April 6, 2016

Macromolecular Crowding Effects of LDH

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Background

For the past 50-60 years, *in vitro* kinetic studies on enzymes have traditionally been performed in dilute solutions. However, in recent years within biochemical studies, there has been a surge of interest in a high potential focus area called macromolecular crowding. According to A.P. Minton, a pioneer in this field, macromolecular crowding is defined as, "the observed effect of excluded volume between one soluble macromolecule and another"[1]. This effect is thought to influence enzyme activity, protein folding, and other areas that have yet to be fully explored or understood.

Within a cellular matrix, macromolecules consist primarily of nucleic acids, proteins, and polysaccharides, with the former two being the most prevalent. It is believed that there is a high concentration of macromolecules within a cell, with 30-40% in dry weight being macromolecules[1]. Therefore, it is crucial to explore enzyme activity in an environment that more appropriately simulates the crowded environment of the inside of the cell.

Macromolecular crowding hinges on the excluded volume effect. The excluded volume effect refers to the fact that a segment of a long chain molecule, such as a protein, cannot inhabit the same space as another segment of a molecule[1]. This physical property of an individual molecule reduces the solvent volume of the other molecules, increasing the effective concentration of macromolecules in solution[1]. This phenomenon may have significant effects on diffusion reactions, cellular aggregation, and protein folding[1].

This study focuses on the reactivity of enzymes in crowded solutions. The attention of this study is directed at an enzyme called lactate dehydrogenase (LDH). LDH is a cytosolic enzyme that is responsible for converting pyruvate into lactate[2]. Pyruvate is a product of glycolysis that is normally converted into acetyl CoA during aerobic respiration to be processed in the citric acid cycle. However, during anaerobic respiration, pyruvate is reduced to lactate (or lactic acid) catalyzed by LDH in a process called lactic acid fermentation[2, 6]. Lactic acid fermentation only produces 2 units of ATP versus the 36

units of ATP created through glycolysis coupled with the citric acid cycle, and oxidative phosphorylation[2]. And yet, lactic acid fermentation is an essential process in producing energy when little or no oxygen is available to replenish the aerobic cycle. Lactic acid fermentation might be more familiarly known as the build up of lactic acid, or the muscular fatigue, that accumulates after intense strenuous exercise. The conversion of pyruvate into lactate is also accompanied by the oxidation of NADH into NAD^+. NADH is a coenzyme that serves as a reducing agent, or electron donor[2].

The structure of LDH is pertinent because structure dictates function. As mentioned, LDH is a cytosolic enzyme, which is an important distinction to make because our study also looks at mitochondrial enzymes[2]. The cytosolic matrix is considerably less crowded than the tiny, compartmentalized, mitochondrial matrix. LDH is a tetrameric protein, which means that it is constituted of four smaller monomer subunits or polymeric chains[2]. The molecular weight of LDH is 140,000 Daltons, which is considerably larger than the molecular weights of its substrates, NADH and pyruvate, having molecular weights of 663 and 88 Daltons respectively[2]. Our work centers around mammalian LDH extracted from rabbit muscle.

Although bacterial LDH typically displays cooperativity, to date no mammalian LDH has ever been shown to exhibit cooperative behavior. Cooperativity is the increase in affinity of the subunits of an enzyme to bind with an active site when one subunit has been already bound by that substrate. A classic example is the binding of oxygen in hemoglobin. Pyruvate and NADH are the two substrates that bind to LDH in order for the conversion to occur.

The clinical importance of LDH cannot be understated. LDH has been shown to be an effective tumor marker in specific germ line cancers such as ovarian and testicular cancer[4]. High levels of LDH can also be indicative of other negative conditions such as heart failure, anemia, meningitis, and HIV[4]. In more recent studies, high ratios of LDH-A to LDH-B have been implicated to high levels of lactate in the brain, which is a potential biological marker of Alzheimer's in older patients[5].

The enzymatic activity of LDH in *in vitro* solution has already been determined and can be found in literature. We have replicated these findings in our lab. To examine crowding effects, we tested

enzymatic activity in Ficoll-70 and bovine serum albumin (BSA). Ficoll-70 is a soluble, globular polysaccharide with a molecular weight of 70,000 Daltons[7], about half the molecular weight of LDH. Slightly smaller, BSA is a globular protein that has a molecular weight of 66,500 Daltons[8]. These are examples of model macromolecular crowders.

