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Changes in the natural abundance of $^{13}\text{CO}_2/^{12}\text{CO}_2$ in breath due to lipopolysacchride-induced acute phase response

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Abstract

The natural abundance of carbon-13 in blood proteins increases during the cachectic state and may be a biomarker for disease status. We hypothesized a corresponding drop in the relative abundance of ^{13}C in breath CO_2 . Using the lipopolysacchride (LPS)-induced endotoxemia model of the acute cachectic state, we demonstrated that the acute phase response causes shifts in the stable isotopes of carbon in exhaled CO_2 ($^{13}\text{CO}_2/^{12}\text{CO}_2$ delta value) shortly after administration of LPS while glucocorticoid treatment does not. Mice were injected with LPS and stable isotopes of blood amino acids and carbon in exhaled CO_2 were monitored. An increase in the relative isotopic mass of serum alanine, proline and threonine was observed at 3 h after LPS injection. Breath delta values began dropping immediately after administration of LPS, and were 4–5 delta values lower than those of the control animals by 2.5 h after injection. A corresponding drop in delta value was not observed with dexamethasone treatment. Thus protein synthesis during the acute phase response probably caused the fractionation of stable isotopes observed in the plasma amino acids and in exhaled breath $^{13}\text{CO}_2$ delta values. The exhaled breath $^{13}\text{CO}_2$ delta value may be a valuable real-time biomarker of cachexia associated with an acute phase response due to endotoxemia.

Acute systemic injection of lipopolysacchride (LPS) results in the release of inflammatory cytokines (TNF α and IL-1) within the first hour of exposure. In turn, increased plasma TNF α and IL-1 result in rapid changes in whole body metabolism that includes the mobilization of amino acids from muscle tissue (net muscle protein breakdown was increased by 28% with no effect on protein synthesis¹). Amino acids mobilized from tissues are used for the synthesis of acute phase proteins (1000-fold increase in serum amyloid proteins^{2–4}) or metabolized, with carbon ultimately expired as CO_2 in breath. Carbon-12 and carbon-13 are naturally abundant isotopes in breath. The relative ratio of $^{13}\text{C}/^{12}\text{C}$ has been shown to be dependent on diet as well as bodily substrates burned for fuel.^{5–9} The relative abundance of

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carbon isotopes in diet and stored tissues is not equally distributed, and the flow of this carbon through enzymatic reactions is not uniform (termed fractionation).¹⁰ Fractionation of carbon isotopes in breath during an inflammatory event was the basis for the hypothesis tested in the experiments described below.

Stable isotopes of carbon have been used to monitor nutrient metabolism during exercise,¹¹ and starvation.¹² We previously showed enrichment of ^{13}C in hemoglobin during chronic dietary restriction.¹² Diet restriction for a period of 3 weeks produced a marked stunting of growth in chicks and produced significant weight loss in adult hens. Hemoglobin, a protein critical for survival, from restricted chickens was found to become naturally enriched with ^{13}C compared with *ad libitum* fed chickens. This observation implies that fractionation (discrimination of the ^{13}C versus the ^{12}C isotopes) occurs in amino acids used to build hemoglobin during a catabolic state. Recent advances in measuring the natural relative abundance of $^{13}\text{C}/^{12}\text{C}$ in breath CO_2 now permit the passive monitoring of carbon isotope fractionation in real time.¹³

This study focuses on the fate of the stable isotopes of carbon in breath and blood plasma during the endotoxin-induced acute phase response. Since amino acids containing the heavy carbon isotope (^{13}C) are synthesized into acute phase proteins during the endotoxin-induced catabolic state,¹² we hypothesized that amino acids containing the light carbon isotope (^{12}C) would be preferentially metabolized and ultimately expelled in breath as CO_2 . For fractionation to occur, the synthesis of new proteins (e.g. acute phase proteins) must occur, whereas in catabolic states with no new protein synthesis (such as is found with acute glucocorticoid treatment), fractionation of carbon would not be observed; hence, the relative abundance of breath $^{13}\text{C}/^{12}\text{C}$ would not change.

EXPERIMENTAL

Animals and sample collection procedures

All animal experiments were approved by the University of Wisconsin-Madison, College of Agricultural and Life Sciences and/or the College of Letters and Sciences Animal Care and Use Committees (Madison, WI, USA). Sexually mature male ND4 mice were purchased from Harlan (Indianapolis, IN, USA).

