

Metformin Inhibition of Pyruvate Carboxylase in the Gluconeogenesis Pathway

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Abstract

Metformin is an antihyperglycemic drug prescribed for the treatment of type II diabetes. While it is known to inhibit gluconeogenesis, debate abounds regarding the exact mechanism by which metformin enacts this inhibition. The present study hypothesized that metformin, specifically in concentrations ranging from 250 μ M - 500 μ M to simulate hepatic portal vein conditions, inhibits gluconeogenesis through inhibition of pyruvate carboxylase (PC), the enzyme responsible for catalysis of the first committed step of gluconeogenesis. This hypothesis was tested through observation of reaction kinetics, assessed with a diode array spectrophotometer measured at a loss of absorbance of 340nm. Enzyme-linked reactions were treated with various concentrations of metformin ranging from 250 μ M - 5000 μ M of metformin, as well as a control with 0 μ M of metformin. Spectrophotometric data was graphed, and linear relationships between absorbance and time were observed in order to ascertain the effects of metformin concentration on reaction rate. 13 C-NMRs were performed to assess the functional groups of metformin, pyruvate, and biotin. A loss of a carbonyl group for pyruvate, oxaloacetate, and biotin was expected when these substances were in the presence of metformin. Metformin was found to inhibit PC at concentrations above 1000 μ M, and a loss of a carbonyl group was found for biotin when mixed with metformin. Although this does not fully support the hypothesis, these data offer significant implications for the future of metformin study as well as for public health, as type II diabetes is a growing global problem which demands improved patient outcomes and treatment methods, and it is of benefit to study the leading prescribed drug for the treatment of this disease.

Keywords: type II diabetes, gluconeogenesis, pyruvate carboxylase, metformin

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Introduction

Metformin is the most widely prescribed drug for the treatment of type II diabetes. This drug is administered for the purpose of lowering blood glucose; yet, the mechanism of this effect remains unclear (Baur & Birnbaum, 2014). Metformin is generally known to inhibit gluconeogenesis (Hundal et al., 2000; Lee et al., 2013; Stumvoll et al., 1995), a pathway that creates glucose out of building blocks amassed from the liver. Through this, blood glucose levels rise, contributing to hyperglycemia in type II diabetic patients (National Institute of Diabetes and Digestive and Kidney Diseases [NIDDK], 2016). Several theories exist regarding the mechanism by which metformin works (Lee et al., 2013; He & Wondisford, 2015; Mráček, Drahota & Houštěk, 2012; Madiraju et. al, 2014), but a definitive mechanism remains to be identified. This study hypothesizes that metformin inhibits gluconeogenesis through the inhibition of pyruvate carboxylase (PC), an enzyme involved in the first step of gluconeogenesis. This study will observe the potential effect of metformin in this initial step of the gluconeogenic pathway.

During gluconeogenesis, lactate is converted into oxaloacetate, which is later reduced to form malate (Kiran, 2015). This initial conversion of lactate into oxaloacetate is catalyzed by PC, and NADH is a coenzyme necessary for the later conversion of oxaloacetate into malate, catalyzed by malate dehydrogenase (MDH). Using a diode array spectrophotometer, NADH concentrations will be measured over time under varying concentrations of metformin. Data will be used to describe the rate of PC catalysis on gluconeogenesis, helping to indicate the effect of metformin on catalysis of

this reaction. Furthermore, ^{13}C -NMRs will be performed in order to assess the functional groups of pyruvate, oxaloacetate, and biotin, a prosthetic group of PC which interacts with PC and enables it to function (Chaambra, 2015). These substances were then each mixed with metformin, and the ^{13}C -NMR of the mixture was compared with the individual ^{13}C -NMRs for metformin and the appropriate substance to ascertain changes to the molecules once combined.

The purpose of this study is to observe the effects of metformin on PC catalysis during gluconeogenesis. This study hypothesized that metformin in concentrations ranging from $250\mu\text{M}$ - $500\mu\text{M}$ inhibits gluconeogenesis through inhibition of pyruvate carboxylase (PC), as well as that pyruvate, oxaloacetate, and biotin will each lose a carbonyl group and gain an amine group once mixed with metformin. Understanding of the metformin mechanism of action will allow for greater understanding of type II diabetes itself, as well as pave the way for improved treatment of this disease and even a potential cure, as well as will allow for better understanding and mitigation of metformin side effects, such as lactic acidosis, a debilitating and often fatal condition which is associated with metformin consumption in rare cases (Duong et al., 2013). Furthermore, observation of the effect of metformin on PC and general understanding of the regulatory role of PC on the gluconeogenesis pathway will help amass support for the metabolic control theory, which posits that major metabolic pathways like gluconeogenesis are not simply controlled by one primary enzyme, such as phosphoenolpyruvate carboxylase (PEPCK) in the case of gluconeogenesis, but rather are controlled by several enzymes and molecules working in tandem with one another in response to cellular stresses and

environmental changes (Fell, 1997).

The implications of this research extend beyond purely academic advancement. Type II diabetes is on the rise, and is becoming more prevalent among younger demographics (CDC, 2017). It is of utmost importance to address this disease and to understand the mechanisms by which current treatments work to lower blood glucose levels, such that greater understanding of type II diabetes may be gained as well as novel treatments for this disorder may be developed. Type II diabetes can affect any person of any age, but is most likely to affect obese or overweight adults, and most commonly afflicts black, Native American, and Hispanic communities (Spanakis & Golden, 2014). These three ethnicities face the highest rates of poverty in the United States of America (Macartney, Bishaw & Fontenot, 2013), and it is well-documented that health outcomes are poorer among lower socioeconomic levels. Diabetes is a public health crisis which disproportionately affects communities that face internal and external pressures such that health outcomes and general quality of life are already reduced compared to the national average. Understanding the mechanism of metformin action will not only add to the growing body of knowledge regarding type II diabetes, but has human implications as well, and will add to the growing body of hope and optimism in afflicted communities across the nation as well as globally.

Review of Literature

Diabetes Mellitus

Diabetes mellitus, more commonly known as diabetes, is a disorder in which

insulin resistance, insufficient production of insulin, or the absence of insulin production, results in the failure of body cells to take up blood glucose and convert this glucose into usable forms of energy (NIDDK, 2016). This disease is further classified into two categories, diabetes type I and diabetes type II. Type I diabetes is a genetic autoimmune disorder in which insulin-producing cells known as beta islet cells are mistakenly recognized as foreign and destroyed by the immune system, and this disease is typically diagnosed in children and youths (Joslin Diabetes Center).. Type II diabetes is characterized by lessened sensitivity or inability for insulin receptors to recognize insulin, and is typically diagnosed in middle-aged persons (NIDDK, 2016). In normal physiology, specialized cells known as the beta islet cells of the pancreas secrete a hormone known as insulin, which stimulates body cells, adipose cells, and liver cells to take up excess blood glucose for use in cellular respiration, storage as fat, and storage as glycogen, respectively. In type II diabetes, cell receptors for insulin become less responsive to this signal, causing the insulin signal in the body to be weakened (Diabetes Teaching Center at the University of California, San Francisco [DTCUCSF]). This weakened cell response results in diminished uptake of glucose by muscle, liver, and fat cells, which leads to an excess of blood glucose, also known as hyperglycemia, in type II diabetic patients.



Figure 1: Insulin resistance in type II diabetic cells leads to lessened glucose uptake (DTCUCSF).

Symptoms of type II diabetes include increased hunger, thirst, frequent urination, weight loss, fatigue, blurred vision, slow-healing times, increased rate of infection, and dark patches of skin (Mayo Clinic, 2016). Complications of type II diabetes include heart and blood vessel disease, nerve damage which may include loss of feeling in the limbs, kidney damage which may lead to kidney disease, eye damage including increased risk for cataracts, glaucoma, or potentially blindness, foot damage which may later lead to lower limb amputation, hearing impairments, skin conditions including increased susceptibility to infections, and Alzheimer's disease (Mayo Clinic, 2016). Current treatments for type II diabetes include lifestyle modifications that help to incorporate healthy exercise and diet, as well as oral medications with hypoglycemic effects, such as metformin (George & Copeland, 2013).

Metformin Effects and Uses

Metformin is an antidiabetic drug with hypoglycemic effects, that is, metformin acts to lower blood glucose levels. Known by the chemical name, dimethylbiguanide, metformin belongs to a group of chemicals known as biguanides. Metformin acts as an antidiabetic drug in the treatment of type II diabetes by increasing insulin sensitivity as well as lowering blood sugar, LDL cholesterol, and triglyceride levels (PubChem).

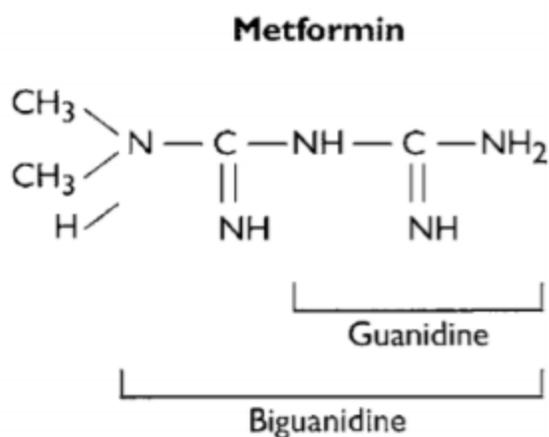


Figure 2: Chemical structure of dimethylbiguanide, a.k.a. Metformin (Stumvoll, Häring, Matthaei, 2007).

The hypoglycemic potential of guanides is well-documented, dating back to medieval Europe, when guanide extracts of *Galega officianalis*, commonly known as goat's rue or French lilac, were used to treat diabetes (Stumvoll, Häring, & Matthaei, 2007). Metformin and other biguanides were first synthesized in the 1920s, and metformin was clinically synthesized in the 1950s and introduced in 1957 under the name of glucophage, or “glucose eater”, as a treatment for type II diabetes (Bailey & Day, 2004; Stumvoll, Häring, & Matthaei, 2007). Metformin was officially approved for use by the U.S. Food and Drug Administration in 1995, and has since been the leading

prescribed drug for the treatment of type II diabetes (Stumvoll, Häring, & Matthaei, 2007).

In type II diabetic patients, increased gluconeogenesis as well as reduced insulin sensitivity contribute to hyperglycemia. Metformin works to inhibit gluconeogenesis as well as improve insulin sensitivity, thereby reducing blood glucose levels in type II diabetic patients (Stumvoll et al., 1995; Hundal et al., 2000).