Although results with Ficoll-70 were promising, since BSA is a protein and proteins are found in greater abundance within the cellular matrix, we chose to pursue experimentation with BSA. BSA is relatively inexpensive, stable in our assay, and very soluble (up to 30-40% weight by volume). Importantly, BSA is also considered inert since interactions with LDH have not been reported.

Hypotheses

There are two central hypotheses to our work. First, the activity of LDH would decrease as the concentration of crowder is increased. Empirically, it has been found that dextrans, a polysaccharide, decrease the activity of LDH[7]. Therefore, Ficoll-70 should also decrease the activity of LDH. In contrast, it has been observed that BSA increases the activity of LDH at concentrations of up to 20-30%, at which point, the activity of LDH starts to decrease[8]. Hence, we contend that the activity of LDH will decrease as the concentration of Ficoll-70 increases, and the activity will increase as the concentration of BSA increases.

The second hypothesis hinges on the conclusions from the first hypothesis. Our experiments suggested that BSA, even at the lower concentrations, decreased the activity of LDH. Furthermore, there was a significant decrease of activity at the higher concentrations of LDH. Deciding to work further with BSA, we hypothesized that the introduction of crowders at 10% by weight will cause inhibitory substrate effects and changes in catalytic properties. Substrate effects with both pyruvate and NADH were explored.

Methods

Procedure Initial rates were determined using a Cary UV-Visible-spectrophotometer with Peltier Temperature Control. LDH activity was obtained by measuring the decrease in absorption of NADH in a quartz cuvette at a wavelength of 340nM.

Linear least squares measurements of initial rates were taken over the first thirty seconds of each time course at 25°C.

Reagents Experiments with varied Ficoll-70 and BSA concentrations were run with 1mL of 42.5mM Tris-HCl buffer, 150μM NADH, 1.6mM pyruvate, and 0.156 units of rabbit muscle LDH. Concentrations of Ficoll-70 used were 0.10, 0.25, 0.50, 1.0, 2.0, 4.0, 7.0, and 10.0%. Concentrations of BSA used were 0.50, 1.0, 2.0, 4.0, 7.0, 12.0, and 20.0%.

For experiments in which pyruvate was the variable substrate, 10μM, 20μM, 30μM, 40μM, 80μM, 160μM, 320μM, 640μM, 1.28mM, and 2.56mM pyruvate were used. These assays were performed in 10% by weight BSA solution. All other condition remained unchanged from the previous experiment.

For experiments in which NADH was the variable substrate, .5μM, 5.0μM, 7.5μM, 10μM, 14.4μM, 18.8μM, 37.5μM, 75μM, and 150μM NADH were used. These assays were performed with 2.56mM sodium pyruvate. This was also observed in 10% by weight BSA solution. All other conditions remained the same.

The addition of BSA was found to increase the absorbance at a linear rate with the increase of BSA concentration. Ficoll-70 did not have this added effect.

