J.N. Andrews
Honors Program
Andrews University

Proposal for Senior Honors Thesis

HONS 497 Senior Honors Thesis **Credits** 4 (2 minimum required)

Directions: Please return signed proposal to the Honors Office **at least one week prior to your scheduled meeting with the Honors Council**. This proposal must be accepted by Honors Council the semester before presentation.

Student's Name: Rayford Alva
Primary Advisor: Dr. Ryan T. Hayes
Secondary Advisor: Dr. Brian Y. Y. Wong
Thesis Title: Mutagenicity (Ames) Test Optimization for Evaluating Mutagenicity of Burned Arginine-based Heterocyclic Amines and the Anti-mutagenic Effect of Chinese Medicinal Herbs
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Expected date of Graduation: May 2018

I. Provide goals and brief description of your project or research.

There are two goals to my research project: the first relates to testing the relative mutagenicity of certain paired amino acid syntheses products (classified as heterocyclic amines). The second goal is to determine the efficacy of certain herbal extracts commonly used in traditional Chinese medicine as inhibitors of the mutagenic effects of mutagenic compounds.

The research project is centered around a mutagenic assay developed by Bruce N. Ames and his lab groups in the 1980s. The mutagenicity assay, known as the Ames mutagenicity assay or the Ames test, utilizes a bacterial model and added rat liver S9 enzymatic fraction (to simulate mammalian metabolic activity). Bacterial cell cultures are then incubated in the presence of experimental chemicals. The bacterial strain (*Salmonella typhimurium* TA98) is modified in such a way that test chemicals can easily enter the bacterial cells and insert into the bacterial genome (*rfa* mutation to the bacterial lipopolysaccharide layer). Additional properties (Δ*uvrB* and hisD3052) of this particular strain allow for mutation events occurring at nanoscale to be observed at the macroscopic level. Bacterial colonies that mutate in this assay are known as "revertants," because any visible colonies represent a mutation that led to a reversion—that is, a reversion from the inability to survive in an environment without histidine to the ability to synthesize histidine, an amino acid necessary for bacterial survival. In this way, the Ames test is a useful method to test a wide variety of chemicals using bacteria with a mammalian component to simulate a mammalian model, without the challenges brought on by housing and maintaining a population of rats to conduct testing on. The second goal is easily completed, as it represents the addition of only one more component to the Ames test, namely, an herbal extract in solution in the presence of a mutagenic compound. The number of revertants (the common name for colonies mutated during the incubation period) from treatments with and without the herbal extract (and at various quantities of the herbal extract per treatment) will allow for a percent inhibition to be calculated. This percent inhibition will thus indicate the effectiveness of a given herb to inhibit the mutagenic activity of an otherwise mutagenic compound, and would be compared to the percent inhibition of other herbs.

II. Outline your methodology. **Please be specific**. How does this achieve your goals and how reliable is it?

At the moment, our current methodological plan would follow the recommendations and specifications presented in two papers published by Ames and his associates, which includes guidelines of chemical preparation and storage. Our plans are to use *Salmonella typhimurium* TA98 (TA98) as our tester strain, a strain originally derived from wild-type *S. typhimurium LT2*. Permanently frozen cultures of TA98 would be kept at -80°C, and would only be removed to create testing cultures that could be kept at 4°C. These master plates (agar media on petri dishes) would include the antibiotic ampicillin, which would be used to exclude bacteria that are not ampicillin-resistant (contaminants). Thus, TA98 colonies could be grown and kept for an extended period of time (4-6 weeks) for use in testing. Once bacteria are needed for testing, a single bacterial colony would be isolated and grown in a shaking incubator for a 14-hr period at 37°C and 210rpm in a test tube containing nutrient-rich broth culture. After this period, the absorbance of the bacteria at 650.28nm would be measured using a spectrophotometer, and the reading would then be used to ensure that the bacterial cell density (cells/mL) fell between the range of 10^5 and 10^8 cells/mL, with a target density of 200 million cells/mL. The cell density and absorbance correlation be determined by creating a standard curve of absorbance for a 14-hr cell culture and manual count of cells using a microscope and a hemocytometer.

Additional chemical solutions necessary for the Ames test would include minimum glucose plates (40% glucose solution and a 50X Vogel-Bonner salt solution), minimal histidine and biotin solution (0.5mM of each) which is added to solution of top agar (0.5% NaCl, 0.6% agar) the day of testing, a mix of cofactors (0.4M $MgCl_2$-1.65KCl solution, 0.2M pH 7.4 phosphate buffer) and energy sources (0.1M nicotinamide adenine dinucleotide phosphate [NADP], 1M glucose-6-phosphate [G6P]) for the S9 enzyme fraction (kept at -80°C when not in use; Aroclor 1254-induced male Sprague Dawley rat liver S9 in 0.15M KCl, manufactured by Molecular Toxicology, Inc.). Finally, the test chemicals (synthesized from arginine and phenylalanine, followed by extraction and purification into eleven initial fractions; dissolved in methanol as solutions) would be obtained from the work done by undergraduate students in Hayes' lab group. A known mutagenic heterocyclic amine, 2-amino-1-methyl-6-phenlyimidazo(4,5-b)pyridine (PhIP), would be tested alongside the experimental chemicals (nicknamed RF-HCA-01 all the way to RF-HCA-11) to establish the integrity and validity of the test. Revertant colony data obtained from PhIP-treated bacteria could then be compared to that found in the published literature. Herbal extracts from the Chinese medicinal herbs *Scutellaria barbata* and *Oldenlandia diffusa* would be obtained from Wong and his lab group.

