

Uses of stable isotopes in clinical diagnosis and research in the paediatric population

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The landmark experiments of Schoenheimer and Rittenberg in the 1930s provided the scientific foundation for the ensuing development and application of stable isotope techniques in clinical diagnosis and research.¹

Stable isotopes are non-radioactive atoms of the same chemical element, which differ only in their number of neutrons.² Many elements also have radioactive (non-stable) isotopes. Aspects of macronutrient metabolism have been investigated employing molecules labelled with ²H (D or deuterium), ¹³C, ¹⁵N, and ¹⁸O. Extensive literature is available detailing the use of ²⁵Mg, ²⁶Mg, ⁴²Ca, ⁴⁶Ca, ⁴⁸Ca, ⁵⁷Fe, ⁵⁸Fe, ⁶⁷Zn, and ⁷⁰Zn isotopes in studies of mineral metabolism. The most commonly used radioactive isotopes are ¹⁴C and ³H (tritium).² More than 6000 stable isotope labelled compounds (tracers) are commercially available for use in metabolic studies. Examples of some of these tracers are [1-¹³C] leucine, [1-¹³C, ¹⁵N] leucine, [ring-D₅] phenylalanine, and [6,6]-D₂ glucose. It is currently accepted that these compounds have negligible biological side effects, which renders them ethically acceptable for use in children.³

Following intravascular or oral administration, the tracer is metabolically indistinguishable from the equivalent unlabelled compound of interest (tracee). The metabolic fate of the compound can be assessed qualitatively and quantitatively by measuring the relative abundance of tracer and tracee and/or their respective metabolites with time. The detectable mass difference of tracer and tracee allows the analysis of compounds, extracted from plasma, by gas chromatography-mass spectrometry (GC-MS, picogram sample size, analytical range 0.1-100 mole %, precision ±0.2 mole %).^{4,5} The detection limit is considerably less than 0.1 mole %, when tracers with multiple stable isotope labels (for example ring-D₅ phenylalanine) are used.⁶ Stable isotopes in breath (¹²CO₂ and ¹³CO₂) are analysed using an isotope ratio mass spectrometer (IRMS, microgram sample size, analytical range 0.0001-0.01 atom % excess, precision ±0.0001 atom %).^{4,7} Combustion IRMS has essentially the same analytical capabilities as IRMS but allows the combustion of tissue samples with subsequent analysis of gaseous isotope enrichment.⁴ Stable isotopes of minerals are typically analysed by thermal ionisation mass spectrometry (TIMS) or inductively coupled plasma mass spectrometry (ICP-MS) with high precision and sensitivity.⁸

The advantages of stable isotope labelled compounds compared with their radioactive counterparts are many. Most importantly, several different stable isotope tracers can be safely administered simultaneously to the same

subject without compromising future studies. The plasma volume which is needed from one sample to analyse the isotope enrichment is small, allowing even preterm infants to be studied. On average 0.5 ml of plasma is needed for one study. The intramolecular location of one or more label(s) is determined easily, which allows the mapping of metabolic pathways.⁴ In contrast, no realistic radioactive tracers exist for certain chemical elements (O₂, N₂) and the handling, application, and disposal of radioactive tracers makes such studies hazardous to all participants.⁴

This review is written for (general) paediatricians to provide a concise and up to date overview of the use of stable isotope labelled compounds in clinical diagnosis and research in the paediatric population. For more detailed information on stable isotope models and additional applications the interested reader is referred to recently published reviews.^{4,9,10}

Breath tests

With few exceptions stable isotope breath tests are based on the ingestion of substrates that are labelled with ¹³C atoms.⁷ Following digestion and absorption, the substrates are metabolised and eventually oxidised to yield H₂O, ¹³CO₂, and ¹²CO₂. Increased isotope enrichment in breath (¹³CO₂) can be measured as outlined above and it is assumed that this increase indirectly reflects the intestinal absorption and metabolism of the substrate. The advantages are obvious. Breath tests are non-invasive and can be performed conveniently in an outpatient setting. The tracer is given orally and breath samples are subsequently collected. Breath tests do not provide metabolic information regarding pathways in which the substrate may play a role until it is finally oxidised. This may be of particular significance in patients with perturbed intermediary metabolism.

