Published in final edited form as:

Rapid Commun Mass Spectrom. 2011 December 15; 25(23): 3555–3562. doi:10.1002/rcm.5263.

Mammalian DNA $\delta^{15}\text{N}$ exhibits 40‰ intramolecular variation and is unresponsive to dietary protein level

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Abstract

We report the first high precision characterization of molecular and intramolecular $\delta^{15}\text{N}$ of nucleosides derived from mammalian DNA. The influence of dietary protein level on brain amino acids and deoxyribonucleosides was determined to investigate whether high protein turnover would alter amino acid ^{15}N or ^{13}C . Pregnant guinea pig dams were fed control diets, or high or low levels of dietary protein throughout gestation, and all pups were fed control diets. Cerebellar DNA of offspring was extracted at 2 and 120 days of life, nucleosides isolated and $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ characterized. Mean diet $\delta^{15}\text{N} = 0.45 \pm 0.33\text{‰}$, compared to cerebellar whole tissue and DNA $\delta^{15}\text{N} = +4.1 \pm 0.7\text{‰}$ and $-4.5 \pm 0.4\text{‰}$, respectively. Cerebellar deoxythymidine (dT), deoxycytidine (dC), deoxyadenosine (dA), and deoxyguanosine (dG) $\delta^{15}\text{N}$ were $+1.4 \pm 0.4$, -2.1 ± 0.9 , -7.2 ± 0.3 , and $-10.4 \pm 0.5\text{‰}$, respectively. There were no changes in amino acid or deoxyribonucleoside $\delta^{15}\text{N}$ due to dietary protein level. Using known metabolic relationships, we developed equations to calculate the intramolecular $\delta^{15}\text{N}$ originating from aspartate (asp) in purines (pur) or pyrimidines (pyr), glutamine (glu), and glycine (gly) to be $\delta^{15}\text{N}_{\text{ASP-PUR}}$, $\delta^{15}\text{N}_{\text{ASP-PYR}}$, $\delta^{15}\text{N}_{\text{GLN}}$, and $\delta^{15}\text{N}_{\text{GLY}}$ $+11.9 \pm 2.3\text{‰}$, $+7.0 \pm 2.0\text{‰}$, $-9.1 \pm 2.4\text{‰}$, and $-31.8 \pm 8.9\text{‰}$, respectively. A subset of twelve amino acids from food and brain had mean $\delta^{15}\text{N}$ of $4.3 \pm 3.2\text{‰}$ and $13.8 \pm 3.1\text{‰}$, respectively, and $\delta^{15}\text{N}$ for gly and asp were $12.6 \pm 2.2\text{‰}$ and $15.2 \pm 0.8\text{‰}$, respectively. A separate isotope tracer study detected no significant turnover of cerebellar DNA in the first six months of life. The large negative $\delta^{15}\text{N}$ difference between gly and cerebellar purine N at the gly (7) position implies either that there is a major isotope effect during DNA synthesis, or that *in utero* gly has a different isotope ratio during rapid growth and metabolism than in adult life. Our data show that cerebellar nucleoside intramolecular $\delta^{15}\text{N}$ vary over more than 40‰ and are not influenced by dietary protein level or age.

Introduction

Natural abundance stable isotopes are widely used to probe the source and chemical reactions involved in the synthesis and degradation of a sample. They are commonly used in

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food web studies to establish the trophic position of consumers because the heavier isotope is usually enriched from diet to consumer, especially for N ^[1]. The molecular mechanisms that induce an increase in isotope ratios are not well understood.

When incorporated into long-lived molecular pools, stable isotopes provide a record of past events. Isotope ratios at the compound-specific or position-specific levels of analysis provide more sensitive and specific information about the sample than do analyses of bulk material ^[2] containing many hundreds of different compounds each with their own unique isotopic signatures. Natural isotopic variability has been shown at the molecular level in lipid synthesis ^[3], protein and amino acid metabolism ^[4–6], and photosynthesis ^[7], among others. These studies have revealed significant differences in ¹³C and ¹⁵N isotopic composition, due to metabolic reactions, of various cellular fractions. For instance, low ¹³C of lipids, first observed in plants ^[8] and microorganisms ^[4], was found to be due to the oxidation of pyruvate to acetyl CoA prior to lipid synthesis ^[3].