On the day of each experiment, the minimal histidine and biotin solution (0.5mM) would be added to liquefied top agar solution kept at ~50°C in a water bath. In this same water bath, the herbal extracts would be warmed and kept for at least 15min prior to use. Each experimental treatment would be done in triplicate and with the necessary controls present (i.e. a treatment that included all Ames components with methanol would be the appropriate baseline control for any of the RF-HCA fractions and PhIP, because the added volume of any given test chemical would only differ in the identity of the chemical dissolved in methanol). For each treatment, a test tube would contain bacteria, cofactor and energy solution (S9 mix), the chemical to be tested, herbal extract (when appropriate), and S9 enzyme. To this, a small quantity of top agar would be added, the contents mixed vigorously, and the contents poured onto minimum glucose plates. Upon hardening of the top agar layer, the plates would be incubated at 37°C for ~48hr. After the incubation period, the number of revertant colonies per plate would be determined. Statistical analyses appropriate to the experimental parameters would follow, with any outliers (extraneous data points) being removed using the methods of Grubbs' outlier test prior to inclusion in the data set.

The above methods are standard practice for mutagenicity testing, and have been used for decades. These methods would allow me achieve my goals of evaluating the mutagenic activity of our chemicals of interest and determine whether the observed counts of revertant colonies represent a statistically significant difference from control. Chemical fractions (which contains any number of compounds) that displayed a significant difference from control could then be purified further to determine the precise nature of

mutagenicity—presumably, which compound(s) in that chemical mixture was/were responsible for causing bacterial colonies to reverse mutate back to the ability to synthesize histidine.

III. Explain in what sense your project is original, unique, or beyond normal senior expectations. How does it relate to current knowledge in the discipline?

This project is unique in that it continues work begun in the 1990s to determine the mutagenic activity of heated binary amino acid mixtures—incomplete, because the compounds thus produced where never fully identified. Additionally, this project goes beyond normal senior expectations in that it (Ames mutagenicity assay) is an experimental procedure that is only mentioned in a handful of biology/chemistry courses at Andrews University, and is seldom discussed at length. Furthermore, this project includes personal preparation of all reagents (chemical solutions, experimental components)—a practice that is rarely, save for a few special instances, done at the undergraduate level during the laboratory phase of a biology/chemistry course. Our endeavor as a research group is to identify mutagenic compounds from heated binary amino acid mixture, which is an initial step in the process of creating a model that might explain why mutagenic compounds form in plant-based proteins (the presence of mutagenic compounds from a variety of animal-based proteins is already well established). Also, our work with heated binary amino acid combinations could lead to predictions concerning proteins that are likely to produce heterocyclic amines upon heating.

IV. Include a substantive annotated bibliography of similar or related work.

Ames BN. 1971. The detection of chemical mutagens with enteric bacteria. In: Hollaender A, editor. Chemical mutagens: Principles and methods for their detection. New York: Plenum Press. p. 267-282.

In this chapter, Ames discusses the possible applications of his mutagenicity assay, outlining the benefits of using a bacterial model. He outlines the relative simplicity of the experimental protocol, the extreme sensitivity of the bacterial tester strains, and the wide range of chemical classes that can be incorporated in to the test. He goes on further and discusses the applicability and reliability of the test, while noting a few chemical exceptions. He affirms the importance and validity of conducting mutagenicity testing, simply because bacteria and humans both contain DNA, and that DNA is susceptible to mutation. He concludes that while mutagenicity does not always lead to carcinogenicity, the correlation is important to consider. This chapter is useful in that it provides a general background for the reliability and scope of Ames' mutagenicity assay.

Knize MG, Cunningham PL, Griffin Jr. EA, Jones AL, Felton JS. 1994. Characterization of mutagenic activity in cooked-grain-food products. Food and Chemical Toxicology. 32(1):15-21.

Knize and his colleagues, working on the idea that some heated or baked (toasted breads and cookies) non-meat foods displayed mutagenicity among bacteria, suspected that they could observe mutagenic activity in an even wider variety of non-meat sources. Using the Ames/Salmonella Test, they sought to investigate the mutagenic activity of a variety of non-meat food products, namely cooked breads and grain products, heated flours, and store-bought meat substitutes. After preparing the various samples and conducting the Ames mutagenicity test, it was observed that longer cooking times and higher gluten content generally resulted in a higher average number of bacterial revertants per gram of original sample. Surprisingly, a compound (PhIP) commonly produced in cooked meat was discovered in one sample, yet only accounted for 4% of the mutagenic activity, leading to the belief that another agent was at work to produce the observed mutagenicity. What this study does is provide me with the scientific justification to study heated non-meat food sources for mutagenic activity. Furthermore, the study's discussion point at potential agents for mutagenic inhibition, an aspect I am now addressing via the medicinal herbs.

