Investigating the Impacts of Growth Rate on Nitrogen Isotope Values in *E.coli*

Ela Kusmierz

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Department of Earth and Environmental Sciences

Advisor: Dr. Jenan Kharbush

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Abstract

Nitrogen (N) is an essential component for the growth of all living organisms, due to the key role it plays in the synthesis of amino acids, proteins, ATP, and nucleic acids. Amino acids are one of the main N-containing compounds in cells and are the primary vehicle for N assimilation in cells, making them useful for studying metabolism and N cycling within and between organisms. The distribution of ^{15}N isotope values in amino acids is used to study trophic relationships between macro-organisms, or as tracers of organic matter processing in sediments. There is also potential for ^{15}N of amino acids to illuminate N cycling within microbial communities, but so far little is known about the intracellular cycling and allocation of N in individual microbes. In preliminary experiments, it was observed that free amino acids in cells were more enriched in ^{15}N than protein-bound amino acids. One hypothesis for why this phenomenon occurs is that there is an unidentified fractionating process associated with protein synthesis, resulting in a kinetic isotope effect where lighter isotopes are incorporated into protein. Because the rate that proteins are synthesized and recycled increases with growth rate (Lahtvee et al., 2014), we attempted to indirectly test this hypothesis by varying growth rates. In this study, we used *Escherichia coli* to investigate whether different growth rates, and in turn different rates of protein synthesis, impact the isotope values between the free amino acids and protein bound amino acids and the magnitude of isotope fractionation.

Growth rates were altered by changing the growth temperature of *E.coli*. Experiments were conducted at different temperatures, including 15°C, 22°C, and 37°C (*E. coli* optimum). Both free and protein bound amino acids samples were purified and analyzed for $\delta^{15}N$ on a GC-IRMS. Bulk samples were freeze dried and run on an elemental analyzer. We found that average N isotopic compositions of individual amino acids differed between temperature treatments. Free amino acids from 37°C cultures were the most enriched in $\delta^{15}N$ whereas those from 15°C cultures were the least enriched. This provides some evidence that growth rate may play a role in controlling the isotope values between free and protein-bound amino acids. There is potential evidence that the magnitude of fractionation is greater at 37°C than at 15°C. Additional work for the future could investigate the effect of growth rate on amino acid $\delta^{15}N$ values in different microbial groups or uncover the specific mechanism during protein synthesis that causes the observed fractionation.

1. Introduction

1.1 Amino acids mediate nitrogen assimilation in cells

Amino acids are one of the most important building blocks of cells due to their role in protein synthesis. As the main nitrogen (N)-containing molecule in cells, amino acids are essential for growth, structure, gene expression, enzyme function, oxygen transport, and many other vital processes. In many organisms, amino acids are also the primary vehicle for N assimilation into cells (Hosios et al., 2016). For example, one of the major pathways utilized is the GS-GOGAT cycle of N assimilation. A nitrogen substrate, such as ammonia, is converted into glutamine with the aid of the enzyme glutamine synthetase (GS), and is then used to form

glutamate through the enzyme glutamate synthase (GOGAT) (Helling, 1998; Sharma, 2023). Glutamate is a central metabolite in cells, and is used for the creation of many of the other twenty proteinogenic amino acids, such as proline and lysine (Idrees et al., 2020).

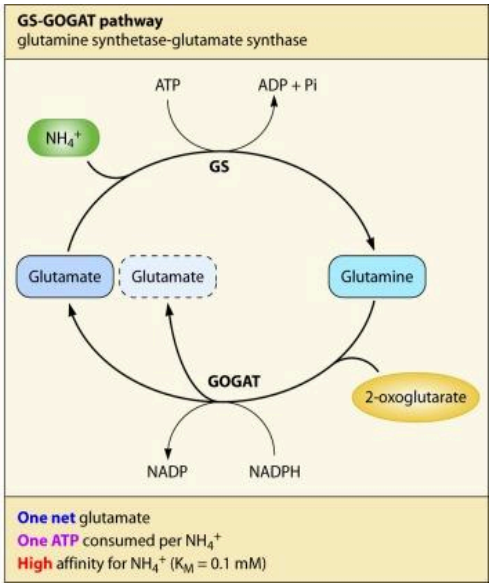


Figure 1. The GS-GOGAT pathway is depicted, which is the main N assimilation pathway in *Escherichia coli*. Figure from Van Heeswijk et al., (2013).

Amino acids from cells are therefore a useful tool for studying N cycling in living organisms. *Escherichia coli* is a prokaryotic heterotrophic bacteria that is able to synthesize all twenty amino acids using inorganic N substrates and carbon sources such as glucose (Elbing, 2019). *E.coli* is a well-characterized model organism, meaning that it has been used in many studies and its metabolic abilities are well-known compared to microbes that are less easy to grow, making it ideal for examining the conditions and processes that affect amino acid biosynthesis. Accordingly, studying N allocation and use in *E. coli* can provide greater insight into microbial N metabolism as a whole, as well as the impact anthropogenic changes to the global N cycle may have on microbial communities.

1.2. Kinetic Isotope Effects

Stable isotope analysis is a valuable tool in the analysis of biological, chemical, physical, geological, and ecological processes. Stable isotopes can be used to study the Earth's past climatic conditions, create intricate food webs and trophic levels, and investigate molecular and biochemical conundrums, such as metabolic processes. The general difference between isotopes and their original element is that their unique masses and nuclear spin cause them to behave in distinct ways; in biological and chemical reactions, this concept appears in the way heavier isotopes react more slowly compared with lighter isotopes (Tiwari et al., 2015). The difference in reaction rate leads to a phenomenon called the kinetic isotope effect, which "sorts"

the heavier and lighter isotopes between reactants and products of a reaction. This differential sorting is called isotopic fractionation.

