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STABLE ISOTOPE TRACER STUDIES OF METABOLIC FUEL TRANSPORT IN INFANCY AND CHILDHOOD

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Approximately 10 years ago, several now classic monographs (1-4) discussed body stores of metabolic fuels and physiologic regulation of their transport in the adult. Despite the fact that infants maintain precarious fuel balance at all times and frequently fail to maintain fuel homeostasis when confronted with the stress of even relatively minor illness (not to mention consequences of the many inborn errors of metabolism) the quantitative information necessary to write a similar monograph for the pediatric age group was unavailable in the early 1970's. This deficit was largely due to the absence of a safe, non-invasive, ethically acceptable approach to obtaining quantitctive fuel transport data in childhood.

Fortunately, technological advances in the last decade have now made non-radioactive, stable isotope tracer methods available to help answer these major pediatric questions. As opposed to the situation 10 years ago, the supply of highly enriched carbon-13, nitrogen-15, oxygen-18, and deuterium now exceeds the biomedical demand for their use and several competent firms in Europe, Canada, and the United States are capable of supplying biochemicals specifically labelled with these isotopes. Likewise, relatively simple gas chromatography-mass spectrometry systems and a wide variety of convenient micromethods for precise quantitative tracer analysis in physiological samples are also readily available. Finally, excluding the initial instrumentation cost which is still substantial, the cost of individual stable isotope tracer studies is now comparable to similar radiotracer studies. Table 1 shows the comparative cost of measuring leucine and glucose turnover in adults with stable or with radiolabelled leucine and glucose tracers. This analysis, based on bulk purchase of $(1-^{13}C)$ leucine and $(6,6-^{2}H_2)$ glucose which drastically reduces their cost, clearly shows that stable isotope tracer studies are cost competitive with conventional radioactive tracer investigations.

Table 1. Comparative Cost of Tracer Studies

	Dose (per kg)	Cost* ($ U.S.)
(1-^{13}C) Leucine	2 mg	30
(1-^{14}C) Leucine	0,3 μ Ci	40
(6,6-$^{2}H_2$) Glucose	5 mg	25
(6-^{3}H) Glucose	1 μ Ci	8
(3-^{3}H) Glucose	1 μ Ci	25

* 4 hr study in 70 kg adult. Isotopes purchased in bulk

Furthermore, a $ 25-30 (U.S.) isotope cost represents only a small fraction of the total overall cost of a single human research study. Naturally, a similar study in a young child would be cheaper still.

While there is little difficulty convincing pediatricians that stable isotope tracers should be used in children for ethical reasons, few pediatric investigators are aware of additional advantages of stable isotope tracers for research purposes (Table 2). First, there are no practical radiotracer alternatives for certain elements. For example, if one is interested in studying body nitrogen dynamics (5) and its relationship to protein turnover, ^{15}N is the only convenient tracer choice since ^{13}N radiotracer has a half-like of only 10 minutes. Secondly, it is commonplace for clinical investigations to ignore isotope effects when using radioactive tracers in vivo even though isotope effects may be substantial for ^{3}H and present (albeit to a lesser degree) for ^{14}C. Corresponding effects for ^{2}H and ^{13}C labelled substrates will be less since their differences from the major naturally occuring nuclides ^{1}H and ^{12}C are less. Third, some human studies would be difficult if not impossible to carry out with radioisotopes even if one could ethically use a radiotracer. This is particularly true for substances that are present at very low plasma concentrations but which have very fast turnover rates. In such circumstances, one must either administer isotope in an amount which exceeds a safe dose or one must draw large blood samples thus limiting pediatric investigations, particularly in small neonates.