Natural C isotope fluctuations are better studied than N. Gaebler and colleagues ^[5] reported ¹⁵N data that showed dispensable (nonessential) amino acids to be more enriched in ¹⁵N than indispensable (essential) amino acids isolated from rat liver proteins, and when compared to the dietary amino acids. Macko and colleagues ^[6] carried out enzymatic experiments *in vitro* that revealed isotope effects during reversible transamination reactions that transfer NH₂ groups from glutamate to oxaloacetate to form aspartate and α-ketoglutarate. In either direction, the transfer of ¹⁴NH₂ was favored over ¹⁵NH₂. More recently, Sacks and Brenna ^[9] demonstrated that the peptide-N is depleted in ¹⁵N relative to the side chain-N in several commercially available amino acids. Additionally, new data show that N isotopic composition of some amino acids can be used for estimating trophic position of animals ^[10].

Remarkably little is known about the isotopic composition of nucleic acids. With the exception of one report on δ¹³C values of human DNA ^[11], only a handful of studies have reported both δ¹⁵N and δ¹³C of nucleic acids isolated from soil samples ^[12], microorganisms ^[13], and plants ^[14]. There is only one study of human DNA, in which the DNA ¹³C/¹²C ratios among American-born Americans (A), European-born Europeans (E), and European-born Americans (I) were compared ^[11]. ¹³C content of the E and I groups was similar with δ¹³C of –20.3‰ and –19.6‰, respectively, while ¹³C in the A group at δ¹³C of –16.1‰ was enriched relative to the E and I groups. These data demonstrate that cerebellar DNA does not turnover throughout the human lifespan and that δ¹³C largely reflect those of the maternal environment during gestation^[11], a result that has recently been confirmed and extended by ¹⁴C measurements ^[15, 16]. Observational studies showing near negligible neuronal DNA turnover have never been replicated under experimental conditions.

The *fetal origins of adult disease* hypothesis holds that *in utero* conditions predispose a fetus to chronic diseases in adult life ^[17]. The hypothesis implies that inherently environmental conditions distinct from the genome influence phenotype via a variety of mechanisms, such as reduced nephron number leading to later hypertension ^[18]. While tools for evaluating genetic variation are routine, long term biomarkers of the fetal environment are rare. Isotope ratios in long-lived molecules of long-lived cells, such as neuronal DNA, are candidate

biomarkers because they are known to be responsive to environmental factors in other contexts, and available data suggest that they are retained in the long term.

We report on two experiments designed to explore DNA C and N isotope ratio behavior in developing animals. We investigated the natural abundance ^{15}N and ^{13}C isotope ratios for mammalian cerebellar genomic DNA and deoxyribonucleosides hydrolyzed from cerebellar DNA in guinea pigs consuming diets defined isotopically at the bulk level. In a separate tracer study using enriched $^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$ diets, we investigated the relative turnover of cerebellar tissue and cerebellar DNA over the first months of life. We also explored whether dietary protein levels influence ^{15}N isotope ratios using a fetal origins experimental model, designed to determine whether metabolic cycling of N due to varying dietary protein levels is reflected in $\delta^{15}\text{N}$ of DNA.

Materials/Methods

Animals and Diets

Tracer Experiment—Females were fed control diets. From days 18 to 39 of gestation when the fetal guinea pig brain is growing rapidly [19], diets were supplemented with algal (*Spirolina*) biomass artificially enriched at the level of $\delta^{15}\text{N} = 1300\text{‰}$ and $\delta^{13}\text{C} = 115\text{‰}$ (Cambridge Isotope Laboratories, Andover, MA, USA). At 5, 45, 90, and 180 d of postnatal life, pups (n=3) were sacrificed and cerebellar tissue collected.

Dietary Protein Experiment—One-month-old female and nine-month-old male Hartley guinea pigs were purchased from Elm Hill Breeding Labs (Chelmsford, MA, USA). Females were randomly divided into three treatment groups (group size, 6) immediately upon arrival: control (CN, 25% protein), low protein (LP, 15% protein) and high protein (HP, 60% protein). Defined experimental diets obtained from Research Diets (New Brunswick, NJ; Diet numbers, CN: D04052004, LP: D04052003; HP: D04052005) were fed *ad libitum*. Our measurements showed that the three diets averaged $\delta^{15}\text{N} = 0.45 \pm 0.33\text{‰}$ and were not significantly different. The means for $\delta^{13}\text{C}$ were -17.6 ± 0.2 , -19.1 ± 0.3 , and -23.2 ± 0.1 for the LP, CN, and HP diets, respectively, and all were significantly different. Soy (C3 plant) was used as the protein source, and cornstarch (C4) was the carbohydrate source, and these varied reciprocally in the diets leading to the relative ^{13}C depletion in the high protein diet.

