionnaire (TEE_q), an accelerometer (TEE_{acc}), a heart rate recorder (TEE_{HR}), and by the doubly-labeled water method (TEE_{DLW}) (mean, SD)

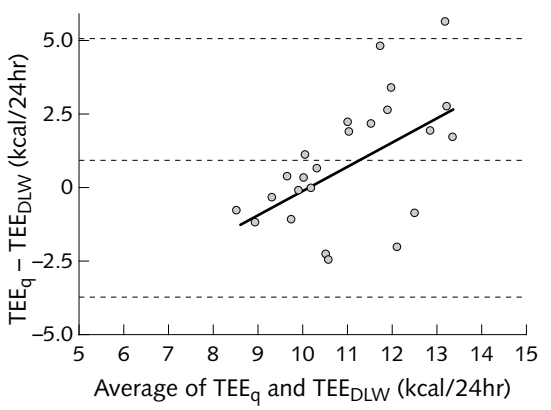

FIG. 2. Comparison of total energy expenditure obtained by the questionnaire (TEE_q) and by the doubly-labeled water method (TEE_{DLW}) according to Bland and Altman. The correlation coefficient of the regression line shown in the figure is 0.56 ($p < .05$)

ratio between TEE, measured by the DLW method, and BMR, measured by indirect calorimetry. The PAL values of the 24 subjects were distributed into low, moderate, or high categories. Each category contained eight subjects. For each method, we calculated the number of individuals who were classified in the same category by the simple method and by the DLW method. We also calculated the number of subjects who were classified one or two categories too low or too high by the simple method (fig. 5). For the questionnaire, the bar in the middle of the histogram shows the number of subjects who were classified in the correct PAL-category by this simple method. This was the case for 12 subjects (that is 50% of the total number of subjects). Four subjects (17%) were classified in a PAL-category that was one step below the correct one and another four subjects (17%) were classified in a category that was one step too high. Two subjects (8%) were classified in a PAL-category that was two steps below the correct one and another four subjects (8%) in a category that was two steps too high. The results obtained by the heart rate recorder and the accelerometer showed a similar pattern. No matter what method we used only about half of the subjects were classified correctly. Even if we used the mean of all three simple methods only about two-thirds of the subjects were classified correctly.

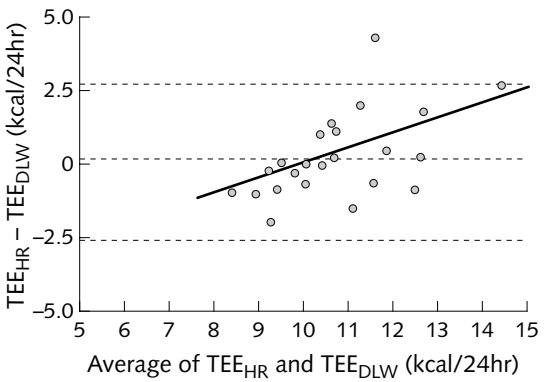

FIG. 3. Comparison of total energy expenditure obtained by the heart rate recorder (TEE_{HR}) and by the doubly-labeled water method (TEE_{DLW}) according to Bland and Altman. The correlation coefficient of the regression line shown in the figure is 0.51 ($p < .05$)

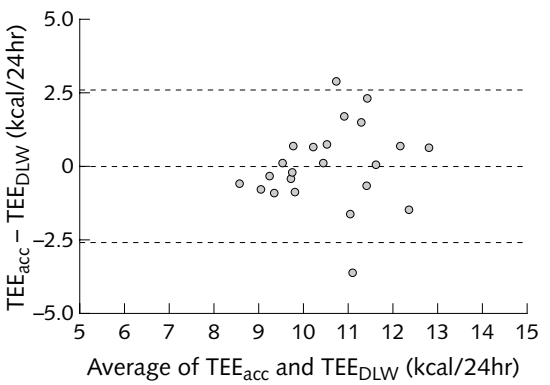

FIG. 4. Comparison of total energy expenditure obtained by the accelerometer (TEE_{acc}) and by the doubly labelled water method (TEE_{DLW}) according to Bland and Altman

Conclusion

All three simple methods showed poor precision. Thus, they cannot be used on individuals. The accelerometer and the heart rate recorder were able to assess mean TEE. They may therefore be used on groups. Results obtained by the accelerometer had no systematic bias. This method was therefore superior to the others.

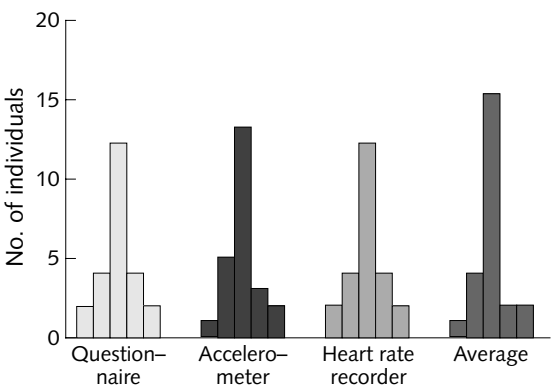

FIG. 5. Distribution of 24 women into different activity categories (low, moderate, high) assessed by a questionnaire, a heart rate recorder, and an accelerometer as compared to the corresponding activity categories obtained by the doubly-labeled water method. The average results of the questionnaire, the heart rate recorder, and the accelerometer are also shown (or further explanation, see the text)

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Validity of deuterium oxide dilution for the measurement of body fat among Singaporeans

Mabel Deurenberg-Yap and Paul Deurenberg

Abstract

Body fat percent (BF%) was measured in 108 adult Chinese, 76 Malays, and 107 Indians in Singapore by densitometry, deuterium oxide dilution (hydrometry), dual energy x-ray absorptiometry (DXA) and a chemical four-compartment model (BF%4c). The hydration of the fat-free mass (FFM) was calculated. Subjects ranged in age from 18 to 69 years and their body mass index ranged from 16 to 40 kg/m^2. BF%4c for the various subgroups were: Chinese females (33.5 ± 7.5%), Chinese males (24.4 ± 6.1%), Malay females (37.8 ± 6.3%), Malay males (26.0 ± 7.6%), Indian females (38.2 ± 7.0%) and Indian males (28.1 ± 5.5%). Biases were found between BF%4c and BF% measured by 2-compartment models (hydrometry, densitometry, DXA), with systematic underestimation by DXA and densitometry. On a group level hydrometry had the lowest bias while DXA gave the highest bias. When validated against BF%4c, 2-compartment models were found to be unsuitable for accurate measures of body fat due to high biases at the individual level and the violation of assumptions of constant hydration of FFM and density FFM among the ethnic groups. On a group level the best 2-compartment model for measuring body fat was found to be hydrometry.

Key words: body composition, body fat percent, total body water, deuterium oxide dilution, four compartment model, validity, ethnic, Singapore, Chinese, Malays, Indians

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Introduction

Body composition can be measured at various levels, ranging from atomic, molecular, cellular, tissue to whole body level [1]. One of the components of body composition that has always attracted interest is body fat. Various methods can be used to measure or to assess the amount of body fat [2]. Historically densitometry (underwater weighing), dilution techniques (like deuterium oxide, tritium oxide, or ^{18}O labeled water) and ^{40}K-counting have been used as reference techniques, densitometry being the most popular. Assessment methods like skinfold thickness measurements and bioelectrical impedance are most suitable in population studies, where the large number of subjects limits the expensive and laborious use of the before mentioned reference techniques [2 ,3].

All body composition methods rely on assumptions. For the reference methods these assumptions are based on chemical analyses of a few (Caucasian) carcasses only, performed in the 1940s. Assuming that the body consists of two distinct compartments, a fat mass (consisting of triglycerides only) and a fat-free mass (chemically consisting of water, protein, minerals, and a negligible amount of carbohydrates), the assumption for densitometry is a constant density of the fat-free mass and the fat mass [4]. For dilution techniques it is assumed that the hydration of the fat-free mass is constant at 73% and for ^{40}K-counting it is assumed that the potassium concentration in the fat-free mass is constant. There are indications in the literature that assumptions may differ between age, gender, and ethnic groups [5, 6]. Using incorrect assumptions leads to systematic bias.

Combination of techniques, like dual energy x-ray absorptiometry (DXA), deuterium oxide dilution, and densitometry, enables the use of more-compartment models of body composition [7]. More-compartment models allow the measurement of composition (in terms of water, protein, and minerals) and density of the fat-free mass and are used to test the validity of assumptions used in simpler two-compartment models.