Additional advantages accrue from the fact that selected ion monitoring GCMS assays measure both the stable isotope enriched material and its corresponding unlabelled substrate at the same time in the same assay (6-9). The stable isotope method, then, is frequently simpler because it is not necessary to have separate assays both for radioactivity and for content in order to subsequently calculate specific activity with a radiotracer. Furthermore by inclusion of a second stable isotopically labelled internal standard, identical to the substrate under

Table 2. Additional Advantages of Stable Isotope Tracers in Biological Studies

1. No practical radiotracer alternative exists for certain elements.
2. Isotope effects are less than with corresponding radiotracer.
3. Some human studies not possible within constraints of safe radiotracer dose.
4. Stable isotope method frequently simpler than radioisotope technique.
5. Substrate content can be measured at the same time as isotope enrichment.
6. Stable isotope method generally more precise.
7. Confidence in assay specificity is frequently greater with a stable isotope.
8. Intramolecular location of label can be determined easily.
9. Several stable tracers can be used simultaneously in the same subject.
10. The same subject can be studied repeatedly.

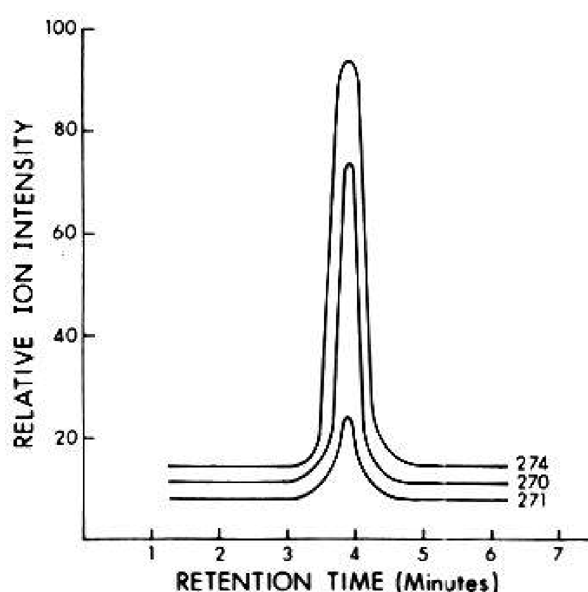

Fig. 1. Electron impact, selected ion chromatogram of the molecular ion of palmitic acid methyl ester. The signal at m/z 270 represents natural plasma palmitic acid. The ion at m/z 271 includes both the natural carbon-13 background as well as the signal from (1-^{13}C) palmitic acid used as a tracer in vivo. The ion at m/z 274 is due to (5,5,6,6-$^{2}H_4$) palmitic acid internal standard added in vitro to quantify natural palmitic acid content.

investigation in all ways except for its isotopic composition, one can measure substrate content, as well at the same time one determines tracer isotope enrichment. Thus, Figure 1 shows an ion monitoring tracing of the molecular ion region of plasma palmitic acid chromatographed as its methyl ester derivative (molecular weight = 270). The ion tracing at mass 170 is from the natural palmitic acid present in plasma. The ion at 271 is due, in part, to (1-^{13}C) palmitic acid employed as a tracer, and the ion at mass 274 comes from (5, 5, 6, 6 - $^{2}H_4$) palmitic acid added to plasma as the assay internal standard. Using this approach one can measure plasma fatty acid turnover in the same 0.1 ml plasma samples from human newborns (10). Because mass spectrometry can measure the ratios of these peaks to each other