Guinea pigs were bred one month after their arrival and continued consuming their respective diets throughout gestation (68 d). All dams were switched to CN diet two days after parturition. At postnatal day 2, half of the pups from each litter were euthanized and tissues collected; all remaining pups were weaned to the CN diet, euthanized at 120 d of age, and tissues were collected, frozen, and stored at -80°C until analyses.

Sample Preparation for Isotopic Analysis

Ten mg of frozen cerebellar tissue were dehydrated in a Savant SpeedVac Concentrator (Thermo Fisher Scientific; Waltham, MA, USA) for 6 to 8 h, and then stored at room temperature until isotope ratio mass spectrometry (IRMS) analysis. Representative portions of each diet (CN, LP, HP) were separately ground into a fine powder and stored at room temperature until IRMS analysis.

DNA Isolation and Hydrolysis

Genomic DNA was isolated from cerebellar tissue using Blood and Cell Culture DNA Midi kits (Qiagen; Valencia, CA, USA) according to the manufacturer's recommendations with minor modifications. Tissue samples (100 mg) were thawed on ice, manually homogenized (Wheaton Science Products; Millville, NJ) and then incubated at 50°C overnight in the manufacturer's digestion buffer plus 250 µL proteinase K (20 mg/mL; Qiagen) and 19 µL RNase A (100 mg/mL; Qiagen). DNA was precipitated using room temperature isopropanol and pelleted by centrifugation at $6100 \times g$ for 20 min at 4°C. The DNA pellets were washed twice with 2 mL cold (−20°C) 70% (v/v) ethanol and centrifuged at $6100 \times g$ for 15 min at 4°C. DNA was dissolved in nuclease-free water (Ambion; Austin, TX, USA) at an estimated concentration of $2 \mu\text{g } \mu\text{L}^{-1}$. The DNA purity and quantity was determined by spectrophotometry (Model DU730, Beckman Coulter, Inc.; Fullerton, CA, USA). DNA with an A_{260}/A_{280} ratio between 1.7 and 1.9 was considered pure. Dissolved DNA was stored at −20°C until further analysis. A total of two to four DNA preps were done per animal, depending upon the amount of cerebellar tissue available. Intact genomic DNA pellets were allowed to completely air dry at room temperature and stored at room temperature until IRMS analysis.

DNA hydrolysis was carried out as previously described [20, 21] to yield deoxyribonucleosides. Briefly, fresh hydrolysis buffer (pH 5.3; 50 mM sodium acetate; 0.2 mM zinc chloride; 0.2 mM erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA); Sigma-Aldrich; St. Louis, MO, USA) was added in equal volume to DNA dissolved in nuclease-free water. EHNA was added to the buffer to prevent the formation of deoxyinosine from dA via adenosine deaminase [21]. Enzymatic DNA hydrolysis was carried out by the addition of nuclease P1 ($0.1 \mu\text{g } \mu\text{L}^{-1}$; Sigma-Aldrich) and calf intestine alkaline phosphatase ($0.01 \text{ unit } \mu\text{L}^{-1}$; Roche Diagnostics; Indianapolis, IN, USA). Samples were incubated for 4.5 hr at 50°C and then transferred to Microcon YM-10 filters (Millipore; Bedford, MA, USA) and centrifuged at $14,000 \times g$ for 20 min at 23°C. Within-animal duplicate DNA preps were pooled after centrifugation. Hydrolyzed DNA samples were stored at −20°C until preparative high performance liquid chromatography (HPLC).

Preparative HPLC for deoxyribonucleosides

A Summit HPLC system (Dionex Corporation; Sunnyvale, CA, USA) with UV/VIS detector was used to purify deoxyadenosine (dA), deoxyguanosine (dG), deoxythymidine (dT), and deoxycytidine (dC) from hydrolyzed DNA. Samples were thawed and 90–125 µL were injected at a time. A Discovery C18 HPLC column (25 cm $\times$ 4.6 mm, 5 µm; Supelco; Bellefonte, PA, USA) was eluted at a rate of 0.8 mL min^{-1} using a water-methanol gradient [21]. Fractions were manually collected in 4 mL glass vials (Wheaton; Millville, NJ, USA) and care was taken to ensure that every nucleoside peak was collected in total. Isotope ratios have been shown to vary across HPLC peaks in a manner similar to that familiar in GCC-IRMS, thus quantitative peak collection is a critical factor to maintain accurate isotope ratio determinations in downstream IRMS [22]. Fractions were stored at room temperature until further processing. Deoxyribonucleoside standards (dA, dG, dC, and dT; Sigma-Aldrich) were injected initially to determine nucleoside retention times and then periodically thereafter to verify the times.

