Hydrogen isotopes in individual amino acids reflect differentiated pools of hydrogen from food and water in *Escherichia coli*

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Hydrogen isotope (δ^2H) analysis is widely used in animal ecology to study continental-scale movement because δ^2H can trace precipitation and climate. To understand the biochemical underpinnings of how hydrogen is incorporated into biomolecules, we measured the δ^2H of individual amino acids (AAs) in *Escherichia coli* cultured in glucose-based or complex tryptone-based media in waters with δ^2H values ranging from −55‰ to +1,070‰. The δ^2H values of AAs in tryptone spanned a range of ∼250‰. In *E. coli* grown on glucose, the range of δ^2H among AAs was nearly 200‰. The relative distributions of δ^2H of AAs were upheld in cultures grown in enriched waters. In *E. coli* grown on tryptone, the δ^2H of nonessential AAs varied linearly with the δ^2H of media water, whereas δ^2H of essential AAs was nearly identical to δ^2H in diet. Model calculations determined that as much as 46% of hydrogen in some nonessential AAs originated from water, whereas no more than 12% of hydrogen in essential AAs originated from water. These findings demonstrate that δ^2H can route directly at the molecular level. We conclude that the patterns and distributions in δ^2H of AAs are determined through biosynthetic reactions, suggesting that δ^2H could become a new biosignature for studying novel microbial pathways. Our results also show that δ^2H of AAs in an organism's tissues provides a dual tracer for food and environmental (e.g., drinking) water.

hydrogen isotopes | aminio acids | diet | *Escherichia coli*

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 (δ^2H) in precipitation and groundwater that intrinsically label animal tissues. Continental-scale models of precipitation δ^2H patterns, or isoscapes (1), are invaluable for these efforts; however, significant intrasite variation (10–20‰) in animal tissue δ^2H values impedes our ability to resolve movement patterns at subcontinental, regional scales. Approximately 15–30% of the hydrogen in animal tissues is derived from environmental water (2–4), whereas the remainder is sourced from food. Because of ecological and physiological factors, such as the type and variety of food in the diet as well as the geographic source of drinking water, the relationship between tissue δ^2H values and δ^2H values of local precipitation can become blurred or even nonexistent (5), even for resident species (6).

Studies of captive animals have shown that when fed diets with a constant δ^2H composition, the δ^2H of animal tissues are depleted in deuterium (^2H) relative to water consumed by ∼20–30‰ (e.g., ref. 4). The biochemical mechanism for this isotopic discrimination has yet to be described. This gap in knowledge contrasts with our more comprehensive 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 is essential to refine the use of δ^2H to characterize animal movement patterns, and to explore further the potential of δ^2H as a tracer of energy within and among ecosystems (7).

Metabolic processes that fractionate hydrogen have been described primarily in microbial and plant tissues. Principal among these studies is the body of literature on δ^2H fractionation during lipid synthesis (8–10). Fatty acids, hydrocarbons, and alkenones synthesized by plants, phytoplankton, and microbes are depleted in ^2H (i.e., have lower δ^2H values) relative to bulk tissue. Such molecules, found in geological settings such as lake sediments, are used extensively as paleoclimate proxies and can have δ^2H values that are 50–90‰ lower than environmental water (10, 11).

Because proteins comprise the bulk of animal tissues of interest to ecologists, an understanding of δ^2H fractionation in the amino acids (AAs) from which they are synthesized has the potential to offer significant insights into how animals assimilate resources and use them to build tissues. Stable isotope analyses of $\delta^{13}C$ and $\delta^{15}N$ in AAs have revealed that extensive fractionation occurs both between and among AAs (e.g., ref. 12). These fractionations are related to biochemical pathway in autotrophs and to a combination of biochemical pathway and direct incorporation in heterotrophs. Certain AAs (e.g., glutamate, alanine) can be synthesized by any eukaryotic organism (nonessential AAs), whereas others (e.g., valine and leucine) must originate from an animal's diet (essential AAs) or gastrointestinal microbiota (13). As an animal incorporates

Significance

Hydrogen isotope (δ^2H) values of bulk tissues have become valuable tracers for studying migration and movement patterns in animals, but the biochemical mechanism for how hydrogen is incorporated into heterotrophic organisms is not well understood. We grew *Escherichia coli* as a model organism on two different substrates, and then measured δ^2H values of individual amino acids (AAs) in cellular material. The δ^2H values of AAs were highly variable within a simple microbial culture. Using isotopic fractionation models, we show that AA δ^2H provide tracers of an organism's environmental (e.g., drinking) water, as well as its food, information of prime interest to ecologists. The work is also of significance to microbial physiologists studying metabolic pathways in microbes from extreme environments.