Because of the kinetic isotope effect, almost all of biology preferentially uses lighter isotopes in metabolic reactions rather than heavier isotopes. This is mainly due to the difference in reaction rates which is related to the strength of the chemical bonds they form (Tiwari et al., 2015). The activation energy is greater for molecules that contain the heavier isotope, due to the stronger bonds they form compared to molecules that contain the lighter isotope, which means the bonds formed are more difficult to break or form. During a fractionating reaction process, the biologically mediated product will be depleted in the heavy isotope, and the reactants will be enriched in the heavy isotope.

1.3. Application of ^{15}N of Amino Acids to trace N assimilation and cycling

The invention and methodology of gas chromatography isotope ratio mass spectrometry (GC-IRMS) in the 1990's helped to propel advancements and studies in compound-specific ^{15}N analysis of amino acids, which have mostly focused on proteinaceous amino acids (Metges et al., 1996). N isotope fractionation patterns in amino acids are used for applications such as estimating trophic position, creating food webs, and assessing N sources and diet shifts. These food web and trophic transfer applications are based on empirically defined patterns in ^{15}N distribution among the various amino acids. When one organism eats another, some amino acids become more ^{15}N-enriched, while others do not undergo much enrichment at all. Amino acids are labeled as either 'source' or 'trophic' amino acids depending on whether they do or do not become ^{15}N enriched with trophic transfers, respectively (Ohkouchi et al., 2023). These patterns in amino acid ^{15}N can also be used to reconstruct N cycles in past environments; for example, signals of eutrophication were observed in the amino acids of formalin-preserved fish in one study (Ogawa et al., 2001). ^{15}N patterns in amino acids have also been utilized in medical research, especially in regard to cancer research. Amino acid metabolism in cancer cells often shifts relative to non-cancerous cells, and understanding why helps in the development of cancer therapies (Krishnamurthy et al., 2017).

Most of the applications described above focus on higher level organisms like animals or humans. We still understand little about the underlying processes that produce these patterns. In contrast, there is limited exploration of isotope amino acid patterns in the microbial world. Microbes often form complex communities that are important in the biogeochemical cycling of elements like N, and may be important in setting the "baselines" for ^{15}N patterns that are propagated through food webs. Microbial activities are also largely responsible for N transformations that mediate the global N cycle. Therefore, determining how microorganisms allocate and use N at the cellular level will allow for greater understanding of their metabolisms, how they will respond to greater anthropogenic climate change in the future, and the potential future impacts on the global N cycle.

1.4 Is ribosomal protein synthesis an ^{15}N-fractionating process?

Recent but unpublished work identified a possible isotope effect between “free” amino acids and protein-bound amino acids: the intracellular free amino acids were more enriched in ^{15}N isotopes than protein-bound amino acids, and the data closely fit a model for Rayleigh isotope fractionation in a closed system (Kharbush, unpublished). The underlying physiological or biochemical mechanism is unknown, but one hypothesis is that the synthesis of free amino acid into protein is a fractionating process.

Protein synthesis is composed of five steps, including transcription, translation, initiation, elongation, and termination. As a cell undergoes protein biosynthesis, the free amino acids floating in the cytoplasm are carried by tRNA to the ribosome, translated into proteins, and become a peptide chain and then protein (Scheper et al., 2007). By analyzing the compound-specific N isotope composition of both free and protein-bound amino acids, we are able to investigate possible fractionation during this process.

In order to indirectly study protein synthesis and the potential kinetic isotope effect, we decided to examine whether growth rates have an impact on the kinetic N isotope effect between free and protein-bound amino acids. There is a relationship between the rate that proteins are synthesized and growth rate; more proteins are synthesized and recycled at higher growth rates, and less at lower growth rates (Lahtvee et al., 2014). A correlation between the magnitude of the isotope effect and growth rates would support the connection between protein biosynthesis and fractionation between these amino acid pools in the cell.

In this study we measured compound-specific ^{15}N isotope values for both free and protein-bound amino acids of *E.coli* cultures grown at different temperatures, and therefore different growth rates. The magnitude of the N isotope fractionation was calculated by fitting the resulting data to a Rayleigh isotope fractionation model. To use this model we assume the reactants would be free amino acids and the products would be protein-bound amino acids. Results demonstrate that growth rate impacted the ^{15}N values of free amino acids, but did not impact the ^{15}N values of protein-bound amino acids, supporting an effect of growth rate on isotope fractionation during protein synthesis.

2. Methods

2.1 Culturing and growth experiments

E.coli was chosen as the model bacterium for this project because of its status as an easy to grow model organism. Additionally, preliminary work showed that the kinetic isotope effect of $\delta^{15}N$ was especially pronounced compared to other bacteria (Kharbush, unpublished). A cryopreserved sample of non-pathogenic *E.coli K-12* was streaked onto an LB-media agar plate and grown for 24 hours. A single colony was isolated and inoculated into a test tube filled with a few milliliters of MOPs Minimal Media (Neidhart et al., 1974; see appendix for detailed recipe). This minimal media contains only essential vitamins together with glucose as a carbon source (0.1% concentration) and NH_4Cl (10mM) of known ^{15}N composition as the nitrogen source.

The test tube cultures were scaled up into 25 mL cultures and then into 1500 mL cultures which were used for the growth experiments. Starter cultures for the large flasks were grown at 30°C, and growth experiments were conducted at three temperatures: 15°C, 22°C, and 37°C (*E.coli's* optimal growth temperature). Cultures were sampled during the exponential and early stationary growth phases of the bacterial growth model seen below; aiming to collect over a range of cell densities.