HPLC fractions were evaporated under a gentle stream of N₂ until reduced to approximately 200 µL and then in 3.5 × 9 mm tin capsules (Costech; Valencia, CA, USA). Minimal IRMS sample size requirements were determined in preliminary experiments, and required pooling of nucleoside fractions from two samples at the evaporation step. Therefore, every nucleoside sample destined for IRMS was a pooled sample from a total of four DNA preps, including the pooling following enzymatic hydrolysis prior to HPLC analysis as discussed above. Nucleoside fractions were pooled from pups of the same dietary treatment and time point.

The tin capsules containing nucleoside fractions were dried in an oven at 60°C for 2 h and weighed. Dehydrated intact genomic DNA, dehydrated bulk cerebellar tissue (~500 µg), and ground diet samples were weighed (~500 µg) and stored at room temperature in a desiccator until analysis.

C and N Stable Isotope Analyses

An elemental analyzer (EA)-IRMS system consisting of an AS200 autosampler, a Carlo Erba NC2500 elemental analyzer, and a Finnigan MAT Delta Plus isotope ratio mass spectrometer was used for all isotopic analyses. The EA and isotope ratio mass spectrometer were coupled via a Conflo II open split, and IRMS data were acquired and processed with ISODAT version 2.0 software (Thermo Fisher, Bremen, Germany). Natural abundance stable isotope ratios are expressed using the δ notation, with units of per mil (‰), using the following equation:

$$\delta^n X = (R_{\text{Spl}} - R_{\text{Ref}}) / R_{\text{Ref}} \times 1000$$

where n = the atomic mass of the minor isotope, X = the element of interest, and $R_X = [H/L]$, with H = heavy isotope and L = light isotope. The isotope ratio of the sample is expressed relative to the isotope ratio of a reference material; atmospheric N₂ is the reference for N and Vienna Pee Dee Belemnite is the reference for carbon.

Amino Acid analysis

One sample of feed from each of the three diets, and one sample of brain tissue from the 120 d samples were processed for amino acid analysis according to our routine protocol [23]. Briefly samples were hydrolyzed with 12 N HCl and derivatized with N-pivaloyl/isopropyl esters, and analyzed with a gas chromatography-combustion isotope ratio mass spectrometer (GCC-IRMS) consisting of an Agilent Technologies (Santa Clara, CA, USA) 6890N gas chromatograph interfaced to a ThermoFinnigan Delta plus XP. $\delta^{15}\text{N}$ are expressed against $\delta^{15}\text{N}$ for N₂ in air, and a mixture of eight amino acids of known $\delta^{15}\text{N}$ is routinely run every few samples for quality control purposes. Analytical precision is standard deviation (SD) ($\delta^{15}\text{N}$) < 0.5‰.

Statistical Analyses

Statistical analyses were performed using JMP, version 7.0 (SAS Institute; Cary, NC, USA). Results are expressed as means ± SD unless noted otherwise. One way analysis of variance

(ANOVA) was used to test treatment effects in the C and N delta values in the diets, bulk cerebellum tissue, DNA, deoxyribonucleosides, and the intramolecular isotope positions. One way ANOVA was also used to test differences among the six different types of samples analyzed in the study. Tukey's test was used as the post-hoc test for all ANOVA analyses. Student's *t* test was used to test the main effect of age in C and N delta values in each of the six sample types. Comparisons among $\delta^{15}\text{N}$ of amino acids were performed with Tukey's HSD test. The level of significance for all tests was set at $P < 0.05$.

Results

Tracer Experiment, DNA Turnover

Figure 1 shows the $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ for whole cerebellum and liver, and for DNA derived from these tissues, over the pup's first six months of life. Consumption of labeled biomass by the dams during the pup's brain growth spurt resulted in labeling of tissue and DNA. Both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ decline in whole tissues, reaching baseline by three months (Figure 1A–C). Figure 1A demonstrates that the rapidly dividing and regenerating liver cell DNA is replaced at nearly the same rate as for whole liver tissue. In contrast, Figures 1B and 1C show that linear least squares slopes of cerebellar DNA $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ are modestly negative and are not significantly different than zero, indicating that DNA of cerebellum tissue does not detectably lose label over six months.

