Hydrogen Isotopes in Individual Amino Acids Reflect Differentiated Pools of Hydrogen from Food and Water in Bacteria

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ABSTRACT: Hydrogen isotope (δH) analysis is widely used in animal ecology to study movement and migration strategies because δ H can trace precipitation and climate. To understand the biochemical underpinnings of how hydrogen is incorporated into biomolecules, the δ H of individual amino acids (AA) were determined in E. coli cultured in glucose-based or complex media (tryptone) in media waters with δ H ranging from -55‰ to +1070‰. The δH values spanned a range of ~350‰ in cells grown in lab distilled water (δH = -55‰) and in tryptone digest proteins. In glucose-grown cells, 33-56% of their hydrogen originate from media water, with the remainder coming presumably from carbon-bonded hydrogen in glucose. These findings are based on an isotopic fractionation model that accounts for potential independent fractionation of hydrogen coming from both water and glucose. In E. coli grown on tryptone, the δH of nonessential AAs (e.g. alanine) varied linearly with the δH of the media water, whereas δH of essential AAs was nearly identical to that in diet. Model calculations determined that as much as 53% of hydrogen in some nonessential amino acids originated from water while no more than 14% of hydrogen in essential amino acids originated from water. This demonstrates that hydrogen isotopes can route directly at the molecular level. We conclude that the patterns and distributions in δH of individual amino acids are determined through biosynthetic reactions, opening up the possibility that δH could become a new biosignature for studying novel microbial pathways. At the molecular level, the δH of AA in an organism’s proteinaceous tissues provides a dual tracer for both food and drinking water, ultimately providing answers as to how and why the δH of organisms vary over landscape scales.
INTRODUCTION

Animal movement is a defining, not to mention fascinating, characteristic of many diverse vertebrate and invertebrate groups that has been a major topic of research in animal ecology for centuries. For the large majority of animals, which are small in size, it is difficult to characterize movement, seasonal or otherwise. Over the past two decades, ecologists have turned to natural spatial gradients in stable hydrogen isotopes ($\delta^2$H) in precipitation and groundwater that intrinsically label animal tissues. Continental-scale models of precipitation $\delta^2$H patterns, or isoscapes (1), are invaluable for these efforts, however, significant intra-site variation (10–20‰) in animal tissue $\delta^2$H values collected from known origin samples impedes our ability to resolve movement patterns at sub-continental, regional scales (2). Animals incorporate ~15–30% of hydrogen into their tissues from water (3-5), sourcing the remainder from food. Because of ecological and physiological constraints, such as the type and variety of food in the diet, as well as geographic source of drinking water, the spatial isotopic patterns in animals related to precipitation $\delta^2$H values tend to become blurred or even non-existent (6), even for resident species (7).

Studies with captive animals have shown that when fed diets with a constant $\delta^2$H composition, the $\delta^2$H of animal tissues are depleted in deuterium ($^2$H) relative to water consumed by ~20–30‰ (5, 8). The biochemical mechanism for this isotopic discrimination has yet to be described. This is in contrast to our understanding of the carbon ($\delta^{13}$C) and nitrogen ($\delta^{15}$N) isotope composition of animal tissues relative to their diets, for which a
biochemical framework of isotopic discrimination has been described and tested in controlled feeding experiments. Determining the magnitude and variation in diet or water to tissue discrimination factors are essential to the application of $\delta^2$H to characterize animal movement patterns, and the use of hydrogen isotopes to trace the flow of energy within and among ecosystems (9).

Metabolic processes that fractionate hydrogen have been primarily described in microbial and plant tissues. Principal among these studies is the body of literature on hydrogen isotope fractionation during lipid synthesis (10-12). Fatty acids, hydrocarbons, and alkenones synthesized by plants, phytoplankton, and microbes are depleted in deuterium relative to bulk tissue, which is the typical manner in which isotopic discrimination within an organism is described. Such molecules, found in geological settings such as lake sediments, are used extensively as paleoclimate proxies and can have $\delta^2$H values that are 50–90‰ lower than environmental water (12, 13).