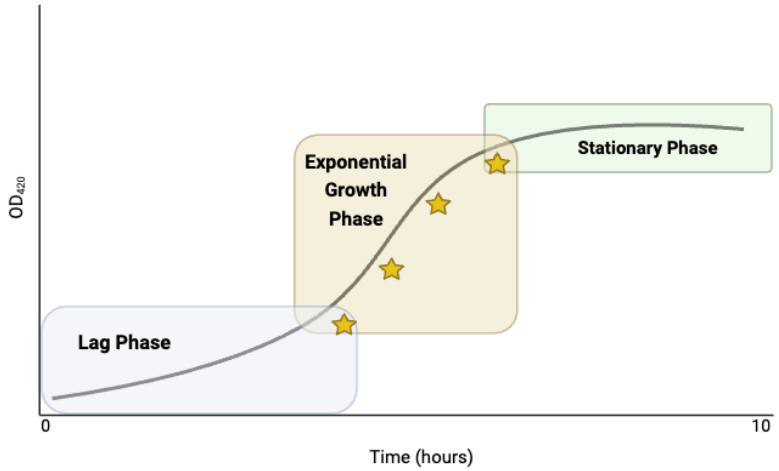


Figure 2. Bacterial growth model, showing the different phases of bacterial growth over time. We aimed to sample during the late lag phase, exponential phase, and early stationary phase, marked by the stars on the graph, in order to collect a range of cell densities.

Culture growth and cell density were monitored using optical density (OD) that was measured with a UV-VIS Thermo Scientific spectrophotometer set to a wavelength of 420nm. Optical density is a measure of light attenuation which is a proxy for cell density. Cells were harvested at ODs of approximately 0.5, 0.7, 0.8, and 0.9. Harvested cells were divided into separate aliquots for bulk biomass, free amino acids, and protein amino acids. 800 milliliters of culture were portioned for the free amino acids at the early exponential growth points, including OD's 0.5 and 0.7, while 500 milliliters of culture was collected at the later growth points, OD's 0.8 and 0.9. They were spun down in a centrifuge at 5000xg at 10°C for 5 minutes and then washed three times with an N-free saline solution with approximately the same ionic strength as the MOPs media (about 0.0708 M), before being transferred to pre-weighed eppy tubes and stored in a -80°C freezer.

Ammonia assays were conducted to measure the assimilation of ammonia in each growth experiment. Small samples of media were taken after the first spin down of the culture in the centrifuge and syringe filtered using a 0.2 micron filter. They were frozen and analyzed later using an Sigma-Aldrich ammonia assay kit.

2.2 Extraction and purification of amino acids for isotope analysis

The free amino acid samples were lysed using sonication for the samples at 15°C, 22°C, and 37°C and the lysate was dried under an N_2 stream. The protein amino acid samples were

hydrolyzed with 12N HCl at 110°C for 20 hours in order to break apart the proteins into individual amino acids; these were then dried under an N_2 stream. Both the free amino acid and protein amino acid samples were then purified using a BioRad resin column to isolate the amino acids and get rid of any impurities using cation exchange. This was followed by drying down the samples and storing them in a -20°C fridge.

2.3 Isotope analysis of amino acids and bulk samples

After purification, the samples were derivatized according to the methods outlined in *Molero et al.* (2011) in order to prepare them to be run on the GC-IRMS. Derivatization was performed using N-methyl-N-(tert-butyl-dimethylsilyl) trifluoroacetamide MTBSTFA which produces a volatile species that is able to be run on the instrument. The $\delta^{15}N$ values of the amino acids were calibrated using an internal reference standard called AABA (α-Aminobutyric acid) in addition to an external standard of in-house mixture of amino acids which allowed for corrections in compound specific variations in combustion and reduction (Yarnes & Herszage, 2017). The internal and external amino acid standards were acquired from Sigma-Aldrich and measured on the EA-IRMS; all free amino acid (FAA) and protein-bound amino acid (PAA) samples were run at least 3 times per sample. The standard deviations were ≤ 1‰ for each amino acid measured.

Bulk samples were freeze dried, weighed out into tin capsules using a microbalance, and run on the elemental analyzer in order to see the overall N isotope signal in the biomass of the samples. The $\delta^{15}N$ values of the biomass were calculated using standard reference materials USGS40 and USGS41 in addition to in-house laboratory standards glutamic acid and tyrosine.

N isotope values are reported as δ values in units of permil (‰) and measured relative to air, as seen in the equation below (Sigman & Fripiat, 2019).

$$\delta^{15}N = \left(\frac{(15N/14N)sample}{(15N/14N)air} - 1\right) \cdot 1000 \qquad \text{(Equation 1)}$$

The N isotope ratio for the free amino acid pool is represented by $\boldsymbol{\delta^{15}N_{FAA}}$ and the ratio for the protein bound amino acid pool is represented by $\boldsymbol{\delta^{15}N_{PAA}}$. These ratios are included in **Figure 3**, which helps to visualize the flow of N in this system.

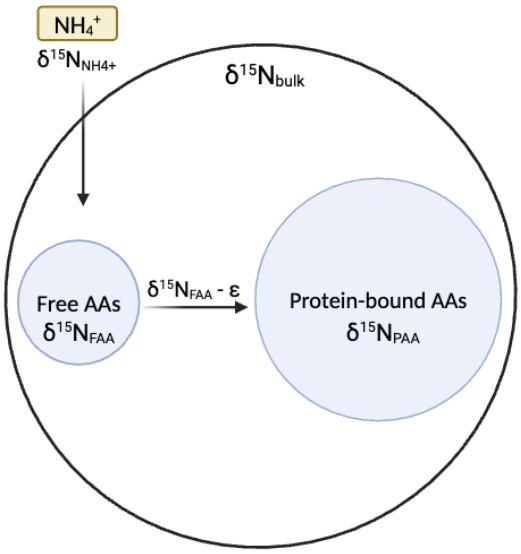


Figure 3. Diagram showing the flow of N moving through the cell during protein synthesis, considering the cell as a closed system for N and assuming an irreversible reaction between free amino acids and protein-bound amino acids. The cell is represented by the large black circle outline. N isotope ratios are denoted by $\delta^{15}N$, including the ratios for the free amino acid pool, $\delta^{15}N_{FAA}$, and the protein bound amino acid pool, $\delta^{15}N_{PAA,}$; the isotope effect is represented by ε. The middle arrow represents protein synthesis and the corresponding isotope effect that impacts the free amino acid values. The import of NH_4^+ into the cell also has an isotope effect that changes the $\delta^{15}N$ value of cellular biomass ($\delta^{15}N_{Bulk}$) as the cell grows and uses external NH_4^+. To take this effect into account when evaluating the potential isotope effect between free and protein-bound amino acids, we measured $\delta^{15}N_{Bulk}$ and used it to normalize $\delta^{15}N_{FAA}$ and $\delta^{15}N_{PAA}$ values. (Figure modified from Ohkouchi et al., 2023).