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an AA directly from its food, the isotopic composition of that AA 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 AA δ^2H. First, bacteria were grown in water of varying δ^2H composition in both glucose and protein-based media. Second, we wanted to quantify the extent to which hydrogen in AAs was directly routed from diet versus synthesized from other nonprotein sources (water and glucose). We incubated *E. coli* in the two media described above whose waters ranged in δ^2H from −55‰ to +1,070‰, which allowed us to calculate the fraction of hydrogen that originated from organic food (i.e., medium) versus media water (Fig. S1).

Results

δ^2H Values of AAs in Tryptone. To test methods for analyzing δ^2H in AAs, we hydrolyzed the tryptone protein that was used as the organic source for growing *E. coli* by three different methods: (*i*) hydrolysis in 6N hydrochloric acid (HCl) for 20 h at 110 °C, (*ii*) vapor hydrolysis with 12N HCl for 20 h at 110 °C, and (*iii*) hydrolysis with ^2H-spiked 6N HCl (+1,070‰ water plus 12N HCl) for 20 h at 110 °C. Following hydrolysis, AA mixtures were dried under N_2 at 110 °C and 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. Because the derivatization reactions are carried out without any liquid water present, it is unlikely that this hydrogen atom exchanged during the derivatization process. The δ^2H of AAs that we report represent what we refer to as "intrinsic" hydrogen: nonexchangeable hydrogen bonded to carbon in the AA and one hydrogen atom bonded to nitrogen that remains following derivatization.

The δ^2H of individual AAs in tryptone varied by >250‰, and among the three treatments, the isotopic compositions of derivatized AAs showed the same pattern for the majority of the AAs (Table S1). Exceptions for the ^2H-spiked hydrolysis treatment were aspartic and glutamic acids, in which δ^2H was more positive by >100‰ relative to the other treatments. Given the nature of the derivatized AAs, 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 and had higher δ^2H values than its counterpart in the control treatment by ∼55‰. Serine and glycine, two of the structurally simplest AAs, should be the AAs with the greatest likelihood for hydrogen exchange, because 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 12N HCl and used it to hydrolyze all of the samples analyzed in our study for 20 h at 110 °C.

The more remarkable information from these tests was the range in δ^2H of the AAs from mammalian tryptone proteins regardless of hydrolysis method. Nonessential AAs had more positive δ^2H values (−21 to −209‰), whereas essential AAs had more negative δ^2H values (−160 to −307‰). A similar pattern in isotopic fractionation has been observed in $\delta^{13}C$ values of AAs from a variety of organisms (12), 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 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‰), NH_4Cl (−124‰), and water (−55‰ to +1,070‰) (Fig. 1). In these experiments, *E. coli* needed to synthesize the full suite of AAs through biosynthetic pathways, even those AA that are considered essential for higher organisms. The range in δ^2H for

