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Measuring in vivo ureagenesis with stable isotopes ☆

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ABSTRACT

Stable isotopes have been an invaluable adjunct to biomedical research for more than 70 years. Indeed, the isotopic approach has revolutionized our understanding of metabolism, revealing it to be an intensely dynamic process characterized by an unending cycle of synthesis and degradation. Isotopic studies have taught us that the urea cycle is intrinsic to such dynamism, since it affords a capacious mechanism by which to eliminate waste nitrogen when rates of protein degradation (or dietary protein intake) are especially high. Isotopes have enabled an appreciation of the degree to which ureagenesis is compromised in patients with urea cycle defects. Indeed, isotopic studies of urea cycle flux correlate well with the severity of cognitive impairment in these patients. Finally, the use of isotopes affords an ideal tool with which to gauge the efficacy of therapeutic interventions to augment residual flux through the cycle.

Introduction: stable isotopes and the dynamic nature of body constituents

Fundamental to current understanding of biochemistry is the notion that virtually all molecules of life participate in an incessant cycle of synthesis and degradation that concludes only with death. This conceptualization, so familiar to contemporary science, was not fully grasped until the middle of the 20th Century, when researchers first used isotopic tracers in an effort to determine the origin and fate of the molecules that constitute the dense skein of human metabolism.

Two developments facilitated this revolution. The first of these was the discovery by Urey of deuterium at Columbia University in 1933 [1]. The second innovation was the application of isotopic tracers to the study of biochemistry, an effort in large measure spearheaded by Rudolf Schoenheimer, who had emigrated from Germany to the United States. In 1935 he utilized deuterium as a tracer with which to scrutinize intermediary metabolism [2]. Working with the American biochemist David Rittenberg, Schonheimer quickly appreciated the potential utility of the isotopic method for characterizing precursor–product relationships and measuring reaction rates:

> The difficulty in following physiological substances in the course of their transportation in the body and their conversion into other substances, accounts for our ignorance with respect to many of the most fundamental questions concerning intermediate metabolism. The solution of the problems will be possible only when direct methods for tracing such substances will be available... As the chemical properties of the various isotopes of an element are almost identical, it is to be expected that the properties of an organic molecule will remain unaltered if one or even several of the atoms were replaced by their isotopes [1].

The discovery of the urea cycle in 1932 by Krebs and Hensleit [3] was a seminal event, not only because it provided a clear schema by which liver converts waste nitrogen into urea, but because it posited a novel structure – a cycle – that mediated a fundamental biochemical process. Indeed, the urea cycle was the first of the major biochemical cycles to be discovered. As such, it offered a critical insight into the nature of metabolic organization, particularly what Schoenheimer termed the "dynamic state of body constituents" [4]. Now it became apparent that the chemistry of life commonly conformed to a cyclical rather than a linear pattern. The biochemist Joseph Fruton nicely captured the conceptual significance of this paradigm shift:

> This work marked a new stage in the development of biochemical thought. Not only was an explanation of a biochemical synthesis offered for the first time in terms of chemical reactions identified in the appropriate biological system and not merely inferred by analogy to the known chemical behaviour of the presumed reactants, but also the paper provided a clue to the

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organisation of metabolic path ways in living cells. This became evident in 1937 with the appearance of the Krebs citric acid cycle, whose conceptual relation to the earlier ornithine cycle was obvious [5].

Isotopic studies of urea turnover

A fortuitous development was the discovery of the urea cycle just prior to the availability of ^{15}N and ^{13}C as metabolic tracers [6,7]. Among the first applications of the isotopic method was the demonstration [7] with a $^{13}CO_2$ probe that the carbon atom of urea derived from carbon dioxide (actually, HCO_3^- is the source). The objective of most early isotopic (usually ^{15}N) studies of urea synthesis was not to measure flux through the urea cycle but to measure rates of total body protein synthesis [8–10]. In the typical experiment a ^{15}N-labeled amino acid was administered to human subjects and the appearance of the label in urinary ^{15}N (most of which is comprised of [^{15}N]urea) was determined with isotope ratio-mass spectrometry [10]. Whole body protein synthesis is then derived from the assumption that isotope retained in the body corresponds to that incorporated *de novo* into body proteins [8].

Technological advances enabled the use of stable isotopes to study flux through the urea cycle in youngsters with inborn errors of metabolism. Most early studies utilized isotope ratio-mass spectrometry as an analytic tool with which to measure isotopic enrichment. This approach had the advantage of exquisite sensitivity with regard to the detection of isotopic label, but it was far less sensitive with respect to the amount of material that is needed for analysis. In addition, isotope ratio-mass spectrometry requires the complete separation of the analyte of interest and the combustion of this analyte to a gaseous form (CO_2 or N_2) in order to determine the $^{13}C/^{12}C$ or $^{15}N/^{14}N$ isotopic ratio. The coupling of the mass spectrometer to a gas chromatograph facilitated pediatric investigations, since reproducible measurements now could be obtained even with relatively small samples sizes (often <100 μl of plasma or serum). In addition, the great resolving power of gas chromatography, which can separate hundreds of metabolites in a single analysis, greatly amplifies the repertoire of compounds now susceptible to study.