3. Results

3.1 $\delta^{15}N$ values between FAAs and PAAs

The growth rates for each temperature experiment are displayed in **Table 1** and show that at 37°C (E.coli's optimal growth temperature), the growth rate is the highest, decreases at 22°C, and is the lowest at 15°C. To analyze the $\delta^{15}N$ values of both the free amino acid and protein bound amino acid pool, we first looked at their distributions relative to biomass in order to more accurately compare between the different temperatures and the past experiment data (**Figures 4 and 5**). We then investigated whether growth rate impacted the magnitude of isotope fractionation **(Figure 7**).

Figure 4 and 5 visualize the differences in the $\delta^{15}N$ values between free and protein-bound amino acids and the relative differences in each respective pool in order to evaluate whether growth rate affected the $\delta^{15}N$ values. Generally, we found that free amino

acids were more enriched in ^{15}N than the protein bound amino acids, which supports the previous work that there is an isotope effect associated with protein synthesis. Furthermore, we saw in the new experiments that the free amino acids at 37°C were the most enriched in ^{15}N while the 15°C free amino acid samples were the least enriched. The $\delta^{15}N_{PAA}$ values and directionality are generally consistent with past studies, and they did not change much between different temperatures.

Table 1. Approximate growth rates for each temperature experiment are shown in the table above, which were measured by calculating the slope of the cultures during their exponential growth phase using OD measurements against hours.

Temperature	Growth Rates (OD/hour)
37 C	0.183
22 C	0.088
15 C	0.034

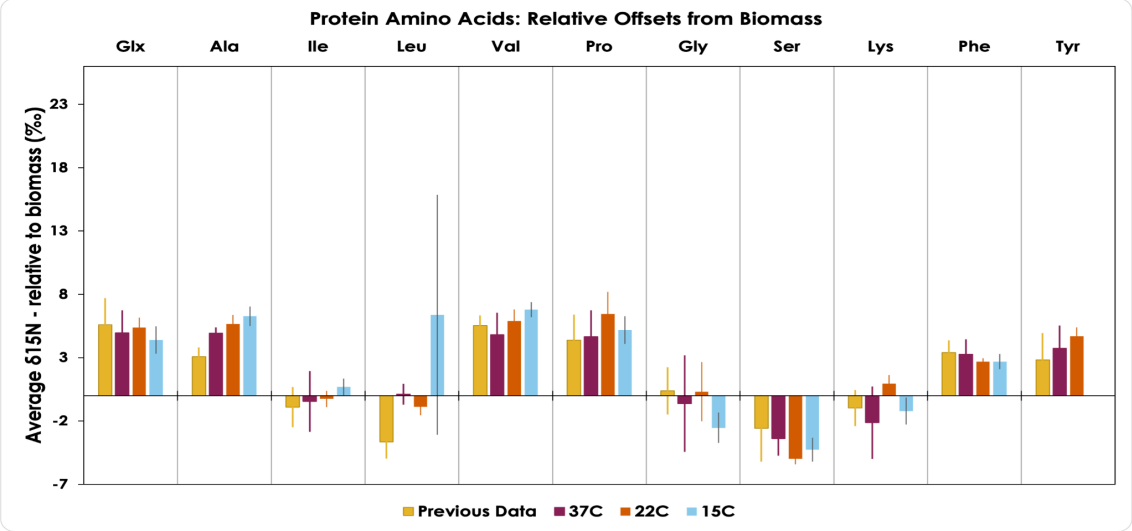


Figure 4. Average $\delta^{15}N_{PAA}$ values relative to $\delta^{15}N_{Bulk}$ from each temperature experiment were recorded and show slight differences with each other and compared to values from previous experiments. This data was normalized to biomass in order for more accurate comparison among the different experiments. Error bars represent ± 1 standard deviation.

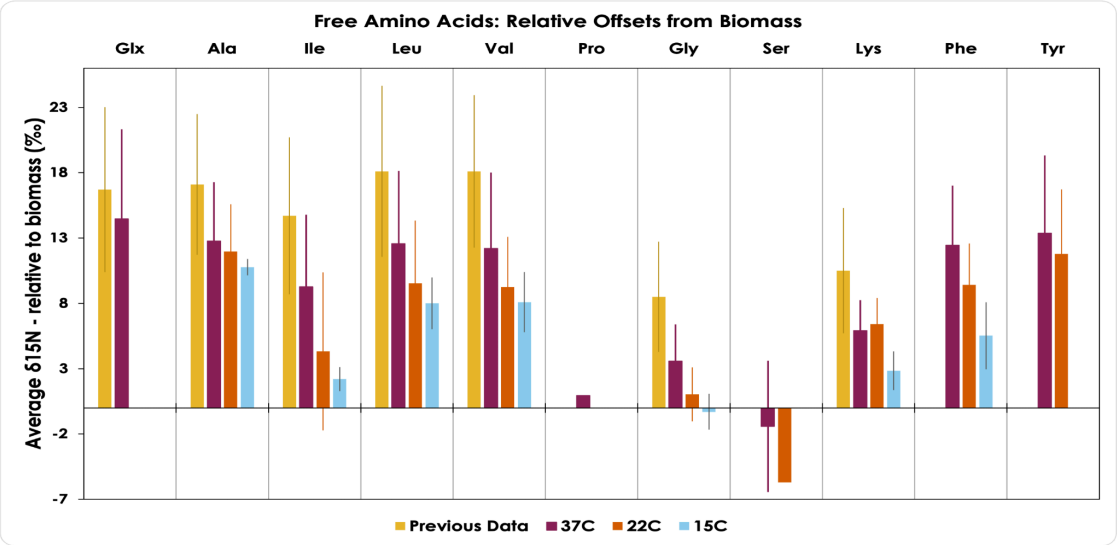


Figure 5. Average $\delta^{15}N_{FAA}$ relative to $\delta^{15}N_{Bulk}$ from each temperature experiment were recorded and show on average greater enrichment in ^{15}N relative to biomass than the protein bound amino acid samples (Fig.4). Like the protein data, this data was normalized to biomass in order for more accurate comparison between temperature treatments. Error bars represent ± 1 standard deviation.