Experiment 1 (1 mg/kg LPS treatment)

All animals were given *ad libitum* access to water and standard mouse chow and were housed on corn cob bedding. Mice were allowed to adjust to the bedding for 24 h prior to initiation of the injection protocol. Assays were conducted serially each day with one mouse in the metabolic chamber at a time. There were four saline-injected and five LPS-injected mice. At the time designated zero, the mice were injected intraperitoneally with 1 mg/kg body weight *E. coli* 055:B5 LPS (Sigma-Aldrich Corp., St. Louis, MO, USA) in sterile saline solution or saline alone. Metabolic chamber measurements were taken once every 15 min for 1 h before injection and 6 h after injection.

Experiment 2 (5 mg/kg LPS or DEX)

All mice were given *ad libitum* access to mouse chow and water and were housed on aspen chip bedding. Assays were conducted serially as in experiment 1. At the time designated zero, the mice were injected with 5 mg/kg dexamethasone (DEX) (n = 3), 5 mg/kg LPS (n = 3), or sterile saline (n = 3). Metabolic chamber measurements were taken once every 15 min for 24 h before and after injection.

Metabolic chamber

During sampling, animals were placed individually in a metabolic chamber and air was drawn through the chamber at a rate to maintain 1% CO₂ concentration (0.04% atmospheric CO₂ and 0.96% expired CO₂) in the exhaust gas. Exhaust air was sampled approximately every 15 min for isotopic analysis using an isotopic CO₂ cavity ring-down spectrometer (CRDS, Picarro, Sunnyvale, CA, USA). The ¹³C delta value was calculated using the following formula with Pee Dee Belemnite (PDB) as a standard. Data were expressed as delta values or parts per mil (‰):

$$\delta = \frac{\left(\frac{^{13}\text{C}}{^{12}\text{C}} \text{ sample} - \frac{^{13}\text{C}}{^{12}\text{C}} \text{ PDB} \right)}{\frac{^{13}\text{C}}{^{12}\text{C}} \text{ PDB}} \times 1000 \quad (1)$$

The metabolic chamber was made of a standard shoebox cage fitted with a sealed Plexiglas lid. The intake and exhaust manifolds were made of stainless steel tubing with ¼" Swage-loc™ fittings. A diaphragm air pump and a Brooks Instruments 5850 mass flow controller (Brooks Instruments, Hatfield, PA, USA) were used to maintain airflow.

Mass spectrometry for amino acid analysis

Blood was collected from mice with heparinized capillary tubes via retro-orbital bleeding immediately before, and at 3 and 24 h after LPS or saline injection. The samples were centrifuged at 14000 *g* for 5 min, and the plasma was transferred to a new tube. One volume of ice-cold acetonitrile was added to the samples to precipitate plasma proteins and the samples were spun at 14000 *g* for 15 min. The sample supernatants were removed and analyzed by conventional liquid chromatography-mass spectrometry (LC-MS). Each sample was processed by C₁₈ reverse-phase LC utilizing a Dionex capillary LC (Dionex Corp., Sunnyvale, CA, USA) coupled to the capillary electrospray ionization source of a MicrOTOF time-of-flight mass spectrometer (Bruker Daltonics, Billerica, MA, USA), operated in positive ionization mode. The peaks (protonated molecules) for the 4 most abundant amino acids (AA) with resolvable heavy isotope peaks (AA; alanine, leucine, proline, and threonine) and AA+1 mass unit were identified and quantified using the area under the curve. The percent abundance of the heavy isotope was quantified using the following equation for each AA:

$$\text{Percent Heavy AA} = \frac{(\text{AA} + 1 \text{ mass unit})}{\text{AA}_{\text{total}}} * 100 \quad (2)$$

Statistical analysis

The breath $^{13}\text{CO}_2$ delta value data from experiment 1 were analyzed using the mixed procedure accounting for autocorrelation of repeated measures from the SAS statistical analysis software package (SAS Institute Inc., Cary, NC, USA). Amino acid data were analyzed by comparing each time point with the zero value using the Student's t-test. Body weight data were analyzed using analysis of variance (ANOVA) with least significant difference post-hoc analysis. Differences were considered significant at $p < 0.05$ unless otherwise stated.