Metabolism of L-[^{15}N, 1-^{13}C] Leucine

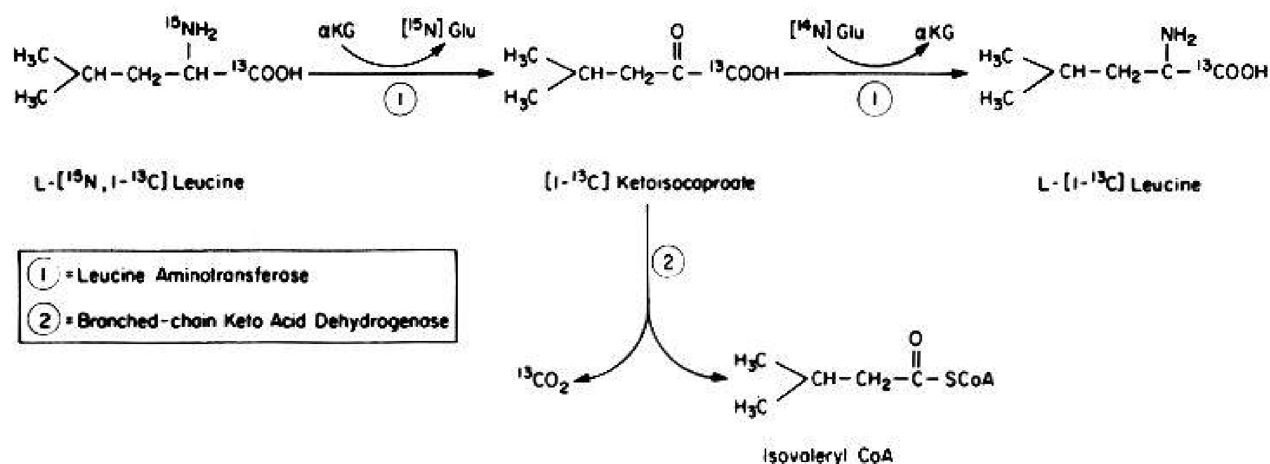

Fig. 2. The metabolism of L-(^{15}N, 1-^{13}C) leucine showing the production of singly labelled L-(1-^{13}C) leucine by reamination (see text).

with a relative precision of ± 0,05 %, and since the sample preparation and assay are identical for all the peaks, stable isotope methods like this reduce propagated errors and are generally more precise than a corresponding radiotracer method where substrate content and radiotracer counting are performed with different sample preparations and under different assay conditions. Furthermore, the investigator's confidence in the specificity of the result (ie. the security of knowing that the enrichment measured is truly only in the compound of interest) is far greater in a stable isotope assay because one can assure specificity at several steps in the assay process including: sample preparation, derivative selectivity, gas chromatographic resolution, choice of mass spectrometer ionization mode, the ions selected for monitoring, and judicious use of the resolving power of the mass spectrometer. These approaches are discussed in detail elsewhere (11).

An additional advantage of stable isotope tracer work is that the intramolecular location of one or several labels can be determined relatively easily. This has major advantages in tracing specific metabolic pathways, in vivo, particularly in inborn errors of metabolism. There are other applications as well. Figure 2 shows the early steps of leucine metabolism traced with L-(^{15}N, 1-^{13}C) leucine. When leucine is transamined, the ^{15}N is lost and (1-^{13}C) ketoisocaproate is formed. Reamination of this ketoisocaproate will produce only (1-^{13}C) leucine since there is far greater chance that the carbon-13 labelled ketoisocaproate will transaminate with a (^{14}N) glutamate than with a (^{15}N) glutamate. With mass spectrometry, it is simple to determine the plasma content of leucine molecules which are unlabelled, labelled with ^{13}C alone, and labelled with both ^{13}C plus ^{15}N. In conjunction with measurements of ketoisocaproate oxidation from expired $^{13}Co_2$, the ability to know the proportion of leucine mole-

cules labelled with only ^{13}C or with $^{13}C + ^{15}N$ allows one to calculate rates of leucine transamination, reamination, and oxidation (11). In postabsorptive man, 90 % of ketoisocaproate formed is reaminated to leucine whereas 10 % is oxidized. In fed subjects, however, the fraction of ketoisocaproate oxidized doubles while reamination is reduced to 80 % (12). Obviously, this approach can be applied to other amino acids as well.