In order to calculate whether there were statistically significant differences between the temperatures, a t-test was performed on the free amino acid results (**Table 2**). We used a two sample t-test with a null hypothesis that assumes there is no difference in the means between the two temperatures being compared. There is very strong evidence against the null hypothesis in phenylalanine when comparing 37-15; this means that there is a statistically significant difference between the means of those two temperatures. There is also moderate evidence to reject the null hypothesis for glycine 37-15, phenylalanine for 22-15, and lysine for 22-15. There is weak evidence to reject the null hypothesis in isoleucine 37-22, and glycine at 37-22. Although there is insufficient evidence to reject the null hypothesis for many of the amino acids, this is likely due to the very small sample size for each amino acid. This also shows that there are more significant differences between 37-15 than 37-22 or 22-15.

Table 2. P-values from a two-sample t-test analysis of the $\delta^{15}N_{FAA}$ values are listed; the temperatures being compared are in the left most column and the amino acid is in the top row. The t-test was equal variance except in instances where the ratio of variance between the two sets of data were greater than 4 (Swinscow, 1997).

	Ala	Ile	Leu	Val	Gly	Lys	Phe	Tyr
37°C, 15°C	0.220	0.080	0.154	0.182	0.034	0.087	0.019	
37°C, 22°C	0.379	0.063	0.211	0.192	0.068	0.372	0.154	0.337

22°C, 15°C	0.230	0.290	0.253	0.301	0.146	0.038	0.043

The $\delta^{15}N$ values of individual amino acids changed with growth stage (**Figure 6**). As cell density and OD increases, the $\delta^{15}N_{FAA}$ becomes increasingly enriched while the $\delta^{15}N_{PAA}$ and the $\delta^{15}N_{Bulk}$ do not increase by the same magnitude. While the bulk isotope values increase with culture growth, they do not approach the $\delta^{15}N$ value of the ammonium substrate, which was -1‰. This shows that the ^{15}N enrichment seen in the FAAs is not only due to substrate usage resulting in Rayleigh fractionation. Instead the relatively greater ^{15}N enrichment seen in the FAAs compared to PAAs at later growth stages suggests an isotope effect is occurring. If there were no isotope effect occurring, the FAAs would have a trend similar to that of PAA and bulk, meaning there would not be an increase in ^{15}N as growth increases.

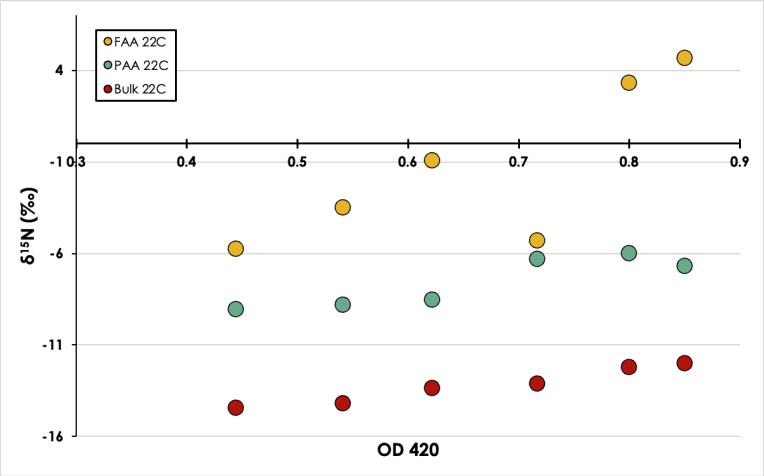


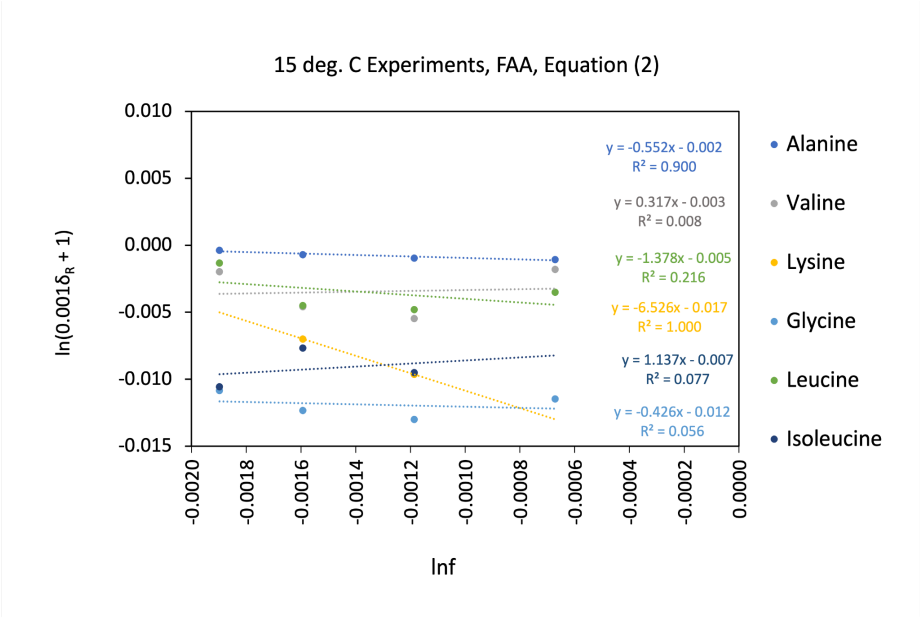
Figure 6. Changes in $\delta^{15}N$ values with OD of cultures grown at 22°C. Data shown include $\delta^{15}N_{FAA}$ and $\delta^{15}N_{PAA}$ from a representative amino acid (Alanine) compared to bulk values.