Because proteins comprise the bulk of animal tissues of interest to ecologists, an understanding of hydrogen isotope fractionation in the amino acids (AAs) from which they are synthesized has the potential to offer significant insight animal diets. Stable isotope analyses of carbon ($\delta^{13}$C) and nitrogen ($\delta^{15}$N) in AAs have revealed that extensive fractionation occurs both between and among AAs (e.g., 14). These fractionations are related to biochemical pathway in autotrophs and a combination of biochemical pathway and direct incorporation in heterotrophs. Certain AAs (e.g. glutamate and alanine) can be synthesized by any eukaryotic organism (nonessential AAs), whereas others (e.g. valine and leucine) must originate from an organism’s diet (essential AAs) or gastrointestinal microbiota (14). As an animal incorporates an amino acid directly from its food, the
isotopic composition of that amino acid influences the isotope value of the animal’s bulk tissue.

Accordingly, we designed a series of experiments with the model bacterial heterotroph *Escherichia coli* to measure the impact of environmental water and diet on amino acid $\delta^2$H. First, the organism was grown in water of varying $\delta^2$H composition in both glucose and protein-based media. Secondly, we wanted to quantify the extent to which hydrogen in AA was directly routed from diet versus synthesized from other non-protein sources, here water and glucose. To this end, we incubated *E. coli* in the two media described above whose waters ranged in isotopic composition from -55‰ to +1070‰, which allowed us to calculate the fraction of hydrogen that originated from organic food (i.e., medium) versus media water.

RESULTS

**Hydrogen Isotopic Composition of Amino Acids in Tryptone:** To test methods for analyzing $\delta^2$H in amino acids we hydrolyzed the tryptone protein that was used the organic source for growing *E. coli* by three different methods: (#1) hydrolysis in 6N hydrochloric acid (HCl) for 20 hours at 110°C, (#2) vapor hydrolysis with 12N HCl 20 hours at 110°C, and (#3) hydrolysis with deuterium-spiked 6N HCl (+1070‰ water plus 12N HCl) for 20 hours at 110°C. Following hydrolysis, amino acid mixtures were dried under $N_2$ at 110°C, then derivatized. The derivatization steps remove exchangeable hydrogen atoms from the carboxyl and amine side groups. One hydrogen atom on the amine group is retained and likely exchanges during hydrolysis and drying. Since the derivatization reactions are carried out strictly without any liquid water present, it is unlikely that this hydrogen atom
exchanged during the derivatization process. The $\delta^2$H of amino acids we report represents what we refer to as “intrinsic” hydrogen: non-exchangeable hydrogen bonded to carbon in the AA and one hydrogen atom bonded to nitrogen that remains following derivatization.

The $\delta^2$H of individual amino acids in tryptone varied by >350‰, and among the three treatments, the isotopic compositions of derivatized amino acids showed the same pattern for the majority of the amino acids. Exceptions for the $^2$H-spiked hydrolysis treatment were aspartic and glutamic acids, in which $\delta^2$H was more positive by >100‰ relative to the other treatments. Given the nature of the derivatized AA, hydrogen atoms at the alpha position of both carboxyl groups must have been susceptible to exchange. In the treatment hydrolyzed by vapor phase, serine was altered the most with $\delta^2$H values more positive by about 75‰. Serine and glycine, two of the structurally simplest AAs, should be those with the greatest likelihood for hydrogen exchange, as they contain only two (glycine) or three (serine) nonexchangeable hydrogen atoms. Following these results, we prepared 6N HCl by mixture of laboratory distilled water and 12 N HCl and used it to hydrolyze all of the samples analyzed in our study for 20 hours at 110°C.

The more remarkable information from these tests was the range in $\delta^2$H of the amino acids from mammalian tryptone proteins regardless of hydrolysis method. Nonessential amino acids had more positive $\delta^2$H values (-60 to -160‰), whereas essential amino acids had more negative $\delta^2$H values (-160 to -400‰). A similar pattern in isotopic fractionation has been observed in carbon isotope values of AA from a variety of organisms (15), and suggests that the carbon skeleton of essential AAs must have originated directly from the protein carbon in the animal’s diet. Whether or not the hydrogen in these
molecules is associated directly with the carbon via direct routing from the protein fraction of diet to animal is unknown at this time.