A complete description of all breath tests applied in the paediatric population is clearly beyond the scope of this review. A recently published *Gut* supplement presents an extensive series of papers reporting a wide range of diagnostic breath tests in children.¹¹ However, we will exemplify the usefulness and clinical impact of stable isotope breath tests in several relevant clinical areas.

Depending on the type of tracer, the isotope enrichment in breath is an indirect reflection of gastrointestinal and/or liver function. The most commonly used substrates in this context are triotanooin, triolein, octanoic acid, aminopyrine, and caffeine respectively.^{7,12,13}

The ¹³C urea breath test exemplifies a stable isotope breath test which is used for the diagnosis and management of *Helicobacter*

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pylori associated gastrointestinal disease in the paediatric population.⁷ *H pylori* synthesises and secretes significant amounts of urease, an enzyme that catalyses the oxidation of urea to NH_3 and CO_2 . Consequently, oral administration of ^{13}C -urea will lead to increased excretion of $^{13}\text{CO}_2$ in a child with *H pylori* associated gastrointestinal disease.⁷ This stable isotope breath test is very sensitive and allows the reliable identification of *H pylori* with only two breath samples following an oral bolus of ^{13}C -urea.¹⁴

Body composition and energy expenditure

From a clinical standpoint, accurate measurement of total body water can provide indirect information relating to whole body composition and energy balance. The mechanisms of energy balance control are unclear and the study of this balance is complicated by the fact that alterations in body weight may reflect a change in energy stores, an alteration in total body water, or both.

Total body water can be calculated by employing standard isotope dilution techniques. Knowledge of the "fixed" water content of the fat free mass permits calculation of this body compartment. Subtraction of this mass from body weight provides indirectly the mass of body fat. Both $^2\text{H}_2\text{O}$ and H_2^{18}O have been used to estimate total body water in renal insufficiency, cystic fibrosis, malnutrition, and in endocrine disorders.¹⁵⁻¹⁹

The doubly labelled water method (employing $^2\text{H}_2\text{O}$ and H_2^{18}O) has become established, over the past 20 years, as the only method currently available for measuring total energy expenditure in completely free living subjects.²⁰ Following isotope administration and equilibration, isotopically labelled hydrogen will leave the body as water (urine plus evaporative losses), whereas isotopically labelled oxygen will leave the body both as water and as carbon dioxide (action of carbonic anhydrase). Monitoring the individual enrichment decay curves for these two isotopes over 7-14 days will result in different slopes. The difference in slopes is directly proportional to total body carbon dioxide production. This direct estimate of metabolic rate can then be converted to units of heat production by incorporating knowledge or estimates of the chemical composition of the foodstuffs being oxidised. This influences the energy equivalence of each litre of carbon dioxide produced.

The method has been validated in infants.^{21, 22} Application of the method to determine energy expenditure in small for gestational age infants, and growth and development in normal infancy and in malnourished children have been reported.²³⁻²⁵ Published data relating to energy expenditure in short and normal stature Guatemalan children and obese and non-obese adolescents (12-18 years) are available.^{26, 27}

Inborn errors of metabolism

Inborn errors of metabolism are ideal study areas for the use of stable isotopes. Many

biochemical pathways have intermediary compounds of which stable isotopes are available, representing different enzymatic activities. Consequently children with inborn errors of metabolism were among the first to be studied with stable isotopes.^{28, 29}

Stable isotope breath tests were used in inborn errors of metabolism for non-invasive diagnosis and reliable semiquantitative estimation of whole body enzyme capacity in subjects with galactosaemia and maple syrup urine disease.³⁰⁻³³ Response to therapeutic intervention and genotype-phenotype correlations were assessed in children with thiamine responsive maple syrup urine disease³⁴ and galactosaemia, respectively.³⁰

Stable isotope studies involving infusions of tracer and blood sampling have been undertaken in many different inborn errors of metabolism, including glycogen storage diseases,³⁵⁻³⁷ galactosaemia,³⁰ phenylketonuria (PKU),^{38, 39} organic acidurias,³³ and urea cycle defects.^{40, 41} Thompson *et al* have shown that phenylalanine or propionate are metabolised in children with PKU or certain organic acidurias (methylmalonic aciduria and propionic aciduria), respectively.³³

Significant phenylalanine hydroxylation in children with classical PKU was recently contradicted by van Spronsen *et al* who showed clearly reduced (whole body) enzyme activities when compared with normal healthy control subjects.³⁹ They also concluded that dietary phenylalanine tolerance did not correlate with *in vivo* hydroxylation of phenylalanine, an important finding which increases the significance of adequate substrate disposal (for protein synthesis).