Regression analysis

The time-series data in experiment 2 were not suitable for the mixed procedure in the SAS program due to the large number of time points being considered. Nearby points of a time series are correlated and a direct test of significance on the individual time points of a series is not advisable. In order to estimate the statistical significance of the time-series trends, we use linear regression to model the data. This approach is reliable if (a) the residual errors from different time series are of comparable value, (b) the residual vectors obtained by subtracting the linear regression line from the corresponding time series are not correlated, and (c) the terms n_i are do not have a strong autocorrelation structure. If the above criteria are met, it is reasonable to assume that the data is represented by the linear trend. Specifically, we model each time series Y_i as follows:

$$Y_i = m_i t + b_i + n_i \quad (3)$$

In this formula, m is the slope of the regression line, b is the intercept of the line, n is the noise term, and i is an index for each time series. We used this formula to perform linear regression on each time series. After obtaining the slope, and intercept, the noise term can be obtained by subtracting the regression line from the original time series. We used the results and verified that the norm of noise term n_i and the correlation structure $\langle n_i, n_j \rangle$ satisfy conditions (a) through (c). We performed ANOVA with least significant difference post-hoc analysis on the slope and intercept values.

RESULTS

Experiment 1: Measurements of amino acids in blood of LPS-treated animals

The most abundant amino acids in serum with resolvable heavy isotope peaks after LPS treatment were alanine (ALA), proline (PRO), threonine (THR), and leucine (LEU). From time zero to 3 h post LPS injection there was an enrichment of the heavy isotope of ALA (11%; $p = 0.06$), PRO (9%; $p = 0.02$), and THR (20%; $p = 0.06$) compared with a 2%, 5%, and 4% enrichment of ALA, PRO, and THR for the saline-injected mice during the same time period (Table 1). The relative abundance of ALA, PRO, and THR with heavy isotopes increased in the LPS-injected group by 12%, 5%, and 19%, respectively, compared with samples from the saline injected controls at 3 h post-injection. The relative isotopic mass of LEU tended to be reduced 3 and 24 h after injection in the saline group compared with in the

LPS-injected mice (Table 1). Hence the relative differences at 3 h for LEU are not due to an LPS-induced increase as observed with the other amino acids.

Measurements of stable isotope of carbon in exhaled CO₂

The initial delta value of breath CO₂ for healthy mice housed on aspen chips was approximately −20‰ before the mice were switched to corncob bedding. After equilibration on corncob bedding for 24 h the healthy delta value was −13‰ (data not shown). Differences in the healthy baseline delta values between aspen shavings and corncob bedding are a function of the isotopic consistency of the diet.^{5,14} After injection of 1 mg/kg of LPS, the delta value became increasingly negative (increased level of ¹²CO₂) after about 1 h and attained a minimum value of −18‰ or a significant decrease in delta value of −5‰ within 2.5 h after injection (Fig. 1). The patterns were similar in data collected from chickens (5.5‰ drop from baseline within 3 h; data not shown), and illustrate species conservation of the response. In contrast to the −5 to −5.5‰ decrease in delta value due to endotoxemia, fasted mice exhibited a decreased delta value of only −2.5‰ after feed was removed from the chamber (data not shown).

Experiment 2: Measurements of breath markers in DEX-treated animals

LPS is a catabolic inflammatory stimulus where protein degradation provides precursors for acute immune protein synthesis. To test that the observed breath fractionation is due to an immune stimulant in response to LPS, we used DEX as a control for an acute organ-specific ‘catabolic’ response (i.e. affecting protein breakdown but not synthesis^{15,16}). We examined the exhaled delta values of a 5 mg/kg DEX treatment versus a 5 mg/kg LPS treatment. As expected, animals treated with DEX and LPS lost body weight (6.1% and 9.1%, respectively) in the 24 h following injection (Fig. 2). Mice treated with DEX did not exhibit a change in delta value beyond diurnal variation (Fig. 3). Mice treated with LPS exhibited a 5% decrease in delta value as seen in experiment 1 even after accounting for diurnal variation, supporting the hypothesis that changes in delta value are due to discrimination of natural isotopes during protein synthesis.