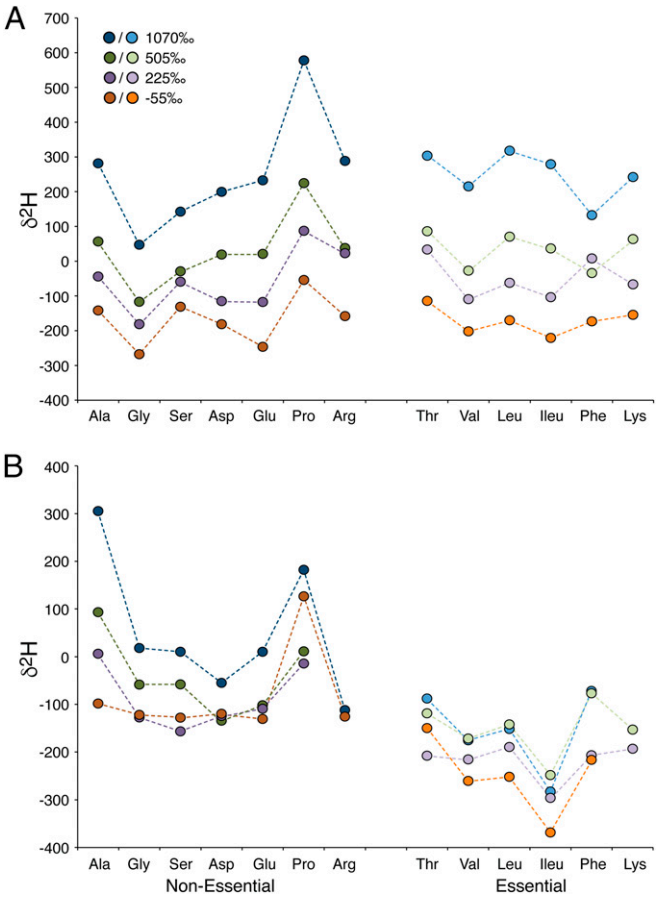

Fig. 1. (*A*) δ^2H of individual AAs from glucose-grown *E. coli* cells. (*B*) δ^2H of individual AAs from tryptone-grown cells. The AAs classified as nonessential for eukaryotes are plotted on the left side of the graph, and AAs considered to be essential are plotted on the right side. Parallel lines indicate similarities in biosynthetic δ^2H fractionation.

intrinsic hydrogen in individual AAs in cells grown in water (−55‰) on the glucose medium, which was required to synthesize all of their AAs, was almost >200‰ (Fig. 1*A* and Table S2). Cells were cultured in three independent experiments and then analyzed in triplicate. Proline was the most enriched AA (δ^2H = −54 ± 40‰), whereas the most depleted AAs were glycine (δ^2H = −268 ± 34‰) and isoleucine (δ^2H = −220 ± 36‰). The range in δ^2H is similar to the range measured in the tryptone digest, and is another example of the extensive range in δ^2H 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 (14).

The relative distribution of δ^2H in the various AAs was largely upheld in the cultures grown in ^2H-enriched media waters (Fig. 1*A*). Glycine had the lowest δ^2H values, and proline had the most positive δ^2H values. In all of the experiments, the intrinsic hydrogen δ^2H values were always more negative than the δ^2H value 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 water can be estimated using the slope of the relationship between the δ^2H of AA hydrogen and the δ^2H of water. Because we do not know the isotope fractionations associated with hydrogen incorporation from glucose or from water exactly, a model was constructed using fractionation factors for water (α_W) and food (α_F) estimated from the literature (9, 15) (Table 1). For example, the model shows that 47–64% of the hydrogen in glutamic acid originates from glucose, whereas the remainder

Table 1. Calculated percentages of hydrogen coming from H_2O based on model calculations using isotope fractionation (α) estimates with upper and lower potential α values

AA	Glucose, $\%H_{H2O}$	Tryptone, $\%H_{H2O}$
Ala	49.1 ± 7.2	46.1 ± 6.8
Gly	36.1 ± 5.3	19.8 ± 2.9
Ser	50.8 ± 7.4	17.3 ± 2.5
Pro	45.4 ± 6.6	11.6 ± 1.7
Asp	55.4 ± 8.1	18.6 ± 2.7
Glu	73.3 ± 10.7	22.2 ± 3.3
Thr	71.6 ± 10.5	12.4 ± 1.8
Val	57.0 ± 8.3	9.0 ± 1.3
Leu	48.3 ± 7.1	8.3 ± 1.2
Ileu	58.2 ± 8.5	3.8 ± 0.6

was incorporated from water. For proline, the model shows that 16–38% originated from glucose, with the remainder derived from water (Figs. 2 *A* and *B*, 3, and 4). The mean proportion (±SD) of nonessential AA hydrogen originating from glucose varied from 27 ± 11% to 64 ± 5%, which was similar to the amount of essential AA hydrogen originating from glucose (42 ± 8% to 53 ± 7%).