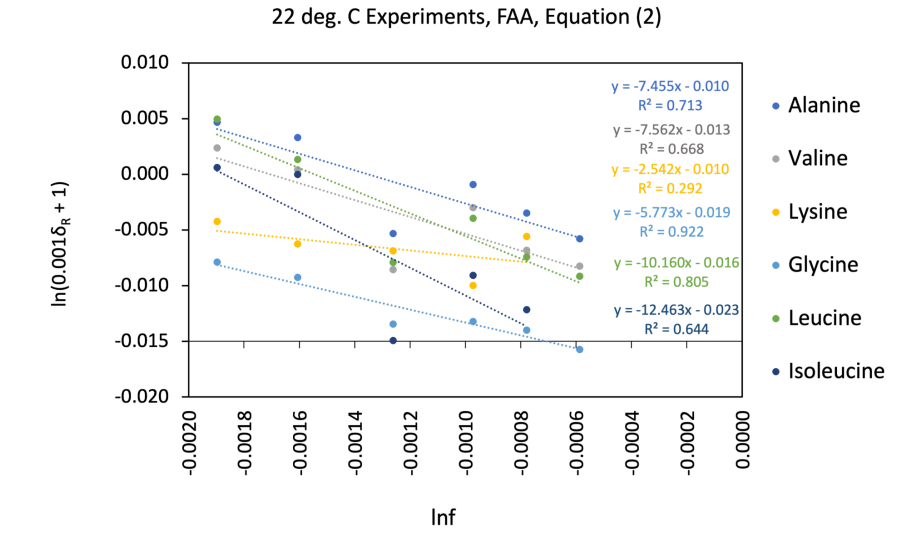
3.2 $\delta^{15}N$ Fractionation Trendlines

In order to estimate the isotope fractionation occurring at each temperature during protein synthesis, equations derived in *Introduction to Isotopic Calculations* (Hayes, 2004) were adapted and used to help visualize the isotope effects for each amino acid. In the equation, δ_R is the isotope value of the reactant (free amino acid) at a given time point, $\delta_{R,0}$ is the isotope value of the reactant at time zero, f is the fraction of unused ammonium substrate remaining in the media, and $\varepsilon_{P/R}$ is the isotope effect between product and reactant, in this case protein-bound and free amino acid, respectively. $\varepsilon_{P/R}$ and $\delta_{R,0}$ are obtained by plotting the measured $\delta^{15}N_{FAA}$ values against the fraction of ammonium substrate remaining. f was obtained by measuring the amount of ammonium remaining in the culture media after harvesting of cells.

$$\ln(0.001\delta_R + 1) = \ln(0.001\delta_{R,0} + 1) + \varepsilon_{P/R} \ln f \qquad \text{(Equation 2)}$$

In theory, using the equation above, the slope of each trendline in **Figure 7** should represent the isotope effect between the free and protein bound version of each amino acid. However, these plots show that the data was more variable than expected, as seen in the low R-squared values for many of the amino acids, especially the ones at 15°C. Although the fits may be poor, generally it is still true that the slopes of the free amino acids at 37°C are larger than the slopes of 15°C, showing that there potentially may be greater fractionation occurring at higher growth rates and less fractionation at lower growth rates. The slopes between 37°C and 22°C were very similar.





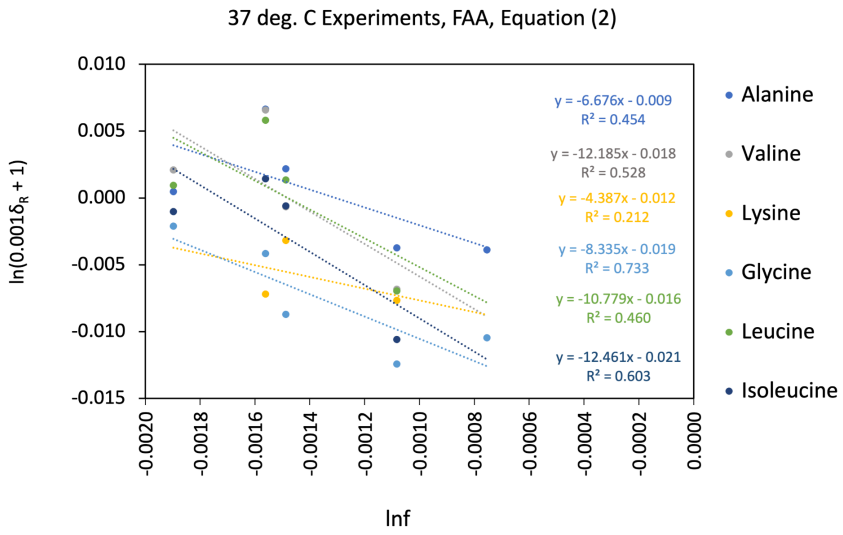


Figure 7. The $\delta^{15}N$ values of the free amino acids were plotted according to Equation 2 for each of the temperature treatments. The isotope effects for each amino acid are represented by the slopes of the lines.

4. Discussion

4.1 Suggested explanations for observations

This work reinforces previous work that showed an apparent isotope effect between FAAs and PAAs, and that growth rate apparently influences the $\delta^{15}N_{FAA}$ values, while the $\delta^{15}N_{PAA}$ did not change much between different temperatures. For an isotope effect and the corresponding fractionation to occur, there must be a reaction that forms and/or breaks chemical bonds with the fractionated element (N, in this case), creating a selective process that preferentially takes up lighter isotopes and leaves behind heavier isotopes (Tiwari et al., 2015). It is currently unknown whether these types of processes occur during protein synthesis.