Macronutrient metabolism

Protein, lipid, and glucose kinetics in infants and children have been measured using a range of labelled substrates: [$1-^{13}\text{C}$] leucine and [D_3] phenylalanine; [^{13}C] labelled glycerol, palmitic or linoleic acids; and [^{13}C] glucose respectively.

van Goudoever *et al* studied protein kinetics in two groups of preterm infants.⁴² The first group was supplemented with amino acids while the second group received only glucose infusions. Nitrogen and leucine balance improved significantly in the first group, secondary to increased protein synthesis and decreased protein breakdown, a clear indication of the benefits of early administration of amino acids in preterm infants. These findings confirmed earlier published results.⁴³⁻⁴⁵ More recently Clark *et al* investigated the effects of parenteral nutrition on leucine and phenylalanine kinetics in preterm infants and showed an acute increase in whole body protein synthesis as well as a reduction in protein breakdown.⁴⁶ A comparison of whole body protein turnover in appropriate for gestational age and small for gestational age infants using two labelled amino acids gave encouragingly similar quantitative results.⁴⁷

Nitrogen balance and protein turnover have been investigated during growth failure in low birthweight infants,⁴⁸ and in sick premature neonates during the first few days of life.⁴⁹

Phenylalanine hydroxylase status was investigated in sick preterm neonates with respiratory distress syndrome.⁵⁰ The results obtained in this study did not support the hypothesis that the enzyme activity was low in these preterm infants.

Albumin synthetic rate has been measured in premature infants and in oedematous and non-oedematous protein energy malnourished children.^{51, 52} In the first study it was found that both the fractional and absolute synthetic rate for albumin in the infants were much higher than rates found in young adults. The results from the latter study suggested that repletion of the albumin pool of children with protein energy malnutrition was not mediated by changes in the fractional synthetic rate. Additionally it was shown that the oedema of malnutrition was not solely a result of the hyperalbuminaemia.

Whole body protein metabolism has been measured in children with cancer using both labelled leucine and phenylalanine.⁵³ Both tracers gave similar results which indicated that both the rates of synthesis and breakdown were higher than in normal children. The effects of feeding on protein turnover in children with cystic fibrosis and healthy controls have been compared.⁵⁴ From their results the authors suggest that feeding may affect protein turnover differently in children with cystic fibrosis when compared with control children. It was suggested that the differences might be independent of plasma insulin concentration.

Studies of plasma arginine and leucine kinetics in paediatric burns patients suggested that the former amino acid was conditionally indispensable in terms of maintaining protein homeostasis in severely burned children.⁵⁵ In healthy children recovering from burn injury, rates of whole body protein synthesis in relation to basal energy expenditure have been explored.⁵⁶ Perhaps surprisingly it has been shown that infants and children do not increase whole body protein turnover or metabolic rate after major operations.⁵⁷ Respiratory quotient fell during the operative period, reflecting mobilisation of endogenous fat. It was speculated that the lack of catabolism observed in these subjects was a result of the switching of protein synthesis from growth to tissue repair. Other studies have looked at amino acid and protein metabolism in critically ill children.^{58, 59}

A significant early study quantitating palmitate and glycerol turnover and the contribution of glycerol to neonatal hepatic glucose output showed the potential of stable isotopes in studies of lipid metabolism.⁶⁰

It has long been speculated that premature infants who are fed formulas that are low in long chain polyunsaturated fatty acids (LCP) have low plasma LCP levels as a result of immature biosynthetic pathways. Carnielli *et al* addressed this issue in an elegant study.⁶¹ They gave five preterm infants formula mixed with [¹³C] linoleic and linolenic acids (both unsaturated C18 fatty acids) and showed a significant increase in ¹³C enrichment in LCP derivatives originating from the C18 precursors. Selective release of fatty acids from lipolysis such as the

release of palmitic or linoleic acids may play an important role in the overall response to critical illness.⁶² The rate of appearance of [U-¹³C] linoleic acid was significantly greater than that of [U-¹³C] palmitic acid when measured in critically ill infants. However, it is not clear whether a selective release of fatty acids may also be present in healthy infants.

