Bio-Mimicking Pyruvate to Inhibit Lactate Dehydrogenase

Honors Project

In Fulfillment of the Requirements for

The Esther G. Maynor Honors College

University of North Carolina at Pembroke

By

Davita Nicole Brockington

Department of Chemistry and Physics

November 2012

Davita Brockington — 11-29-12

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Honors College Scholar — Date

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Siva Mandjiny, Ph.D.
Faculty Mentor — Date

Mark Milewicz — 11/29/2012

Mark Milewicz, Ph.D.
Dean, Esther G. Maynor Honors College — Date

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The completion of this project would not be possible if it were not for all of the support, guidance, and encouragement provided by my faculty mentor, Dr. Sivanadane Mandjiny. All that he has done has been greatly appreciated. I would also like to thank the Pembroke Undergraduate Research and Creativity Center for their financial support. Last, but not least, I am thankful for the direction that I received in support of my project from Dr. Mark Milewicz, the dean of the Esther G. Maynor Honors College.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

BIO-MIMICKING PYRUVATE TO INHIBIT LACTATE DEHYDROGENASE

by
Davita Nicole Brockington
Esther G. Maynor Honors College Senior Thesis
The University of North Carolina at Pembroke
May 2013

Lactate dehydrogenase is an enzyme that catalyzes the reaction of converting pyruvic acid into lactic acid. This reaction took place at pH 7.0. Molecules, such as acetamide and oxamic acid, which are believed to mimic pyruvate in terms of hydrogen bonding characteristics and ionic interactions with enzymes, were used to mimic pyruvate. Therefore, it is expected that acetamide and oxamic acid will both mimic pyruvate in order to inhibit the activity of lactate dehydrogenase. The activity of the enzyme with and without inhibition was measured and as expected it was determined that both molecules are inhibitors of lactate dehydrogenase. As expected oxamic acid is a competitive inhibitor of lactate dehydrogenase and acetamide is a noncompetitive inhibitor. The results suggest that the binding of pyruvate to lactate dehydrogenase occurs either at the carboxylic acid functional group and/or the adjacent carbonyl group.

IMPORTANT STRUCTURES

Figure 1. Pyruvic Acid[1]

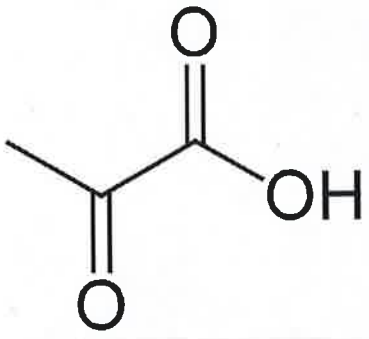


Figure 2. Oxamic Acid[2]

Figure 3. Acetamide[3]

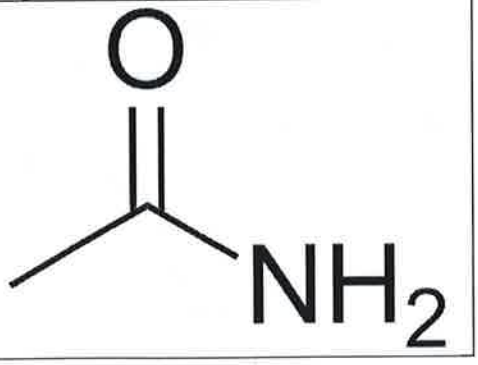


Figure 4. Lactate Dehydrogenase[4]