Although the specific mechanism behind what causes the difference between FAA and PAA isotope values is unknown, there are a few potential hypotheses. One possible explanation is that FAAs are used in many biosynthetic processes in addition to protein synthesis (e.g. the synthesis of heme) and one or more of these processes is responsible for the observed fractionation of the FAA pool. Additionally, the size of the intracellular free amino acid pool is much smaller compared to PAAs, which may result in greater enrichment of ^{15}N in free amino acids that we measured.

An alternative hypothesis is related to the elongation factor, EF-TU, which is used in the elongation process during protein synthesis that is responsible for lengthening the polypeptide chain at the ribosome (Morse et al., 2020). Protein biosynthesis is often assumed to be a “committed step” where every amino acid bound to its corresponding tRNA molecule (aminoacyl-tRNA) is incorporated into the growing peptide chain in the ribosome. However, a

unique phenomenon has been observed during the elongation phase of peptide synthesis where EF-TU acts as a proofreading mechanism when selecting aminoacyl-tRNA. At lower rates of peptide bond formation, as would occur with an amino acid mismatch, EF-TU rebinding to the ribosome increases the number of GTP hydrolyzed per peptide bond (Morse et al., 2020). This proofreading mechanism leads to the release of any incorrect aminoacyl-tRNA before peptide bond formation, because incorrect aminoacyl-tRNA has a different molecular weight than the correct versions, causing the formation of the bond to have a different reaction rate. Fractionation of N isotopes could potentially occur here because the rate difference in bond formation between heavier and lighter isotopes in the amino acids could trigger EF-TU to preferentially remove the compounds with heavier isotopes, leading to a pool of protein bound amino acids that are lighter than the free amino acids. This proofreading process may be impacted at different temperatures, because at greater temperatures there is more protein synthesis using this process. In contrast at lower temperatures, there is a large pool of non-translating ribosomes in *E.coli* suggesting that lower growth temperatures cause a defect in initiation during protein synthesis, leading to less protein synthesis overall (Farewell & Neidhart, 1998). This provides some evidence that temperature-specific processes may impact the isotope effect and the enrichment of the FAA pool.

There are also examples of certain N-fractionating metabolisms that are affected by temperature changes, such as denitrification, which has been found to have an isotope effect of −29.4±2.4‰ at 20°C and −24.6±0.9‰ at 30°C (Mariotti et al., 1981). Although this is the opposite direction from our results (more fractionation at higher temperatures), this provides some evidence that other processes, such as protein synthesis, could potentially have differing levels of fractionation at different temperatures.

4.2 Limitations and unexpected variability in data collected

While we obtained sufficient amino acid $\delta^{15}N$ measurements for the above analysis, there was an unexpected gap in the free amino acid dataset. For all but two of the free amino acid samples, no or very little glutamate was detected on the mass spectrometer and we were unable to obtain $\delta^{15}N$ values for free glutamate. This is strange because glutamate is central to all of protein production in *E.coli*; it is a major N donor for biosyntheses and roughly 80% of the cell's N flows through glutamate (Reitzer, 2004). It is also one of the first amino acids made during N assimilation in *E.coli*. Therefore, a sizable glutamate peak was expected in these samples, as was found in previous iterations of this experiment.

To investigate this issue, we implemented several changes to experimental methods to determine whether our procedures were affecting the downstream measurement of glutamate on the mass spectrometer. We attempted another cell lysis procedure, bead beating new samples of free amino acids instead of the previously-used sonication procedure. We also omitted the sample filtration step prior to the BioRad purification column in order to preserve as much of the free amino acids as possible. However, these changes still did not produce glutamate. We finally did another growth experiment using a different strain of *E.coli* called *E.coli DH5-*α. Finally, glutamate signature was seen in all of these samples.

This leads to the conclusion that it was the initial strain of *E.coli K-12* that was likely the cause of the disappearance of glutamate. This particular strain was cryo-preserved for over ten

years, although we do not know how or why the strain changed over time. This may give more context to the inconsistencies of the data, such as the poor fits to the trendlines representing fractionation and the large error bands in **Figures 4** and **5**. Perhaps using different strains or fresher samples would show more consistent results and trends. Furthermore, the unpublished experiment data used as a comparison to the new temperature experiments was done with varying levels of ammonia added (both 10mm and 5mm) at 30°C, so the comparison to these new experiments may not be perfect, although both the previous and the new experiments agree that an isotope effect is occurring.

4.3 Methodology constraints and future work

These experiments were conducted using batch culture method, which may explain some of the large error bands seen in **Figures 4** and **5**. A batch culture is a closed system in which there is a defined amount of media and the nutrients essential for growth are added at the beginning of the experiment and are progressively depleted throughout the experiment (Pirt, 1972). This method of microbial culturing introduces potential for human error, particularly during harvesting and subsequent processing steps. In addition, there is Rayleigh fractionation associated with depleting the ammonium substrate in the media that can partly obscure the isotope effect that may be occurring during protein synthesis.

Using batch cultures, it is difficult to harvest at correct ODs because the harvesting process must be simultaneously juggled with tracking OD measurements of the growing culture on the spectrophotometer. At higher temperatures, such as 37°C, there is not enough time during the exponential phase to harvest multiple timepoints in one batch culture due to the fact the growth rate is extremely high. Splitting up the harvesting of the cultures on different days also may lead to some inconsistencies in the data, as the samples would use the same starter culture but would be separate cultures grown on different days.

In larger batch cultures, limitations of gas exchange may also cause inconsistencies in OD measurements. In smaller cultures, the ratio of the surface area of the culture compared to the volume of the flask is large, so oxygen is able to dissolve easily. However, when sizing up to 1500 mL cultures, the surface area to volume ratio is smaller so there is less gas exchange that is able to occur within the flask. This may impact the growth of the aerobic *E.coli*, which requires plenty of oxygen and gas exchange to thrive. The requirement for oxygen may also change with different temperatures which affects metabolic rates (Rubalcaba, 2024).