*E. coli* grown in Glucose and Inorganic Nutrient Media: Hydrogen sources available to *E. coli* included the following: glucose (-4‰), NH₄Cl (-124‰) and water (-55‰ to +1070‰) (Fig. 1). The range in δ²H for intrinsic hydrogen in individual AAs in cells grown in lab-distilled water (-55‰) on the glucose medium, which were required to synthesize 100% of their AA, was almost 300‰ (Table 1). Cells were cultured in three independent experiments, then analyzed in triplicate. Proline was the most enriched amino acid (δ²H= -37±40‰), while the most depleted amino acids were glycine (δ²H= -315 ± 34‰) and isoleucine (δ²H= -220 ± 36‰). The range in δ²H is similar to that measured in the tryptone digest and is another example of the extensive range in δ²H of individual AAs from two completely different organisms, which spans almost the entire range of values found in surficial waters or organic bulk material in terrestrial and marine environments (16).

The relative distribution of δ²H in the various AAs was largely upheld in the cultures grown in deuterium-enriched media waters (Fig. 1): glycine had the lowest δ²H values and proline the most positive. In all of the experiments, the intrinsic hydrogen δ²H values were always more negative than that of the water in the culture medium. The contribution of glucose-derived hydrogen atoms to *E. coli* AAs relative to the contribution of hydrogen from H₂O can be estimated using the slope of the relationship between the δ²H of AA hydrogen and the δ²H of water. Because we do not know the isotope fractionations associated with hydrogen incorporation from glucose or from H₂O exactly, a model was constructed using fractionation factors for water (α₇) and food (αₑ) estimated from the literature (11,17) (Table 2; Supplemental Information). For example, the model shows that
36–53% of the hydrogen in glutamic acid originates from glucose, while the remainder was incorporated from water. For proline, the model shows that 16-38% originated from glucose, with the remainder derived from water (Fig. 2A and B, Fig. 4). The mean proportion (±SD) of nonessential AA hydrogen originated from glucose varied from 41±14% to 56±11%, which was similar to the amount of essential AA hydrogen originating from glucose (33±11% to 50±8%).

Pairs of AA that are related biosynthetically provide further information on hydrogen metabolism. For example, alanine and aspartate are closely related through steps that connect the glycolytic pathway with the tricarboxylic acid cycle (TCA cycle). Their δ²H values are nearly identical in all treatments, suggesting a common hydrogen pool available for synthesis. Conversely, nonessential proline, which is synthesized from glutamate, is always more enriched than its parent glutamate by X-Y‰. Likewise, aspartate is the first amino acid on the pathway to isoleucine synthesis; there is a sizeable (yet variable) isotopic fractionation between these two AAs.

**E. coli grown with Tryptone as Sole Organic Hydrogen Source:** Hydrogen sources available to the *E. coli* in these experiments included tryptone (-65‰) and water (variable, -55‰ to +1070‰). The δ²H of intrinsic hydrogen from individual AA from these cells ranged from +26‰ (proline) to -312 ‰ (isoleucine) in cultures grown on lab-distilled water (n=4 separate cultures) (Fig. S1). These values can be compared to those AAs in the proteinaceous “food” source tryptone. Proline in the tryptone digest had a δ²H of -64‰ compared to a δ²H of +26‰ in *E. coli*, demonstrating net positive isotope fractionation between proline in the diet and microbial biosynthesis by *E. coli* including the incorporation of hydrogen from water and other organic intermediates during metabolic
processing. Approximately half of the amino acids have $\delta^2$H values more positive than their counterparts in tryptone, with the remainder slightly more negative than those in tryptone. At the other end of the spectrum, isoleucine ($\delta^2$H = -294‰) in tryptone and in E. coli, ($\delta^2$H = -312‰) indicates total routing of hydrogen with little influence of fractionation during hydrogen exchange or tissue biosynthesis.

Using a similar model as explained above, our calculations show that isoleucine has the lowest percentage of 3.3 to 4.4% of hydrogen incorporation from media H$_2$O, which makes sense because this amino acid is one of the most complex AA and the majority of its hydrogen is in the intrinsic form (15) (Table S2, Fig. 3, Fig. 4). Other AAs that show low hydrogen incorporation from media water include leucine (7.1–9.5%) and valine (7.7–10.3%). Interestingly, these two AAs – like isoleucine – are also branched chain amino acids whose de novo biosynthesis requires catabolism of other amino acids, and whose direct incorporation from media would be energetically favorable.