A related advantage of stable isotope tracer use is that several stable isotopically labelled compounds can be administered simultaneously to the same subject, a luxury not generally possible with radioactive tracers where safe dosage limits will usually be exceeded if more than a single labelled material is given. We have used this advantage to simultaneously study various aspects of the metabolism of several amino acids (13-18), hepatic recycling of glucose carbon (19-20), and the interrelationships between amino acid and glucose transport (21-24) in vivo. For example, if (ring-$^{2}H_5$) phenylalanine and (1-^{13}C) tyrosine are infused in tracer quantities at the same time and plasma deuterated phenylalanine, ^{13}C-tyrosine, and (ring-$^{2}H_4$) tyrosine (formed from $^{2}H_5$-phenylalanine by the action of phenylalanine hydroxylase) are measured, one can calculate the transport rates of both amino acids and the absolute rate of phenylalanine conversion to tyrosine by simple precursor-product tracer equations. Furthermore, if one collects expired $^{13}CO_2$ tyrosine oxidation can also be assessed. Using this approach, we were able to show that phenylanine and tyrosine flux each averaged nearly 40 μmoles $kg^{-1}hr^{-1}$ in postabsorptive young adults and that approximately 6 μmoles $kg^{-1}hr^{-1}$ of phenylalanine (16 % of its turnover) was converted to tyrosine (18). These results indicate that the normal basal activity of phenylalanine hydroxylase in vivo may be significantly lower than that suggested by the results of unlabelled phenylalanine loading studies or studies employing loading doses of ($^{2}H_5$)-phenylalanine alone. The usefulness of this combined isotope approach to study physiologic regulation of this important enzyme and to answer questions of appropriate dietary intake regulation in the hyperphenylalaninemias is apparent.

A final practical advantage of stable isotope tracer work is that the same subject can be studied repeatedly, an option also generally not possible with radiotracers because of dosage limitations. This not only allows the subject to serve as his own control for investigative manipulations but also permits determination of intrasubject variability. The latter is important both for understanding the fine control of metabolic events as well as for allowing the investigator to assess what magnitude of change a given intervention must produce in order to be perceived. In other words, knowledge of intrasubject variance lets one calculate the so-called "power" of the test circumstance employed and whether one is likely to decide that no difference exists between two circumstances when a real difference does exist (Type II error).

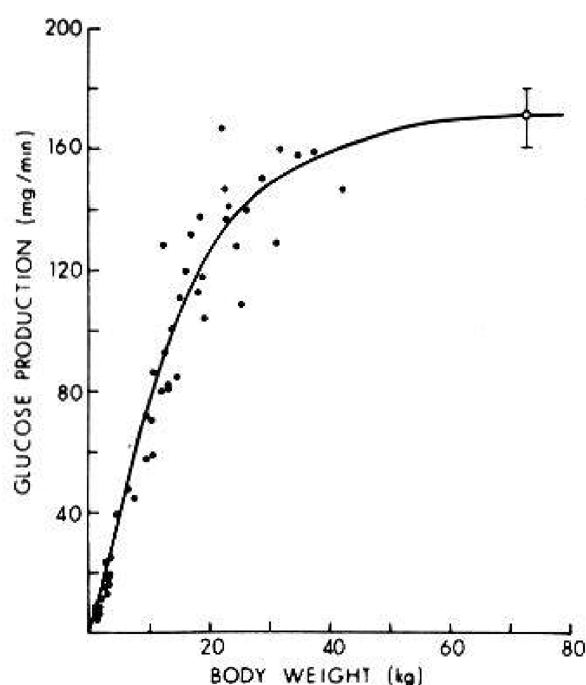

Fig. 3. Hepatic glucose production as a function of body weight throughout childhood measured with (6,6-$^2H_2$) glucose tracer. The average adult value (± 2 S.D.) is represented by an open circle.

Not surprisingly, there has been little information on the intrasubject variability in basal metabolic fuel transport rates reported in radiotracer studies. We have been able to obtain such data employing (6,6-$^2H_2$) glucose, (1-^{13}C) palmitic acid, and amino acids labelled with ^{13}C and ^{15}N (25-27). Glucose trnasport is remarkably constant, varying by less than 4 % in each of five individuals each studied six times in the postabsorptive state (25). Amino acid transport is somewhat more variable, generally on the order of ±5-7 % and usually less than ±10 % (27). Palmitic acid flux, however, varied between 13-28 % when repeatedly measured in the same subjects over a period of weeks (26).