Metformin is generally preferred to other hypoglycemic drugs due to its relative safety, low cost, and accessibility (PubChem). Metformin also has been shown to possess anticancer properties, as well as weight loss effects for obese patients (Inzucchi et al., 2012; University of Pennsylvania School of Medicine, 2016). However, certain adverse effects associated with metformin have been identified as well. These side effects include flatulence, diarrhea, abdominal pain, and lessened absorption of vitamin B12 (He et al., 2009). Metformin users with underlying liver or kidney complications are at risk for a serious and oftentimes fatal condition known as lactic acidosis. This condition is characterized by severe vomiting, diarrhea, and kidney damage (Duong et al., 2013).

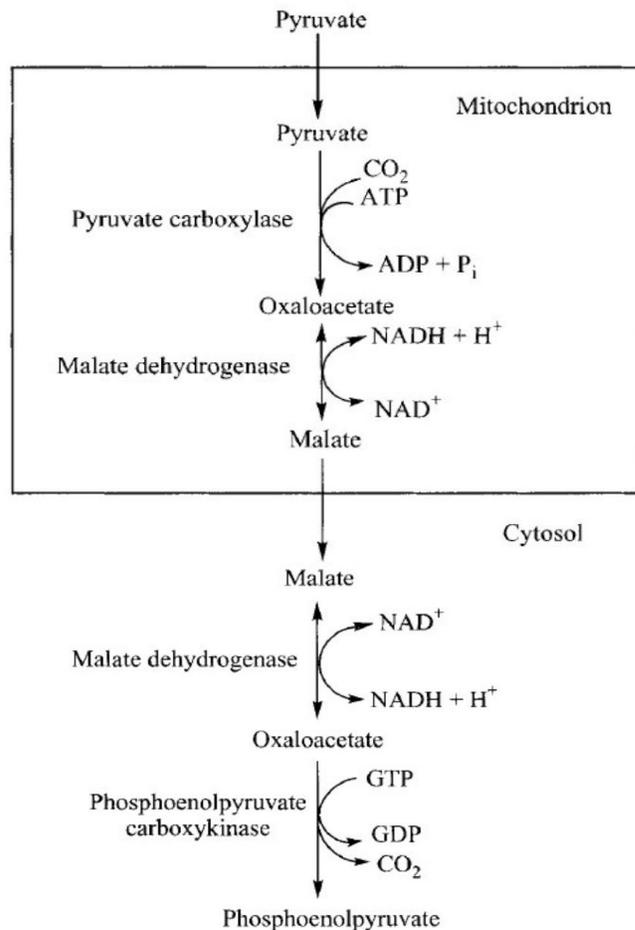
Pharmacokinetics of Metformin. Clinical dosages of metformin are not to exceed 35 mg/kg of body weight (Ismail, Soliman and Nassan, 2015). After being ingested, metformin is absorbed by the gastrointestinal tract and is then transported to the liver via the hepatic portal vein. In the hepatic portal vein, metformin is found in concentrations ranging from 40-70 μM (Duong et al., 2013). Metformin concentrations then rise to 220 μM once inside the liver (Jin et al., 2009). After entry into the liver, metformin is distributed to the rest of the body, and can be found in concentrations

ranging from 40-10 μM (He and Wondisford, 2015). Mimicking the concentrations of metformin in certain target organs is crucial in studies which assess its method of action. Debate regarding the mechanism by which metformin inhibits gluconeogenesis is in part due to studies with ranging metformin concentrations; too high or too low study concentrations which do not accurately represent pharmacological concentrations may yield inaccurate results. In the present study, concentrations above, below, and consistent with pharmacologically realistic metformin concentrations, ranging from 250 μM to 5000 μM of metformin, are used to ensure accurate results (He & Wondisford, 2015).

Mitochondrial Gluconeogenesis

Gluconeogenesis is a pathway involving the synthesis of glucose, and is present in the liver and the kidneys of all mammals. (Hanson & Owen, 2014). In this pathway, pyruvate and lactate are converted into oxaloacetate by PC. Several steps in the gluconeogenesis pathway then convert oxaloacetate into the final form of glucose, one of which involves the use of malate dehydrogenase (MDH) to catalyze the conversion of oxaloacetate to malate. This conversion involves the oxidation of NADH into the reduced form, NAD⁺. Other enzymes involved in this process include phosphoenolpyruvate carboxylase (PEPCK), which converts oxaloacetate into phosphoenolpyruvate (Kiran, 2015).

GLUCONEOGENESIS



Intracellular localization of the initial steps of gluconeogenesis.

Figure 3. (Kiran, 2015)

PEPCK Control of Mitochondrial Gluconeogenesis. Hepatic gluconeogenesis is controlled by several enzymes and occurs in two main locations: the mitochondria and the cytosol. Mitochondrial gluconeogenesis is controlled mainly by PC, whereas cytosolic gluconeogenesis is controlled mainly by PEPCK. PEPCK has been established as the main controlling enzyme of hepatic gluconeogenesis, dominant over PC (Rognstad, 1979; Veneziale, Donofrio & Nishimura, 1983). PEPCK's dominant role in

the gluconeogenesis pathway has even allowed for its use as an indicator of gluconeogenic activity in many studies (Chakravarty et al., 2005). However, recent literature suggests that PEPCK is not as closely related to gluconeogenesis activity as is generally proposed. In studies involving hyperglycemic mice with high-fat diets, there was no significant correlation between PEPCK mRNA transcription and blood glucose levels, and *in vitro* protein analysis of hepatic cells suggested a weak correlation between PEPCK protein expression and hyperglycemia in diabetic patients (Burgess et al., 2007). This data challenges the supposition that PEPCK is the dominant enzyme for hepatic gluconeogenesis control.

PC Control of Mitochondrial Gluconeogenesis. PC catalyzes the first step of gluconeogenesis, converting pyruvate into oxaloacetate (Jitrapakdee et al., 2008). This step is enabled by biotin, a prosthetic group which activates PC and enables it to function (Chaambra, 2015). PC exerts control on mitochondrial gluconeogenesis, but is alleged to be inferior to PEPCK in control of gluconeogenesis as a whole. However, data suggests greater control of gluconeogenesis by PC during type II diabetes. Specialized cells of the pancreas known as beta islet cells produce insulin, the hormone which downregulates hepatic gluconeogenesis and whose signal is resisted by receptors in type II diabetes (Ip et al., 2012). Literature suggests that PC is highly expressed in beta islet cells of the pancreas, whereas PEPCK is not significantly expressed in these cells (Weir & Bonner-Weir, 2004; Sugden et al., 2011). Moreover, rats exposed to high-fat diets exhibited reduced hyperglycemia during fasting, reduced insulin concentrations during fasting, and reduced glucose production when PC function is lost (Kumashiro et al.,

2013). This data suggests that the loss of PC is a powerful inhibitor of gluconeogenesis, suggesting greater gluconeogenic control by PC than is traditionally assumed.

Gluconeogenesis in Type II Diabetes. In normal physiology, the gluconeogenesis pathway is only active during periods of starvation or cellular stress, but in diabetic patients, this pathway is overactive even under feeding conditions (Hanson & Owen, 2014). Insulin resistance combined with upregulated gluconeogenesis leads to hyperglycemia in type II diabetes. Therefore, it has been established that metformin works to lower blood glucose in type II diabetic patients through control of hepatic gluconeogenesis, however, the exact mechanism of this effect is contested.

Metformin Effect on Gluconeogenesis

It is well-known that metformin works to lower blood glucose levels through inhibition of hepatic gluconeogenesis (Hundal et al., 2000; Lee et al., 2013; Stumvoll et al., 1995). There are several proposed targets for metformin action, the prevalent theory involving metformin association with the adenosine monophosphate activated protein kinase (AMPK) pathway. This paper proposes metformin action through inhibition of PC, and will evaluate prevalent opposing theories.

Metformin Action Through PC Inhibition. Literature reveals that PC assumes a greater controlling role in the gluconeogenesis pathway, especially during type II diabetes, than is traditionally posited. Recent studies also reveal mounting evidence for a PC-related pathway for metformin action. In a study involving mice with high-fat diets, the test subjects produced lower amounts of malate when exposed to metformin (Lee et al., 2013). Given that malate is an intermediate product of the gluconeogenesis pathway,

upstream inhibition of PC may lead to lessened malate yield under metformin action. This data suggests a possible mechanism for metformin control of hepatic gluconeogenesis through PC inhibition.

Metformin Action Through AMPK Pathway. Adenosine monophosphate activated protein kinase (AMPK) is a master regulatory enzyme which maintains cellular energy balance. As a kinase, AMPK turns on or off other proteins and molecules, known as target proteins, by attaching a phosphate group to the surface of the molecules in a process known as phosphorylation (Grahame, 2014). AMPK is a sensor of low energy levels, and once activated, works to activate energy-releasing pathways and inhibit energy-using pathways, one of which is gluconeogenesis.

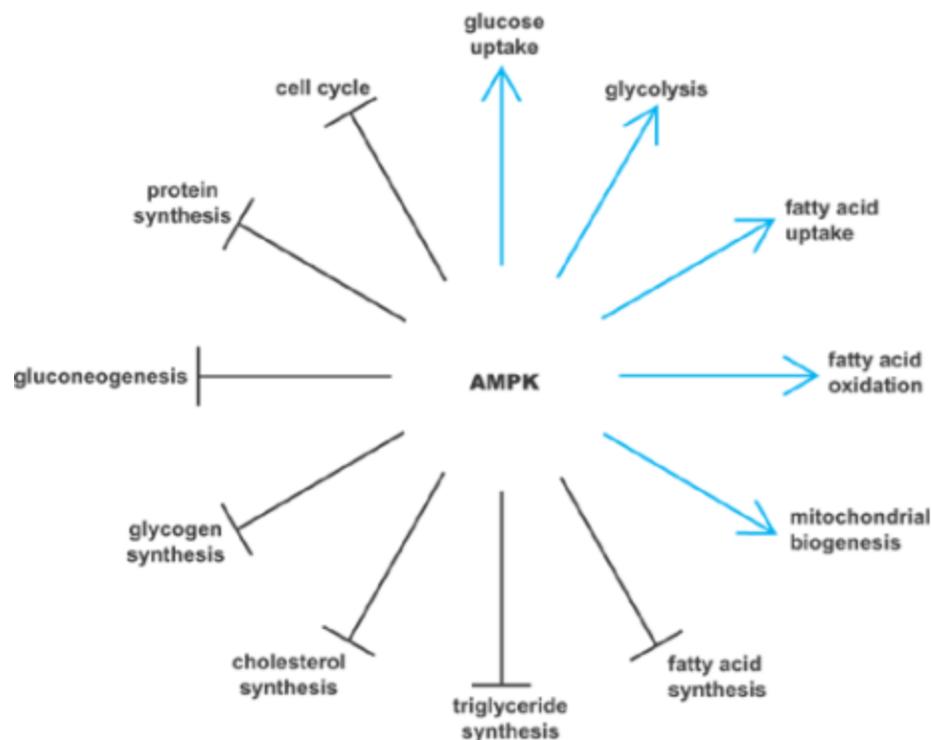


Figure 4: Arrows indicate processes that are switched on by AMPK activation, lines indicate processes that are switched off by AMPK activation (Grahame, 2014).