Dietary Protein Experiment, Cerebellar Tissue and DNA isotopes

All the following results pertain to the dietary protein experiment. Figure 2 presents analyses of $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ at 2 and 120 d of age for cerebellar tissue and DNA relative to the dietary levels. $\delta^{15}\text{N}$ is presented in terms of absolute numbers because ^{15}N did not vary significantly among the three diets, while ^{13}C is presented to adjust for the differential dietary ^{13}C levels. There were no significant differences due to dietary protein level at either age, thus $\delta^{15}\text{N}$ data were pooled within treatments at each time point. At 2 d of age, cerebellar tissue was enriched in ^{15}N relative to the diet and had a $\delta^{15}\text{N} = +4\text{‰}$, while cerebellar DNA was depleted in ^{15}N relative to diet with a by about $\delta^{15}\text{N} = -4\text{‰}$, to yield a difference of $^{15}\text{N} = +8\text{‰}$. There was a small but significant effect of age on the $\delta^{15}\text{N}$ of cerebellar tissue ($P < 0.0001$) and DNA ($P < 0.01$), yielding a significant increase to $^{15}\text{N} = +10\text{‰}$ at 120 d.

Figure 2 also shows that cerebellar tissue $\delta^{13}\text{C}$ was very similar to that of the diet (that is, ^{13}C near 0‰), although there was a 2‰ gradient from LP to HP at 2 days of life. Relative to tissue, cerebellar DNA was enriched in ^{13}C by +2–4‰, and no differences were detected among the diet groups at either time point. By 120 d of age, cerebellar tissue ^{13}C differences were significant and a reverse trend was observed, but much attenuated so that only the LP and HP groups were different by about $\delta^{13}\text{C} = 0.5\text{‰}$. Cerebellar DNA maintained a $\delta^{13}\text{C} = +4\text{‰}$, which is enriched relative to bulk cerebellar tissue, and was also significantly enriched by about $\delta^{13}\text{C} = +2\text{‰}$ compared to the 2 d cerebellar DNA time point.

Deoxyribonucleoside IRMS Analyses

There were no significant differences in $\delta^{15}\text{N}$ among the deoxyribonucleosides due to treatment or age. Figure 3 presents pooled $\delta^{15}\text{N}$ means and SDs for the deoxyribonucleosides, bulk cerebellar tissue, DNA, and the diet. As expected, the experimentally determined $\delta^{15}\text{N}$ of DNA was a mean of the four deoxyribonucleoside $\delta^{15}\text{N}$. There were highly significant differences in $\delta^{15}\text{N}$ among the four nucleosides, with $\delta^{15}\text{N}_{\text{dT}} > \delta^{15}\text{N}_{\text{dC}} > \delta^{15}\text{N}_{\text{DNA}} > \delta^{15}\text{N}_{\text{dA}} > \delta^{15}\text{N}_{\text{dG}}$, with an average $\delta^{15}\text{N}$ of 1.40‰, -2.10‰, -4.47‰, -7.18‰, -10.4‰, respectively ($P < 0.0001$). All nucleosides were depleted in ^{15}N relative to cerebellar whole tissue, and all except dT were depleted in ^{15}N compared to the diet (Figure 3).

There were also significant differences among the mean ^{13}C values of the four deoxyribonucleosides (Table 1). All deoxynucleosides were enriched in ^{13}C compared to the diet, and the order of most ^{13}C enriched deoxynucleoside to least enriched was different than that for ^{15}N enrichment.

Calculation of Amino Acid-Specific $\delta^{15}\text{N}$ Values in Purine and Pyrimidine Bases

All N atoms in DNA bases are derived from one of three dispensable amino acids: aspartate (asp = D), glutamine (gln = Q), or glycine (gly = G), as shown in figure 4. We assume that base positions derived from the same amino acids have the same $\delta^{15}\text{N}$, and can then write four mass balance equations for base $\delta^{15}\text{N}$ in terms of the three amino acid source $\delta^{15}\text{N}$. Asp donates its N atoms to pyrimidines and purines in different ways. The pyrimidines incorporate the entire asp molecules, one N and three C, losing only H in the process. In contrast, asp donates only N to the purines: two to adenine and one to guanine. The difference in these N incorporation mechanisms are a likely source for different isotope effects, and thus ratios for the N atoms in the pyrimidines and purines. We therefore treated asp-derived N as having different isotope ratios, and can write four mass balance equations with four unknowns, as follows:

$$\delta_{\text{THY}} = \frac{1}{2}\delta_{\text{gln}} + \frac{1}{2}\delta_{\text{asp(pyr)}} \quad (1)$$

$$\delta_{\text{CYT}} = \frac{2}{3}\delta_{\text{gln}} + \frac{1}{3}\delta_{\text{asp(pyr)}} \quad (2)$$

$$\delta_{\text{ADE}} = \frac{2}{5}\delta_{\text{gln}} + \frac{2}{5}\delta_{\text{asp(pur)}} + \frac{1}{5}\delta_{\text{gly}} \quad (3)$$

$$\delta_{\text{GUA}} = \frac{3}{5}\delta_{\text{gln}} + \frac{1}{5}\delta_{\text{asp(pur)}} + \frac{1}{5}\delta_{\text{gly}} \quad (4)$$

where THY is thymine, CYT is cytosine, ADE is adenine, GUA is guanine, PUR is purine, and PYR is pyrimidine.