Now, to return to the child, I would like to present some of the data which have allowed us to begin to understand the magnitude of transport of various metabolic fuels in the pediatric age group. Since infantile hypoglycemia is such a common manifestation of deficient fuel transport due to almost any event which impairs oral nutrient intake, our first efforts were aimed at determining hepatic glucose production in childhood using (6,6,-$^2H_2$) glucose tracer (28). Figure 3 shows the results of such measurements in more than 50 children and demonstrates that glucose production has reached adult values (open circle ± 2 S.D.) by about 6 years of age (30 kg). Before this age, glucose production is approximately 5-6 mg $kg^{-1}min^{-1}$ or nearly 3 times the adult value on a body weight basis. The reason for this increased rate is apparent in Figure 4 where the same data are replotted versus estimated

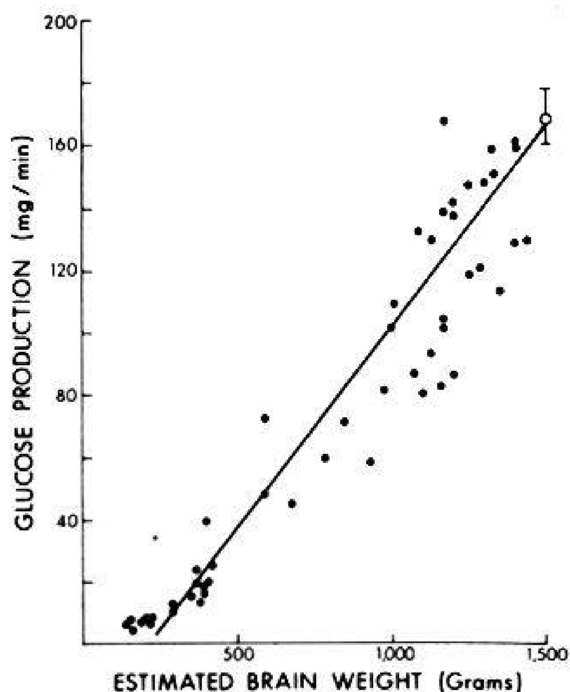

Fig. 4. Hepatic glucose production as a function of estimated brain weight throughout childhood. The average adult value (± 2 S.D.) is depicted with an open circle.

brain weight, since the brain is by far the leading consumer of glucose, metabolizing 50-70% of daily glucose production. Since the brain weight of a 6 year old child is on the order of 1250 grams and that of the adult is only 1400-1450 grams, it is not surprising that the 6 year old must produce glucose at such a rapid rate.

This accelerated glucose production rate is also present in newborns as small as 650 gms. If hepatic glycogen were the only source of blood glucose in the neonate, the infant would not be able to maintain normoglycemia for more than a few hours after birth. Thus, gluconeogenesis must be active early in neonatal life. Unfortunately, estimates of the onset of gluconeogenesis in the newborn extrapolated from animal data are not helpful in this regard since the intracellular localization of key gluconeogenic enzymes and the course of their maturation varies from species to species. We have had the opportunity to measure gluconeogenesis in the human newborn by assessing the fraction of metabolized glucose carbon-13 recycled from lactate, alanine and pyruvate (19) and by directly measuring the incorporation of $(2,3,-{}^{13}C_2)$ alanine into glucose (29). These studies showed that gluconeogenesis was active soon after birth and implied that it may even be active at birth since our earliest measurements were at 4-6 hours of age. Furthermore, they demonstrated that 10-15 % of hepatic glucose output comes from alanine alone. Therefore the overall contribution of gluconeogenesis to hepatic glucose output is substantially higher than this if one adds in the potential