A prevalent theory regarding the mechanism of action of metformin involves regulation of the AMPK pathway. This theory posits that metformin activates AMPK, which then phosphorylates two downstream target proteins, CBP and CRTC2, which are involved in the disassembly of the CREB co-activator complex, thereby inhibiting gluconeogenic gene expression (He & Wondisford, 2015). In a study involving knockin mice models with mutated acetyl-coA carboxylase 1 and 2 such that AMPK was prevented from phosphorylating these enzymes, the mice exhibited insulin resistance as well as increased gluconeogenesis. This suggests that the AMPK pathway plays an important role in downregulating gluconeogenesis as well as insulin resistance, and it is therefore suggested that metformin works to mitigate these effects through the AMPK pathway.

Metformin Action Through Mitochondrial Glycerophosphate

Dehydrogenase (mGPDH) Inhibition. Another theory regarding the action of metformin posits that metformin works to inhibit mGPDH, an enzyme involved in the reoxidation of electron transport molecules and thereby involved in bridging the gap between the mitochondria and the cytosol in processes such as gluconeogenesis (Mráček, Drahotka & Houštěk, 2012). The metformin–mGPDH method of action proposes that metformin inhibits this redox shuttle enzyme, mGPDH, which alters the redox state of the liver, lowers the rate of conversion of lactate and glycerol to glucose, and ultimately lowers the rate of hepatic gluconeogenesis (Madiraju et. al, 2014).

Type II Diabetes, Gluconeogenesis, and Metformin

Type II diabetes is a disease that can cause serious damage when left untreated.

The interplay of complex processes such as insulin signaling and gluconeogenesis which contribute to the disease's characteristic hyperglycemia allude to the overall complexity and sensitivity of homeostatic pressures in the body. Through greater understanding of the mechanism by which metformin works, side effects such as lactic acidosis, overall themes of type II diabetes, and potential improvements for treatment of this disease may be better understood and identified. This project aims to augment the growing body of knowledge regarding type II diabetes and metformin in the hopes that with increased knowledge comes increased awareness of this disorder, as well as increased chances of discovering a cure for type II diabetes.

Methods and Materials

Studies involving diode array spectrophotometry and ^{13}C -NMR analysis with metformin, PC, and other related inputs and products of gluconeogenesis were conducted in order to ascertain the enzymatic effect of metformin on gluconeogenesis. These studies were performed with assistance, supplies, and lab space provided by the Department of Chemistry and Biochemistry at the University of Northern Colorado, Greeley.

Diode Array Spectrophotometry of PC

Assays of PC and metformin were prepared for analysis in a diode array spectrophotometer provided by the University of Northern Colorado Department of Chemistry and Biochemistry. Protocol for these assays was modified from several pre-existing procedures (Bahl et al., 1997; Duggelby et al., 1982; Jitrapakdee et al., 2008; Warren and Tipton, 1974). Several reagents were prepared for the enzyme-coupled

reaction, including reaction cocktail, PC solution, ATP Sodium Bicarbonate solution, and metformin solution. Assays were run in order to determine the activity of purified bovine PC in the presence and absence of metformin. Metformin was added to test assays in concentrations above, below, and consistent with normal pharmacokinetic concentrations. Conditions among all assays such as temperature, volume, spectrophotometry conditions, pH, etc. were kept constant throughout the experiment.

Reaction Cocktail Preparation. A reaction cocktail comprising of several different solutions was prepared and added to each assay of the spectrophotometry. 72.7% of the cocktail volume was comprised of a solution containing 135 mM Triethanolamine Buffer (TEA), 7mM Magnesium Sulfate, 9 mM Pyruvic Acid, and 0.15% (w/v) Bovine Serum Albumin in deionized water (diH_2O), maintained at a pH of 8.0 and a temperature of 30° C with 1 M HCl to maintain pH balance as needed. 18.2% of the cocktail volume was comprised of a solution containing 150 units of Malic Dehydrogenase in equal volumes of diH_2O and 0.3 mM Acetyl Coenzyme A solution prepared in diH_2O . The remaining 9.1% of the cocktail volume was comprised of a solution containing 26 mM β -Nicotinamide Adenine Dinucleotide, Reduced Form (β -NADH) in diH_2O . This solution was stored on ice.

PC Solution Preparation. PC solution was prepared by adding 32.5 units/ml of PC to cold buffer consisting of 50mM Tris HCl buffer, 50% (v/v) Glycerol, 2 mM Magnesium Acetate, and 1 mM Ethylenediaminetetraacetic Acid (EDTA) in diH_2O at a temperature of 30° C and a pH of 7.4 maintained by 1 M KOH as needed. This solution was stored on ice.

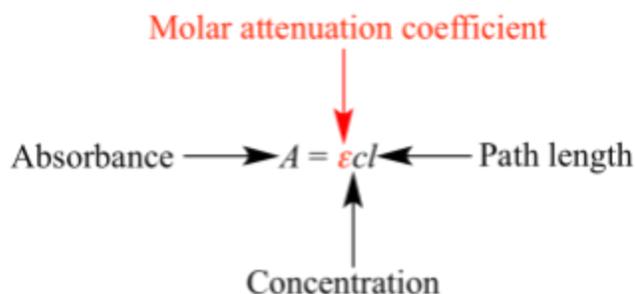
ATP/NaHCO₃ Solution Preparation. A solution of 135 mM TEA, 30 mM Adenosine 5' Triphosphate (ATP) , and 450 mM Sodium Bicarbonate in diH₂O was prepared. Temperature was maintained at 30° C and a pH level of 8.0 was maintained with 1 M KOH. This solution was stored on ice.

Metformin Solution Preparation. Solutions of metformin were prepared at concentrations of 5000μM, 2500μM, 500μM, and 250μM using powdered metformin and diH₂O. These solutions were stored on ice.

Diode Array Spectrophotometry. The spectrophotometer was allowed to equilibrate for at least 30 minutes before use. After equilibration, the spectrophotometer was blanked with a 1 ml, clean cuvette containing 900μL of reaction cocktail, 5μL of Tris/EDTA buffer (50mM Tris HCl buffer, 50% (v/v) Glycerol, 2 mM Magnesium Acetate, and 1 mM EDTA in diH₂O), and 100μL of ATP/NaHCO₃. The assay temperature was maintained at 30° C with a total run time of 1 minute and a cycle time of 5 seconds. In order to blank the spectrophotometer, a 1 mL cuvette was cleaned with a KimWipe, appropriate volumes of solutions were pipetted into the cuvette such that the final solution was appropriately concentrated, the cuvette was again cleaned with a KimWipe, the cuvette was inserted into the diode array spectrophotometer, and the assay reading was initiated by selecting the appropriate icon on the connected computer. After an appropriate graph for the blank was obtained, the cuvette was removed and a new assay was run with the same protocol. For test assays, 800μL of reaction cocktail, 5μL of PC solution, 100μL of ATP/NaHCO₃ solution, and 100μL of the appropriate concentration of metformin solution were added to a 1 mL cuvette, and the assay was run

as normal. For control assays, 900 μ L of reaction cocktail, 5 μ L of PC solution, and 100 μ L of ATP/NaHCO₃ solution were added to a 1 mL cuvette. The cuvettes were cleaned out with diH₂O and acetone after assays were run. In total, 9 test assays and 3 control assays were run.

Analysis of Diode Array Spectrophotometry Data. Beer's Law was used to analyze spectrophotometry data such that useful information regarding the concentration of NADH and the PC reaction rate under the influence of metformin could be determined.



The diagram shows the Beer's Law equation $A = \epsilon cl$ centered on the page. Four arrows point to the variables in the equation: a red arrow points down to ϵ with the label "Molar attenuation coefficient" above it; a black arrow points left to A with the label "Absorbance" to its left; a black arrow points right to l with the label "Path length" to its right; and a black arrow points up to c with the label "Concentration" below it.

Figure 4. Beer's Law. For NADH concentration, $\epsilon=6220\text{m}^{-1}$. $L = 1\text{cm}$. (UCLA Chemistry and Biochemistry).

Reaction rate data from the diode array spectrophotometry was graphed in Microsoft Excel, and a line of best fit was found using linear regression. The slope of this line was considered "A" in the above equation. Using this equation, c , or concentration of NADH, was calculated for each assay. These calculations were performed for each assay in order to determine a rate for the reaction and a concentration value for NADH.

¹³C-NMR Analysis

¹³C-NMR analysis was performed in order to identify the structures of biotin, oxaloacetate, pyruvate, as well as biotin mixed with metformin, oxaloacetate mixed with

metformin, and pyruvate mixed with metformin. Each substance was dissolved in 1 mL of deuterium oxide (D_2O Sigma-Aldrich, 99.9 % analytical grade) and heated in a water bath to a temperature of 32-36° C for 10 minutes. For samples consisting of two substances (metformin mixed with sample substances), equal volumes of each substance were mixed and similarly heated in a water bath to a temperature of 32-36° C for 10 minutes. Final samples for NMR analysis consisted of 450 μ L of sample solution added to 150 μ L of D_2O . ^{13}C -NMR was run using the Bruker Avance I 360 MHz FT-NMR spectrometer provided by the University of Northern Colorado Department of Chemistry and Biochemistry. The ^{13}C -NMRs were maintained at ambient temperature. 15,000 scans were taken per NMR overnight. Graphs were obtained and printed, and the resulting peaks were analyzed in order to ascertain changes to the functional groups of pyruvate, oxaloacetate, and biotin under metformin interaction.

Statistical Analysis

NADH concentrations obtained from Beer's Law calculations for six groups (*i.e.*, group 1 = control, group 2 = 1000 μ M, group 3 = 2500 μ M, group 4 = 250 μ M, group 5 = 5000 μ M, group 6 = 500 μ M) were compared. A test of one-way analysis of variance (ANOVA) through the SAS 9.4 statistical program (Cary, NC) was performed. Further, if the ANOVA was significant, the Duncan-Waller's post hoc analysis was performed to determine which groups differed significantly.