The aim of this study was to validate deuterium oxide dilution as a relatively simple technique to determine body fat percent (BF%) in the three main ethnic groups in Singapore.

Subjects and methods

During the1998 National Health Survey (NHS), 300 of the 4,700 subjects were invited to participate in a body composition study [8]. They fulfilled the inclusion criteria which included an age range of 18 to 69 years, a wide range of body mass index with representation from the various gender and ethnic groups (Chinese, Malays, and Indians). Of the final participants who underwent all measurements108 were Chinese, 76 were Malays and 107 were Indian (table 1). The study was approved by the National Medical Research Council of Singapore.

Body height (0.1 cm) without shoes and body weight (0.1 kg) with subjects wearing only a swimsuit was measured and body mass index (BMI, kg/m^2) was calculated as weight divided by height squared.

Total body water (TBW) was determined using deuterium oxide dilution [9]. An accurately weighed dose of 10 to 15 g deuterium oxide was taken orally by the subjects. After 2.5 to 3 hours dilution time, a 10 ml venous blood sample was drawn. Plasma was separated and stored at –20°C until analysis. Deuterium was determined by infrared spectroscopy after sublimation of the plasma. TBW (kg) was calculated using a 0.95 correction factor for non-aqueous dilution of deuterium [9]. From TBW, body fat percent (BF%) was calculated as 100 × (weight-tbw/0.73)/weight. Body density was derived from air displacement plethysmography (BODPOD®, Body Composition System, Life Measurement Instruments, Concord, Calif., USA) and body volume was calculated. A concise description of the principles of the air displacement plethysmography is provided by Sardinha et al. [10]. From density BF% was calculated using Siriís formula [11]. Bone mineral content (BMC) was measured using a Hologic whole body dual energy x-ray absorptiometer (QDR-4500, Hologic, Waltham, Mass., USA; software version V8.23a:5). The Hologic software also provides a measure for soft tissue and BF%.

BF% was also calculated using the four-compartment model as described by Baumgartner et al. [12]:

$$BF\%_{4C} = 100 \times (2.75 \times BV - 0.714 \times TBW + 1.148 \times M - 2.05 \times BW)/BW$$

where BV= body volume, TBW = total body water, M = total body mineral and BW = body weight. Fat-free mass (FFM, kg) was calculated as BW (kg) – fat mass (FM, kg). The water fraction of the FFM was calculated as TBW/FFM. All methods are described in more detail elsewhere [13].

Statistical analyses were performed using SPSS for Windows [14]. The bias of BF% from two-compartment models (densitometry, hydrometry, and DXA) compared to BF% from four-compartment models was calculated and tested against zero with one sample t-test. The hydration of the FFM was tested against the assumed value values (one sample t-test). Differences between sex and age groups were tested using ANOVA. Bland and Altman plots [15] were constructed and the correlation between bias and BF% and age was calculated. Values are expressed as mean ± SD unless otherwise stated. Significance is set at $p < .05$.

Results

Table 1 gives some characteristics of the subjects by gender and ethnic groups. As expected males were taller and heavier than females. Malays and Indians had significantly higher BMIs than the Chinese. Table 2 presents BF% from density, deuterium oxide, DXA, and four-compartment model for males and females. The underestimation of BF% from density and from DXA in females was observed in all three ethnic groups (fig. 1). In males the underestimation of BF% from density was most obvious in Indians and Malays, whereas in Chinese the bias of BF% from density was not significant. BF% from DXA was significantly

TABLE 1. Characteristics of the subjects

	Chinese				Malays				Indians			
	Females (N = 61)		Males (N = 47)		Females (N = 33)		Males (N = 43)		Females (N = 53)		Males (N = 54)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Age (yr)	36.3	12.8	40.7	13.6	35.6	13.9	41.4	12.3	36.6	10.1	43.4	12.8
Weight (kg)	54.7	11.1	65.0	10.8	58.1	11.5	69.0	12.4	61.2	13.9	69.8	11.9
Height (cm)	157.8	5.9	168.9	5.2	154.1	6.1	165.9	6.4	156.5	6.0	168.3	6.9
BMI (kg/m^2)	22.1	4.8	22.8	3.5	24.5	4.8	25.0	3.7	24.9	5.2	24.2	3.6
BF%4C	33.5	7.5	24.4	6.1	37.8	6.3	26.0	7.6	36.2	7.4	26.2	6.5

BMI, body mass index (kg/m^2); BF%4C, body fat percent from four-compartment model.

underestimated in all male ethnic groups.

The hydration of the fat-free mass in all gender and ethnic groups was close to the assumed value of 0.73 (fig. 2) except for Chinese and Indian males where there is a slight but significant difference from the assumed value. The small differences in hydration fraction result in systematic bias of BF% from deuterium oxide dilution, but the bias is small (in all groups less than 1 percent point) and hardly biologically relevant (fig. 1).

In all gender and ethnic groups the bias of BF% from deuterium oxide is much smaller than that of BF% from body density or from DXA (fig. 2) and is not dependent on the level of body fatness or age (fig. 3).

Discussion

In this body composition study Malay and Indian males and females had higher BMIs (table 1) than their Chinese counterparts. This is in accordance with the findings from the general NHS [16] in which a representative sample of the Singapore population participated.

TABLE 2. Body fat percent as measured by different techniques

	Females (N = 147)		Males (N = 144)	
Body fat percent from:	Mean	SD	Mean	SD
Deuterium oxide	36.4	6.9	26.2	6.5
Body density	33.4	8.7	24.8	7.6
Dual energy x-ray absorptiometry	33.9	6.2	22.5	5.5
Four-compartment model	36.2	7.6	26.2	6.5

The results of this study confirmed concerns in the literature that many assumptions normally used in body composition techniques that were extracted from chemical analyses of a few Caucasians carcasses only, are not valid across ethnic groups. It is known that the chemical composition of the FFM differs across ethnic groups [5, 6, 13,17] and this has consequences for the validity of the assumption that the density of the FFM is constant at 1.1 kg/L. For the three ethnic groups in Singapore it can be calculated that the density of the FFM exceeds 1.1 kg/L, resulting in an underestimation of BF% when using Siri's formula [13]. Surprisingly the hydration fraction of the FFM is a relatively constant (fig. 2) and in all six gender and ethnic groups very close to the generally assumed value of 0.73 [18]. Consequently the bias in BF% when using deuterium oxide dilution alone in a two-compartment model of body composition is small (fig. 1). It did not exceed

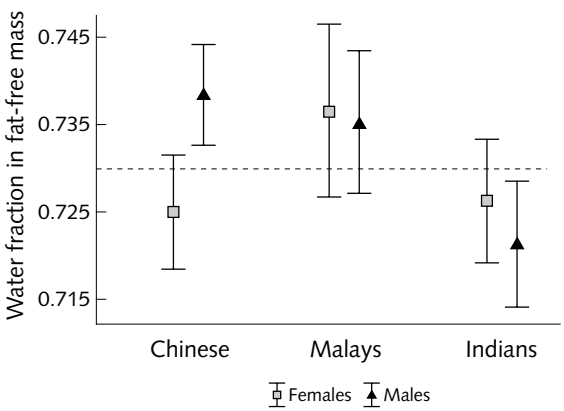

FIG. 2. Water fraction in fat-free mass (mean ± 95% CI) in Chinese, Malays and Indian males and females 95% CI, 95 percent confidence interval. Dotted line is assumed constant hydration of 0.73.

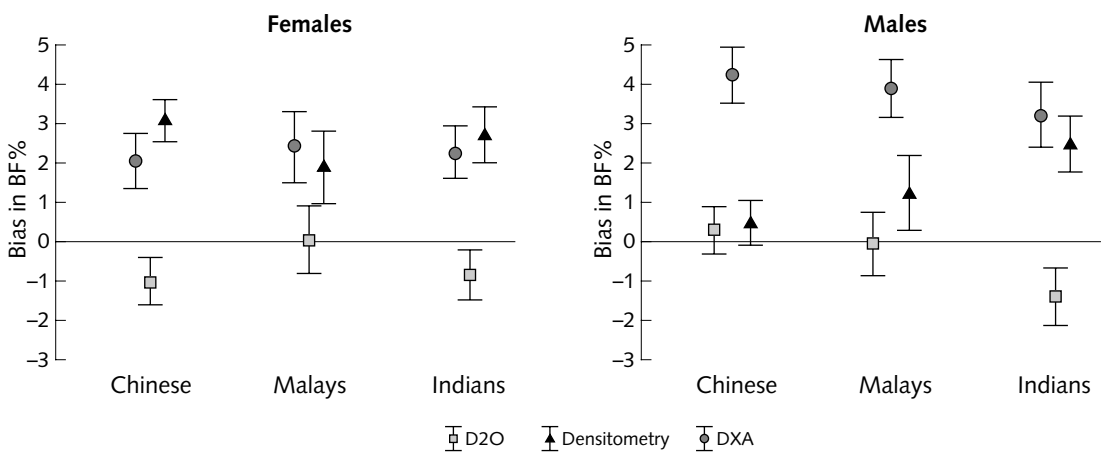

FIG. 1. Mean bias (± 95% CI) in body fat percent from various two-compartment models in Chinese, Malays, and Indian males and females. Bias, Body fat percent (BF%) from four-compartment model minus BF% from deuterium oxide; 95% CI: 95 percent confidence interval.