Results

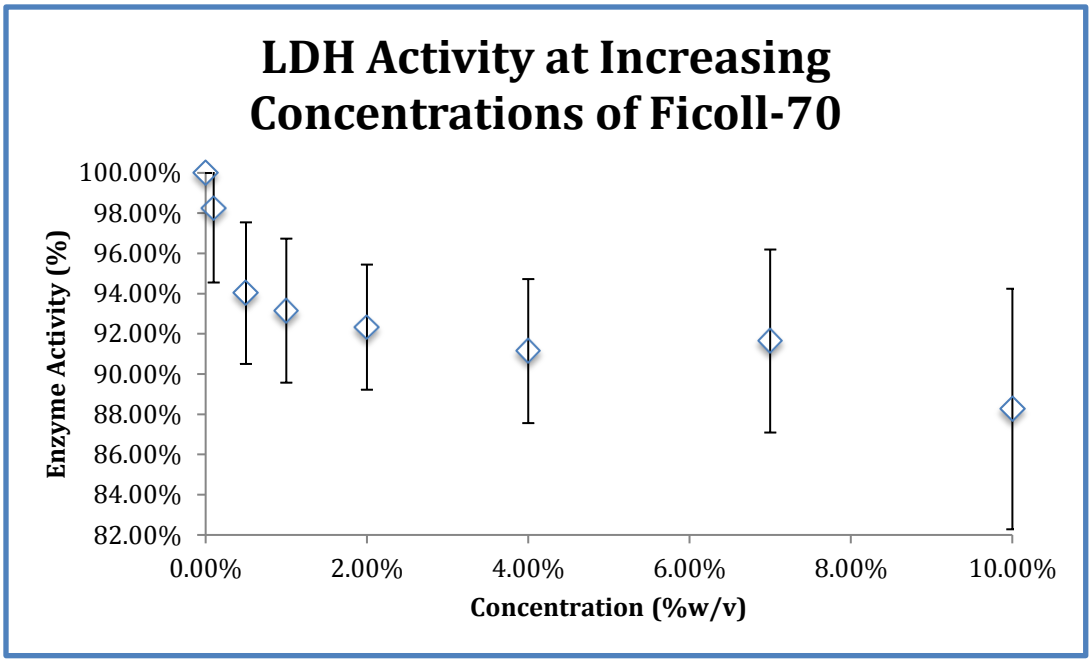

Figure 1: Observed inhibition in LDH activity with the introduction of increasing concentrations of Ficoll-70.

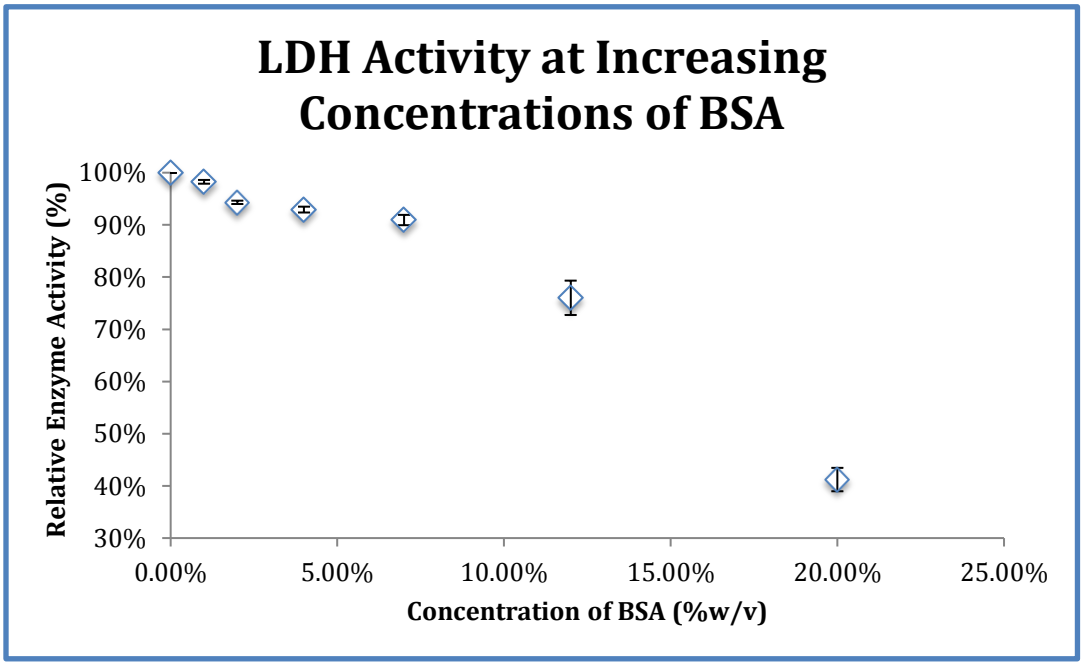

Figure 2: Observed inhibition in LDH Activity with the introduction of increasing concentrations of BSA.