Pairs of AAs that are related biosynthetically provide additional information on hydrogen metabolism. For example, alanine and aspartate are closely related through steps that connect the glycolytic pathway with the tricarboxylic acid (TCA) cycle (Fig. 5). Their δ^2H 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 195–355‰. Likewise, aspartate is the first AA 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 (−55‰ to +1,070‰). The δ^2H of intrinsic hydrogen from individual AAs from these cells ranged from +126‰ (proline) to −368 ‰ (isoleucine) in cultures grown on laboratory distilled water ($n = 4$ separate cultures; Fig. 1*B* and Table S2). These values can be compared with those AAs in the proteinaceous "food" source tryptone. Proline in the tryptone digest had a δ^2H value of −77‰ compared with a δ^2H value of +126‰ 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. Four of the AAs have δ^2H values more positive than their counterparts in tryptone, with the remainder more negative than their counterparts in tryptone. At the other end of the spectrum, low but similar δ^2H values in isoleucine ($\delta^2H = -294‰$) in tryptone and in *E. coli* ($\delta^2H = -346‰$) indicate isotopic 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 9.4 ± 1.4% of hydrogen incorporation from media water (Table 1), which makes sense because this AA is one of the most complex AAs and the majority of its hydrogen is in the intrinsic form (Figs. 3 and 4 and Table 1). Other AAs that show low hydrogen incorporation from media water include leucine (12.1 ± 1.8%) and valine (7.9 ± 1.2%). Like isoleucine, these two AAs are also branched chain AAs whose de novo biosynthesis requires catabolism of other AAs, and whose direct incorporation from media would be energetically favorable.

Alanine, unlike isoleucine, has a very simple structure and can be synthesized from pyruvate during glycolysis. Alanine has the highest proportion (46 ± 7%) of hydrogen derived from media water. Nonessential AAs generally had greater proportions of hydrogen originating from water (20 ± 14%), whereas the more complicated essential AAs contained significantly less hydrogen from water (8.7 ± 3.4%). Finally, the influence and proportion of hydrogen atoms from water in these experiments were considerably less than when *E. coli* was cultured on simple glucose and was required to synthesize all of its AAs. This finding 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 δ^2H analyses of AAs from heterotrophic microbes hold information related to biosynthetic pathways and nutritional status. Our experiments with *E. coli* demonstrate that a majority of the hydrogen intrinsic to AAs (carbon-bonded or *N*-linked) is derived from the hydrogen in organic dietary substances. Not only have we shown dietary hydrogen incorporation 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 an explanation as to why and how much organic hydrogen derived from diet directly influences animal tissues commonly analyzed by ecologists to characterize animal movement and trace the flow of energy within and among ecosystems (1, 7, 16).

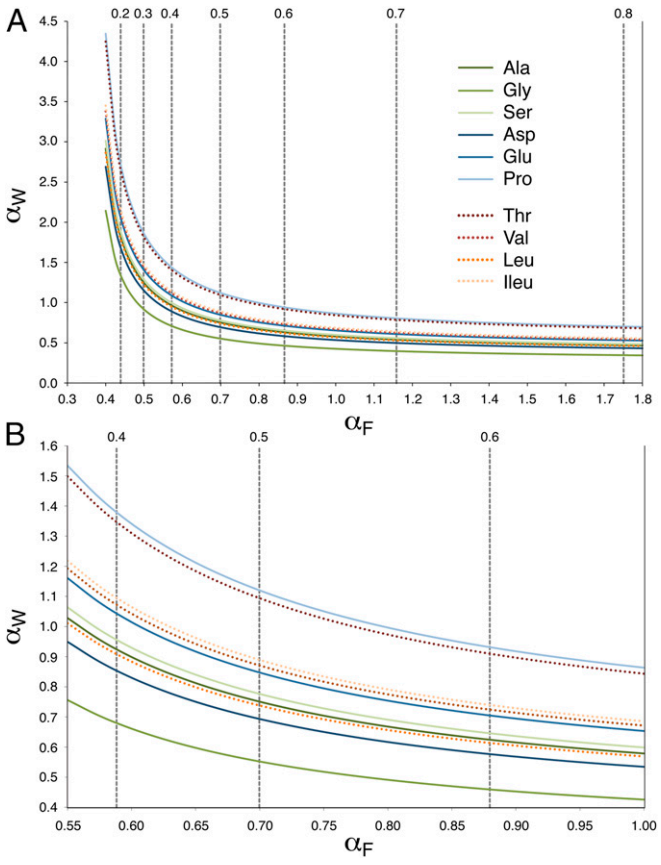

Fig. 2. (*A*) Modeled contribution of hydrogen from water versus organic hydrogen source based on δ^2H of individual AAs from *E. coli* grown on glucose. (*B*) Expanded view. Isotope fractionations for water (α_W) and food (α_F) are estimates following the method of Session and Hayes (15), using the linear regression slope associated with water- and glucose-labeling experiments.