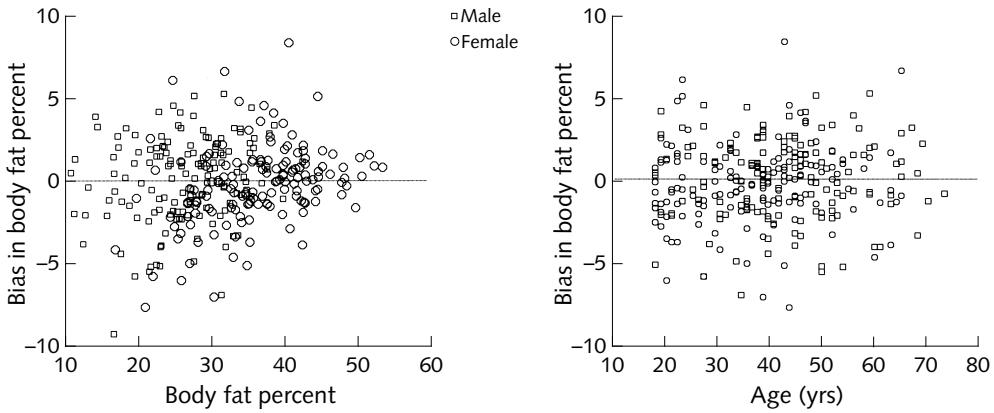

FIG. 3. Relation of bias of BF% from deuterium oxide with level of body fatness and with age. Bias, Body fat percent (BF%) from four-compartment model minus BF% from deuterium oxide.

one percent point of body fat percent in any of the groups. Also, the bias of BF% from deuterium oxide was not dependent on level of BF% and not dependent on age (fig. 3).

The overall good validity of BF% from deuterium oxide dilution makes the technique a suitable reference method for body fat percent among adult Singaporeans of Chinese, Malay, and Indian ethnic backgrounds. In addition, the method is 'portable' and does not require much cooperation of the subjects (in contrast to densitometry) and is less expensive and less laborious than the use of a four-compartment model.

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Measurement of deuterium oxide by infrared spectroscopy and isotope ratio mass spectrometry for quantifying daily milk intake in breastfed infants and maternal body fat

Graciela Caire, Ana María Calderón de la Barca, Adriana Verónica Bolaños, Mauro E. Valencia, Andy W. Coward, Gabriela Salazar, and Esther Casanueva

Abstract

*Measurements of deuterium oxide concentrations (DOC) in saliva by infrared spectroscopy (IRS) and isotope ratio mass spectrometry (IRMS) were compared for the calculation of infants' milk intake and maternal body fat. Deuterium oxide (30g) was given to the mother. DOC by IRS and IRMS were 340 ± 209 and 345 ± 202 ppm. The mean difference between both methods (4.62 ± 76.3) was not different from zero (*p = *.57). Bland and Altman analysis showed no significant overall bias (r = 0.10;* p = *.33), but dispersion within the limits of agreement. The mean milk intake in the infants' by IRS and IRMS were 624 ± 296 g/day and 634 ± 327 g/day (*p = *.642) and the mothers' mean body fat were 31.3 ± 12% and 31.5 ± 13% (*p = *.755). The results appear to be the same for milk intake and body fat by the two methods and no evidence of bias was found.*

Key words: deuterium oxide, infrared-spectroscopy, human-milk intake, body composition.

Introduction

Safeguarding the nutritional status of breastfeeding infants and of their mothers, depends on the period of lactation and the quantity and quality of the mothers' milk. Mother's body reserves may not be enough to meet the traditional long periods of lactation in deprived populations [1]. Consequently, it is essential to accurately assess the amount of human milk produced, as well as the mother's body fat content during the lactation period.

Isotope dilution methods to measure infants' milk intake have been validated against the direct weighing of formula bottles and with the test-weighing technique in the case of breastfeeding [2–3]. Using deuterium as a tracer, measurements can be carried out with minimum interference on the lactation process. The dose-to-the-mother deuterium-dilution method to measure both infant milk intake and mother's body composition uses isotope ratio mass spectrometry (IRMS) to quantify deuterium in body fluids [4]. In spite of its potential advantage in field studies of human lactation, it has been limited because of mass spectrometry availability. This equipment is expensive and requires specialized operation and maintenance. Alternative methods have been proposed for deuterium determination in water [5, 6]. These include infrared spectroscopy [7–9], which is widely available.

An inexpensive technique to quantify deuterium oxide (D_2O) by infrared spectroscopy (IRS) in saliva samples was developed and compared with isotope ratio mass spectrometry determination of the same samples. This was applied to measure average daily milk intake of breastfed infants as well as maternal body fat.

Methods and materials

Subjects

Ten low-income lactating women and their infants from Hermosillo, Sonora, Mexico, participated in the study. They had full term (breastfed) infants of appropriate gestational size and age. Written informed consent was obtained from the mothers to participate in this study, which was approved by the Ethical Com-

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mittee of Centro de Investigación en Alimentación y Desarrollo, where the study took place.

Doses and sample collection

The study was conducted over a 15-day period. At days 0 and 14 weight and length measurements were taken. Saliva samples were obtained at home for milk intake and total body water estimation by the deuterium (D_2O) dilution method. On the first day, mothers received an oral dose of 1.5 mol (30g) of D_2O 99.8% (Cambridge Isotope Laboratories, Andover, Mass., USA). Saliva samples were collected from mothers and infants (1 ml) at days 1, 6 and 14; and 1, 2, 5, 6, 13, and 14, respectively, after dosing the mothers. The samples were frozen at –70°C.

Sample sublimation

A simple sublimation system connected to a freeze drier was used [10] to obtain water from saliva. 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). D_2O in different concentrations was completely recovered on condensation flasks to avoid fractionation. Time of sublimation for saliva was 1 ml/hour.

D_2O measurements by infrared spectroscopy (IRS)

Deuterium abundance was measured by IRS with a CaF_2 cell (Miran 1FF, Foxboro Co., Mass., USA). Measurements were taken after filtration (0.22μm) at 2513 cm^{-1} under controlled temperature (15°C). A programmable voltmeter (Mod. 83 III, Fluke Corporation, Everett, Wash., 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 samples were measured in duplicate after adjusting the absorbance to 0 with water or basal saliva.

D_2O measurements by isotope ratio mass spectrometry (IRMS)

Sublimated samples were analyzed by IRMS at the Instituto de Nutrición y Tecnología de Alimentos (INTA) in Chile. The samples were isotopically equilibrated with hydrogen for three days, using platinum on alumina powder as a catalyst [11]. The deuterium content of the resultant gas was compared with that of known calibrators, using HYDRA (Europe Scientific, Crewe, UK) and the results were converted to the V-SMOW (Vienna Standard Mean Ocean Water) scale [12].

Milk ingestion and body composition

The dose-to-mother D_2O-dilution method was applied and babies' milk intakes were estimated by a program modified to obtain a better curve adjustment using the IRS concentration values. Mothers' and babies' data samples were introduced to the program to calculate milk intake based on *in vivo* tracer kinetics. The computer program also calculated the mothers' D_2O enrichment at the equilibration time. Thus, total body water and percentage of body fat were obtained from the program as well.

Statistical methods

Statistical analysis was done (NCSS®, 1997; Kaysville, UT, USA) and results were expressed as means ± standard deviations (SD). Accuracy of the technique was examined by a Student's *t*-test ($p < .05$) and regression procedures. The comparison was considered accurate if the regression between IRMS and IRS had a slope not significantly different from 1 and an intercept not significantly different from zero. Precision was assessed by the model R^2 and the standard error of the estimate (SEE) from the regression procedures described above. Bias was examined using the procedures of Bland and Altman [13].