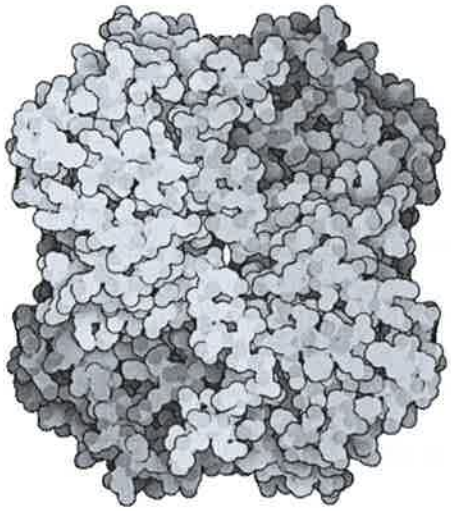
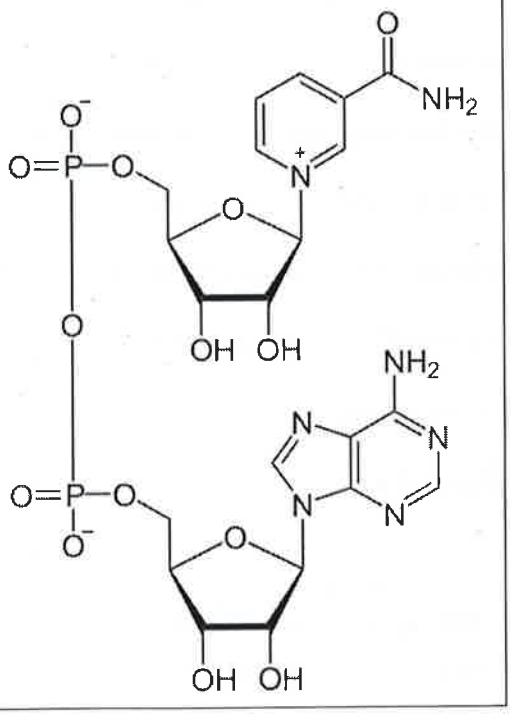


Figure 5. NAD^+ [5]

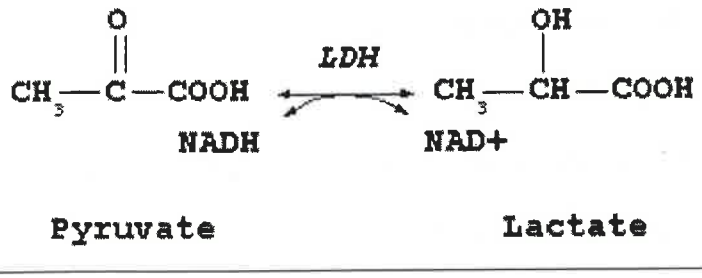


INTRODUCTION

The end product of glycolysis is pyruvate. In the reductive pathway NAD^+ is converted into NADH. Under aerobic conditions the NADH is transported to the electron transport chain, where ATP is made from NADH and molecular oxygen is reduced to water. Under anaerobic conditions, NADH builds up. If NADH builds up instead of being oxidized to NAD^+ then glycolysis cannot run because of the lack of NAD^+.[4] This problem is easily solved with the assistance of an enzyme.

Lactate dehydrogenase is an enzyme that converts the substrate pyruvate into the product lactate, this reaction is shown below in **Figure 6.** Lactate dehydrogenase is found in most tissues in the human body. Lactate dehydrogenase needs a coenzyme in order function properly; it has been proven that lactate dehydrogenase will not bind to pyruvate in the absence of a coenzyme.[7] That coenzyme is NADH. Lactate dehydrogenase allows for NADH to be oxidized to NAD^+. The conversion of pyruvate to lactate is reversible.

Figure 6. Conversion Between Pyruvate and Lactate[6]



Lactate dehydrogenase has three types of subunits.[8] However, only two of the types will be discussed in this paper. The M subunit of lactate dehydrogenase is responsible for converting pyruvate to lactate. The M subunit is found in skeletal muscle. The H subunit of lactate dehydrogenase catalyzes the opposite reaction, which converts lactate to pyruvate. The H subunit is found in cardiac muscle.[8,9]

Lactate dehydrogenase is a tetramer, meaning that each molecule of lactate dehydrogenase has four subunits bound together. These subunits are called monomers. The M and H forms of lactate dehydrogenase are the monomers that join together to create the enzyme. Isozymes are different forms of the same enzyme; the difference is in the structure. Lactate dehydrogenase has five isozymes, which are named numerically. The isozymes are listed below[8,9]:

- LDH1 is made up entirely of the H form and is found within the cardiovascular system.
- LDH2 is made up of one M subunit and three H subunits. It can be found in all tissues, but is concentrated in white blood cells.
- LDH3 is made up of equal portions of M and H subunits and is concentrated in the lungs, even though it is found in all tissues.
- LDH4 is comprised of one H subunit and three M subunits. Like LDH2 and LDH3, LDH4 is found in all tissues. However, it is

concentrated in placenta, and internal organs such as the kidneys and the pancreas.

- LDH5 only has M subunits and is found in the organs that can perform glycolysis, the liver and skeletal muscles.