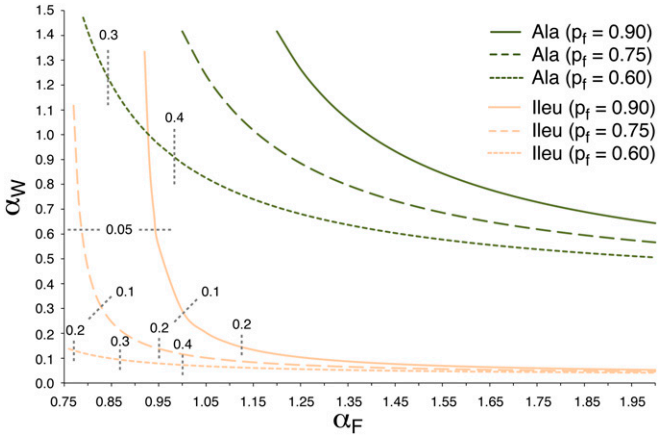

Fig. 3. Calculated %H (f_w) originating from water in microbes grown on either glucose or tryptone. Dashed lines represent the values of f_w that correspond to that point on the curve at α_F or α_W. Estimates of α_W and α_F follow the method of Session and Hayes (15), using the linear regression slope associated with water- and tryptone-labeling experiments.

Glucose Metabolism and AA Synthesis. Some AAs, especially nonessential ones like alanine, serine, and glycine, show strong evidence for large contributions of hydrogen from water during de novo synthesis with glucose as the sole carbon source. Glucose is transported into *E. coli* by the phosphotransferase 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 water 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 isotopic 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 to one of the primary activated carrier molecules for general cellular biosynthesis.

The second major alteration of hydrogen atoms during glycolysis occurs when a H_2O molecule is removed during the phosphoglycerate enolase step to form phosphoenolpyruvic acid. During the final step of glycolysis, pyruvate kinase adds one hydrogen atom to a nonexchangeable position on the methyl carbon of pyruvate. During transamination, a carbon-bonded hydrogen atom is added to the methylene carbon, in addition to the NH_3^+ group. Our method for AA 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, only two (33%) are the original hydrogen atoms from glucose: Two come from transamination, and the remaining four come from water. Our results for both the glucose and tryptone treatments are roughly consistent with this model: Alanine, which should be the most direct AA 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 water in serine and a 31–41% contribution in glycine.

Aspartate, which is synthesized by transamination of oxaloacetate, has two carbon-bonded hydrogen atoms that should originate from water 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 and 33% from H_2O), two from the TCA cycle (H_2O), and two from transamination. For glutamate from *E. coli* grown on glucose, 47–64% of the hydrogen originates from water, which also overlaps with theoretical predictions.

Isotope fractionations 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 water but, instead, may be influenced by a different hydrogen pool. Hydrogen atoms in NH_4^+/NH_3 should be in isotopic equilibrium with media water. Once in the cytosol of *E. coli*, however, the δ^2H of these N-H atoms might be reequilibrated with metabolic water 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. Glucose-6-phosphate dehydrogenase, for example, is one of the primary reactions forming NADPH in bacteria (19). The hydrogen transferred to $NADP^+$ comes from glucose. By the time glucose enters the TCA cycle as acetyl-CoA, only three of the original 12 hydrogen atoms remain. In the TCA cycle, an additional eight hydrogen atoms are cycled into NADH, NADPH, and $FADH_2$, 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. δ^2H effects in enzyme reactions have traditionally been determined using ^2H-substituted reactants and measuring rate constants. Many enzymatic reactions, however, manifest quantum effects in biological electron transfer. For example, L-α-AA transferase reactions occur by the ping pong bi bi mechanism (20) in which two substrates (e.g., aspartate, glutamate) are transformed into two different reactants (bi bi; e.g., oxaloacetate, α-ketoglutarate) and the substrate–enzyme complex and the activated enzyme complex take place in two distinct steps (ping pong). These enzymes

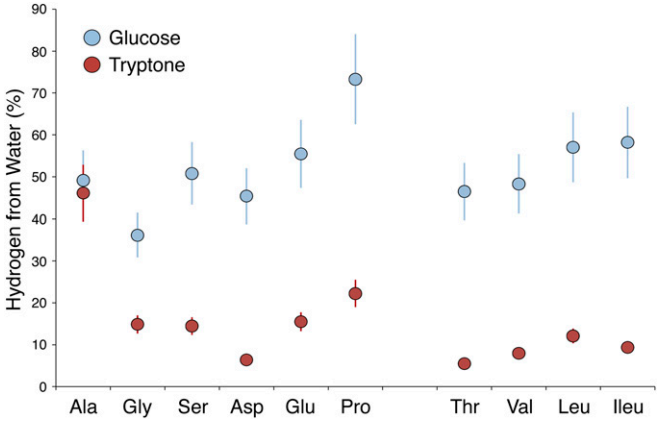

Fig. 4. Calculated proportion of hydrogen originating from water in microbes grown on either glucose or tryptone based on models presented in Figs. 2 and 3.