Results

The mean age of the 10 healthy lactating mothers was 21.7 ± 3.4 years (range 16.9 to 27.5 years) and their weight was 62.7 ± 8.1 kg (range 49.6 to 74 kg). Mean weight of the infants under three months old, was 4.5 ± 0.9 kg (range 3.6 to 6.4 kg).

D_2O analysis by IRS and IRMS

Accuracy and precision of D_2O concentration analyzed by infrared spectroscopy (IRS) in comparison to isotope ratio mass spectrometry (IRMS) was studied for 88 sublimated saliva samples. Mean D_2O concentrations obtained by IRS and IRMS were 340 ± 209 and 345 ± 202 ppm, respectively, within a range of 21 to 1,022 ppm. The mean difference between the methods was 4.62 ± 76.3, which was not different from zero ($p = .57$). The standard deviations of the difference showed a considerable dispersion by the Bland and

Altman analysis (fig. 1). The discrepancy between the measurements on both instruments showed no significant overall bias (r = 0.10; p = .33), however it showed a wide range of dispersion within the 95% limits of agreement (mean ± 2SD). The tendency to bias was more pronounced at lower concentrations of deuterium in the range of 200 to 500 ppm.

The relationship between D_2O concentration by IRS and IRMS (fig. 2) deviated significantly from the line of identity in terms of the intercept ($p < .01$) but not of the slope ($p > .90$) that was not different from 1. This relationship was explained by the following correction equation: Actual D_2O concentration = 0.896 × D_2O concentration by (IRS) + 39.89 ppm (table 1).

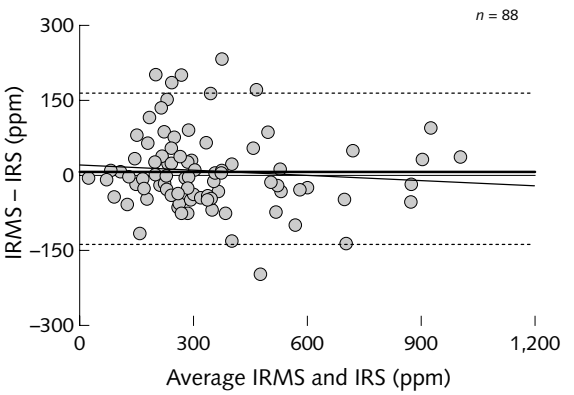

FIG. 1. Individual differences versus the averages for deuterium enrichment (ppm) measured by mass spectrometry (IRMS) and infrared spectroscopy (IRS). The mean difference is represented by the solid line and the dotted lines represent ± 2SD from the mean.

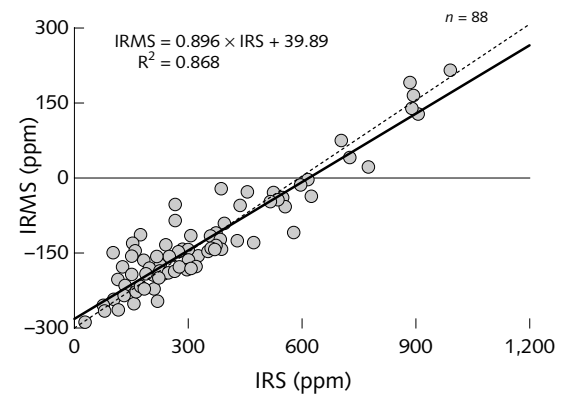

FIG. 2. Relationship between deuterium enrichment (ppm) measured by infrared spectroscopy (IRS) versus mass spectrometry (IRMS). The identity line (slope = 1, intercept = 0) is the dotted line, while the solid line represents the linear regression of the total ppm data.

Infant's milk intake and mother's body fat

Milk intakes using data obtained by IRS ranged from 280 to 1,290 g/day and the mean (624 ± 296 g/day) was not different (p = .64) from that determined by IRMS (634 ± 327 g/day). The mean mother's percent body fat by IRS and IRMS were 31.3% ± 12.4 and 31.5% ± 13.1 (p = .755), respectively. For milk intake, the difference between the methods was 10 ± 65.7 g/day, with 95% limits of agreement from –121 to +141. The discrepancy analysis showed a strong tendency to bias, however non-significant with an intercept of –54.7 (p = .26) and a slope of 0.10 (p = .15). The relationship of milk intakes' means by both methods and the difference between them showed a correlation of 0.48; p = .15.

The difference in the mothers' percent body fat by the IRS and IRMS was 0.194 ± 1.9% and was not different from zero (p = .74). Limits of agreement were from –3.6 to +4.0. The discrepancy analysis for mother's body fat showed an intercept and a slope of –1.67 (p = .33) and 0.059 (p = .25), respectively. The relationship of the body fat's mean by both methods and the difference between them showed a correlation of 0.38; p = 0.25.

Regression analyses of infants' milk intake and mothers' body fat were performed using IRS and IRMS data. There was a significant relationship between measurements obtained for milk intake (r = 0.983, p = .0000) and percent body fat (r = 0.991, p = .0000). Precision of IRS determined from the model R^2 and standard error of the estimate (SEE) from the regression procedures is listed in table 1. The IRS technique explained 96% and 98% of the variance for milk intake and percent body fat, respectively.

Discussion

The use of IRS to quantify deuterium oxide concentration in physiological fluids has the potential to be an alternative technique to IRMS for lactation and body composition studies by dilution and wash-out kinetics principles. In this study, D_2O concentration between

TABLE 1. Summary of regression of deuterium concentration in saliva samples, milk intake, and percent body fat by IRS against IRMS

Measurement	R^2	Intercept	Slope	SEE[a]
Deuterium in saliva (ppm) (n = 88)	0.868	39.89	0.896	73.7
Milk intake (g/day) (n = 10)	0.966	–45.3	1.09	63.9
Body fat (%) (n = 10)	0.981	–1.41	1.05	1.9

a. Standard error of the estimate.

the IRS and IRMS methods were not different, however, the standard deviations were large. Bias although not significant, did show a wide dispersion. Accuracy from the standpoint of the line of identity, showed that the intercept was different from zero and a correction equation was applied.

One of the limitations of the IRS technique is the precision (CV, 7.17%) for repeated measurements of the same sample. By IRMS or Fourier transform infrared spectroscopy (FTIR) it is possible to obtain CV < 1.0% [7, 9]. Another limitation for IRS technique is the quantity of sample required for the analysis, 400 µl of sublimated sample was required to fill the cell and ports. Even most important, is the poor sensibility of the IRS, several times lower than that of the IRMS.

Although there was no significant bias in either milk intake or in the mothers' percent body fat derived from D_2O concentration examined by IRS and IRMS, large standard deviations were observed especially with regard to milk intake. The limits of agreement for individual estimates indicated that both methods could differ by as much as 130 g/day, which would be equivalent to 21% of the overall mean milk intake. Mean values for body fat were essentially identical and the 95% limits of agreement represents about 13% of the mean body fat observed in these women. Although there were no differences detected between the means or the mean difference was not different from zero, the large variation could be the reason for not detecting these differences. An increase in sample size could help to resolve this problem.

The IRS methodology described herein is a potentially good alternative to those laboratories that do not have IRMS or FTIR facilities. However, further analysis using a large sample should be done. The cost of the IRS instrument, like the one used in this study plus voltmeter that increases sensitivity, is approximately US$5,200. The sublimation may be avoided by carefully loading saliva samples into the IR cell, just after centrifugation [9]. The comparison of the IRS and IRMS techniques for measuring deuterium oxide enrichment showed a reasonable agreement and provided similar estimates of milk intake and maternal percent body fat in the mother-infant pairs studied.