The human body normally has higher levels of LDH2 than LDH1. If this is not the case, and LDH1 levels are higher then LDH2 levels then there is a cause for concern. High levels of LDH1 are indicative of a myocardial infarction, which is better known as a heart attack. Total lactate dehydrogenase levels can tell us the state of our tissues. Elevated levels are indicative of tissue damage. By further determining which isozymes are elevated can assist the physician in determining the location of the tissue damage.[8,9]

This experiment is being conducted in order to gain valuable information about what portion of pyruvate binds to lactate dehydrogenase. Choosing molecules that are similar in structure to pyruvate will allow for inferences about the interaction between pyruvate and lactate dehydrogenase to be made.

MATERIALS

- Diode Array Spectrophotometer
    - Agilent 8453
- L-Lactic dehydrogenase:
    - Type III, From bovine heart
    - Sigma Chemical Co.
    - Lot 70K7435
- Pyruvic acid:
    - F.W. 110.04
    - M.P. > 300^{0}
    - Aldrich Chemical Company, Inc.
    - Lot No. 08014ES
- Oxamic Acid (Aminooxoacetic acid)
    - Sodium Salt
    - Approx. 98%
    - Sigma Chemical Co.
    - Lot 86H3910
    - $C_2H_2NO_3Na$
    - F.W. 111.0

- β-Nicotinamide Adenine Di-Nucleotide (β-NADH)
    - Reduced Form
    - Disodium Salt
    - $C_{21}H_{27}N_7O_{14}P_2Na_2$
    - F.W. 709.4
    - Purity 95% based on H_2O 7%, solvent 3%
    - Sigma-Aldrich Co.
    - Lot 025K7006
- Acetamide:
    - CH_3CONH_2
    - FW-59.07
- Sodium Phosphate Monobasic
    - $NaH_2PO_4*H_20$
    - F.W. 137.99
    - Fisher Scientific
    - Lot No. 976562

METHODOLOGY

A 0.1 M sodium phosphate buffer of pH 7.0 was made. The commercially produced enzyme, lactate dehydrogenase (LDH3), was purified through a process called dialysis. 1 mL of lactate dehydrogenase was placed into a dialysis membrane. The membrane containing the enzyme was soaked overnight in the buffer, allowing the salt to diffuse out of the enzyme solution. Then the enzyme was collected.

The substrate, sodium pyruvate, was made in a concentration of 30mM. The coenzyme, NADH (ε_{NADH} = 6200 M^{-1} cm^{-1}), was made in a concentration of 6.6 mM. The other reagents were the inhibitors, oxamic acid and acetamide. Both oxamic acid and acetamide were made in concentrations of 30 mM. (The concentrations of oxamic acid and acetamide in the reaction cuvette are both 1 mM)

A diode array spectrophotometer was used to monitor the reactions. The reaction was performed in a cuvette. The cuvette contained 2.8 mL buffer, 100 µL NADH, 100 µL pyruvate, and 10 µL of enzyme. In order to determine the optimal concentration of enzyme, the lactate dehydrogenase was diluted 1000X, 100X, 10X, and 1X. The 10X dilution had the highest activity and was used for all subsequent reactions.

First the activity of the enzyme was measured without inhibition. The cuvette contained 2.8 mL buffer, 100 µL NADH, 100 µL pyruvate, and 10 µL of enzyme. In order to perform pseudo first order reactions first the NADH concentration of each trial was changed eight times; the NADH concentrations were 0.0066 mM, 0.066 mM, 0.132 mM, 0.33 mM, 0.66 mM, 1.32 mM, 3.3 mM, and 6.6 mM. Then the sodium pyruvate concentration was changed; the sodium pyruvate concentrations were 0.03 mM, 0.3 mM, 0.6 mM, 1.5 mM, 3 mM, 6 mM, 15 mM, and 30 mM.