Intramolecular N calculations for equations 1 through 4 were completed for every individual sample analyzed by IRMS at the deoxyribonucleoside level. Errors therefore propagate through the means. There were no treatment or age effects on any of the intramolecular $\delta^{15}\text{N}$ values. The calculated values within each intramolecular N position were then pooled to test for differences among the intramolecular $\delta^{15}\text{N}$ values. As shown in the right panel of Figure 4, all intramolecular $\delta^{15}\text{N}$ values were highly significantly different ($P < 0.0001$), with mean values for $\delta^{15}\text{N}_{\text{ASP-PYR}}$, $\delta^{15}\text{N}_{\text{ASP-PUR}}$, $\delta^{15}\text{N}_{\text{GLN}}$, and $\delta^{15}\text{N}_{\text{GLY}}$ of $+11.9 \pm 2.3\text{‰}$, $+7.0 \pm 2.0\text{‰}$, $-9.1 \pm 2.4\text{‰}$, and $-31.8 \pm 8.9\text{‰}$, respectively.

Food and Brain Amino acid $\delta^{15}\text{N}$

Results for $\delta^{15}\text{N}$ analyses of food and a subset of three brain samples are presented in Figure 5 for ten amino acids derived from 12 residues. Food amino acids ranged from $\delta^{15}\text{N} = -0.7 \pm 1.3\text{‰}$ for ser to $9.1 \pm 1.2\text{‰}$ for pro, with an average $\delta^{15}\text{N} = 4.3 \pm 3.2\text{‰}$ and many differences are significant. There were many fewer significant differences among brain amino acids, which averaged $+13.8 \pm 3.1\text{‰}$, and ranged from $\delta^{15}\text{N} = -7.8 \pm 1.7\text{‰}$ to $18.0 \pm 0.3\text{‰}$ for ser and pro, respectively. Brain amino acids were substantially enriched in ^{15}N compared to diet for all but phe. The average enrichment between food and brain, ^{15}N , for the nine other amino acids is $8.7 \pm 4.6\text{‰}$ with phe ^{15}N not significantly different. The two amino acids that contribute alpha amino N to DNA, gly and asx, had $\delta^{15}\text{N}$ of $2.5 \pm 0.4\text{‰}$ and $5.2 \pm 0.7\text{‰}$ in food, respectively, and in brain had $\delta^{15}\text{N} = 12.6 \pm 2.2\text{‰}$ and $15.2 \pm 0.8\text{‰}$, respectively. The amide N of gln, metabolically incorporated into DNA, is lost during acid hydrolysis and thus was not measured.

Discussion

Our tracer experiment is the first *experimental* demonstration that mammalian cerebellar DNA that acquires isotopic label *in utero* retains that label through at least six months of life, while tissue rapidly loses isotopic label. Cerebellar tissue consists primarily of neurons that synthesize DNA during mitosis associated with the prenatal brain growth spurt of the guinea pig. The turnover of label in whole cerebellar tissue is expected based on the known turnover over most cellular components. The stability of label within cerebellar DNA is consistent with previous observational reports showing that human neuronal DNA turnover is undetectable after infancy, determined in postmortem studies of $^{13}\text{C}/^{12}\text{C}$ [11] or ^{14}C incorporated from the $^{14}\text{CO}_2$ appearing in the atmosphere from above ground nuclear testing in the 1940–1960 era [16]. Turnover of liver DNA was found, as expected, because of regular mitosis of hepatocytes.

The dietary protein experiment explores changes in natural abundance and shows that cerebellar whole DNA is significantly depleted in ^{15}N but enriched in ^{13}C relative to bulk cerebellar tissue. At the compound-specific level, $^{15}\text{N}/^{14}\text{N}$ ratios varied significantly among deoxyribonucleosides, with purines depleted in ^{15}N relative to pyrimidines. At sacrifice, ^{15}N of amino acids was about 10‰ enriched compared to feed, and there were relatively few differences among amino acids.