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Use of stable calcium isotopes (^{42}Ca & ^{44}Ca) in evaluation of calcium absorption in Beijing adolescents with low vitamin D status

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Abstract

Female adolescent populations with low calcium intakes and sub-optimal vitamin D status are found in northern China. Whether these individuals are able to adapt by enhancing calcium absorption and reducing calcium excretion for maximizing calcium retention is unknown. This study examined the association between true-fractional-calcium-absorption (TFCA), plasma 25-hydroxy-vitamin-D, and urinary calcium excretion among adolescents in Northern China. Twelve healthy girls 9 to 17 years old were recruited from Beijing during a winter. Calcium intake, anthropometry, pubertal status, plasma 25-hydroxyvitamin-Ds (25-OHD), serum calcium, and urinary calcium were determined. TFCA was evaluated by dual stable calcium isotopes. The mean ± SD calcium intake, 24-hour urinary calcium excretion, plasma 25-OHD and TFCA were 591 ± 164 mg/day, 79.9 ± 49.6 mg/day, 30.5 ± 9.8 nmol/L, and 60.4 ± 14.4%, respectively. TFCA was inversely correlated with 25-OHD ($r = –0.73$, $p = .008$). Urinary calcium was correlated with the onset of menarche ($r = 0.63$, $p = .027$). Post-menarcheal girls had a higher urinary calcium output than pre-menarcheal girls ($p = .03$). Adolescents from north China with sub-optimal vitamin-D status are able to adapt by enhancing TFCA and reducing urinary calcium excretion to retain calcium for bone development. TFCA was inversely correlated with plasma 25-OHD. Whether a higher efficiency of calcium absorption is sustainable if the shortfall of vitamin D persisting remains to be studied.

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Key words: calcium intake, calcium absorption, stable isotopes, 25-hydroxyvitamin D, adolescents, Chinese

Introduction

Nutritional rickets, whether clinical or sub-clinical, still prevails in certain areas of China where vitamin D status is sub-optimal and calcium supply is limited [1, 2]. In 1987, the prevalence of rickets among children aged zero to five years old was 11.3%, with the peak onset by one to two years of age (27.8%) [3]. The prevalence of rickets in North China is still high among children under three years of age (6% to 44%) and adolescents (7% to 24%), which is attributable to low sunshine exposure and a poor source of vitamin D in foods [2, 4]. In the rural area of Beijing the mean plasma 25(OH)-vitamin D_3 (25-OHD) level of schoolchildren 12 to 14 years of age during the winter was only 13.4 nmol/L, 45.2% were categorized as sub-clinically vitamin D-deficient with a plasma 25-OHD level below 12.5 nmol/L [5]. Plasma 25-OHD levels and calcium intakes were found to be strong predictors of bone mass in Beijing female adolescents [5]. The short duration of sunshine exposure and low intensity of ultraviolet light during the long cold winter may explain the higher prevalence of clinical and sub-clinical rickets among infants and children in northern China as compared with that in the south [6, 7]. It has been suggested that vitamin D deficiency might occur more frequently in populations where there is a limited vitamin D supply along with diets low in calcium [8, 9].

Average calcium intakes of Chinese children are low which is attributable to their non-milk based diets, with mean calcium intakes below 400 mg/day) [10, 11]. In a controlled calcium supplementation trial in the early 1990s Chinese children with normal plasma vitamin D levels (85 nmol/L) were able to enhance true fractional calcium absorption even though their calcium intake was low [12, 13]. The true-fractional-calcium absorption (TFCA) of these Chinese children was significantly higher than that of their counterparts in the United

States (54% to 63% vs. 25% to 34%) who consume 925 mg of calcium per day [14]. Adequate circulating vitamin D among these children allows nutritional adaptation to occur [12, 13]. The calcium absorption study in Chinese children [12, 13] was conducted in South China (latitude: 21°N) where there is plenty of sunshine throughout the year. Children could manufacture most of their vitamin D underneath their skin through regular outdoor activities. Nonetheless, the calcium absorptive capability of children residing in North China with both sub-optimal vitamin D and calcium status has never been investigated.

We studied the association between TFCA, plasma 25 (OH)-Vitamin D_3 (25-OHD) concentration, and 24-hour urinary calcium excretion among growing adolescents residing in the temperate region of North China (latitude: 40°N).

Subjects and methods

Twelve adolescent girls aged 9 to 17 years participated in the study in Beijing city from December, 1999 to February, 2000. Plasma vitamin D levels of children have been found to be lowest in North China during the winter [4]. All the girls were healthy and had not suffered from recent fractures or any endocrinal diseases that would interfere with calcium and vitamin D metabolism. Informed consent was obtained from their parent. The study was approved by the Human Research Ethics Committee of the University of Newcastle, New South Wales, Australia.

Puberty and on-set of menarche

Pubertal status (pubic hair distribution and breast development) was determined by a physical examination according to Tanner's staging [15, 16]. Pubertal development was categorized into three groups: prepuberty (Tanner stage I), early puberty (Tanner stages II and III), and late-puberty (Tanner stages IV and V) [15]. The time for the onset of menarche was also obtained from the subjects.

Calcium absorption study

The doubly-labeled stable isotopic technique [17–19] was used to determine the TFCA for each subject. Stable calcium isotopes ^{42}Ca (87.8 atom% enrichment) and ^{44}Ca (93.8 atom % enrichment) in the form of calcium carbonate were made soluble in 1 N Spectrograde Hydrochloric acid (Beijing Chemical Company, Beijing). The pH of ^{42}Ca doses dissolved in normal saline solution was adjusted to 6 for intravenous injection while the pH of ^{44}Ca in Millipore water was adjusted to 4 for oral administration by adding drops of 1 N Spectrograde sodium hydroxide solution (Beijing Chemical Company, Beijing). ^{42}Ca solution for injection was sterilized and dispensed aseptically into vials at the Clinical Pharmacological Research Center, Peking Union Medical College Hospital. The concentrations of the solutions were assayed by the colorimetric method (Beckman Synchron CX3 Clinical System, Fullerton, Calif., USA).

The subjects fasted overnight before the calcium absorption test. The ^{44}Ca (0.5 mg/kg) dose pre-mixed with 120 ml of cow's milk (122.4 mg Ca/ml) was refrigerated for at least 24 hours prior to the study. The ^{44}Ca dose was administrated orally to each subject along with the three main meals of the day. The oral tracer (^{44}Ca) was divided into three smaller doses in proportion to the usual daily calcium intake. By giving smaller doses of the oral tracer along with the meals has been shown to better estimate the TFCA from the diets [20]. A nurse administered the ^{42}Ca (0.05 mg/kg) intravenously via the antecubital vein over at least one minute immediately after the first oral dose. A 5 ml of venous blood sample was drawn immediately before the IV injection for the determination of 25-OHD and serum calcium concentration. Details of isotope administration to the subjects have been described elsewhere [12, 13]. A 24-hour urine collection started exactly 24 hours after the isotope administration to determine the recovery of the two isotopes in the urine.

Mass spectrometric analysis for isotopic recovery in urine

To avoid contamination of the urine samples, the treatment of urine before mass spectrometric analysis was performed in a laminar-flow hood. All the glassware was acid washed and then oven dried before use. Organic matters of urine were removed by digestion upon heat (70°C) with Spectrograde concentrated nitric acid (Beijing Chemical Company, Beijing) [2]. Calcium oxalate was formed by adding alkaline ammonium oxalate solution (pH = 10) to the digested urine samples at 70°C for 15 minutes. Calcium oxalate was precipitated by setting the urine samples in an ice-bath for one hour. The calcium oxalate precipitate was then washed several times with 0.1% diluted ammonium oxalate solution to remove cations especially potassium ions [21]. A standard solution ($CaNO_3$) for the determination of the background natural abundances of various stable calcium isotopes was prepared by replacing carbonate ions from spectrograde calcium carbonate (Beijing Chemical Company, Beijing) with nitrate ions from spectrograde nitric acid (Beijing Chemical Co., Beijing). All the samples were analyzed by thermal ionization mass spectrometry using a Finnegan MAT 261 Magnet Sector Mass Spectrometer (Finnegan-Mat GmbH, Bremen, Germany). The ion beam intensities of ^{42}Ca and ^{44}Ca were compared to

that of ^{40}Ca. Each urine sample was scanned 10 times (1 block of data) to obtain a mean and a relative standard deviation for the ratio of $^{42}Ca/^{40}Ca$ and $^{44}Ca/^{40}Ca$. The final ratios of $^{42}Ca/^{40}Ca$ and $^{44}Ca/^{40}Ca$ were obtained by the mean of three blocks of the data (30 scans). The coefficient of variation (CV) of multiple scans on the same double-filament with respect to the ratios of $^{42}Ca/^{40}Ca$ and $^{44}Ca/^{40}Ca$ were 0.31% and 0.33%, respectively. Whereas the CV of sequential scans on different double-filaments with respect to the ratios of $^{42}Ca/^{40}Ca$ and $^{44}Ca/^{40}Ca$ were 0.1% and 0.06%, respectively. The CVs were comparable to those found by others [21–23]. Calculation of the TFCA was based on the fact that both intravenously and orally administered calcium isotopes are metabolized at the same rate once achieving equilibrium 24 hours after dosing. The TFCA was determined according to the following equation developed by Yergey et al. [17]:

$$\%\ \mathrm{TFCA} = \frac{(\mathrm{na}\ ^{44}\mathrm{Ca})(^{42}\mathrm{Ca\ IV}) \times \Delta\ \%\mathrm{Xs}\ ^{44}\mathrm{Ca} \times 100}{(\mathrm{na}\ ^{42}\mathrm{Ca})(^{44}\mathrm{Ca\ oral}) \times \Delta\%\mathrm{Xs}\ ^{42}\mathrm{Ca}}$$

where na is the natural abundance of the two isotopes, IV and oral, the exact dose administered, and Δ %Xs, the degree to which a particular ratio differs from the natural ratio.