Fatty acid oxidation was studied in 12 patients (3–19 years) with seizure disorders, who were receiving valproic acid, predominantly as monotherapy, before and following L-carnitine supplementation.⁶³ Results suggested that, in patients at low risk of valproic acid induced hepatotoxicity, the acid caused metabolic abnormalities that resembled those observed in inborn errors of mitochondrial beta oxidation that were not corrected by the L-carnitine supplementation.

The mechanisms controlling fat intake and fat oxidation are poorly understood. A recent study looked at fat oxidation and adiposity in prepubertal children.⁶⁴ Using a combination of the ingestion of [¹³C] labelled mixed fatty acids (in an ice cream meal) and indirect calorimetry permitted the partitioning of exogenous and endogenous resting fat oxidation rate over a nine hour postprandial period. Exogenous and endogenous fat oxidation, expressed as a proportion of total fat oxidation, was directly related to and inversely related to fat mass respectively. It was suggested that the enhanced exogenous fat oxidation with increasing adiposity in the dynamic phase of obesity may represent a protective mechanism. This would act to prevent further increase in the fat mass and maintain fat oxidation at such a level when the body ingests a large amount of dietary fat, as is often encountered in obese children.

Quantitation of various aspects of glucose kinetics can be achieved using several available glucose tracers: [6,6 ²H₂], [1-¹³C], or [U-¹³C] glucose.⁶⁵ Gluconeogenesis from lactate has been described in fasting infants using a sophisticated protocol.⁶⁶ In this study 54% of glucose was found to be derived from plasma lactate. Gluconeogenesis in the fetus and neonate has recently been described in depth.⁶⁷ Very low birthweight infants (less than 1100 g) have been studied with respect to endogenous glucose production and disappearance.⁶⁸ It was shown that these very low birthweight infants utilise some three to four times more glucose per kilogram body weight than adults. This presumably reflects their high ratio of brain to body weight. Endogenous glucose production provided only about 30% of the glucose needed by these infants; the authors suggested this fact provided a mandate for the exogenous infusion of glucose to prevent hypoglycaemia developing.

Mineral metabolism

Studies employing stable isotopes of zinc, iron, calcium, and/or magnesium have been performed to assess absorption, bioavailability, or balance of these minerals in healthy children or children with disorders affecting the gastrointestinal tract.^{69–73} Both intrinsic and extrinsic labelling techniques have been employed in these studies. The latter technique is far less

expensive in that the isotope is simply mixed with the food before consumption. In this instance it has to be assumed that the extrinsic label equilibrates completely with the native intrinsic inorganic element in the diet, and is absorbed and metabolised in an identical fashion. Plant and animal foods can be labelled intrinsically by the incorporation of the chosen isotope biosynthetically into the growing food. Several procedures to achieve this end have been described.⁷⁴⁻⁷⁵ When calcium and magnesium metabolism was assessed in prepubertal and pubertal girls and boys, Abrams *et al* showed that total calcium absorption from milk was increased in pubertal compared to prepubertal children.⁷² Almost 50% of the children studied had negative magnesium balances despite magnesium intakes based on the recommended dietary allowance for magnesium. A more detailed review relating to stable isotopes and mineral metabolism in children was recently published.⁷⁶

Conclusion

Stable isotopes are ideal "tools" for the dynamic assessment of *in vivo* metabolism in the paediatric population. Not only are these tracers safe and therefore ethically justifiable, in addition they may be particularly important for the validation of new treatment modalities, such as novel drug treatment or gene therapy. Many more exciting studies are currently underway to enhance our knowledge of paediatric metabolism and (patho)physiology, an important factor for the continued reduction of paediatric morbidity and mortality.

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FETAL AND NEONATAL EDITION

May 2001 issue

The following articles—being published in the May 2001 issue of the *Fetal and Neonatal* edition of the *Archives of Disease in Childhood*—may be of general interest to paediatricians.

LEADING ARTICLES

Management of the neonate with symptomatic congenital heart disease

D J Penny, L S Shekerdemian

ORIGINAL ARTICLES

Community based universal neonatal hearing screening by health visitors using otoacoustic emissions

M Owen, M Webb, K Evans

REVIEWS

Hepatitis C in pregnancy

Y Nako, A Tachibana, T Fujiu, T Tomomasa, A Morikawa