INHIBITION STUDIES

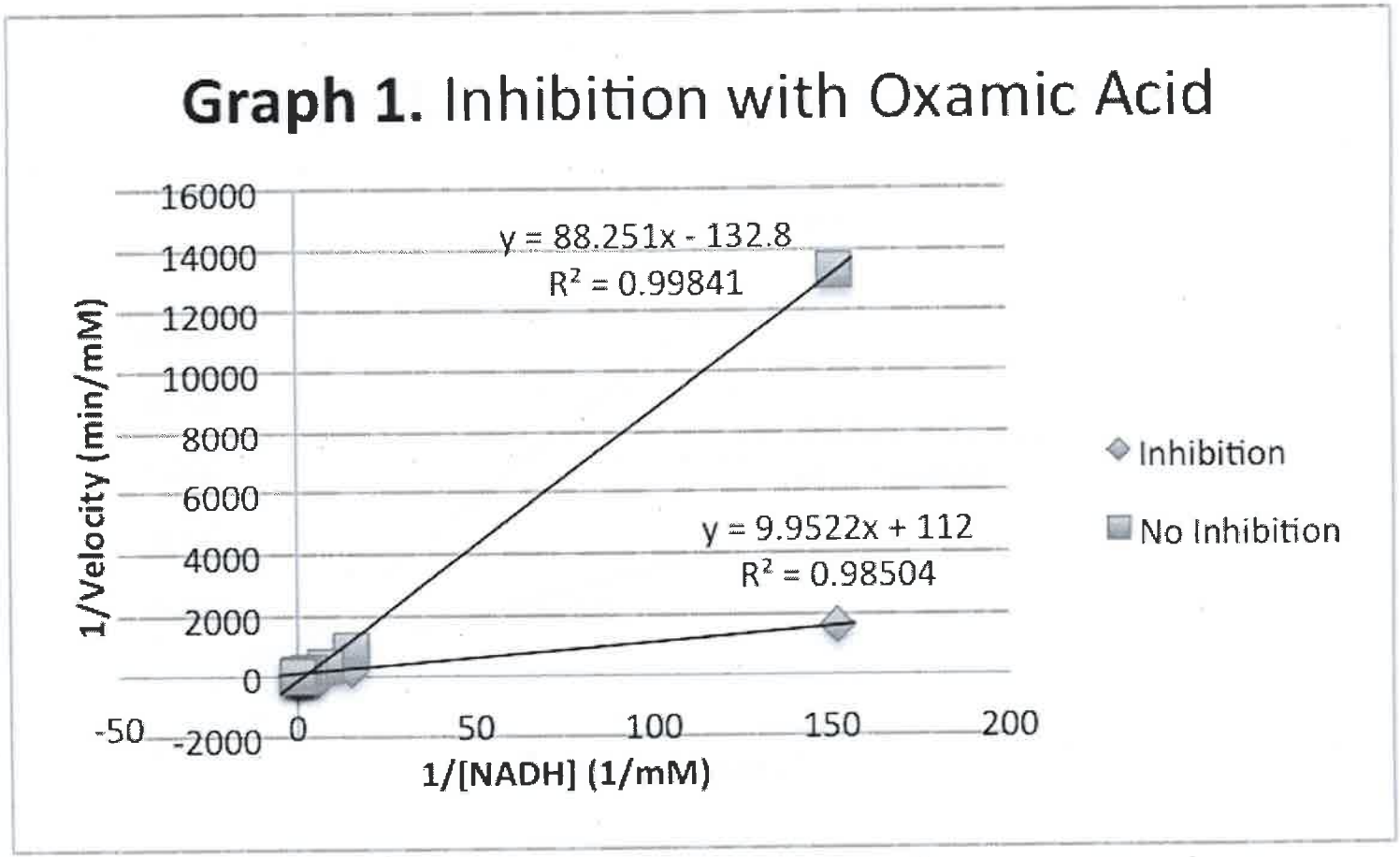
a) These reactions were performed in the presence of oxamic acid. The cuvette contained 2.7 mL buffer, 100 µL NADH, 100 µL pyruvate, 100 µL oxamic acid, and 10 µL of enzyme. The pseudo first order reaction was performed as described above.
b) These reactions were performed in the presence of acetamide. The cuvette contained 2.7 mL buffer, 100 µL NADH, 100 µL pyruvate, 100 µL acetamide, and 10 µL of enzyme. The pseudo first order reaction was performed as described above.