The intramolecular $\delta^{15}\text{N}$ for the position that originates with glycine was extraordinarily low, at $-31.8 \pm 8.9\text{‰}$. $\delta^{15}\text{N}$ for food gly was $4.3 \pm 3.2\text{‰}$, and this apparently increased to

12.6±2.2‰ in brain gly, so that the differences between cerebellar DNA gly and food or brain gly were a startling 36.1 and 44.4‰, respectively. Isotope effects of this magnitude always involve primary isotope effects, involving bond-making or breaking, at the fractionated atom, and require a branch point where reactants and products of differing isotope ratio can exit. The entry of gly into purine biosynthesis occurs via condensation at the carboxyl with 5-phospho-D-ribosylamine, mediated by phosphoribosylamine-glycine ligase. The relevant N is two atoms remote from the reaction center and thus this step is unlikely to be involved. The next step, mediated 10-formyltetrahydrofolate:5'-phosphoribosylglycinamide N-formyltransferase, catalyzes transfer of a formyl group from 10-formyltetrahydrofolate to formylate the N. There is, however, no obvious branch point in the pathway for several more steps. Fractionation might also take place during degradation. Purines are catabolized and excreted as uric acid, and there are no reactions involving the relevant N apart from conversion of a double bond to a single bond in the last step, an oxidation from xanthine to uric acid. The origin of the ^{15}N depletion between food gly and DNA is unlikely to be via synthesis or degradation of purines. We can speculate that compartmentally isolated gly may have entered intermediary metabolism isotopically fractionated.

The well known increase in $\delta^{15}\text{N}$ found with trophic level long ago [24] is not characterized on a molecular level. Our data showing a lack of an effect of dietary protein level on tissue or DNA $\delta^{15}\text{N}$ is surprising, considering that much more of the protein in the high protein diet would be deaminated and used for energy compared to the low protein diet, which was near the lower level required to maintain guinea pig reproduction. Based on these data, metabolic transformations that enrich ^{15}N appear to be largely independent of cellular respiration.

The range for the purine intramolecular $\delta^{15}\text{N}$ value equaling nearly 40‰ is the largest difference in intramolecular $\delta^{15}\text{N}$ values in any biologically relevant molecule reported to date. In the purine bases, N atoms donated by glu are light (−9.1‰) while those donated from aspartate are significantly heavier (7.0‰). The differences between the $\delta^{15}\text{N}$ values for the positions derived from asp in pyrimidine and purine molecules is 5‰. During purine synthesis, aspartate donates its N atom for incorporation into the adenine and guanine rings. This transfer is catalyzed via SAICAR synthetase. Asp also donates its alpha NH_2 group to inosine monophosphate to form adenine monophosphate, and this reaction is catalyzed by adenylosuccinate synthetase. The straightforward transfer of the relatively small molecule would be expected to be associated with normal kinetic isotope effects. In contrast, the N from asp found in pyrimidine bases arises from the initial reaction of asp with carbamoyl phosphate at the asp N, mediated by aspartate transcarbamoylase. This reaction, also involving reaction at the relevant N, should be less sensitive to kinetic isotope effects because it involves two large molecules, and because there are no branch points in the pathway. As a parent donor of N, tissue asx with $\delta^{15}\text{N}$ of 15.2±0.8‰ yielded a pyridine N value of +11.9‰ and purine N of +7.0‰, consistent with the expected influences on isotopic reaction rates.

A striking trend to emerge from the $\delta^{15}\text{N}$ analysis of feed and brain is the mean increase of about 10‰, as contrasted with the reverse finding for ^{13}C . The molecular origin of these observations cannot be discerned from our data.

Tissue levels of ^{13}C were close to dietary $\delta^{13}\text{C}$ and varied with dietary protein, in contrast to DNA which was elevated by about 3‰ compared to diet and was not sensitive to dietary protein. At two days, the effect on tissue was more pronounced than at 120 d and the trends were reversed at the two ages. For deoxyribosides, five C originate with the deoxyribose group, which in principle should have the same ^{13}C value for all, and thus diluting out any changing isotope ratios from the five or six C atoms contributed by the base. More sensitive analysis of variability in physiological $\delta^{13}\text{C}$ would require removal of the deoxyribose with analysis of the bases alone. However, the base C atoms are donated by six different sources, specifically amino acids, CO_2 , bicarbonate, and methyl groups from the one C pool, and with four bases and 6 sources, calculation of intramolecular isotope ratios from $\delta^{13}\text{C}$ of bases would be underdetermined. Analysis of intramolecular isotope ratios requires a different strategy, possibly involving pyrolysis methods as we have applied previously to several classes of compounds [25–27].