Additionally, attaining sufficient biomass for free amino acid extraction at lower ODs sometimes poses a problem. Unavoidable sample loss during sample processing exacerbates this issue. During the harvesting process, there are several washes that occur using N-free media for each sample in order to remove any N-containing media stuck on the cells. With each wash, the sample continuously becomes slightly smaller; this is also true for the processing steps, such as sonication, sample filtration, purification, and derivatization. At lower cell densities, such as an OD of 0.5, it is even more difficult to get enough material for measurement.

Finally, changing temperature may have other effects on cell physiology we are not accounting for, such as changing the molecular composition or volume expansion of the cell (Knapp & Huang, 2022).

One way to ameliorate these factors in the future would be to use a chemostat, where conditions can be held constant and the growth rate can be controlled. This would aid in achieving controlled steady states of biomass that would allow for more precise sampling and collecting more material for analysis.

5. Conclusion

This project investigated whether growth rate impacted $\delta^{15}N$ isotope values of free and protein bound amino acids in *E.coli* as well as the magnitude of isotope fractionation during protein synthesis. We found that the free amino acids were more enriched than the protein bound amino acid pool, supporting that there may be an isotope effect occurring during protein synthesis. At 37°C, we observed the free amino acids were the most enriched in $\delta^{15}N$ while the free amino acids at 15°C were the least enriched in $\delta^{15}N$, which provides evidence that growth rate impacts the $\delta^{15}N$ values. A t-test was performed to clarify whether the difference between the means among the various temperatures were statistically significant, but generally showed there was insufficient evidence to support this claim; this is likely due to the small sample size. The isotope fractionation was also examined, but the results were disorderly with poor R^2 values; however, the trendlines, which represented the magnitude of isotope fractionation, had larger slope values for 37°C and 22°C while the trendlines for 15°C had much lower values. This could potentially show that there is greater isotope fractionation at higher temperatures and less at lower temperatures. This study helps to provide further insight into intracellular N cycling in microbes (using compound specific N isotope amino acid analysis) which has not been extensively studied before and it investigates the unknown fractionating mechanism during protein synthesis. N is an essential component to life and understanding how organisms utilize and allocate N in their cells is important in the face of climate change where varying temperatures might impact how they use N. It can also help us better understand microbial mediation of the biogeochemical N cycle. Future work may investigate the effect of growth rate on amino acid $\delta^{15}N$ values in different microbial groups, and the specific mechanism during protein synthesis that causes the observed isotope effect.

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Appendix

F. C. Neidhardt, P. L. Bloch, and D. F. Smith. 1974. Culture medium for enterobacteria. *J Bacteriol* 119(3): 736-747 [PubMed Central].

Mops Minimal Medium

10X MOPS mixture	100 ml	
0.132 M K_2HPO_4	10 ml	
milliQ H_2O	880 ml	
1mg/ml thiamine	0.1 ml	(*optional* - we do not use thiamine because it does not affect the growth rate of *E. coli* K-12 MG1655 in this medium.)

TOTAL	990 ml	

1. Mix ingredients above and adjust the pH to 7.2 with approximately 300 microliters 10 M NaOH.
2. Filter sterilize. Can be stored at 4 degrees for up to 1 month.
3. Before use add 10 ml 100X carbon source (as appropriate - we typically use a final concentration of 0.1% glucose).

10X Mops Mixture

1. In a 1 L beaker with a stir bar, add the following to ~300 ml milliQ H_2O:

Component	FW	grams
MOPS	209.3	83.72
Tricine	179.2	7.17

2. Add 10 M KOH to a final pH of 7.4 (10 to 20 ml)
3. Bring total volume to 440 ml
4. Make fresh $FeSO_4$ solution and add it to the MOPS/Tricine solution:

Component	FW	grams	H_2O vol (ml)	stock conc (M)
$FeSO_4 \cdot 7H_2O$	278	0.028	10	0.01

5. Add the following solutions to the MOPS/tricine/$FeSO_4$ solution (see below how to make each of these):

Mix in the order shown!

Component	Volume

1.9 M NH_4Cl	50 ml
0.276 M K_2SO_4	10 ml
0.02 M $CaCl_2•2H_2O$	0.25 ml
2.5 M $MgCl_2$	2.1 ml
5 M NaCl	100 ml
Micronutrient stock	0.2 ml
Autoclaved milliQ H_2O	387 ml
TOTAL	1000 ml

6. Filter sterilize with 1 L capacity 0.2 micron filter
7. Aliquot into sterile 100 or 200 ml plastic bottles and freeze at -20°.

Stocks used in the 10X Mops Mixture

Make each separately, mixing the amount indicated into the specified volume. Store at room temp.

Component	FW	stock conc (M)	grams	vol (ml)	note
NH_4 Cl	53.49	1.9	50.82	500	

K_2SO_4	174.3	0.276	4.8	100	
$CaCl_2•2H_2O$	147	0.02	0.294	100	
$MgCl_2$	203.3	2.5	50.75	100	
NaCl	58.44	5	292.2	1000	

Micronutrient stock (100 ml)

Mix everything together in 40 ml autoclaved milliQ H_2O, bring up total volume to 50 ml. Store at room temp.

Component	Formula	FW	Grams for 50 ml
ammonium molybdate	$(NH_4)_6 Mo_7O_{24}• 4H_2O$	1235.9	0.009
boric acid	H_3BO_3	61.83	0.062
cobalt chloride	$CoCl_2$	237.9	0.018
cupric sulfate	$CuSO_4$	249.7	0.006
manganese chloride	$MnCl_2$	197.9	0.040
zinc sulfate	$ZnSO_4$	287.5	0.007

Potassium phosphate K_2HPO_4 Solution:

NOTE!! Make sure you use dibasic K_2HPO_4, and do NOT use monobasic KH_2PO_4 !!!!! Can be stored at room temperature after autoclaving.

Component	FW	stock conc (M)	grams	Vol (ml)	note
K_2HPO_4	173.2	0.132	23.0	1000	autoclave

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