DISCUSSION

The acute phase response (APR) is defined by a set of events initiated by an inflammatory stimulus. Animals given an infectious insult, for example lipopolysaccharide from gramnegative bacterial cell wall, quickly respond with the systemic release of tumor necrosis factor (TNFα) and interleukin-1 (IL-1).¹⁷ These cytokines have been shown to cause muscle catabolism within 20 min of the insult,^{18,19} and unpublished data. The total plasma amino acid efflux from skeletal muscle doubles following an injection of TNFα,²⁰ with alanine, proline, threonine, and leucine being the most abundant amino acids with resolvable heavy isotope peaks observed. Amino acids released from the catabolism of peripheral tissues^{21,22} become available for the synthesis of acute phase proteins^{21,23} or are used for energy metabolism and/or hepatic gluconeogenesis.²⁴ Increased hepatic gluconeogenesis during the APR²³ has been shown to provide glucose for anaerobic glycolysis in hypoxic tissues of immune-stimulated animals.^{25,26} Plasma lipids are also increased during the APR. TNFα inhibits the uptake of fat by adipose tissue through the inhibition of lipoprotein lipase and

increased adipose lipolysis.²⁷ However, lipid is underutilized as a fuel source during the APR owing to the systemic hypoxic nature of the inflammatory response.^{25,26} Hence, during the APR, the amino acids from systemic protein catabolism serve both the need for synthesis of acute phase proteins and the need for energy metabolism through direct metabolism. The conversion of an amino acid into an acute phase protein only involves the loading of tRNA while the amino acids that are oxidized for energy undergo multiple enzymatic steps before their carbon is ultimately expired as CO₂ (Fig. 4). Each step in the oxidation process of substrates that originated from amino acids offers the opportunity to discriminate against the ¹³C-laden substrates while ¹²C-containing substrates move through substrate oxidation relatively unimpeded.²⁸ The flux of the amino acids toward the synthesis of proteins versus energy metabolism provided the basis for our hypothesis: fractionation of the stable isotopes of carbon naturally present in body amino acids occurs during the acute catabolic state, and during the acute catabolic response to LPS, exhaled CO₂ in breath would become isotopically lighter than the breath of a healthy animal (discussed below).

This study is the first to demonstrate that, 3 h after an LPS injection, there is an increased relative isotopic abundance in plasma amino acids (alanine, proline and threonine) relative to their levels prior to LPS injection and relative to the levels of saline-injected controls. Asai *et al.* demonstrated that plasma concentrations of individual amino acids quickly decrease then increase in the first 6 h of the response to endotoxin with a pivotal point at 3 h after injection in rats.²⁹ Phenotypic variation due to the LPS stimulus and the timing of the 3-h sample, a potential inflection point in plasma amino acid concentration, may have led to a high degree of variability in the observed shift in amino acid isotope abundance. We did not observe an enrichment of heavy amino acids at 24 h, as expected, because plasma amino acids turnover is fast³⁰ and animals begin to recover from the 1 mg/kg LPS stimulus within 24 h of the injection.¹⁸ These data are congruent with reports¹² that wasting due to calorie restriction caused increased abundance of heavy isotopes of carbon in body proteins. Hatch *et al.*¹² reported that body weight loss due to severe diet restriction resulted in an increase of ¹³C into hemoglobin. They hypothesized that the increased ¹³C in hemoglobin was due to fractionation, based on isotopes naturally present, of the amino acids liberated during weight loss toward either fuel (light amino acids) or newly synthesized proteins (heavy amino acids).

The foundation of the hypothesis relies on the kinetic isotope effect (KIE). Amino acids are composed of the elements carbon, nitrogen, oxygen, and sulfur, which exist naturally in several isotopic states. The most prevalent heavy isotope of carbon in nature is carbon-13, which represents 1% of all carbon on the planet, while carbon-12 makes up almost 99% of the stable isotopes of carbon. Molecules containing heavy isotopes (¹³C) require higher activation energy and thus react more slowly than those containing the lighter isotopes (¹²C).^{31,32} In our hypothesis, application of the KIE *in vivo* to plasma amino acids derived from LPS-induced muscle catabolism should result in a fractionation of pooled amino acids as they are used for either protein synthesis or as a source of energy.

In our study, we examined the isotopic composition of carbon in the end product of energy production (CO₂) using the CRDS as a sensitive technology to measure ¹³CO₂/¹²CO₂ in breath. The CRDS allowed for a continuous monitoring of breath CO₂ isotopes in real time.