Calcium intake and biochemical analysis

A quantitative food frequency questionnaire was used to evaluate habitual calcium intake [24]. Fasting plasma 25-OHD was determined by radioimmunoassay (Diasorin Inc., Stillwater, Minn., USA.) [12]. Serum calcium and 24-hour urinary calcium concentrations were analyzed by colorimetry (Beckman Synchron CX3 Clinical System, Fullerton, Calif., USA).

Statistical analysis

The mean ± SD and range were determined for all the variables. The Mann-Whitney test was used to test group difference. Pearson and Spearman correlation analyses were performed to examine the correlation between variables. Linear regression analysis was employed to study the relation between different variables and the TFCA. The level of significance was $p \leq .05$, 2-tailed. Statistical tests were carried out by using SPSS 10.0 for Window (SPSS Inc. Chicago, Ill., USA).

Results

Characteristics of the 12 girls are shown in Table 1. Their mean age was 12.4 ± 2.4 years. The mean calcium intake of the subjects was 591 ± 164 mg/day. Milk and dairy products contributed to 54% of their daily calcium intake, whereas vegetables and beans and bean products contributed 9% and 5%, respectively, to their daily calcium intake.

The mean TFCA, plasma 25-OHD, serum calcium, and 24-hour urinary calcium levels of the subjects were 60.4 ± 14.4 %, 30.5 ± 9.8 nmol/L, 2.29± 0.16 mmol/L and 79.9 ± 49.6 mg/24-hours, respectively (table 2). There was no correlation between the TFCA and age (r = –0.11, p = .73), weight (r = 0.10, p = .75), height (r = 0.16, p =.93), dietary calcium (r = 0.13, p = .69) or 24-hour urinary calcium excretion (r = –0.17, p = .59). However, there was a significant inverse correlation between TFCA and plasma 25-OHD (r = –0.73, p = .008) (fig. 1).

TABLE 1. Characteristics of all 12 adolescent girls

	Mean ± SD	Range
Age (yr)	12.4 ± 2.4	9–17
Weight (kg)	42.1 ± 9.2	28–55
Height (m)	1.52 ± 0.09	1.33–1.63
Dietary calcium (mg/day)	591 ± 164	402–847

TABLE 2. True fractional calcium absorption (TFCA), serum 25-OHD, serum calcium, and 24-hour urinary calcium of adolescent girls (N = 12)

	Mean ± SD	Range
TFCA (%)	60.4 ± 14.4	41.3–83.0
Plasma 25-OHD (nmol/L)	30.5 ± 9.8	15–47.25
Serum calcium (mmol/L)	2.29 ± 0.16	1.9–2.5
24-hour urinary calcium (mg/day)	79.9 ± 49.6	22.4–185.6

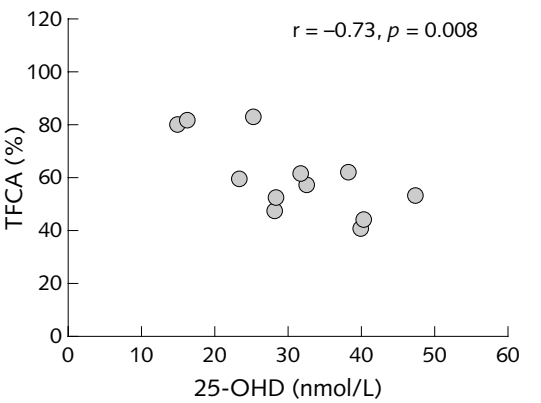

FIG 1. Correlation between true fractional calcium absorption (TFCA) and 25-(OH) vitamin D_3 (25-OHD) among the 12 Beijing adolescent girls

Three girls were at the pre-puberty stage, 4 girls were at Tanner stage II, three girls were at Tanner stage III, and two girls were at Tanner stage IV. Five girls had already started menarche. Urinary calcium excretion was positively correlated with pubertal stage ($r = 0.68$, $p = .015$) and onset of menarche ($r = 0.63$, $p = .027$). The subjects who had started menarche also had a significantly higher urinary calcium excretion than those who have not (115.5 ± 53.5 mg/day vs. 54.5 ± 28.2 mg/day, $p = .03$). There was no association between pubertal development and the TFCA in the subjects ($r = –0.209$, $p = .514$) which may be attributable to the small sample size.

Discussion

The mean calcium intake of the subjects was 591 mg/day or 59% of the Chinese adequate intake (AI) of calcium for adolescents [25]. The mean TFCA of adolescents in this study was comparable to that of a previous study in Hong Kong (TFCA: 57.4%, calcium intake: 693 mg/day and 25-OHD: 84.25 nmol/L) and South China (TFCA: 63%, calcium intake: 381 mg/day and 25-OHD: 82 nmol/L) [12] despite the fact that circulating 25-OHD of the subjects in the present study was remarkably lower. Furthermore, TFCA in the current study was higher than that of the counterparts in the United States (25–34%) with a mean calcium intake 925 mg/day [14]. Findings from the present study and our previous studies [12, 13] indicated that there is a difference in TFCA between the Chinese and American children.

Results of a recent report on vitamin D deficiency involving 1,250 adolescents during the winter in Beijing [4] compared with those of the present study (table 3).

Table 3. A comparison of results between subjects from the current study and 108 school girls aged 12–14 years in urban area of Beijing during winter

	Du et al., 2001 (N =108) [4]	This study (N=12)
	Mean ± SD	Mean ± SD
Age (y)	12.7 ± 0.4	12.4 ± 2.4
Weight (kg)	46.8 ± 10.2	42.1 ± 9.2*
Height (m)	1.54 ± 0.07	1.52 ± 0.09**
Dietary calcium (mg/day)	396 ± 91	591 ± 164***
Serum calcium (mmol/L)	2.26 ± 0.12	2.29 ± 0.16[a]
Plasma 25-OHD (nmol/L)	13.9 ± 9.6	30.5 ± 9.8***

* $p = .0002$; ** $p = .05$, *** $p < .001$
a. $n = 96$.

Plasma 25-OHD concentration of the present study was more than two times greater than that of Du et al. [4]. However, the serum calcium concentration in the current study was not statistically different, and calcium intake was significantly higher. Milk intake contributed to 54% of total calcium in the present study as compared to 35.4% in Du et al.'s study. The dietary calcium and vitamin D status of our subjects were not worse than that of the Du et al. study which was a large-scale randomized study on calcium and vitamin D status of female adolescents in China. All the subjects resided in Beijing city and most of the parents were highly educated which may explain for the better nutritional status of the subjects.

Low plasma 25-OHD (< 30 nmol/L) was also observed in adolescent populations from northern Europe, South America, and the tropics [26–29]. In a recent study in northern France 67% of the healthy adolescents had circulating 25-OHD less than 25 mmol/L [30]. Plasma 25-OHD of adolescents has been found lowest in winter and spring [4, 30], particularly among those at puberty growth spurt [30] living in areas with inadequate sunlight or atmospheric pollution.

An adequate vitamin D status is important for increased calcium absorption during adolescent growth spurts [6, 31]. Although the plasma 25-OHD concentration in the present study was approximately 2.5 times lower than that of the previous study conducted in sub-tropical region of China [12], TFCA in the current study was comparable to those of Lee et al. [12]. The significant inverse correlation between TFCA and plasma 25-OHD in adolescents girls has never been reported before. Our previous study conducted in the sub-tropical region did not observe such an inverse association [12]. An enhanced conversion of 25-OHD to $1,25(OH)_2D_3$ may explain the decline in plasma 25-OHD in adolescents during pubertal growth spurts. The phenomenon of a negative association between TFCA and plasma 25-OHD status may be explained by the fact that the escalated demand for calcium for bone growth stimulates the conversion of most of the plasma 25-OHD to $1,25(OH)_2D_3$ under the influence of parathyroid hormone to enhance calcium absorption. The plasma 25-OHD concentration was thus lowered with a corresponding increase in TFCA. However, if the short supply of 25-OHD persists, whether the high level of TFCA is sustainable is unknown. Further study is needed to determine the relationship among hormonal influence, calcium absorption, and sub-optimal plasma vitamin D concentration in this group of growing adolescents.