Alanine, unlike isoleucine, has a very simple structure and can be synthesized from pyruvate created either during glycolysis or from oxaloacetate in the TCA cycle. Alanine has the highest proportion (39.4–52.9%) of hydrogen derived from media H$_2$O. Nonessential amino acids in general had greater proportions of hydrogen originating from water (20±11% to 27±15%), whereas the more complicated essential amino acids contained significantly less hydrogen from water (7.2±3% to 9.6±4%). Lastly, the influence and proportion of hydrogen atoms from water in these experiments was considerably less than when E. coli was cultured on simple glucose and was required to synthesize all of its amino acids. This demonstrates at the molecular level that hydrogen atoms can be routed directly
from the specific molecules in the diet and thus move unaltered up food chains into higher organisms.

**DISCUSSION**

Our δ²H analyses of AAs from heterotrophic microbes hold information related to biosynthetic pathways and nutritional status. Our laboratory experiments with *E. coli* demonstrate that a majority of the hydrogen intrinsic to the molecules (i.e., carbon bonded or N-linked) is derived from the hydrogen in organic dietary substances. Not only have we shown this at the cellular level, but we have also demonstrated that at the molecular level, hydrogen in certain AAs is incorporated directly into protein biomass. This observation provides a novel explanation as to why and how much organic hydrogen derived from diet directly influences animal tissues commonly analyzed in the study of animal ecology to characterize movement/migration patterns and trace the flow of energy within and among ecosystems (5,9,16).

*Glucose Metabolism and Amino Acid Synthesis:* Some AA, especially nonessential ones like alanine, serine, and glycine show strong evidence for large contributions of H from water during *de novo* synthesis with glucose as the sole carbon source. Glucose is transported into *E. coli* by the phosphotranferase system, in which carbon-bonded hydrogen and hydroxyl hydrogen atoms should enter relatively intact (17). Water molecules are brought into *E. coli* via aquaporins, which are enzymes that transport molecular H₂O into and out of cells (18). In the strain of *E. coli* that we used, both transport mechanisms were active.
During the first reactions of glycolysis, in which glucose is phosphorylated and converted to fructose, the exiting hydrogen atom is a hydroxyl hydrogen, the conversion of glucose to fructose goes to completion, so no measurable fractionation should occur. It is not until the glyceraldehyde-3 phosphate dehydrogenase step, that hydrogen atoms are removed from glucose to form NADH + H⁺. Note that the hydrogen coming from glucose is not lost or exchanged, but transferred efficiently to one of the primary activated carrier molecules for general cellular biosynthesis.

The second major alteration of hydrogen atoms during glycolysis occurs when a H₂O molecule is removed during the phosphoglycerate enolase step to form phosphoenolpyruvic acid (PEP). During the final step of glycolysis, pyruvate kinase adds one hydrogen atom to a non-exchangeable position on the methyl carbon of pyruvate. During transamination, a carbon-bonded hydrogen atom is added to the methylene carbon, in addition to the NH₃⁺ group. Our method for amino acid derivatization preserves one hydrogen from the amine group, which is theoretically exchangeable, and all other C-bonded hydrogen. Of the six hydrogen atoms that we analyze by our method, only two (33%) are the original hydrogen atoms from glucose; two come from transamination, and the remaining third from H₂O. Our results for both the glucose and tryptone treatments are roughly consistent with this model: alanine, which should be the most direct amino acid coming from glycolysis, has 41-56% of its hydrogen originating from water in glucose cultures and 39-53% in tryptone cultures.

Glycine and serine are synthesized from 3-phosphoglycerate. Two hydrogen atoms (50%) are added during transamination from glycerate to serine, which has a total of four intrinsic hydrogen atoms, two of which should originate from glucose. Glycine, if
synthesized from serine, should have only one hydrogen atom from the original glucose out of a total of three. In our glucose treatment, we measured a 43-58% hydrogen contribution from H₂O in serine, and 31-41% in glycine.