¹³C The isotopic CO₂ delta values in the exhaled breath of animals were monitored under different conditions such as: fed, fasted, exposed to LPS or DEX, and to saline-injected alone. The CO₂ delta value of fasted mice was 2‰ lower than that of *ad libitum* fed mice after 2 h of fasting, consistent with literature reports of fasted animals.^{5,6} The decrease in delta seen during fasting was probably due to increased ¹³C-depleted lipid utilization during fasting.^{6,33} However, during the APR to LPS, an inflammatory stimulus, animals very quickly become anorexic and catabolic. Correspondingly, the relative abundance of breath ¹³CO₂ began to decrease after LPS injection and was 5‰ lower than that of the saline-injected animals 3 to 4 h after injection. Based on previous reports, the observed change in delta value of breath, in response to acute endotoxin exposure, preceded other physiological changes, such as a drop in mean arterial pressure,^{34,35} gut hypoperfusion³⁶ or blood pO₂, pCO₂ and pH;³⁷ physiological changes which typically become evident as much as 12–24 h after exposure to lethal doses of endotoxin. Interestingly, when a catabolic anti-inflammatory stimulus, DEX, was administered, there was no corresponding drop in delta value. Although both DEX- and LPS-treated animals are hypophagic during the first hours of the response, all the weight lost by the DEX animals occurred during the first 8h post-injection (Fig. 2, and data not shown) indicating a heavy reliance on protein for energy metabolism without an increase in acute-phase protein synthesis. Furthermore, the fractionation hypothesis put forth here posits that heavy amino acids must be built into new proteins for isotopic discrimination to occur. However, if there was no increase in acute-phase protein synthesis (e.g. Dex-induced wasting), a critical step in isotope discrimination was absent and the relative abundance of carbon isotopes in breath remained unchanged. Therefore, we hypothesize that the large decrease in delta value after LPS administration is a combination of an expected drop due to anorexia (~2‰), and fractionation due to acute phase protein synthesis and discrimination against ¹³C structures for energy metabolism (~3‰).

CONCLUSIONS

We were able to detect changes in the stable isotopes of carbon in response to physiological changes in metabolism during the acute phase response. Moreover, the change signals were discernable after as little as 2 h after the insult. Our presented data and discussion strongly support the hypothesis that isotope changes in breath following acute exposure to LPS are dominated by fractionation of molecules in accordance with the kinetic isotope effect (KIE); i.e. it is not simply the result of muscle catabolism. We have demonstrated that endotoxin responses were conserved across birds and mammals. Therefore, the carbon isotopes in serum amino acids, or in exhaled CO₂, are valuable as biomarkers for the catabolic state in acute inflammatory response/infection.

These advances point to potential application in the early detection of the APR that may reduce morbidity and mortality in patients undergoing an acute systemic inflammatory response. The research community has begun to view biomarkers, such as the stable isotope ratio described here, as potential tools for discovery and therapeutic decision making on a personalized or autonomous level. Nonetheless, there are growing concerns that rising expenditures in pharmaceutical research and development may not be sustainable if sufficient gains for industry or society at large cannot be realized.³⁸ Thus, there is new

impetus for the development of methods that go beyond the mere collection of massive amounts of data and instead focus on how biomarkers, personalized medicine, and the industry will interact and create clinical solutions.

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Figure 1.

Experiment 1: Carbon delta values of expired carbon dioxide from male ND4 mice injected with lipopolysaccharide (LPS). Mice were housed on corncob bedding in a sealed metabolic chamber while fresh air was passed through the chamber to maintain CO₂ levels at 1%. Stable isotopes of carbon in CO₂ in exhaust air were measured about once every 15 min. Mice were given an injection of 1 mg/kg LPS in sterile saline, or saline only, at time designated zero. Open circles represent the mean breath ¹³CO₂ delta value of saline-injected animals and filled squares represent LPS-injected animals. Error bars represent the standard error of the mean. Asterisks represent significant treatment differences within a time period with $p < 0.05$. Data were analyzed by using the mixed procedure accounting for auto-correlation of repeated measures from the SAS statistical analysis software package.

Figure 2.