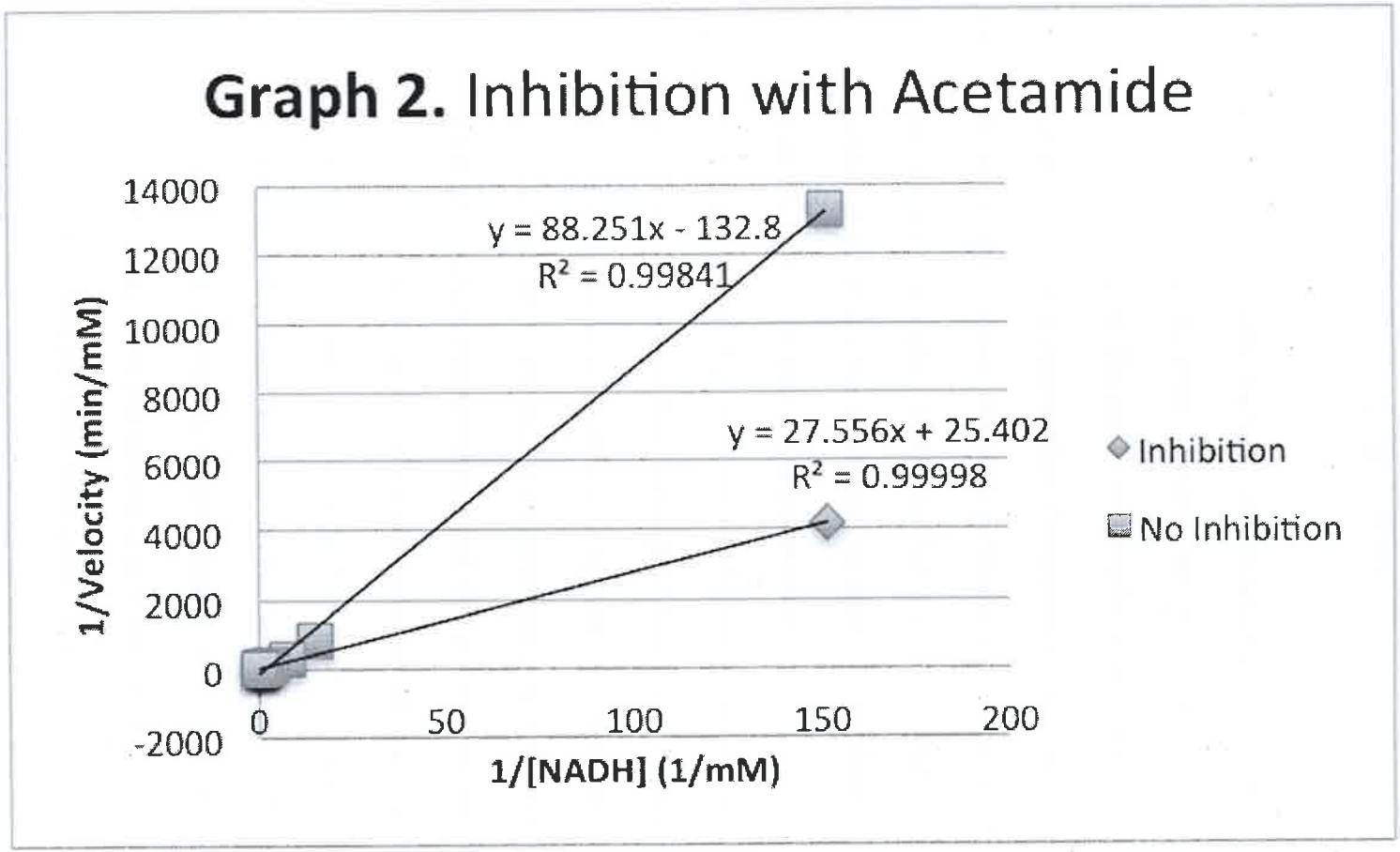
RESULTS

Table 1. Kinetics- No Inhibition		
	Constant Pyruvate	**Constant NADH**
V_{Max}	7.5 μM/min	33.2 μM/min
K_M	0.67 mM	0.42 mM
Specific Activity	4.69 units/mg	

Table 2. Kinetics- Inhibition with Oxamic Acid		
	Constant Pyruvate	**Constant NADH**
V_{Max}	8.9 μM/min	5.4 μM/min
K_M	0.089 mM	0.1 mM
K_I	0.13 mM	
	Inhibition	**No Inhibition**
V_{Max}	8.9 μM/min	7.5 μM/min
K_M	0.089 mM	0.67 mM
Inhibition	Competitive	

Table 3. Kinetics- Inhibition with Acetamide		
	Constant Pyruvate	**Constant NADH**
V_{Max}	39.4 μM/min	43.8 μM/min
K_M	1.08 mM	0.17 mM
K_I	0.45 mM	
	Inhibition	**No Inhibition**
V_{Max}	39.4 μM/min	7.5 μM/min
K_M	1.08 mM	0.67 mM
Inhibition	Noncompetitive	