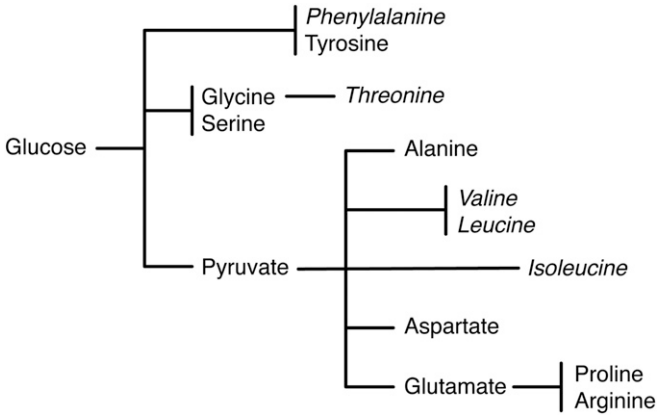

Fig. 5. Conceptual dendrogram of AA relationships. AAs in italics are considered to be essential AAs in eukaryotes. The length of the horizontal lines indicates the relative complexity of steps in the AA biosynthetic pathway.

are highly conserved in eubacteria, yeast, birds, and mammals, and they play central roles in catalysis and biosynthesis. Biochemists have learned about these enzymes by creating mutants, particularly with AA substitutions at the active site. Based on what is known (21), the hydrogen in the α-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 AA reactant or preformed pyridoxal phosphate. We predict then that because AAs are metabolized via aminotransferase reactions, the hydrogen in them should ultimately originate from organically bonded hydrogen.

Some biological reactions exhibit nonclassical 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 (22). 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. (23) found possible evidence of hydrogen tunneling in lipids produced by microbial cultures grown on acetate or succinate. Reactions that transfer hydrogen to $NADP^+$ in the TCA cycle (e.g., succinate dehydrogenase) could produce NADPH that is substantially ^{2}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 (24), which is close enough to allow hydrogen tunneling.

New Isotopic Biosignature? Zhang et al. (23) presented δ^2Hs of lipids from four different microbes using autotrophic or alternative pathways of biosynthesis and found that the δ^2H values 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 δ^2H value. Chemoautotrophic microbes can derive energy from the oxidation of H_2 to protons and electrons; for example, deep-sea microbes have unique sets of hydrogenase enzymes (25). We predict that the δ^2H of AAs from organisms with different hydrogenase enzymes will be useful in elucidating which hydrogenase activities are supporting microbial growth in extreme environments.

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 (26) 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, a minor portion (~10–15%) of hydrogen atoms in proteins and AAs are known to be exchangeable (27), yet available data show that not all δ^2H exchange rapidly in biological conditions (8, 28). A deeper understanding of the cycling of water inside cell membranes (29), including mitochondria, and how this water is connected to external sources of water is needed. Compound-specific δ^2H analysis of AA provides a 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 (30), our data show a significant amount of isotopic routing or direct incorporation of δ^2H from dietary protein (tryptone) into tissue without significant isotopic fractionation or exchange. With the exception of alanine, in which ~39–52% of the intrinsic hydrogen is derived from water, those AAs considered nonessential in eukaryotes had only 6–22% of their hydrogen from media water, with the remainder being derived from that particular AA in the tryptone. Moreover, AAs considered to be essential in eukaryotes had only 5–12% of their intrinsic hydrogen from media water. Although δ^{13}C values of essential AAs in animal tissues are nearly identical to those specific AAs in their diets (e.g., ref. 31), our AA δ^2H results document, for the first time to our knowledge, that isotopic routing occurs with hydrogen as well as carbon.