Knize, MG, Cunningham PL, Avila JR, Jones AL, Griffin Jr. EA, Felton JS. 1994. Formation of mutagenic activity from amino acids heated at cooking temperatures. Food and Chemical Toxicology. 32(1):55-60.

This paper by Knize and his associates is an example of previous work done on single and binary amino acid combinations that used the Ames' "*Salmonella* test" as the method to evaluate mutagenicity. This further builds the argument for the use of this methodology (based on precedence), and provides me with published data using the same tester strain (*Salmonella typhimirium* TA98) in compounds derived from burn products of arginine and phenylalanine.

Maron DM and Ames BN. 1983. Revised methods for the Salmonella mutagenicity test. Mutation Research. 113(3):173-215.

This highly-detailed document forms the theoretical and practical basis for my research. It covers the breadth of the mutagenic assay, presenting possible variations, and provides a detailed procedure for the preparation of stock solutions and media (agar-covered petri dishes.) It provides me with the information necessary for virtually every single aspect of my research project, save for the inclusion of herbal extracts as inhibitors and detailed information on the preparation of a buffered solution.

Skog KI, Johansson MAE, Jägerstad MI. 1998. Carcinogenic heterocyclic amines in model systems and cooked foods: A review on formation, occurrence and intake. Food and Chemical Toxicology. 36:879-896.

This review paper by Skog *et al.* presents a comprehensive approach to the science of heterocyclic formation from whole foods, including data from various studies that give estimates on daily human intake of heterocyclic amines. It also includes a table with heterocyclic amine identities produced from single amino acids burned with creatinine and with or without sugar. This paper, although not solely on mutagenic provides me with a working knowledge on the factors related to heterocyclic amine formation, a topic of interest to my lab group as a whole.

Sugimura T, Wakabayashi K, Nakagama H, Nagao M. 2004. Heterocyclic amines: Mutagens/carcinogens produced during cooking of meat and fish. Cancer Science. 95(4):290-299.

This paper provides substantial evidence for the case that many heterocyclic amines are produced from the cooking of meats and are either mutagenic (bacterial testing), carcinogenic (testing on rats), or both. It provides a literature background for the field of food-sources for heterocyclic amines, namely, animal-based foods. Additionally, it provides me with a target revertants/μg of PhIP with the same tester strain (*Salmonella typhimirium* TA98) value to compare my own work to.

Wong BYY. 1992. Modulation of rat hepatic S9-dependent mutagenesis, DNA binding, and metabolism of aflatoxin B_1 and benzo[a]pyrene by four Chinese medicinal herbs. [Loma Linda]: Loma Linda University.

This thesis written by Wong provides evidence for the efficacy of certain herbs used in traditional Chinese medicine as inhibitors of mutagenic activity produced by mutagen/carcinogen benzo[a]pyrene (BaP). BaP is a polyaromatic hydrocarbon, a class of chemicals similar to heterocyclic amines (HCAs) in structure. On this basis, we are testing PhIP in the presence of two of the four herbs mentioned in Wong's dissertation—*Scutellaria barbata* and *Oldenlandia diffusa*—to observe potential inhibitory effects.

V. Provide a statement of progress to date and list the research methods coursework completed.

To date, I have done preliminary screenings of all eleven fractions at least once. Additionally, I have tested and observed the decreased mutagenicity of PhIP when in the presence of either herb (separately) or in synergy (both herbs). The completed work represents seven separate successful experiments that yielded results. I am currently in discussions with a few professors on campus proficient in statistical analyses to ensure I use the appropriate test—as such, much of the literature on mutagenicity testing is lacking in statistical analyses.

I took Biostatistics & Research Design (BIOL280) in the Spring of 2016 and Research Pro-Seminar (HONS398H) in Fall of 2016 to fulfill my requirements for a courses in research methods. Additionally, relevant coursework that prepared me for my work with bacteria and bacterial testing, chemical laboratory techniques, and the reading/writing of scientific papers and formal lab reports include, in the order taken: General Chemistry I & II (CHEM131 & 132), Cellular & Molecular Biology (BIOL372), Scientific Communication (BIOL305), and Biology of Bacteria (BIOL475).

Department Chair Approval

- **This student's performance in his major field is acceptable.**
- **He has completed the requisite research methods coursework for the research to be pursued.**
- **I understand that he plans to graduate with Honors.**

Department Chair (signature)

Research Advisor Approval

I have read and support this proposal:

Primary Advisor (signature)

I have read and support this proposal:

Secondary Advisor (signature)

NOV 21, 2017

If human subjects or if live vertebrate animals are involved, evidence of approval from the Institutional Review Board or an Animal Use Committee is needed through the campus scholarly research offices (Ext. 6361).