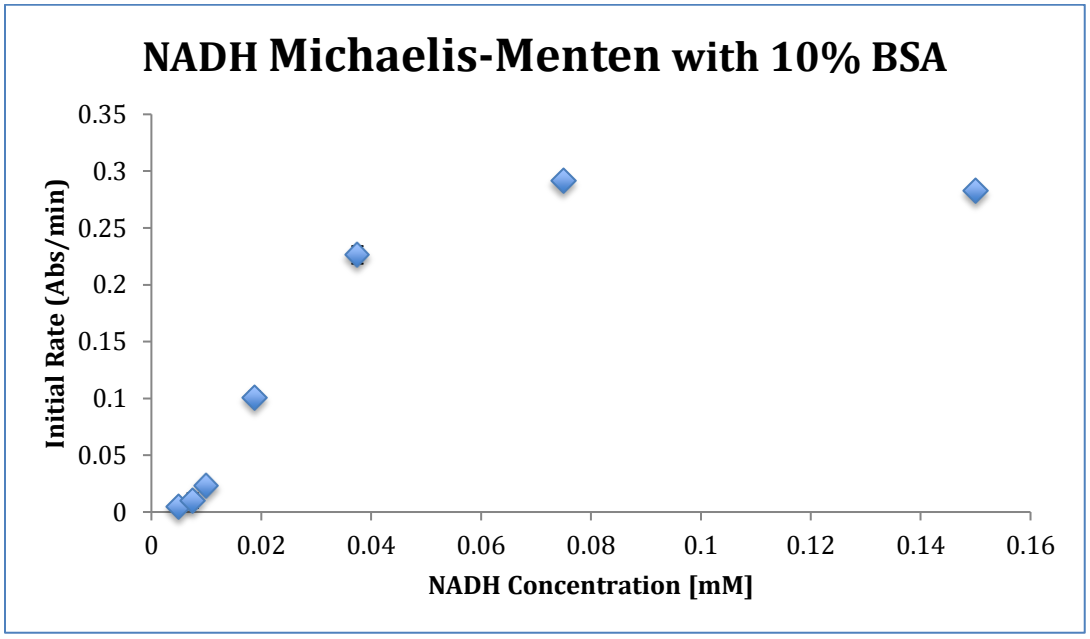

Figure 3: Sigmoidal Michaelis-Menten curve for NADH with 10% BSA (w/v%) solution.

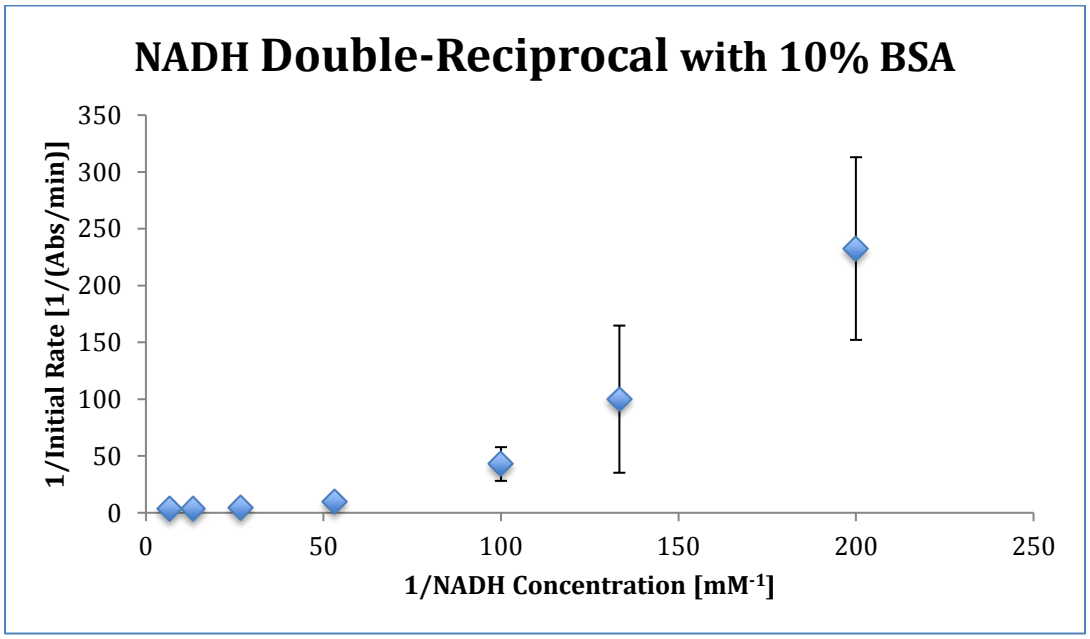

Figure 4: Non-linear Double-Reciprocal Plot for NADH Run 1.