contributions of other gluconeogenic amino acids and lactate. Provocative additional conclusions of the alanine tracer study (29) were 1) that the high alanine levels in neonates, commonly attributed to impaired removal because of immature gluconeogenesis, are at least in part due to a high alanine inflow rate on the order of 15-20 μmoles $kg^{-1}min^{-1}$. Whether this accelerated inflow is due to increased protein breakdown in the newborn remains unsettled since the transport rate of the essential amino acid, lysine, is 70 % greater in the newborn than in the adult (30) but the turnover of another essential amino acid, leucine, is comparable to the adult rate, 2) that alanine turnover is not impaired in normoglycemic small-for-gestational-age infants despite their significantly lower blood sugar levels compared with term infants, and 3) that a high alanine level alone is not sufficient indication of impaired hepatic gluconeogenesis since several infants with alanine levels in the 600 μM range had more than 15 % of their hepatic glucose output coming from alanine.

At all ages, the principal fuel source is lipid. Despite the fact that plasma fatty acid levels rise rapidly after birth, accompanied by a fall in R.Q. close to 0.7, indicating a lipid fuel economy, there are no reported measurements of free fatty acid transport in the human neonate and virtually none in older children. Recently, Dr. Pierre Bougnères (31) from Hôpital des Enfants Malades, Paris, has measured fatty acid transport in human infants using albumin-bound (1-^{13}C) palmitic acid as the representative tracer. The average FFA turnover in infants only two hours after feeding was comparable to that seen in adults after an overnight fast. In infants unfed for 5-12 hours, FFA transport averaged nearly 20 μmoles $kg^{-1}min^{-1}$, a value reached in adults only after 72 hours of starvation. As in adults, neonatal FFA flux correlated directly with plasma FFA levels. Interestingly, the slope of this correlation was virtually identical to the average regression slope seen in adults suggesting that regulatory feedback mechanisms are already maturely established within the first days of life. An important implication of these results is that glycerol transport must also be substantial in the neonate. In fact, the glycerol released from tissue lipolysis necessary to produce the above FFA turnover rates could account for 15-20 % or more of neonatal hepatic glucose output if all the glycerol were converted to glucose. This calculation is likely to underestimate the potential contribution of glycerol to glucose since FFA release rates underestimate triglyceride hydrolysis because some of the FFA are reesterified in adipose tissue whereas all glycerol is released.

The above studies in infants combined with additional related information we have obtained in older children with similar stable isotope tracer techniques have now permitted construction of a preliminary fuel transport table for the pediatric age group (Table 3). Despite the fact that the human neonate is only 5 % the size of an adult, the infant transports glucose

Table 3. Rates of Postabsorptive Fuel Transport in Man

	Infant*	Child+	Adult△
		(g/hr)	
Glucose	1.3	8	9
Amino Acids			
Flux	1	5	10
Net	0.3	1.5	3
Fatty Acids	1	(3.5)	7
Glycerol	(0.1)	(0.4)	0.7
Ketones	(?)	(?)	2
	*3.5 kg	+25 kg	△70 kg

Values in parenthesis are estimates

and fatty acids at 15 %, and amino acids at 10 % the adult rate. Likewise, a young child, one-third the size of an adult, transports glucose at virtually the adult rate and amino and fatty acids at half the adult value. Ketone body transport rates are still unknown in the infant and child. Plasma ketone body levels are low in the newborn and it is commonly assumed that this is a consequence of decreased ketone production. In view of the high FFA transport rates, an equally tenable hypothesis is that these levels are low because ketones bodies are being utilized at an accelerated rate, perhaps by the brain. Nevertheless, Table 3 graphically affirms the reasons why infants and children maintain a precarious fuel balance and why they are frequently incapable of maintaining this balance if exogenous fuel intake is reduced by almost any childhood illness.

In conclusion, the above stable isotope studies demonstrate that in vivo biochemical kinetics can be studied safely in children. Answers to the remaining fuel transport questions posed above and to the host of other questions still unanswered about pediatric metabolism in general can be addressed by judicious application of these techniques in appropriate research situations.

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