Aspartate, which is synthesized by transamination of oxaloacetate, has two carbon-bonded hydrogen atoms that should originate from H₂O catalyzed by fumarase during repeated TCA cycling. The remaining two hydrogen atoms enter the molecule during transamination. In our glucose treatment, 39-52% of hydrogen in aspartate originated from water, similar to the 50% predicted. Glutamate is composed of two hydrogen atoms from acetyl-CoA (66% from glucose; 33% from H₂O), two from the TCA cycle (H₂O), and two from transamination. For glutamate from glucose grown E. coli, our results show 47–64% of the hydrogen originate from H₂O, which also overlaps with theoretical predictions.

Isotope fractionation of hydrogen include many different components, none of which are completely constrained: synthesis reactions, routing, and exchange reactions that may or may not be in equilibrium. An example is the hydrogen atoms added during transamination, which should technically be hydrogen atoms derived from H₂O, but instead may be influenced by a different hydrogen pool. Hydrogen atoms in NH₄⁺/NH₃ should be fully exchanged with media water. Once in the cytosol of E. coli, however, the δ²H of these N-H atoms might be re-equilibrated with metabolic H₂O derived from diet (i.e., glucose).

Another source of hydrogen from glucose metabolism is the formation of NADH and NADPH from NAD⁺ and NADP⁺ with the added hydrogen atoms coming directly from glucose. By the time glucose enters the TCA cycle as acetyl CoA only three of the original twelve hydrogen atoms remain. In the TCA cycle, an additional eight hydrogen atoms are cycled into NADH, NADPH, and FADH₂, such that by the time one full TCA cycle has been
completed, none of the original hydrogen atoms are attached to any of the TCA intermediates. The hydrogen atoms in NADPH carry some of the original hydrogen from glucose and are key donors of hydrogen in many biosynthetic reactions.

Enzymatic Hydrogen Fractionation and Tunneling: Hydrogen isotope effects in enzyme reactions have traditionally been determined using $^2$H-substituted reactants and measuring rate constants. Many enzymatic reactions, however, manifest quantum effects in biological electron transfer. L-$\alpha$-amino acid transferase reactions, for example, occur by the ping pong bi bi mechanism (21) in which two substrates are transformed into two different reactants (bi bi) and the substrate-enzyme complex and the activated enzyme complex take place in two distinct steps (ping pong). These enzymes are highly conserved in eubacteria, yeast, birds, and mammals and play central roles in catalysis and biosynthesis. Biochemists have learned about these enzymes by creating mutants, particularly with amino acid substitutions at the active site. Based on what is known (22), the hydrogen in the $\alpha$-carbon position has a 50:50 chance of coming from intracellular water or pyridoxamine phosphate. The pyridoxamine phosphate hydrogen transferred to a newly formed AA has a 50:50 chance of originating from the amino acid reactant or pre-formed pyridoxal phosphate. We predict, then, that as AAs are metabolized via aminotransferase reactions the hydrogen in them should ultimately originate from organically bonded hydrogen.

Some biological reactions exhibit non-classical isotope effects for hydrogen, implying that hydrogen atoms can tunnel through energy barriers, particularly if the bond distance between the reactants is close enough (<2.8 Å) to permit hydrogen to overcome tunneling barriers (23). These experiments have been conducted with deuterated substrates in vitro in a growing, yet limited number of enzymes. Tunneling at the natural
abundance level has not been readily measured, although Zhang et al. (24) found evidence of hydrogen tunneling in lipids produced by microbial cultures grown on acetate or succinate. Hydrogen-leaving reactions in the tricarboxylic acid cycle (e.g., succinate dehydrogenase) could produce NADPH that is substantially $^2\text{H}$-enriched. The enzyme proline dehydrogenase, which catalyzes the interconversion from proline to D1-pyrroline-5-carboxylate, has a bonding distance at the active site of 2.7 Å (25), which is close enough to allow hydrogen tunneling.

**New Isotopic Biosignature?:** Zhang et al. (24) presented $\delta^2\text{H}$ of lipids from 4 different microbes using autotrophic or alternative pathways of biosynthesis and found that the $\delta^2\text{H}$ of fatty acids vary by as much as 300‰ depending on the biosynthetic pathway and heterotrophic growth substrate, even if the microbes were cultured in media water with a constant $\delta^2\text{H}$ value. We predict that the $\delta^2\text{H}$ of AA from similar types of experiments will elucidate the different hydrogenase enzymes in microbes thriving in hydrothermal vents (e.g., 26).