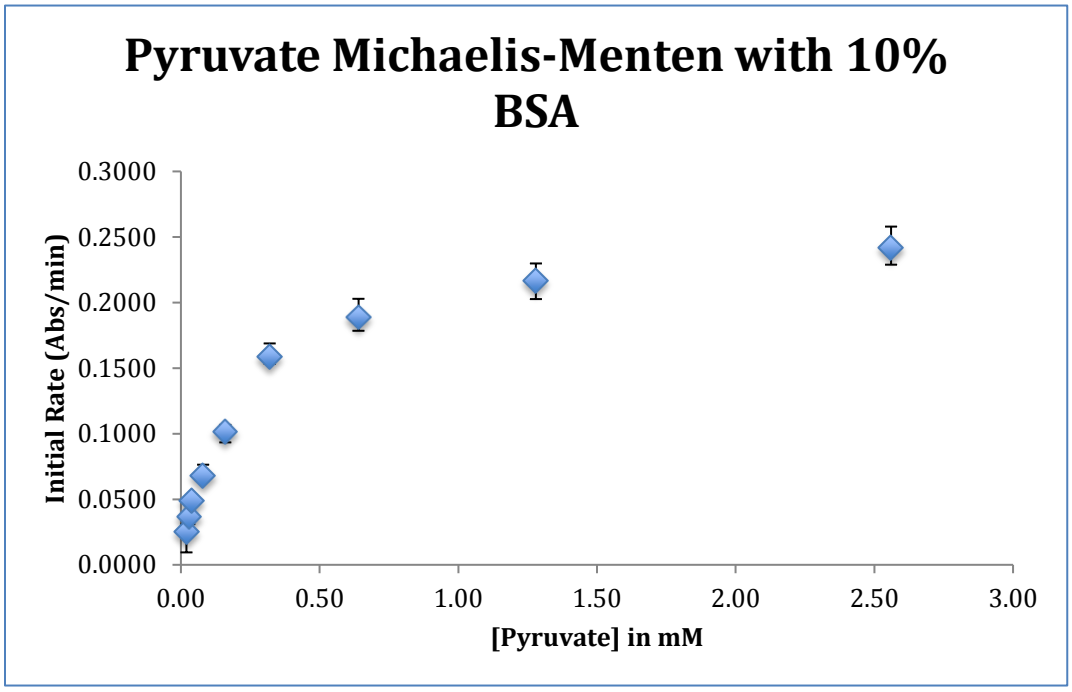

Figure 5: Classic Michaelis-Menten curve with pyruvate in 10% BSA (w/v%)

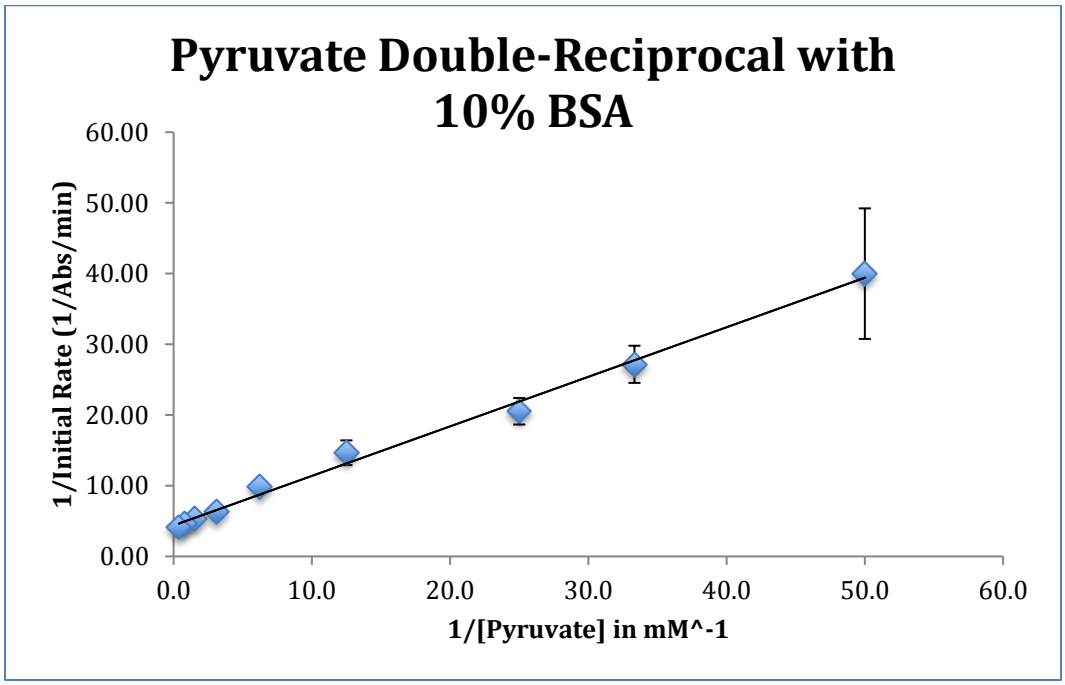

Figure 6: Linear Double-Reciprocal plot for Pyruvate in 10% BSA (w/v%).