The resistance of nucleic acid $\delta^{15}\text{N}$ to metabolic perturbation due to dietary protein levels differences is striking. The $\delta^{15}\text{N}$ of bulk DNA, individual deoxyribonucleosides, as well as position-specific N atoms in deoxyribonucleosides, were all stable against influence due to extreme levels of dietary protein (i.e., 60% kcal from protein vs. 15% kcal from protein). These results are surprising given the well-known ^{15}N enrichment of animals of +3–5‰ compared to their food. Urea is depleted in ^{15}N but feces is enriched in ^{15}N , leaving the mechanism for body $\delta^{15}\text{N}$ in question and an area of active research [28]. N in the urine is primarily carried by urea [29], thus, conditions that increase urea production and protein turnover, such as high dietary protein intake, might be expected to influence *in vivo* ^{15}N . Considerable study is required to clarify physiological factors that control $\delta^{15}\text{N}$ at the bulk, molecular, and intramolecular levels.

A recent report indicates that the $\delta^2\text{H}$ value of bacterial lipids is a long term molecular isotopic record of central metabolic pathways [30]. Our previous report showed that the intramolecular $\delta^{15}\text{N}$ of lysine is very sensitive to growth in an O_2 rich, or depleted, environment in the facultative anaerobe *Paracoccus denitrificans* [31]. These and related studies hint at the richness of metabolic record available in the molecular and intramolecular isotope metabolomes.

Acknowledgments

This work was supported by NIH grants RR031264 and GM49209. YT and NO are supported by grants from Japan Society for the Promotion of Science.

Abbreviations

dA	deoxyadenosine
dG	deoxyguanosine

dC	deoxycytidine
dT	deoxythymidine

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Figure 1.

Loss of label from liver and cerebellum with postnatal age. (A) $\delta^{15}\text{N}$ and (B) $\delta^{13}\text{C}$ rapidly decline to a plateau in liver whole tissue and in liver DNA. Lines are drawn freehand as a visual aid. In cerebellar tissue (C) $\delta^{15}\text{N}$ and (D) $\delta^{13}\text{C}$ drop with a pattern similar to liver, but is retained in cerebellar DNA (slope of least squares fits are **not** significantly different from zero).

Figure 2.

Cerebellar whole tissue and DNA $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ at 2 and 120 days of life in experiment 2. ^{15}N in cerebellar whole tissue is enriched compared to diet, while DNA ^{15}N is depleted relative to diet, and there are no significant differences among groups at either time point.

^{13}C is calculated by difference of samples to the respective in utero dietary $\delta^{13}\text{C}$; ^{13}C of tissue significantly increases with dietary protein at 2 days but the differences are attenuated at 120 days. LP: low protein; HP: high protein; CN: control. See text for details.

Figure 3.

$\delta^{15}\text{N}$ of cerebellar tissue, DNA, and deoxyribonucleosides. $\delta^{15}\text{N}$ were determined in 2 day and 120 day old guinea pigs ($n=16-28$); mean $\pm$ SD, all samples are significantly different from one another ($p<0.05$). The mean $\delta^{15}\text{N}$ for bulk DNA is near the mean of $\delta^{15}\text{N}$ for dG, dA, dC and dT, providing a check for isotopic integrity of the method.

Figure 4.

The amino acid origins of N for the four bases of mammalian DNA (left), and calculated position-specific $\delta^{15}\text{N}$.

Figure 5.

$\delta^{15}\text{N}$ of selected amino acids in food and in brain, arranged from lowest to highest $\delta^{15}\text{N}$ in food. The mean increase from food to brain is almost +10‰, and all amino acid $\delta^{15}\text{N}$ increase except phe. There are no discernable trends with respect to indispensable (leu, ile, val, phe) compared to non-indispensable amino acids.

Table 1

Mean and SD of ^{13}C ($= \delta^{13}C(\text{food } \delta^{13}C) - \delta^{13}C(\text{dX})$) for deoxyribonucleosides among all animals. All means are significantly different from one another by t-tests of adjacent means ($p < 0.05$, two tailed test).

	^{13}C (Mean $\pm$ SD)
dA	$5.8 \pm 1.2\text{‰}$
dG	$4.4 \pm 2.1\text{‰}$
dT	$2.9 \pm 1.5\text{‰}$
dC	$1.3 \pm 1.7\text{‰}$