Results

Diode Array Spectrophotometry

A diode array spectrophotometry was performed in order to ascertain the kinetics of the reaction under varying concentrations of metformin. The following data was collected regarding absorbance versus time measured at 340nm for each trial of the enzyme-coupled reaction, and were graphed using Microsoft Excel 2011. Average absorbances versus time for various tested metformin concentrations against the control are graphed below (see Appendix for raw absorbance versus time graphed for each trial of each concentration group). The slopes of the best fit line for each trial are displayed on the graphs, and demonstrate that the reaction rate for the control was faster than the reaction rates for groups treated with metformin.

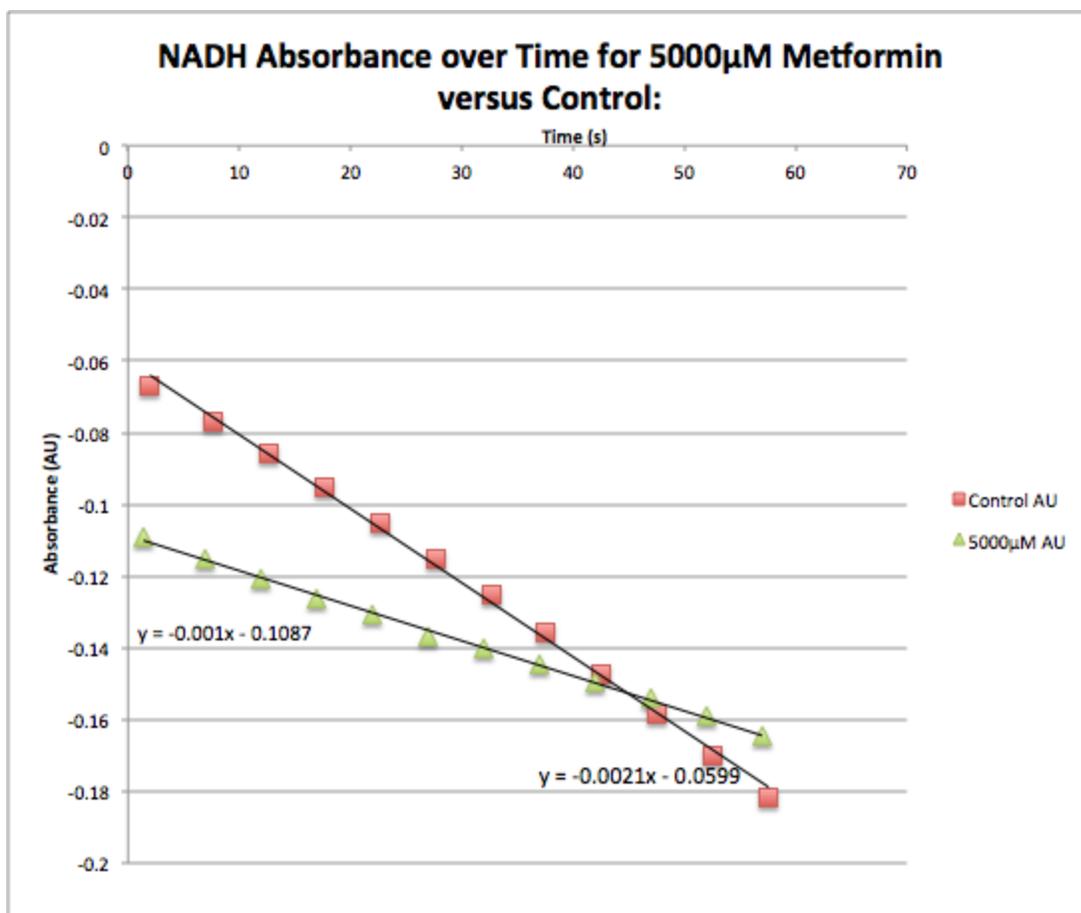


Figure 5: Average absorbance versus time for 5000 μ M metformin versus the

control are graphed above.

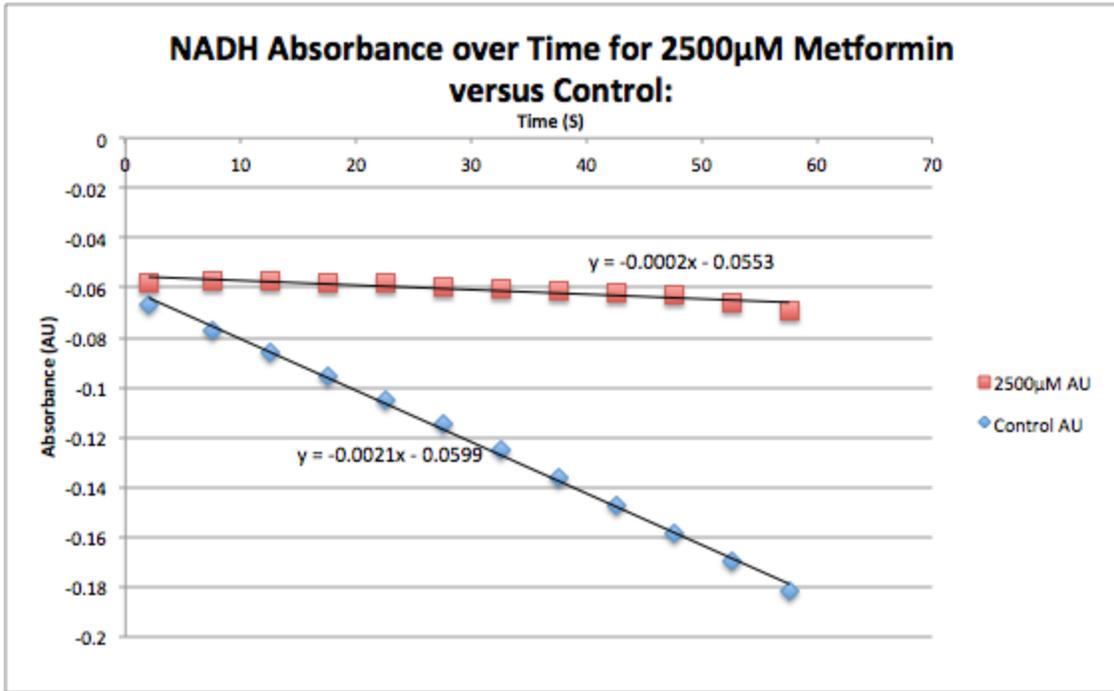


Figure 6: Average absorbance versus time for 2500µM metformin versus the control are graphed above.

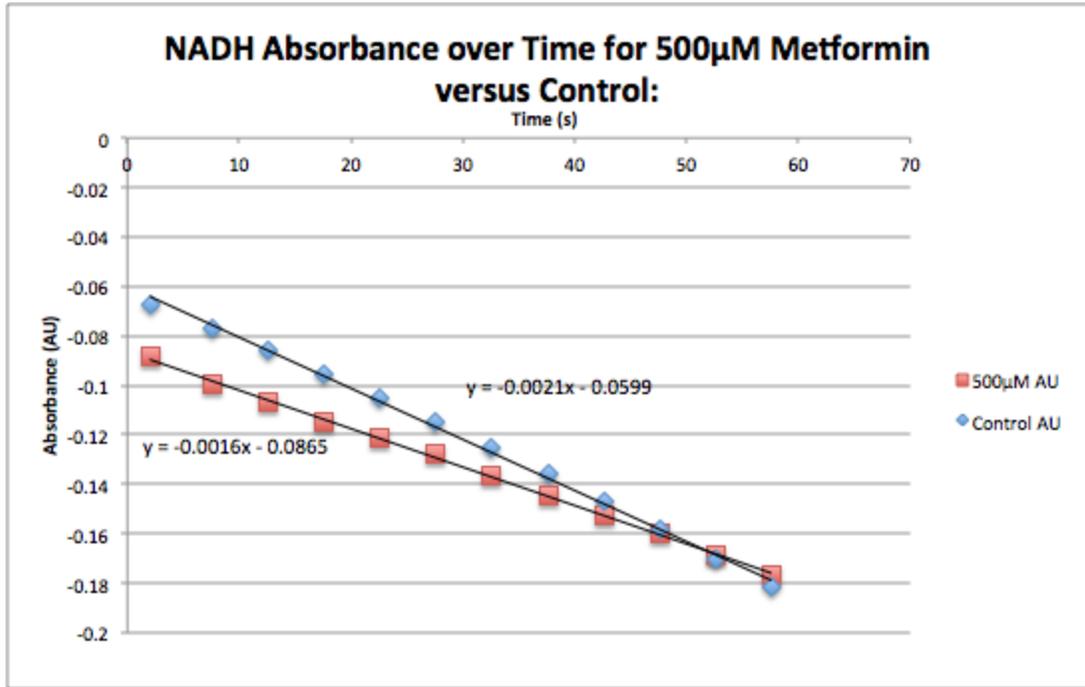


Figure 7: Average absorbance versus time for 500 μ M metformin versus the control are graphed above.

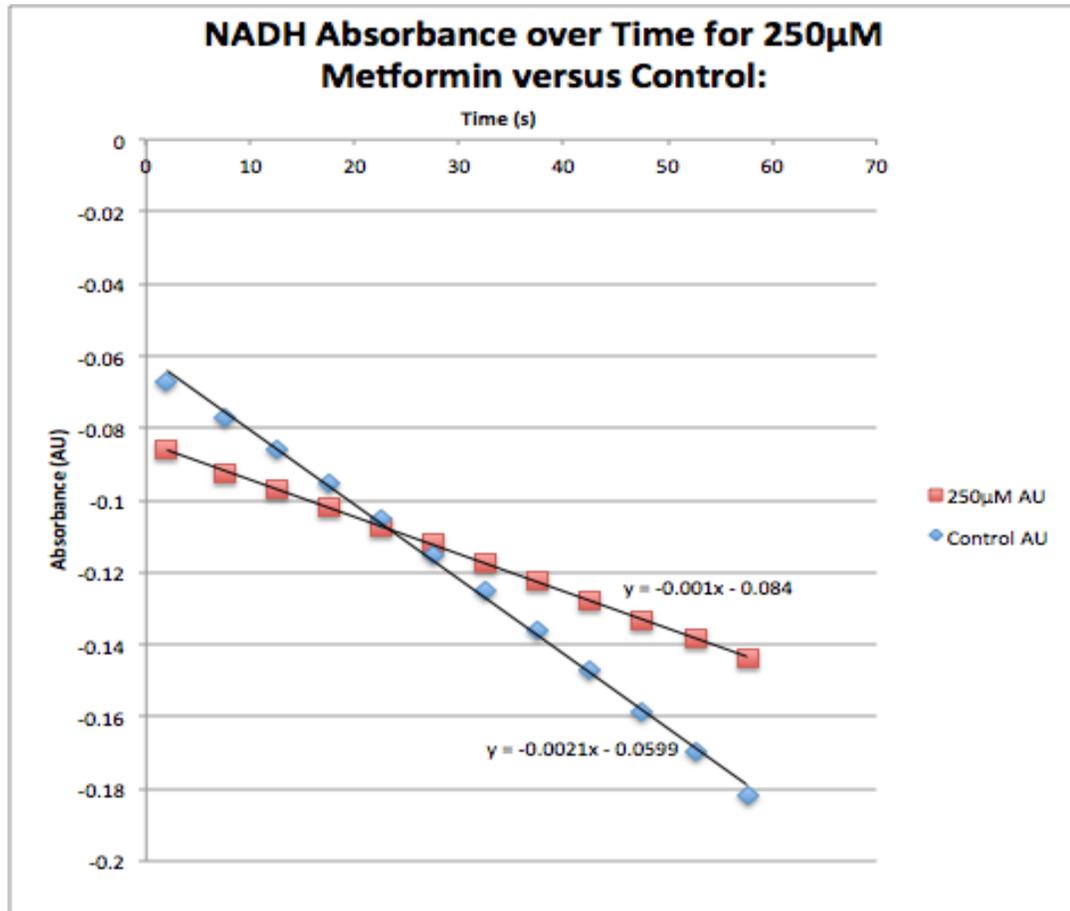


Figure 8: Average absorbance versus time for 250 μ M metformin versus the control are graphed above.