Graph 1. Inhibition with Oxamic Acid
16000
14000
12000
10000
8000
6000
4000
2000
0
-2000
-50
0
50
100
150
200
1/Velocity (min/mM)
1/[NADH] (1/mM)
y = 88.251x - 132.8
R² = 0.99841
y = 9.9522x + 112
R² = 0.98504
Inhibition
No Inhibition


Graph 2. Inhibition with Acetamide
14000
12000
10000
8000
6000
4000
2000
0
-2000
1/Velocity (min/mM)
y = 88.251x - 132.8
R² = 0.99841
y = 27.556x + 25.402
R² = 0.99998
0
50
100
150
200
1/[NADH] (1/mM)
Inhibition
No Inhibition

CONCLUSIONS

There were four kinetic parameters calculated in this experiment. The first is V_{Max}, which is the rate of the reaction. The second is the K_M, which is the michealis-menten constant. The third is the specific activity of the enzyme. The last is K_I, which is the inhibition constant. Comparing the V_{Max} and K_M values of the pseudo first order reactions that were performed will allow for us to determine if the particular type of inhibition being studied is competitive or noncompetitive. If the V_{Max} is the same then the inhibition is competitive. If the K_M is the same then the inhibition is noncompetitive.

It was hypothesized that both oxamic acid and acetamide have the ability to mimic pyruvate in order to inhibit lactate dehydrogenase. It was also hypothesized that oxamic acid would be a competitive inhibitor; and acetamide would be a noncompetitive inhibitor. The experimental data showed that both molecules inhibited lactate dehydrogenase and that as expected oxamic acid is a competitive inhibitor, whereas acetamide is a noncompetitive inhibitor.

The specific activity of the lactate dehydrogenase was measured and it was determined to be 4.69 units/mg. The inhibition constant for oxamic acid was determined to be 0.13 mM. The experimental K_I for acetamide was 0.45 mM.

The results suggest that the carboxylic acid functional group and/or the adjacent carbonyl group on pyruvate is the portion of the molecule that interacts

with lactate dehydrogenase. This is because oxamic acid, a competitive inhibitor, contains both a carboxylic acid functional group with an adjacent carbonyl group like pyruvate does. Acetamide, which binds to lactate dehydrogenase at a different site than pyruvate, does not contain a carboxylic acid functional group

There is more work that could be done with the inhibition of lactate dehydrogenase. In the future these reactions could be conducted under different conditions. The conditions that could be changed are pH, temperature, pressure, and concentrations or substrates. Seeing how the different isozymes of lactate dehydrogenase behave under the conditions used in this experiment could also be useful. Additionally, other molecules could be used to try to inhibit lactate dehydrogenase, in order to hone in on the exact portion of the molecule interacts with lactate dehydrogenase.

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APPENDIX A

RAW DATA

Table 4. Experimental Data Used to determine the optimal concentration of lactate dehydrogenase			
LDH	**Trial 1**	**Trial 2**	**Trial 3**
0.0072mM	1.84×10^{-5}	3.19×10^{-5}	1.05×10^{-4}
0.072mM	2.76×10^{-4}	2.36×10^{-4}	2.79×10^{-4}
0.72mM	2.70×10^{-3}	1.35×10^{-3}	2.37×10^{-3}
7.2mM	2.79×10^{-4}	3.95×10^{-3}	1.61×10^{-3}

Table 5. No Inhibition					
Constant Pyruvate			**Constant NADH**		
	Activity units/mg			**Activity units/mg**	
NADH	**Trial 1**	**Trial 2**	**Pyruvate**	**Trial 1**	**Trial 2**
0.0066mM	4.06×10^{-6}	1.15×10^{-5}	**0.03mM**	3.36×10^{-4}	1.23×10^{-4}
0.066mM	1.22×10^{-4}	1.18×10^{-4}	**0.3mM**	7.45×10^{-4}	8.54×10^{-4}
0.132mM	3.23×10^{-4}	3.24×10^{-4}	**0.6mM**	2.11×10^{-3}	1.69×10^{-3}
0.33mM	8.09×10^{-4}	7.38×10^{-4}	**1.5mM**	2.61×10^{-3}	3.86×10^{-3}
0.66mM	1.45×10^{-3}	1.31×10^{-3}	**3mM**	3.96×10^{-3}	4.27×10^{-3}
1.32mM	1.90×10^{-3}	1.58×10^{-3}	**6mM**	3.53×10^{-3}	3.94×10^{-3}
3.33mM	2.13×10^{-3}	1.93×10^{-3}	**15mM**	3.23×10^{-3}	3.82×10^{-3}
6.6mM	1.99×10^{-3}	2.98×10^{-3}	**30mM**	1.86×10^{-3}	2.33×10^{-3}