The findings that girls who had already started menarche had significantly a higher urinary calcium output than those who have not started menarche agrees with a previous study [32] in which the rate of skeletal calcium accretion was highest is at the perimenarcheal time period when a higher amount of renal

calcium is conserved for bone mineralization.

Adolescent girls with sub-optimal vitamin D status in northern China are able to adapt by enhancing calcium absorption and reducing urinary calcium excretion in order to retain calcium for bone development. The TFCA was inversely correlated with 25-OHD in the circulation. Urinary calcium excretion was lower in pre-menarcheal girls than in post-menarcheal girls. It is not known whether the higher efficiency of calcium absorption can be sustainable if the shortfall of vitamin D continues. Further study is warranted to study the interaction between sub-optimal vitamin D status and calcium homeostasis among adolescents in order to understand the extent of adaptation and its long-term effect on bone growth among these adolescents.

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Bulk and compound specific analysis of stool lipid confirm that the "missing" ^{13}C in the mixed triacylglycerol breath test is not in the stool*

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Abstract

The ^{13}C-mixed triacylglycerol (MTG) breath test is used to measure intraluminal fat digestion. In normal digestion 20% to 40% of the ingested ^{13}C label is recovered in breath CO_2. We aimed to identify the proportions of ingested label excreted in stool as well as breath following ingestion of ^{13}C-MTG by children with impaired exocrine pancreatic function and healthy controls. ^{13}C enrichment of breath samples was measured by continuous flow isotope ratio mass spectrometry (IRMS) and the cumulative percent dose recovered (cPDR) in 10 hours was calculated. Total ^{13}C of a fecal fat extract from each stool was measured by combustion-IRMS, and ^{13}C enrichment and concentration of the tert.-butyldimethylsilyl (TBDMS) derivative of octanoic acid was measured by gas chromatography-mass spectrometry (GC/MS) after hydrolysis of the fat extract. Stool 5-day cPDR was calculated. Mean breath cPDR was 33% for children with cystic fibrosis and 45% for controls. Mean cPDR in stool by combustion-IRMS and GC/MS, respectively, was 0.7% and 0.3% for children with cystic fibrosis and 1.4 and 4.2% for controls.

Key words: mixed triacylglycerol breath test, MTG, stable isotope, ^{13}C breath test, children with cystic fibrosis

Introduction

^{13}C-breath tests have become useful non-invasive tools to measure gastrointestinal functions and to monitor pharmacological and nutritional interventions [1]. Labeling of nutritional substrates with ^{13}C enables investigation of their digestion, absorption, and subsequent metabolism. The labeled compound is taken by mouth, usually as part of a test meal. Thereafter it is digested, absorbed, oxidized and the label appears in breath carbon dioxide (CO_2). ^{13}C-breath tests are particularly attractive for use in children.

The ^{13}C-mixed triacylglycerol (^{13}C-MTG) breath test has been used to measure intraluminal fat digestion [2–4]. 1,3 distearyl 2-[1-^{13}C] octanoyl-glycerol (MTG) is composed of two long-chain saturated fatty acids (C18:0, stearic acid) at the *sn*-1 and -3 positions, and a medium-chain fatty acid (octanoic acid) at the *sn*-2 position of the triacylglycerol. Pancreatic lipases preferentially hydrolyze triglycerol at the *sn*-1 and -3 positions, after which the octanoate is rapidly absorbed and oxidized with release of $^{13}CO_2$.

In normal digestion 20% to 40% of the ^{13}C label is excreted in breath CO_2 [2]. It is not known what proportion of the other 60% to 80% is excreted in stool, or remains after digestion and absorption, in slow-turnover carbon pools within the body. The aim of this study was to identify the proportions of ingested label excreted in stool as well as breath following ingestion of ^{13}C-MTG by children with impaired exocrine pancreatic function. Bulk analysis of stool lipid ^{13}C was compared with compound specific analysis of ^{13}C-octanoic acid in stool.

Methods

Subjects and procedures

The study was performed at the Royal Hospital for

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Sick Children in Glasgow with the informed consent of the children and their parents and the approval of the hospital ethics committee. After an overnight fast, eight children with cystic fibrosis (CF) and two healthy controls ingested 20 mg/kg (CF) or 10 mg/kg (control) ^{13}C-MTG (99 atom% excess ^{13}C, Cambridge Isotope Laboratories, Andover, MA, USA) with a standard breakfast (whole wheat, low sugar breakfast cereal, milk, and beet sugar; about 170 kcal and 7.7g fat), and their normal enzyme replacement therapy. The subjects were asked to avoid foods that are naturally enriched in ^{13}C, e.g., maize, maize products and foods containing cane sugar, for 24 hours prior to the test, and they remained at rest during the test [5]. Breath samples were collected at baseline, half-hourly for six hours, and hourly for four more hours. CO_2 production rate ($VCO2$) was measured hourly during the breath sampling period using a ventilated hood indirect calorimeter (Deltatrac II, Datex, Helsinki, Finland) [6]. All stools were collected before and for five days after ^{13}C-MTG ingestion. Stool wet weight was noted and the samples frozen immediately.

Breath ^{13}C analysis

^{13}C enrichment of breath samples was measured by continuous flow isotope ratio mass spectrometry (CF-IRMS, Hydra, PDZ Europa, Crewe, UK) with reference to a standard gas (3% CO_2 in N_2, Air Products, Crewe, UK), which had been calibrated against international standards [7]. Instantaneous percentage dose recovered (PDR/h) was calculated using the following equation.

$$\text{PDR h}^{-1} = \frac{VCO_2\ (\text{mmole h}^{-1}) \times \text{breath } ^{13}CO_2 \text{ enrichment (ppm excess)} \times 100}{\text{amount MTG taken (mmole)} \times \text{enrichment MTG (atom \% excess} \times 10^4)}$$

The cumulative percent dose recovered in breath (cPDR) was then calculated by integrating instantaneous PDR values over the 10 hour sampling period.

Stool lipid extraction

Lipid was extracted from 0.5g of freeze-dried stool by the method of Jeejeebhoy [8], as described by Slater [9] which ensures good recovery of medium chain fatty acids. One-fourth of the solvent extract was dried under nitrogen gas and weighed to give lipid yield, 25% was frozen for bulk ^{13}C analysis, 25% had internal standards added (nonanoic acid and tri-pentadecanoin (Sigma-Aldrich, Poole, UK) at concentrations expected for octanoic acid, and heptadecanoic acid (Sigma-Aldrich, Poole, UK) at the palmitate level), and was stored at –20°C for compound specific analysis of ^{13}C octanoic acid. An external standard, containing ^{13}C-MTG, internal standards, unlabeled octanoic acid and fatty acids in the relative proportions found in stool [10] was stored with the samples to check for losses during storage.

Bulk analysis of stool lipid ^{13}C

Stool lipid extract containing approximately 2 mg C was pipetted into 150 µl capacity tin boats (8 × 5 mm, Elemental Microanalysis Ltd., Okehampton, UK) containing 20 µl 0.1M di-sodium tetraborate (BDH, Analar grade, Merck, Lutterworth, UK) and an inert support (acid washed Chromosorb W, mesh size 60/80, Alltech Associates, Carnforth, UK), which had been pre-combusted at 450°C for two hours to remove residual carbon. The solvent was removed by drying slowly in a refrigerator overnight and then freeze-drying for two hours. This procedure prevented the sample 'creeping' up the sides of the combustion boat and loss of any free octanoic acid. Stool lipid ^{13}C abundance and total carbon content were analyzed by CF-IRMS (Europa Scientific 20-20 with a biological sample converter [11], PDZ Europa, Crewe, UK). The oxidation stage was held at 980°C, the reduction stage was 550°C, and the gas chromatograph column was held at 75°C. The system was calibrated using beet sucrose, whose ^{13}C enrichment had been independently calibrated against an international standard. The ^{13}C enrichment of the samples was calculated by comparison with working standards comprising 2.375 mg beet sucrose (containing 1 mg C), after subtraction of the blank consisting of a combustion boat with Chromosorb W. The percent dose recovered in each stool sample was calculated and these were summed to determine stool 5-day cPDR.