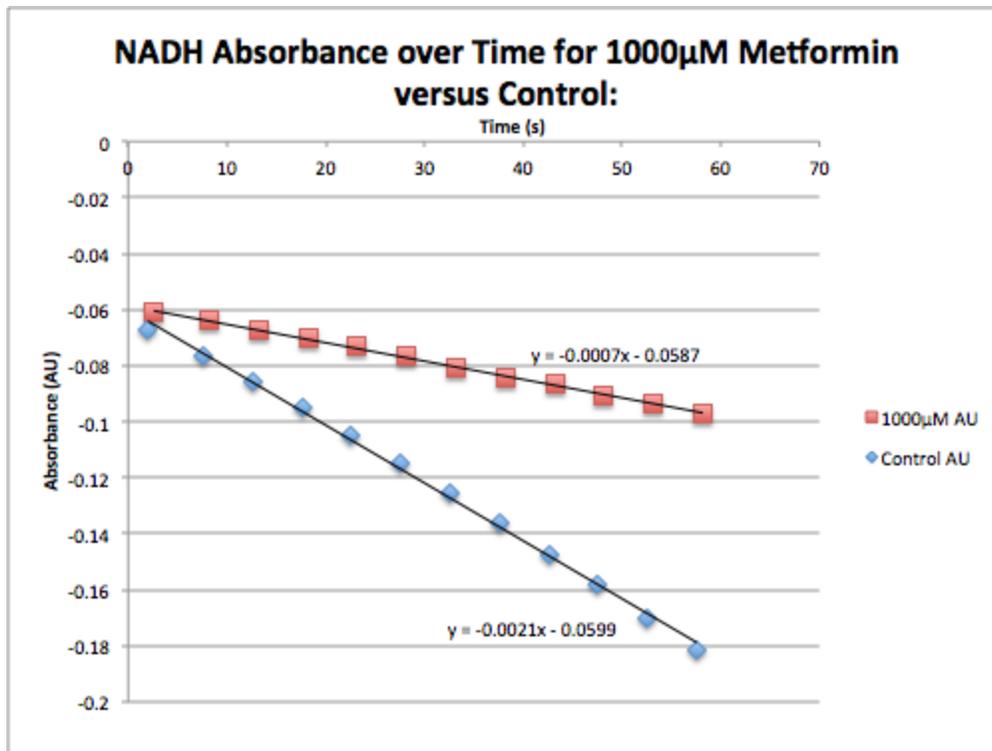


Figure 9: Average absorbance versus time for 1000µM metformin versus the control are graphed above.

Absorbance data was also used to calculate the average net moles of NADH converted into NAD⁺ after a time interval of approximately 60 seconds in order to quantify the average reaction rate for each concentration group. For each trial, absorbance was multiplied by the corresponding time, and total sums of these products were calculated in order to quantify the net total moles of NADH converted to NAD⁺ for each trial. These sums were averaged for each concentration group and are graphed below against the control. For all concentration groups except the 5000µM, the net moles of NADH converted into NAD⁺ was greater for the control than for the experimental group, indicating an overall faster reaction for the control than for most concentration groups of metformin.

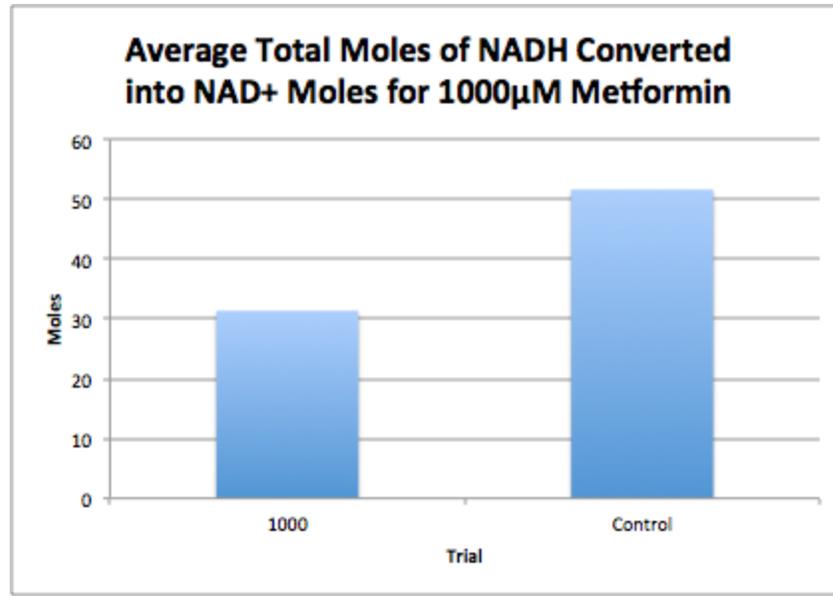


Figure 10: Average net conversion of NADH into NAD⁺ for 1000µM against the control is graphed above.

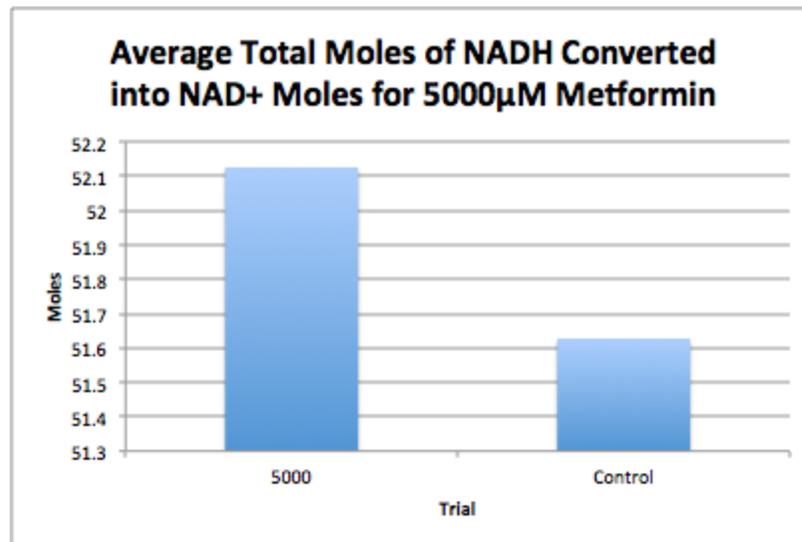


Figure 11: Average net conversion of NADH into NAD⁺ for 5000µM against the control is graphed above.

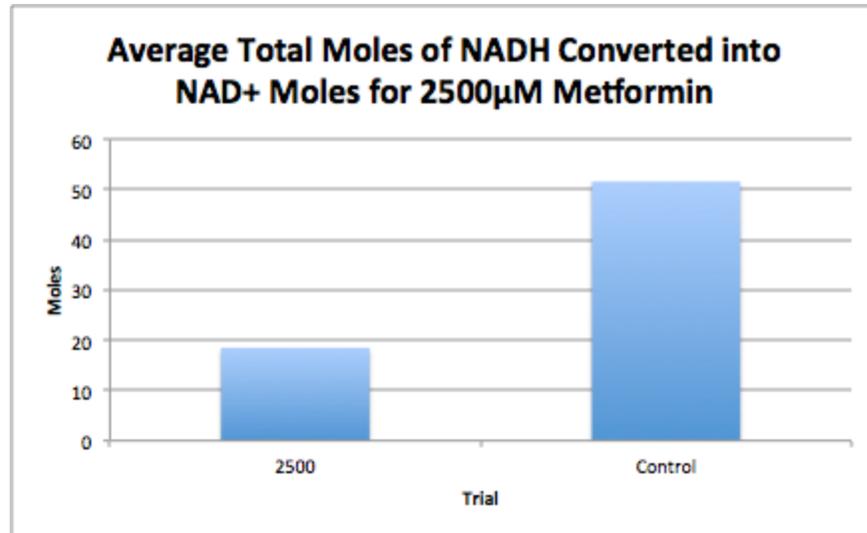


Figure 12: Average net conversion of NADH into NAD⁺ for 2500µM against the control is graphed above.

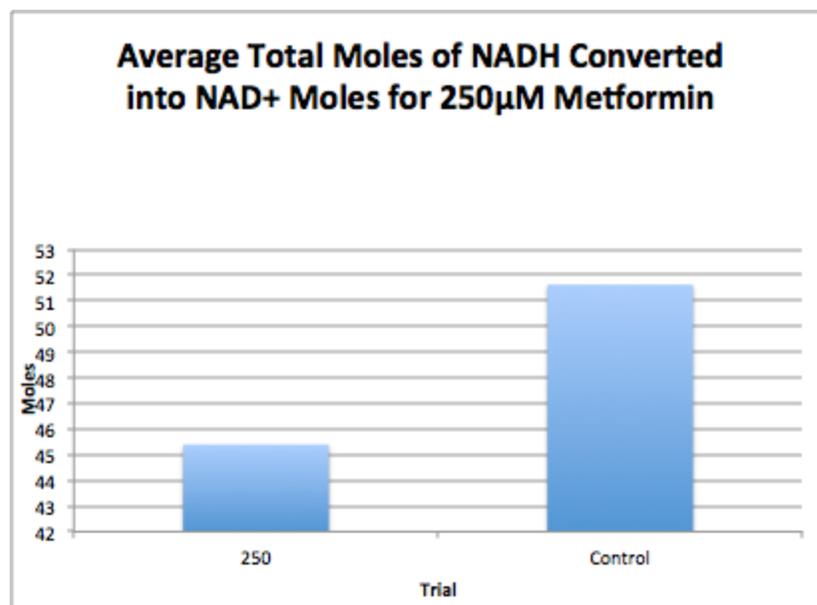


Figure 13: Average net conversion of NADH into NAD⁺ for 250µM against the control is graphed above.