Hydrogen cycling is inherently more complex than either carbon or nitrogen, because hydrogen can enter a molecule from many different points along a metabolic pathway (27) and many organisms have two distinct sources of hydrogen (water and food) available to them, whereas carbon and nitrogen originate from a single source (food). In addition, some hydrogen atoms in proteins and AAs are known to be exchangeable (28) yet available data show that not all hydrogen isotopes exchange rapidly in biological conditions (10,29). A deeper understanding of the cycling of water inside of cell membranes (30), including the mitochondria, and how this water is connected to external H$_2$O pools is needed. Compound specific hydrogen isotope analysis of AA provides a novel tool to
answer these questions, because these molecules are all synthesized in central metabolic processes.

*Isotopic Routing and Implications for Animal Ecology:* Even though the *E. coli* strain we used is theoretically able to synthesize all of its required AAs, our data show a significant amount of isotopic routing or direct incorporation of hydrogen isotopes from the diet into tissue without significant isotopic fractionation or exchange. With the exception of alanine, in which ~39–52% of the intrinsic hydrogen derived from water, those AAs considered non-essential in eukaryotes had only 10–25% of their hydrogen from media water, the remainder being derived from that particular amino acid in the tryptone. Moreover, AAs considered to be essential in eukaryotes had even less input from H2O with only 3–14% of their intrinsic hydrogen from media water. Although carbon isotope values of essential AAs in animal tissues are nearly identical to those specific AAs in their diets (31), our amino acid δ2H results document for the first time that isotopic routing can happen with hydrogen as well as carbon.

We propose that the direct transfer of many of the essential amino acid hydrogen atoms could be used as direct tracers, not only of precipitation, but also of dietary sources for animals. Thus, from one tissue sample (e.g., keratin), the isotopic composition of the drinking water can be determined by knowing the relationship between nonessential AA, like alanine, and local surficial waters (e.g., precipitation). Alternatively, the δ2H of the organic hydrogen sourced from food could be traced by understanding hydrogen fractionation patterns in essential AAs. This approach opens up new doors in ecology. Along with carbon isotopes used to trace the relative inputs of primary producers with different biosynthetic pathways (e.g., C3-C4 plant), and nitrogen isotopes commonly used to
assess trophic level, hydrogen isotopes of AAs provide a measurement to characterize location or habitat (nonessential AA $\delta^2$H) and diet source and quality (essential AA $\delta^2$H) in an individual animal.

The application of $\delta^2$H analysis in many animal movement and migration studies relies on the premise that the $\delta^2$H of precipitation is the main determinant of variation in the $\delta^2$H value of animal tissues. Presumably, precipitation $\delta^2$H is directly transferred to primary producers, then transferred up the food chain to consumers (e.g., 5,32). The assumption of direct transfer implies that there is a linear relationship between $\delta^2$H of animal tissues and precipitation with a slope of 1, and any isotopic offsets related to trophic discrimination are known. In reality, however, the relationship between the $\delta^2$H value of animal tissues and that of precipitation varies considerably (16,33). The variation among studies has been attributed to a variety of factors including differences in lab protocols, variation in the $\delta^2$H of precipitation over time and space (34,35), and inherent ecological factors such as general dietary preference (e.g., herbivore vs. carnivore). Our data show that organic hydrogen from diet can route directly into tissues, supporting earlier work showing the source of hydrogen in animal tissues to be derived from the hydrogen in organic compounds in food and pre-formed water, including free drinking water and water in food (3,5). Compound specific analysis of hydrogen in AAs has the potential to become a more direct way of evaluating the effects of diet versus precipitation for ecologists characterizing animal movement and resource use patterns.

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**Materials and Methods**

*E. coli* Cultures: *E. coli* (MG1655) was grown in defined glucose medium with MOPS (3-(N-morpholino)propanesulfonic acid) as buffer with NH₄Cl as the nitrogen source or in the complex Tryptone Salt Broth, in which dietary hydrogen is derived from the pancreatic casein digest tryptone (Bacto Tryptone, Becton Dickson). Slightly deuterated water ($\delta^2$H = 1070 ‰) was prepared by mixing 98% $^2$H₂O with distilled water ($\delta^2$H = -55 ‰). Desired media water isotopic compositions were achieved by mixing appropriate portions of these two waters (See On-line Supplementary Material).