Body weight change after LPS or DEX treatment. Mice ($n = 3$ per group) were housed in a sealed metabolic chamber while fresh air was passed through the chamber to maintain CO_2 levels at 1%. Mice were given an injection of either 5 mg/kg DEX, 5 mg/kg LPS in sterile saline, or saline only, at the time designated zero. Body weight was measured at the time of injection and 24 h later. Error bars represent the standard error of the mean. Data were analyzed by ANOVA with least significant difference post-hoc analysis. Different letter superscripts indicate significant differences with $p < 0.05$.

Figure 3.

Experiment 2: Delta values of mice on aspen bedding given either lipopolysaccharide (LPS), dexamethasone (DEX), or saline injections. Mice were housed in a sealed metabolic chamber while fresh air was passed through the chamber to maintain CO₂ levels at 1%. Stable isotopes of carbon in CO₂ in exhaust air were measured once about every 15 min. Mice were given an injection of either 5 mg/kg DEX, 5 mg/kg LPS in sterile saline, or saline only, at the time designated zero. Open circles represent the mean breath ¹³CO₂ delta value of saline-injected mice, filled circles represent DEX-injected mice, and filled squares represent LPS-injected mice. Shaded boxes represent dark/night time hours. Error bars represent the standard error of the mean. ANOVA with least significant difference post-hoc analysis was performed on the slope and intercept from linear regression of the data. Different letter superscripts represent significant differences with $p < 0.05$.

Figure 4.

Conceptual model of carbon stable isotope fractionation during the acute phase response to an invading pathogen. Invading pathogens activate the innate immune response by stimulating tissue macrophage activation.^{39,40} Inflammatory cytokines, such as tumor necrosis factor (TNF), interleukin-1 (IL-1) and IL-6, are released into circulation. TNF and IL-1 induce catabolism and amino acid release from skeletal muscle,^{1,23,41} and stimulate acute phase protein synthesis in the liver.^{2,4,42} Amino acids are loaded onto tRNA for protein synthesis in one enzymatic step, or they are metabolized. Carbon from metabolized amino acids requires multiple enzymatic steps before being released as CO_2 . During each enzymatic step heavier carbon sources will react more slowly due to the kinetic isotope effect. Fractionation of the stable isotope of carbon may occur over the entire metabolic pathway, leading to heavier carbon isotopes remaining with the body and lighter isotopes being expired in breath CO_2 .

Table 1.

Percent heavy isotopic abundance of selected amino acids

isotopic abundance of selected serum amino acids									
		% heavy <i>ALA</i> [*]				% heavy <i>THR</i> [*]			
<i>LPS</i>		–	<i>p</i> ⁺	+	<i>p</i> ⁺	–	<i>p</i> ⁺	+	<i>p</i> ⁺
Time (h)	0	4.374 (0.063)	–	4.493 (0.060)	–	5.180 (0.056)	–	5.361 (0.081)	–
	3	4.455 (0.093)	0.41	4.994 (0.469)	0.06	5.445 (0.088)	0.07	6.441 (0.989)	0.06
	24	4.522 (0.152)	0.25	4.548 (0.062)	0.10	5.101 (0.103)	0.08	5.335 (0.047)	0.25
		% heavy <i>PRO</i> [*]				% heavy <i>LEU</i> [*]			
<i>LPS</i>		–	<i>p</i> ⁺	+	<i>p</i> ⁺	–	<i>p</i> ⁺	+	<i>p</i> ⁺
Time (h)	0	5.874 (0.067)	–	5.892 (0.010)	–	6.795 (0.010)	–	6.782 (0.015)	–
	3	6.123 (0.240)	0.09	6.425 (0.341)	0.02	6.448 (0.323)	0.08	6.803 (0.048)	0.34
	24	5.977 (0.155)	0.21	5.887 (0.032)	0.47	6.469 (0.290)	0.07	6.904 (0.132)	0.11

Data are expressed as the mean with standard error in parentheses.

^{*} LPS = lipopolysaccharide, ALA = alanine, LEU = leucine, PRO = proline, THR = threonine⁺ *p*-values were computed by comparing the data at each time point with the value at t = 0 using Student's *t*-test.

Percent abundance of the heavy isotope was quantified using the following equation for each amino acid (AA):

$$\text{Percent Heavy AA} = \frac{\text{AA} + 1 \text{ mass unit}}{\text{AA}_{\text{total}}} \times 100$$