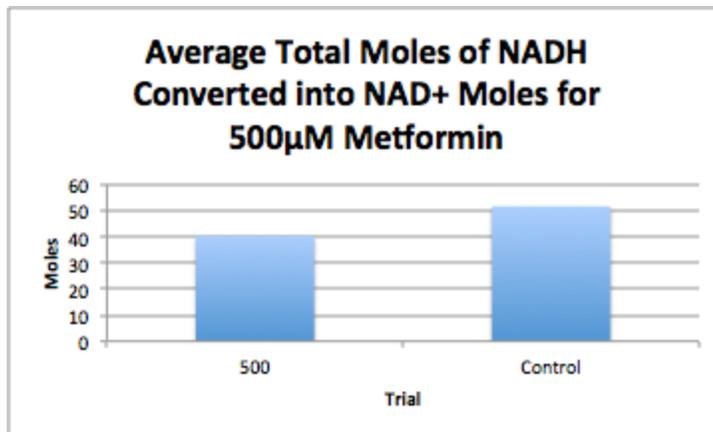


Figure 14: Average net conversion of NADH into NAD⁺ for 500µM against the control is graphed above.

¹³C-NMR Data

¹³C-NMRs were performed in order to analyze the presence and number of functional groups, specifically carbonyls, in metformin, oxaloacetate, pyruvate, and biotin, in order to ascertain the interaction between metformin and gluconeogenesis-related compounds. ¹³C-NMR images for trial 1 of each sample are displayed below (for ¹³C-NMR images for each trial of the samples, see Appendix).