Compound–specific analysis of stool ^{13}C-octanoic acid

Lipid extract equivalent to 5 mg fat was dried under N_2, hydrolyzed using methanolic NaOH, acidified and extracted into 2 ml iso-octane. The *tert.*-butyldimethylsilyl (TBDMS) derivative of the fatty acids was prepared [9]. TBDMS fatty acid enrichment and concentration were analyzed by gas chromatography-mass spectrometry (GC/MS, Hewlett Packard 5890 II GC with an Optic temperature programmable injector, Fisons Instruments A200S autosampler and VG Trio-1000 quadrupole MS with electron impact ionisation, all supplied by Fisons Instruments, Middlewich, UK).

The gas chromatograph was operated in splitless mode with helium (CP grade) as the carrier gas. The injector temperature was initially 80°C, with a 0.75 minutes hold, then ramped to 300°C at 16°C s^{-1}. The transfer line between the GC and the MS was operated at 300°C. The analytical column was a DB5ms (J & W Scientific, Folsom, Calif., USA), length: 30 m, internal diameter 0.25 mm, film thickness 0.25 µm. The temperature program started at 80°C and ramped to 240°C at 20° minutes^{-1}, then from 220°C to 256°C at 2°

minutes^{-1}, and finally from 256°C to 320°C at 20° minutes^{-1}, followed by 30s at 320°C. The solvent delay was 5 minutes. The injection volume was initially 0.2 μL.

The Trio-1000 quadrupole mass spectrometer with electron impact ionization was operated with a trap current of 150 μA and electron energy of –70eV. The source temperature was 200°C, and the detector multiplier was operated at 300 V. The TBDMS derivative has a prominent ion at M-57, i.e., M-(C-$(CH_3)_3$ and a much smaller ion at M-15, i.e., M-CH_3. As there was very little octanoic acid in the samples, selected-ion recording mode of M-57 was used to measure TBDMS-octanoic acid enrichment and concentration, and M-15 was used for the other fatty acids. This enabled the analysis of nmoles octanoic acid in the presence of μmoles of other fatty acids. The quantitation masses for octanoic acid and ^{13}C-octanoic acid were *m/z* 201 and 202 (M-57). M-15 was monitored for all other fatty acids including *m/z* 257 for nonanoic acid and *m/z* 341 for pentadecanoic acid, the internal standards. The dwell time on each mass was 0.08 seconds, with a span of ± 0.1 amu (atomic mass unit).

Samples were screened to determine how much octanoic acid was in each and then re-injected at optimum volume in order to match the amount of octanoic acid in baseline and post-dose samples to avoid problems arising from subtracting the wrong background correction. This will be discussed later. Concentration of octanoic acid was calculated with reference to the internal standards, and related to the wet weight of stool. As any octanoic acid present was relatively highly enriched, the sum of the ion beams at *m/z* 201 and 202 was divided by that of the internal standard and related to the response factor of the external standard to determine the amount of octanoic acid in the lipid extract. From this, the concentration of octanoic acid in the original sample was calculated. Enrichment of 1-^{13}C-octanoic acid (mole percent excess, MPE) was calculated from the background corrected ratio of 202/201 according to the following equation.

$$MPE = \frac{R_E - R_B}{1 + (R_E - R_B)} \times 100$$

where R_E and R_B are the observed ion beam ratios (202/201) of the enriched and baseline samples, respectively.

The PDR in each stool sample was calculated from the product of concentration and enrichment, divided by the product of the MTG dose and enrichment. These were summed to determine stool 5-day cPDR.

The non-esterified fatty acid (NEFA) and triacylglycerol (TAG) fractions of the lipid extract were separated by solid phase extraction (Bond Elute 200 mg amino propyl columns, International Sorbent Technology, Hengoed, Mid Glamorgan, UK) [12] in samples from two subjects with a relatively large amount of octanoic acid.

Results and discussion

The mean (SD) cPDR in breath $^{13}CO_2$ was 33 (13)% for children with CF and 45% for controls. This is comparable with previous studies [2]. The results for individual subjects are shown in table 1.

TABLE 1. Cumulative percent dose recovered in breath and stool of children

Child number	Age (yr)	Breath cPDR 10 hours	Stool 5 day CPDR IRMS	Stool 5 day cPDR GC/MS
Controls				
1	2.3	46.3	0.6	0.4
2	4.2	43.4	2.2	8.0
Mean controls		44.9	1.4	4.2
Cystic fibrosis				
3	7.7	41.9	0.5	0.1
4	13.3	13.3	2.1	0.0
5	2.8	32.4	0.3	0.4
6	12.4	30.5	0.6	0.1
7	9.9	25.0	0.8	0.1
8	6.2	19.7	0.6	0.6
9	11.5	48.7	0.0	0.4
10	8.6	52.3	0.5	0.4
Mean cystic fibrosis		33.0	0.7	0.3
SD cystic fibrosis		13.8	0.6	0.2

Bulk analysis of ^{13}C in the stool lipid extract showed that very little tracer was recovered in stool (cPDR was 0.7 (0.6)%, mean (SD), for children with CF and 1.4% for controls). Extracted lipids were analyzed in preference to whole, homogenized stool because lipid extracts can be analyzed with better precision and greater sensitivity [9]. Compound specific analysis of ^{13}C-octanoic acid showed that there was a median of 6 μmole octanoic acid per stool, compared to about 3 mmole palmitate in our previous study [9]. At very low concentration, the measured ratio (*m/z* 202:201) of natural abundance samples increases compared to the ratio calculated from the natural abundance of the constituent isotopes (fig. 1). Subtraction of the wrong baseline can thus lead to erroneous results when calculating enrichment. At higher sample concentration, the measured ratio is close to the theoretical ratio. Use of matching baseline and enriched samples as described above avoids these problems. The stool cPDR measured by GC/MS was 0.3 (0.2)%, mean (SD), for children with cystic fibrosis and 4.2% for controls.

We are not aware of any other reports of ^{13}C-MTG measurements in stool, but the results presented here agree well with those of Murphy et al. [13], who used ^{13}C-labeled tripalmitin to study gastrointestinal handling of lipids in children recovering from severe malnutrition. When normal nutritional status was achieved, i.e., weight-for-height had reached 90% of the reference, 1.4 (0.4–2.5)% of dietary lipid was found in stool. These data were obtained by bulk ^{13}C analysis of whole, freeze-dried stool. In children with severe malnutrition, the tracer in stool was present in the form of undigested triacylglycerol, but after recovery the label was found as non-esterified fatty acid. The label in MTG is on a medium chain fatty acid, octanoic acid, which is rapidly absorbed directly into the portal circulation and therefore behaves differently from long chain fatty acids. The ^{13}C-MTG test is therefore a test of digestion, rather than absorption.

Given the difficulty of measuring very low amounts of stool ^{13}C lipid, the two analytical methods agree reasonably well. IRMS measures total ^{13}C and analysis of hydrolyzed lipid extract by GC/MS measures ^{13}C-octanoic acid, therefore most ^{13}C must be in the form of free or esterified octanoic acid. Solid phase extraction of the lipid extract from two subjects with a relatively large amount of octanoic acid, showed that it was mainly in the form of undigested triacylglycerol. Thus, there is no evidence of substantial modification by colonic microflora.

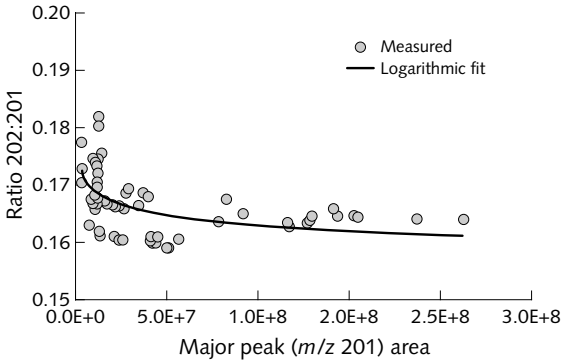

FIG.1. Variation of measured natural abundance ratio with concentration of octanoic acid in stool lipid extract. The theoretical ratio calculated from the natural abundance of the constituent isotopes is 0.162.

Conclusions

Ingestion of ^{13}C-MTG by children with cystic fibrosis on their normal enzyme replacement therapy is followed by almost complete intestinal digestion and absorption of the substrate. The ^{13}C label not identified in breath must remain in the body, and the variation of cPDR in breath is therefore due largely to factors other than efficiency of lipolysis.

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