Compound Specific $\delta^2$H in Amino Acids: Tissues (1–3 mg) were hydrolyzed in 6N HCl at 110°C for 20 hours. Tests with tryptone showed that, with the exception of glutamic and aspartic acid, hydrogen did not exchange during hydrolysis or derivatization. Amino acids were subsequently derivatized with 2-isopropanol and N-trifluoroacetic acid (N-TFA) (29), then analyzed in triplicate for $\delta^2$H after separation on a 50 m DB-5 column in a Thermo-Fisher Trace Gas Chromatograph. Separated AAs were thermally decomposed to H₂ in a
ceramic reactor set at 1400°C. The δ²H of AA were calculated from measured δ²H values on 3–5 separate analyses by mass balance with adjustments being made for hydrogen removed during derivatization. Measured δ²H values include contributions from hydrogen in the isopropanol. Mass balance calculations were based on the number of hydrogen atoms in the derivatized molecule, which includes methyl-hydrogen, aliphatic-H, 1 nitrogen-bound H, and hydrogen from isopropanol. Extensive tests were performed with solid amino acid powders to determine whether or not hydrogen atoms were exchangeable with liquid water vapor at room temperature or steam at 100°C. We used the δ²H of the native amino acid standards to calculate the δ²H and the isotopic fractionation in making the derivative, similar to the method used for determining carbon isotopic compositions of amino acids (14, 15). Because a subset of the hydrogen atoms are removed during derivatization, we needed to know whether the remaining hydrogen atoms had similar δ²H values. At room temperature, about 10% of the hydrogen was exchangeable. At 100°C, 33% of the total hydrogen was exchangeable, however, using ambient (Washington, DC) lab distilled, deionized water changed the δ²H by an average of only +5‰. Changes in δ²H of serine (29‰) and proline (18‰) were significantly greater. Interpretation of exchange experiments are complicated by many factors, most of which are not relevant for the calculations we made in this paper. Therefore, we used the δ²H of the amino acids measured directly from the bottles that were used to make up the standard mixtures. An analytical error of ±5‰ has little influence on the δ²H values we report here.

The proportion of hydrogen originating from H₂O or diet--glucose or tryptone versus single amino acid--can be determined by plotting the δ²H of medium water versus either total cellular (i.e., bulk) δ²H or specific amino acid δ²H . The slope of this line is
roughly, but not exactly, equivalent to the proportion of hydrogen derived from water with the remainder assumed to originate from organic hydrogen in the medium diet. This simple rendition ignores the fact that there are two fractionations involved for both water and dietary uptake, neither one of which is accurately known. We constructed a model using Sessions and Hayes (2005) (17) estimates for isotopic fractionation between media water and fatty acids for photoautotrophs. We determined a potential range in estimates for the proportion of hydrogen coming from media water or diet based on the $\delta^2$H individual AA from our E. coli experiments in which the $\delta^2$H of media water varied substantially (Supplemental Figures 2, 3, and 4).

**Literature Cited**


**Figure Legends**

Figure 1. $\delta^2$H of individual amino acids from glucose-grown *E. coli* cells. The amino acids classified in higher organisms as nonessential for eukaryotes are plotted on the left side of the graph; AA considered to be essential are on the right hand side. Parallel lines indicate similarities in biosynthetic hydrogen isotope fractionation.

Figure 2. (A) Modeled contribution of hydrogen from water vs. organic hydrogen source based on $\delta^2$H of individual amino acids from E. coli grown on glucose. (B) Expanded view. Isotope fractionations for water ($\alpha_W$) and food ($\alpha_F$) are estimates following the method of Session and Hayes (11), utilizing the linear regression slope associated with water- and glucose-labeling experiments.

Figure 3. Modeled contributions of hydrogen from water versus amino acids in tryptone based on the $\delta^2$H of individual amino acids from E. coli grown on tryptone. Isotope fractionations for water ($\alpha_W$) and food ($\alpha_F$) are estimates following the method of Session and Hayes (11), utilizing the linear regression slope associated with water- and tryptone-labeling experiments.

Figure 4. Calculated proportion of hydrogen originating from H$_2$O in microbes grown on either glucose or tryptone based on models presented in Figures 2 and 3.
FIGURE 1