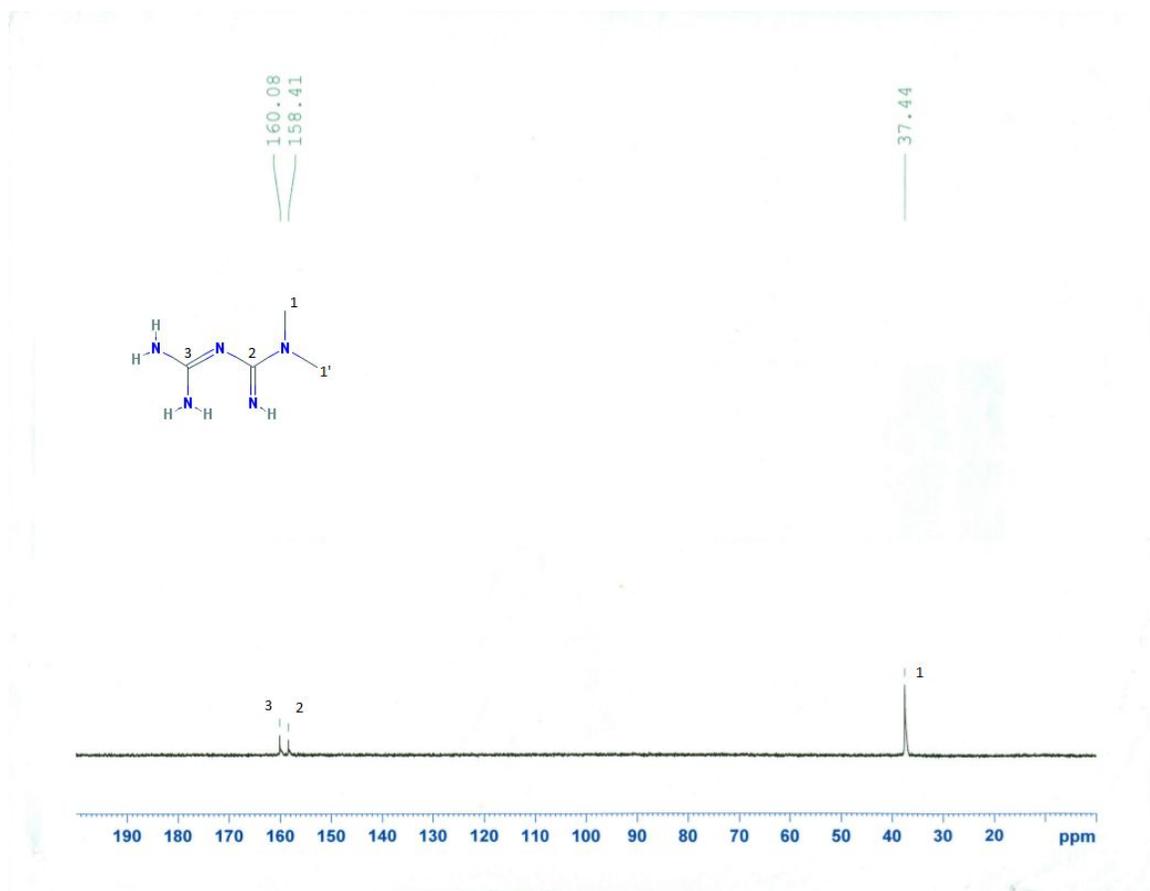


Figure 15: Metformin ^{13}C -NMR Trial 1

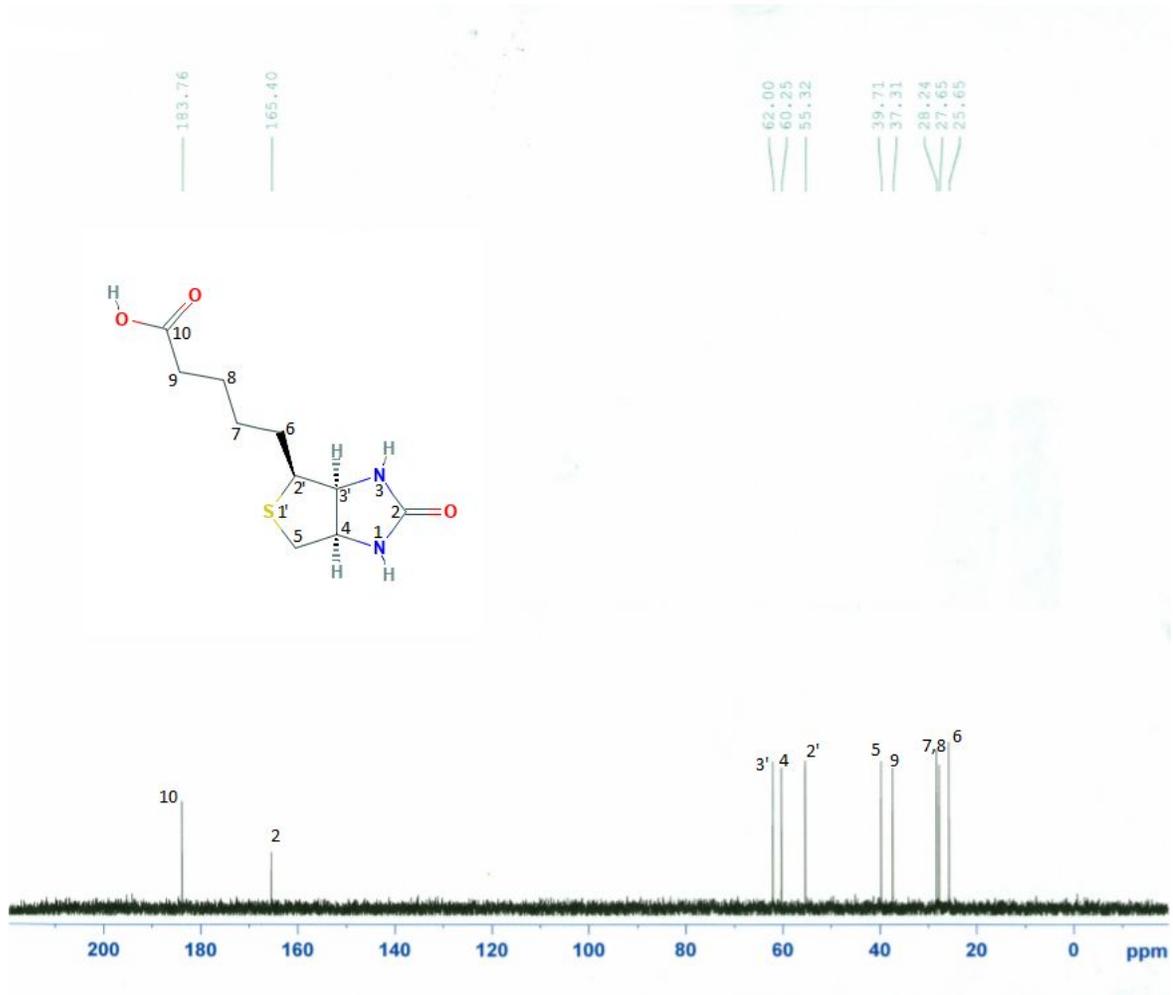


Figure 16: Biotin ^{13}C -NMR Trial 1

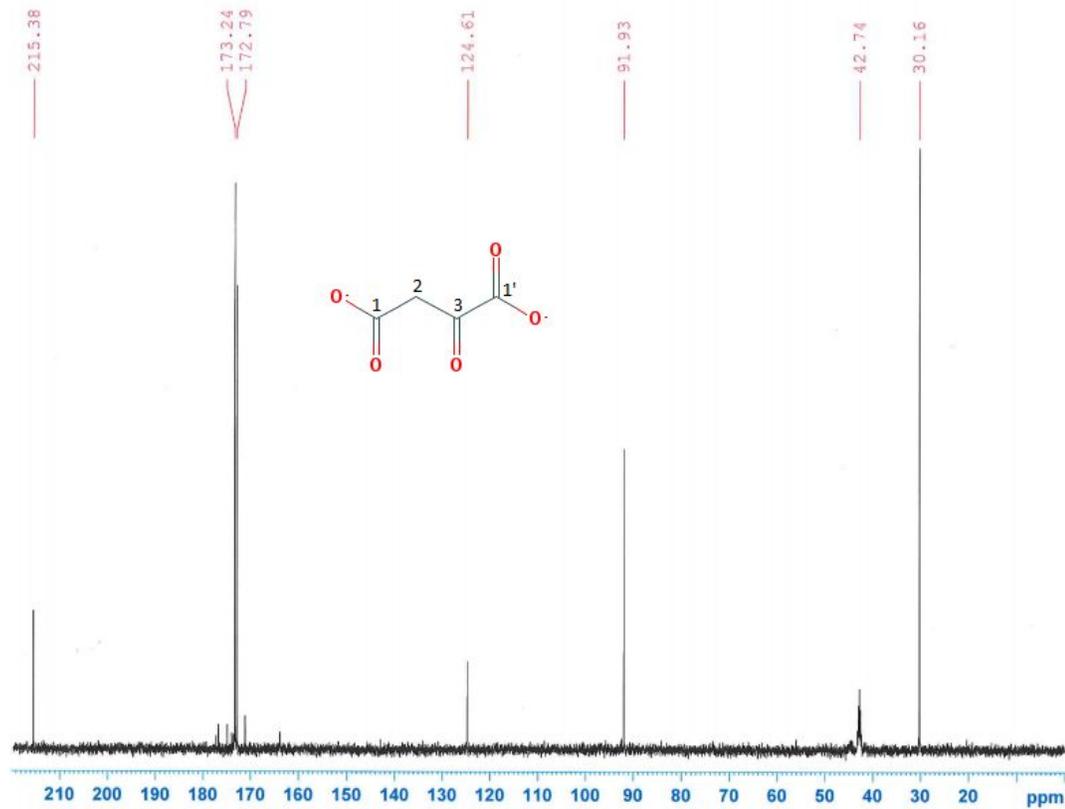


Figure 17: Oxaloacetate ^{13}C -NMR Trial 1

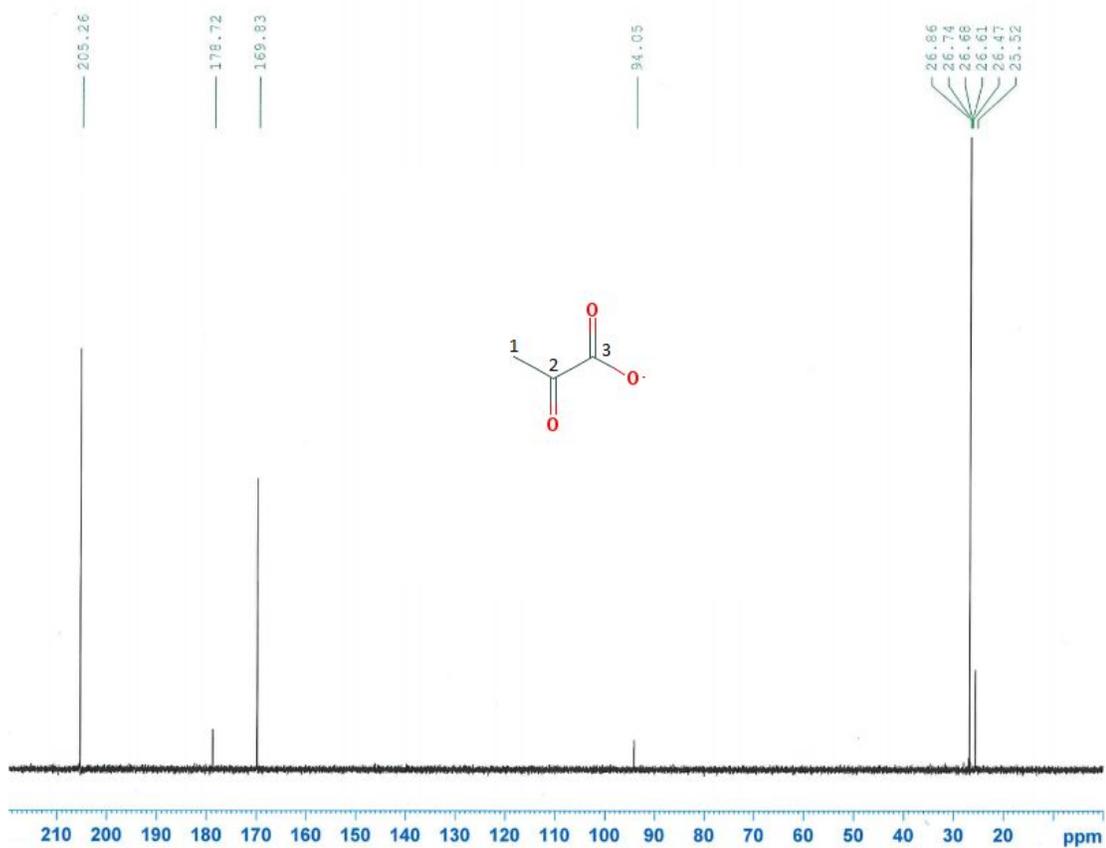


Figure 18: Pyruvate ^{13}C -NMR Trial 1

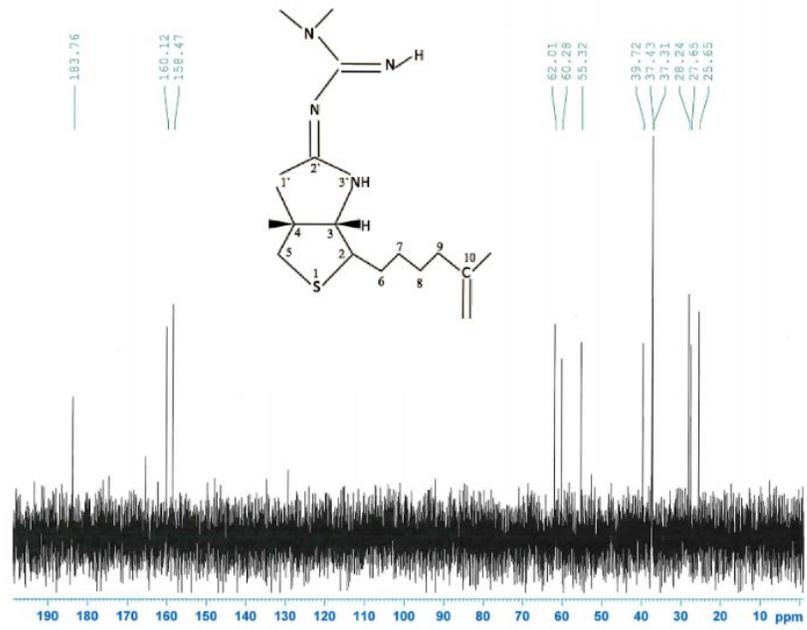


Figure 19: Metformin-Biotin ¹³C-NMR Trial 1

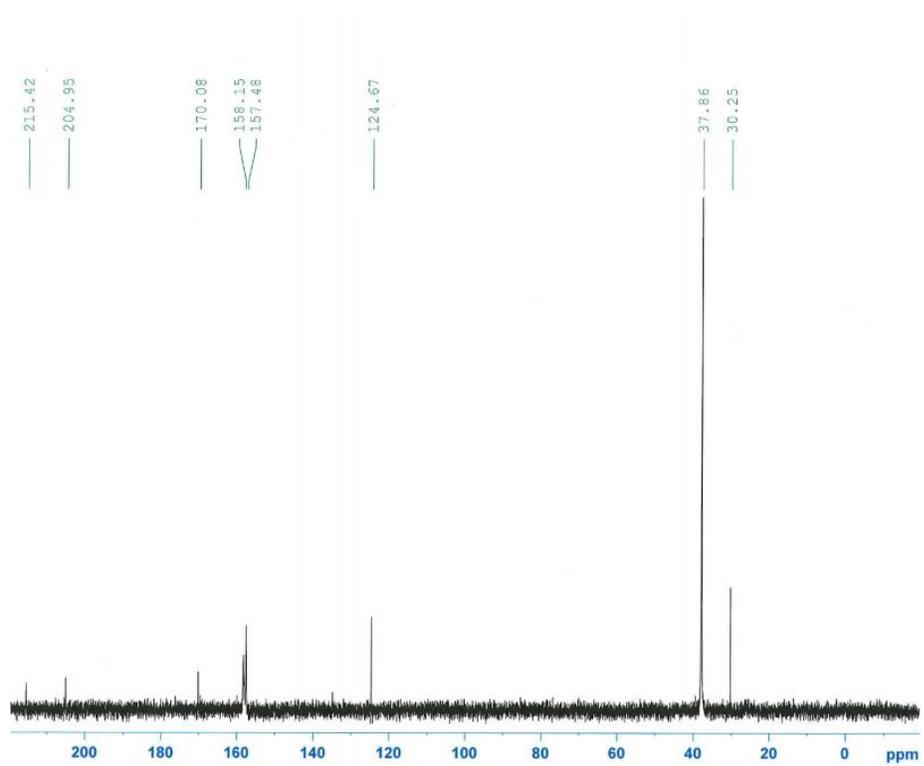


Figure 20: Metformin-Oxaloacetate ¹³C-NMR Trial 1

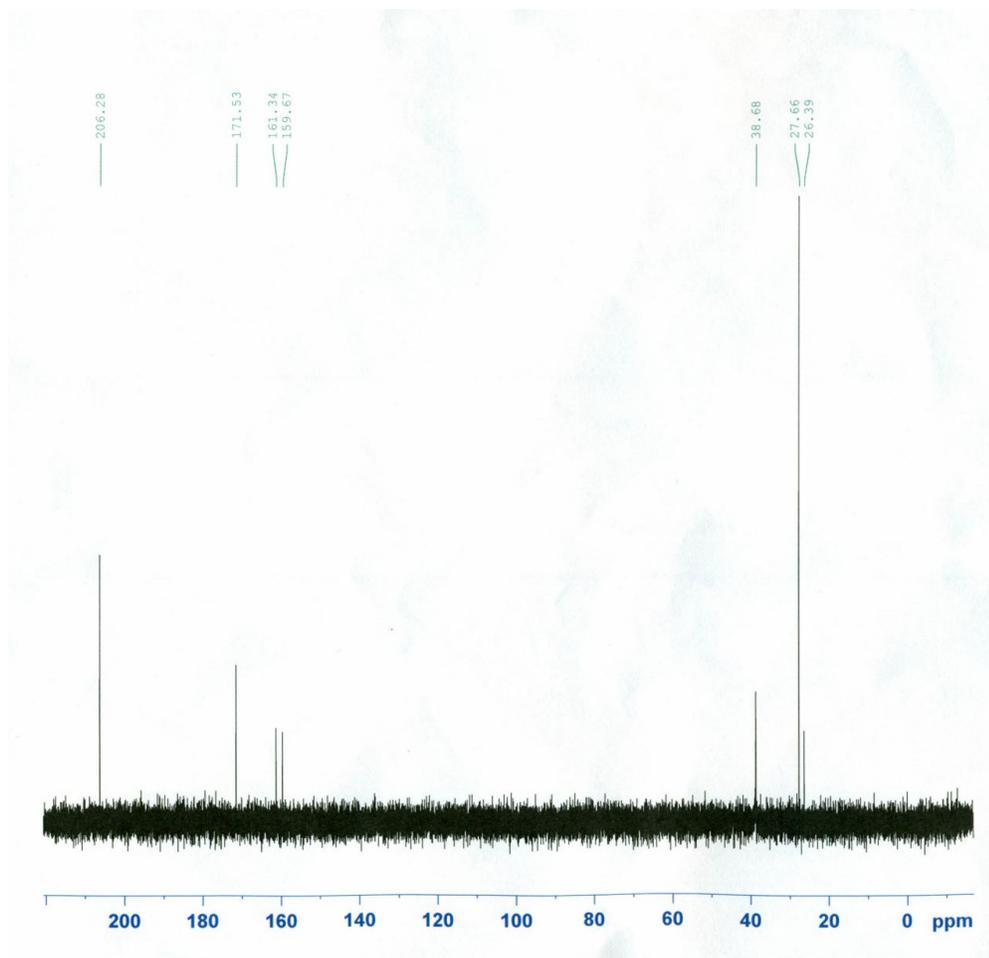


Figure 21: Metformin-Pyruvate ^{13}C -NMR Trial 1

Statistical Analysis of Absorbance Data

Absorbance values for each trial of the spectrophotometry were inputted into Beer's Law to find the concentration values of NADH. These values were inputted into an ANOVA using SAS 9.4 software. The ANOVA was performed at a 95% confidence interval, with $\alpha = 0.05$. The following table represents relevant information obtained from the ANOVA:

Degrees of Freedom	F Value	P Value
5	2.66	0.0473

$$H_0: \mu_i = 0 \quad i = 1, 2, \dots, 6$$

H_1 : at least one of the groups is different, $\mu_i \neq 0$

The table above shows that $p < \alpha$, therefore, the H_0 was rejected, and significance in the data set was found.

A Duncan-Waller Post Hoc test was performed in order to ascertain which groups in the data set differed significantly from the control. The following data was collected:

Rate of PC with different levels of Metformin

The ANOVA Procedure

Waller-Duncan K-ratio t Test for Rate

Means with the same letter are not significantly different.				
Waller Grouping	Mean	N	concentration	
A	3.34941E-7	6	0000Rate	
A				
B	1.79528E-7	6	500Rate	
B				
B	1.66131E-7	6	250Rate	
B				
B	1.1254E-7	3	1000Rate	
B				
B	1.09861E-7	6	2500Rate	
B				
B	1.07181E-7	3	5000Rate	

The post hoc test shows that there are significant differences for several groups: control versus 1000 μ M, control versus 5000 μ M, and control versus 2500 μ M.

Discussion

The data supports the hypothesis that metformin inhibits PC, however, the data does not support the hypothesis that metformin concentrations ranging from 250-500 μ M inhibit PC. Rather, metformin concentrations above and including 1000 μ M of metformin inhibited PC such that there was a significant difference between the NADH concentrations for these groups as compared to the control, according to the Duncan-Waller Post Hoc test.

Analyzing the peaks from the ^{13}C -NMR images reveals an interaction between metformin and biotin such that a carbonyl group normally associated with the biotin C-NMR disappeared. This suggests that metformin and biotin formed a bond when brought into contact with each other, somehow changing the structure of biotin. The proposed method of interaction is a transamination reaction, in which a nitrogen in the metformin chemical structure will attack the carbon in one of the carbonyl groups of biotin such that there will be a loss of that carbonyl group.

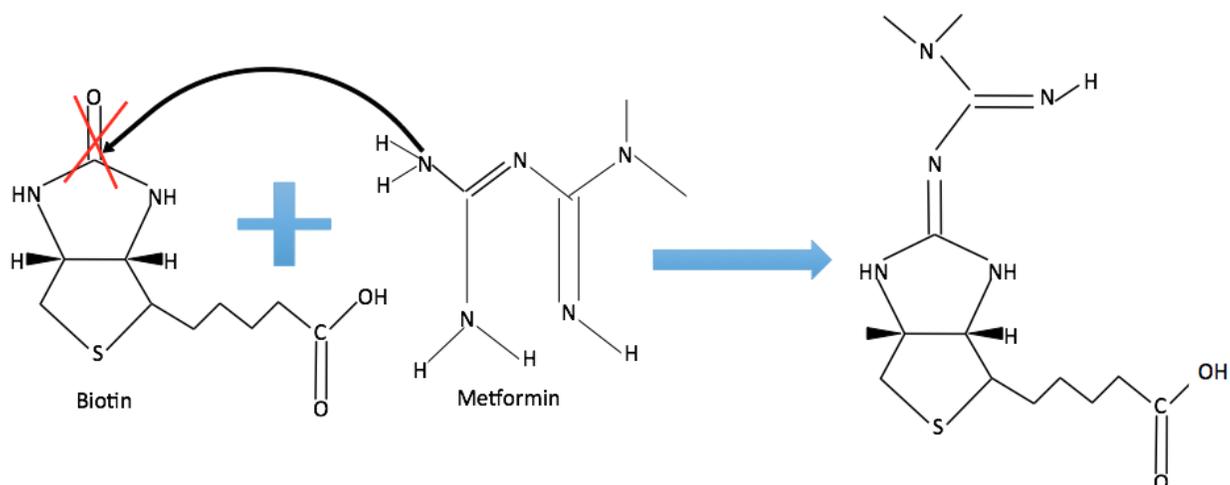


Figure __: Transamination reaction between metformin and biotin.

Biotin is a prosthetic group of PC, meaning that it will interact with PC and allow PC to function. Biotin interacts with PC through a specific carbonyl group, therefore, if metformin interacts with biotin such that the specific carbonyl group is replaced with an amine group, biotin will no longer be able to associate with PC. In this way, metformin may indirectly inhibit PC function. Although the metformin-biotin NMR did not indicate the appearance of an amine group, indicating that the proposed transamination theory is flawed, there was a loss of a carbonyl group, indicating that metformin interacted with biotin. This metformin-biotin interaction supports the metformin PC inhibition theory.

It was hypothesized that concentrations of metformin ranging from 250-500 μ M would best inhibit PC given that metformin is commonly found in concentrations ranging from 40-70 μ M in the hepatic portal vein (Duong et al., 2013; He & Wondisford, 2015). Once within the liver, the metformin concentration rises to 220 μ M (Jin et al., 2009). Given this information, it was expected that lower metformin concentrations would have an inhibitory effect on PC compared to higher concentrations. Future studies should

investigate even lower metformin concentrations ranging from 40-70 μ M to simulate hepatic portal vein conditions. Interestingly, while the 5000 μ M concentration group differed significantly from the control, data such as absorbance over time as well as net NADH to NAD⁺ conversion suggests that this high metformin concentration increased the reaction rate rather than decreased it relative to the control. Much of the controversy surrounding metformin action involves studies which use inaccurate or suprapharmacological metformin concentrations, and it may be that the anomaly of the 5000 μ M trend can be attributed to the excessively-high metformin concentration which may have skewed the data. It is important to note that several studies which support other metformin mechanisms of action, including through AMPK activation, also use suprapharmacological concentrations (He et al., 2009), so these data, although representative of higher than normal concentrations, still merit consideration.

Data collected from the present study may have been influenced by error and study limitations. The 5000 μ M and 1000 μ M concentration groups each had 3 trials, rather than 6 trials, and this may have impacted the distribution of error. Moreover, human inconsistencies, such as variation in starting times for the diode array spectrophotometer, possible contamination in cuvettes or in the NMR tubes, enzyme denaturation, and other such uncontrollable error may have influenced results.

Although this study data can not offer definitive proof for one mechanism of metformin action over another, significant data trends can be identified in the study set which amass support for the PC inhibition theory.

Conclusion

This study investigated the role of metformin in the gluconeogenesis pathway, specifically observing its interaction with PC in the initial steps of mitochondrial gluconeogenesis. Metformin was found to inhibit PC, although not under the pharmacokinetic concentrations of 250 μ M-500 μ M. Additionally, 13 C-NMR analysis indicated an interaction between metformin and biotin, the prosthetic group of PC, such that a carbonyl group was lost. The proposed metformin-biotin interaction is a transamination reaction, in which one of biotin's carbonyl groups, specifically the one which binds to PC and activates it, is lost and is replaced with an amine group. Although an amine group was not found in the 13 C-NMR of metformin mixed with biotin, the loss of one carbonyl in this interaction suggests support for the PC inhibition theory. If metformin somehow interacts with biotin, it is possible that this interaction prevents biotin from interacting with PC in mitochondrial gluconeogenesis, thereby preventing PC from completing its function in the pathway, leading to gluconeogenic inhibition.

Further study should investigate the role of concentrations in metformin action, as several studies which support various mechanisms of metformin action find significance in a wide range of concentrations — some of which are suprapharmacokinetic, or outside the pharmacokinetically realistic range of concentrations. Furthermore, replication studies using a greater number of assay trials should be conducted in order to gain a more complete picture of metformin action in mitochondrial gluconeogenesis. Furthermore, *in vitro* or *in vivo* studies with metformin should be conducted in order to better model biological conditions, as these conditions may affect metformin action. Various other

proposed targets of metformin action, such as AMPK or GPDH, should be investigated, and these findings should be compared to those of the present study. Various other uses of metformin, such as cancer treatment, should be investigated in order to amass more knowledge on the drug profile and various mechanisms of action in the body.

Furthermore, a comparative study between metformin and other biguanides, as well as between biguanides and other antidiabetic drug groups, should be conducted in order to amass more knowledge for the field and help to engender more options for the treatment of type II diabetes as well as other diseases.

These study findings pose important implications for the field of public health. Type II diabetes is a disease which may be caused and worsened by several socioeconomic factors, such as food insecurity, which is the inability to afford or access nutritious foods. This can lead to a diet high in sugars, which can cause obesity and prediabetes, two powerful precursors for the development of type II diabetes. Metformin is a first line drug of choice due to its relative safety and accessibility, and is the most commonly prescribed drug for the treatment of type II diabetes. A common side effect of metformin known as lactic acidosis can lead to severe, debilitating pain and cramping (Duong et al., 2013). Given that type II diabetes disproportionately affects Native Americans, African Americans, and Latinos/Hispanics - the three ethnic demographic groups which face the lowest socioeconomic status (SES) in the United States (Macartney, Bishaw & Fontenot, 2013; Spanakis & Golden, 2014) - lactic acidosis is particularly harmful, as it prevents working people in lower socioeconomic levels from being able to work and earn a livelihood necessary for obtaining basic health care and

other such amenities. Understanding the mechanism of metformin action will not only add to the growing body of knowledge in the field, but will allow for more and better treatment options to be uncovered, as well as will help to explain and ameliorate symptoms such as lactic acidosis.

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