A notable example of this approach is the study of Lee et al. [11], who measured urea turnover in a group of patients with urea cycle defects, including ornithine transcarbamylase deficiency (10 female/5 male), citrullinemia (5) and argininosuccinic aciduria (2). The experimental protocol involved the continuous infusion of [5-^{15}N]glutamine and [^{18}O]urea, which enabled measurement of total body turnover of the urea pool and of the amide-N of glutamine. Measurements of blood [^{15}N]urea defined the rate of nitrogen transfer from 5-N of glutamine to urea. They found that the ratio of [5-^{15}N]glutamine/[^{15}N]urea discriminated the control subjects from those with a urea cycle defect, with this parameter being much higher in patients with disease of neonatal onset.

This study involved a constant infusion of isotope over a relatively prolonged (7.5 h) period. There are advantages to parenteral administration of tracer, which neutralizes the problem of variable absorption from the gastrointestinal tract. The constant infusion method also lends itself to a straightforward kinetic analysis. However, there are unavoidable problems attendant upon this method, which may be difficult to implement in children. A long infusion period is necessary to achieve steady-state in an individual with a urea cycle defect and consequent diminution of urea turnover. In addition, the relatively small vessels of infants and children commonly present a problem with regard to venous access. Children (as well as many adults) have found it difficult to summon the patience to comply with a long period of isotope infusion. The latter difficulty may be especially vexatious in a child (or adult) with a urea cycle defect, in whom impaired cognition and a relatively short attention span are common comorbidities.

A method based upon oral administration of isotope might prove more practicable to pediatric studies. To this end, we devised a protocol [12,13] based upon the oral administration of $^{15}NH_4Cl$. We utilized gas chromatography–mass spectrometry to monitor the appearance of label in [^{15}N]urea and [5-^{15}N]glutamine over a 4-hour time period following the test dose of tracer. Our study cohort for this X-linked disorder included 15 heterozygotes, of whom 6 were ostensibly asymptomatic, 8 were clearly symptomatic, and 1 was a severely affected individual with neonatal onset.

The results are shown in Fig. 1. We found essentially no difference with regard to the formation of [^{15}N]urea (left panel) when we compared the response of the control group with that of asymptomatic carriers. In contrast, symptomatic heterozygotes converted significantly less ^{15}N from ammonia to urea. A single, severely affected hemizygote was able to produce virtually no [^{15}N]urea.

Of note is the finding (Fig. 1, right) that the synthesis of [5-^{15}N]glutamine was greater even in asymptomatic carriers than in the control group. This observation indicates that ostensibly "asymptomatic" carriers are biochemically distinguishable from normal. In a subsequent study [14] we explored this concept in greater detail by performing neuropsychological testing in 19 female heterozygotes for ornithine transcarbamylase deficiency. The carriers displayed normal IQ scores as well as strength in verbal intelligence and reading, but they manifested weakness in fine motor dexterity and lesser impairments in executive skills, nonverbal intelligence and mathematics. Interestingly, we found that the degree of residual urea synthetic capacity, as measured with the $^{15}NH_4Cl$ loading procedure (Fig. 1), often predicted cognitive measures. Thus, the isotopic method underscored the importance of maintaining good metabolic control, even in otherwise "asymptomatic" carriers. There was a significant positive correlation between individual ^{15}N incorporation into urea and performance in math ($p = 0.0013$), attention/executive ($p = 0.001$) and reading ($p = 0.032$). Indeed, the isotopic approach was more predictive of cognitive outcome than the allopurinol challenge test [14].

No method is entirely free of disadvantages. Although the $^{15}NH_4Cl$ loading test proved highly discriminative, it did present problems: (a) The flavor of ammonium chloride is obnoxious and provoked gagging in several subjects. (b) It would be ethically impossible to administer an ammonium salt to an individual who already may have hyperammonemia. (c) Ammonia administration in such a clinical setting is experimentally unsound, since the expanded endogenous N pool would unduly dilute the isotopic tracer. (d) Finally, the method requires gas chromatography–mass spectrometry to analyze enrichment in [^{15}N]urea and [5-^{15}N]glutamine. As noted above, this technology is exquisitely sensitive with regard to sample size, but it is several orders of magnitude less sensitive with respect to the detection of isotopic enrichment. This becomes a confounding limitation in studying patients with severe urea cycle defects, in whom we anticipate very little incorporation of label into urea.

To this end, we endeavored to develop a method that would measure residual competency of the urea cycle by quantifying the synthesis of [^{13}C]urea from a ^{13}C-labeled precursor. The tracer we selected was [1-^{13}C]acetate, the carbon in which is incorporated into urea via the following reaction sequence:

$$[1\text{-}^{13}C]\text{-acetate} \rightarrow \text{TCA cycle} \rightarrow H^{13}CO_3^- \rightarrow {}^{13}\text{C-carbamylphosphate} \rightarrow [^{13}C]\text{urea}$$

In hepatic mitochondria acetate is very quickly oxidized to bicarbonate following condensation with oxaloacetate in the citrate synthetase reaction. Bicarbonate then enters the urea cycle