We propose that the direct transfer of many of the essential AA hydrogen atoms could be used as direct tracers, not only of precipitation but also of dietary sources for animals. Thus, from one tissue sample, the isotopic composition of the drinking water can be determined by knowing the relationship between a nonessential AA (e.g., alanine) and local surficial waters (e.g., precipitation). Alternatively, the δ^2H of the organic hydrogen sourced from food could be traced by understanding hydrogen fractionation patterns in essential AAs (e.g., isoleucine). Our compound-specific approach provides a powerful tool to characterize both diet and water use via analysis of individual AAs in a single tissue. Along with δ^{13}C used to trace the relative inputs of primary producers with different biosynthetic pathways and δ^{15}N commonly used to assess trophic level, δ^2H of AAs provide a measurement with which to characterize location or habitat (nonessential AA δ^2H) and diet source and quality (essential AA δ^2H) in an individual animal.

The application of δ^2H analysis in many animal movement and migration studies relies on the premise that the δ^2H of precipitation is the main determinant of variation in the δ^2H value of animal tissues. Presumably, precipitation δ^2H is directly transferred to primary producers and then transferred up the food chain to consumers (e.g., refs. 1, 32). The assumption of direct transfer implies that there is a linear relationship between the δ^2H of animal tissues and precipitation with a slope of 1, and that any isotopic offsets related to trophic discrimination are known. In reality, however, the relationship between the δ^2H of animal tissues and the δ^2H of precipitation varies considerably (16). This variation has been attributed to a variety of factors, including differences in laboratory protocols; variation in the δ^2H of precipitation over time and space; and inherent ecological factors, such as general dietary preference (e.g., herbivore versus carnivore). Our data show that organic hydrogen from diet can route directly into tissues, supporting earlier work showing that the majority of hydrogen in animal tissues is derived from the hydrogen in organic compounds in food, with a minor component derived from preformed water, including environmental (e.g., drinking) water and water in food (3–5). δ^2H analysis of individual 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.

Materials and Methods

E. coli (MG1655) was grown in defined glucose medium with 3-(*N*-morpholino) propanesulfonic acid as a buffer and with NH_4Cl 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). The most deuterated water we used (δ^2H: +1,070 ‰) was prepared by mixing 98% ^2H_2O with distilled water (δ^2H: −55 ‰). δ^2H values of other media water treatments (δ^2H: +225 ‰, +505 ‰, and +1,070‰) were achieved by mixing appropriate portions of these two waters (*SI Materials and Methods*).

Tissues (1–3 mg) were hydrolyzed in 6N HCl at 110 °C for 20 h. Tests with tryptone showed that, with the exception of glutamic and aspartic acid, hydrogen did not exchange during hydrolysis or derivatization. AAs were subsequently derivatized with 2-isopropanol and *N*-TFA (31), and then analyzed in triplicate for δ^2H after separation on a 50-m DB-5 column (SGE Analytical Science) in a Thermo-Fisher Trace Gas Chromatograph. Separated AAs were thermally decomposed to H_2 in a ceramic reactor set at 1,400 °C. The δ^2H values of AA were calculated from measured δ^2H values on three to five separate analyses by mass balance, with adjustments being made for hydrogen removed during derivatization. Measured δ^2H 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, one nitrogen-bound H, and hydrogen from isopropanol. Extensive tests were performed with solid AA 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 δ^2H of the native AA standards to calculate the δ^2H values and the isotopic fractionation in making the derivative, similar to the method used for determining $\delta^{13}C$ values of AAs (12, 13). Because a subset of the hydrogen atoms is removed during derivatization, we needed to know whether the remaining hydrogen atoms had similar δ^2H values. At room temperature, about 10% of the hydrogen was exchangeable. At 100 °C, 33% of the total hydrogen was exchangeable; however, using ambient laboratory distilled, deionized water increased the δ^2H value by an average of only 5‰. Changes in δ^2H of serine (29‰) and proline (18‰) were significantly greater. Interpretation of exchange experiments is complicated by many factors, most of which are not relevant for the calculations we made in this study. Therefore, we used the δ^2H values of the AAs measured directly from the bottles that were used to make up the standard mixtures. An analytical error of ±5‰ has little influence on the range of δ^2H values we report here.

The proportion of hydrogen originating from water or diet (glucose or tryptone) for individual AAs can be determined by plotting the δ^2H of medium water versus either total cellular (bulk) δ^2H or specific AA δ^2H. 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 estimates for isotopic fractionation between media water and fatty acids for photoautotrophs (15).

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