Discussion

With the confirmation of pyruvate and NADH substrate kinetic effects on LDH, macromolecular crowders were introduced. Both macromolecular crowding agents caused inhibition of LDH activity. Ficoll-70, as exemplified in Figure 1, produced a moderate inhibition of LDH activity at higher concentrations (~10%). At 10% by weight, there was a 12% decrease in activity of LDH using Ficoll-70. Similarly, BSA, as seen in Figure 2, saw a significant inhibition of LDH activity at higher concentrations (12-20%). At 12%, there was a 25% decrease of activity, however, at 20% concentration, there was over a 50% decrease in activity. LDH inhibition with BSA at these concentrations contradicts the literature findings that our hypothesis cited. Our work concluded that BSA has an inhibitory effect on LDH activity.

Experiments were continued with a 10% BSA solution for pyruvate. Figure 5 demonstrates no change in a typical hyperbolic curve. Additionally, the double reciprocal plot in Figure 6 is linear which is not hyperbolic behavior in terms of kinetic modeling. There was no evidence of inhibition at the higher concentrations of pyruvate.

Results with NADH substrate were very different compared to those with pyruvate. Figure 3 illustrates a sigmoidal-shaped "S" curve instead of the expected hyperbolic curve for a Michaelis-Menten plot. The sigmoidal shape of the curve is indicative of cooperativity. The non-linear correlation for the double reciprocal plot in Figure 4 further confirms this sigmoidal behavior. Cooperativity in mammalian LDH has not been previously reported, and these results could be very significant to our understanding of mammalian LDH as it behaves within the cell. Also important to note is that there may have been slight inhibition at the highest concentration of NADH. Inhibition of LDH at higher concentrations has been reported in literature.

It is important to increase our confidence in these results, since the sigmoidal behavior hinges on the influential points of the lower concentration of NADH substrate. The lower the concentration of substrate, the more difficult it was to obtain precise rates. The error bars at these lower concentrations are apparent in Figures 4 and 6, though double reciprocal plots are known to exaggerate the error.

Conclusion

Both Ficoll-70 and BSA were observed to inhibit the activity of LDH, with BSA showing a greater inhibition at equivalent concentrations. Further experimentation with BSA found that nothing unusual occurred with the pyruvate, but sigmoidal behavior resulted with NADH. Sigmoidal behavior with NADH suggests cooperativity with LDH enzyme. Further work must be done to corroborate these results.

Future Studies

It is essential to continue our work in characterizing the kinetic behavior of LDH under crowded conditions. It would be significant definitively demonstrate that mammalian LDH exhibits cooperativity with NADH in crowded solution. In addition, to further validate the results of this work, there are several other aspects of interest. First, it is necessary to increase the concentration of model crowder to 30-40% because this is the concentration of crowders observed in a typical cell. Furthermore, we should explore effects of a mixture of crowders under controlled concentrations, which have not been reported in literature. We will also study the effects of other model crowders not examined in this project such as polyethylene glycol (PEG), and other proteins, such as ovalbumin and hemoglobin. Finally, we should examine the effect of macromolecular crowding during inhibition of LDH through oxalate or oxamate, known inhibitors of LDH, to better understand the potential of these LDH inhibitors in treating cancer.

Acknowledgements

I would like to thank Dr. David W. Seybert, my faculty advisor for guiding me on my project and giving me the chance to work on it. I would like to thank the Duquesne University Research Program and Duquesne University for giving me the resources needed to complete this project. I would also like to thank Caroline Cwalina for helping with experiments on LDH without any crowder and for assisting with the procedure for BSA experiments. Lastly, I would like to thank the Duquesne University Research Symposium for selecting me to give an oral presentation and allowing me to publish this research in their online proceedings.

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