Table 6. Inhibition with Oxamic Acid					
Constant Pyruvate			**Constant NADH**		
	Activity units/mg			**Activity units/mg**	
NADH	**Trial 1**	**Trial 2**	**Pyruvate**	**Trial 1**	**Trial 2**
0.0066mM	1.14×10^{-4}	1.35×10^{-5}	**0.03mM**	1.29×10^{-4}	1.30×10^{-4}
0.066mM	8.94×10^{-4}	1.57×10^{-4}	**0.3mM**	2.14×10^{-4}	5.77×10^{-4}
0.132mM	3.07×10^{-4}	3.52×10^{-4}	**0.6mM**	3.84×10^{-4}	3.21×10^{-4}
0.33mM	4.71×10^{-4}	6.31×10^{-4}	**1.5mM**	3.55×10^{-4}	3.15×10^{-4}
0.66mM	6.72×10^{-4}	7.91×10^{-4}	**3mM**	2.30×10^{-3}	4.89×10^{-4}
1.32mM	2.72×10^{-3}	1.45×10^{-3}	**6mM**	5.69×10^{-4}	5.82×10^{-4}
3.33mM	7.85×10^{-4}	1.33×10^{-3}	**15mM**	1.01×10^{-3}	1.05×10^{-3}
6.6mM	1.01×10^{-3}	9.74×10^{-4}	**30mM**	1.26×10^{-3}	1.71×10^{-3}

Table 7. Inhibition with Acetamide					
Constant Pyruvate			**Constant NADH**		
	Activity units/mg			**Activity units/mg**	
NADH	**Trial 1**	**Trial 2**	**Pyruvate**	**Trial 1**	**Trial 2**
0.0066mM	7.16×10^{-6}	1.74×10^{-5}	**0.03mM**	6.81×10^{-4}	6.72×10^{-4}
0.066mM	2.03×10^{-4}	9.95×10^{-5}	**0.3mM**	8.04×10^{-4}	8.73×10^{-4}
0.132mM	1.99×10^{-4}	3.40×10^{-4}	**0.6mM**	1.76×10^{-3}	1.56×10^{-3}
0.33mM	1.09×10^{-3}	7.27×10^{-4}	**1.5mM**	3.54×10^{-3}	4.83×10^{-3}
0.66mM	2.37×10^{-3}	1.55×10^{-3}	**3mM**	4.75×10^{-3}	4.71×10^{-3}
1.32mM	2.06×10^{-3}	2.61×10^{-3}	**6mM**	4.66×10^{-3}	4.97×10^{-3}
3.33mM	2.48×10^{-3}	2.34×10^{-3}	**15mM**	3.87×10^{-3}	3.45×10^{-3}
6.6mM	3.39×10^{-3}	3.03×10^{-3}	**30mM**	2.61×10^{-3}	2.82×10^{-3}

APPENDIX B

TABLES

Table 8. No Inhibition		
1/V	**1/[NADH]**	**1/[Pyruvate]**
151.51515	13281.9195	-----
15.151515	861.111111	-----
7.5757576	319.422978	-----
3.030303	133.591898	-----
1.5151515	74.8792271	-----
0.7575758	59.3869732	-----
0.3030303	50.9031199	-----
0.1515152	41.5828303	-----
33.333333	-----	450.254176
1.66666667	-----	54.3859649
0.66666667	-----	31.9422978
0.33333333	-----	25.1113811
0.16666667	-----	27.662204
0.06666667	-----	29.3144208
0.03333333	-----	49.3237868

Table 9. Inhibition with Oxamic Acid		
	No Inhibition	**Inhibition**
1/[NADH]	**1/S**	**1/S**
151.51515	-----	1619.6447
15.151515	-----	196.4512
7.5757576	-----	313.13131
3.030303	-----	187.53781
1.5151515	-----	141.16576
0.7575758	-----	49.441786
0.3030303	-----	77.694236
0.1515152	-----	102.31023
151.51515	13281.9195	-----
15.151515	861.111111	-----
7.5757576	319.422978	-----
3.030303	133.591898	-----
1.5151515	74.8792271	-----
0.7575758	59.3869732	-----
0.3030303	50.9031199	-----
0.1515152	41.5828303	-----

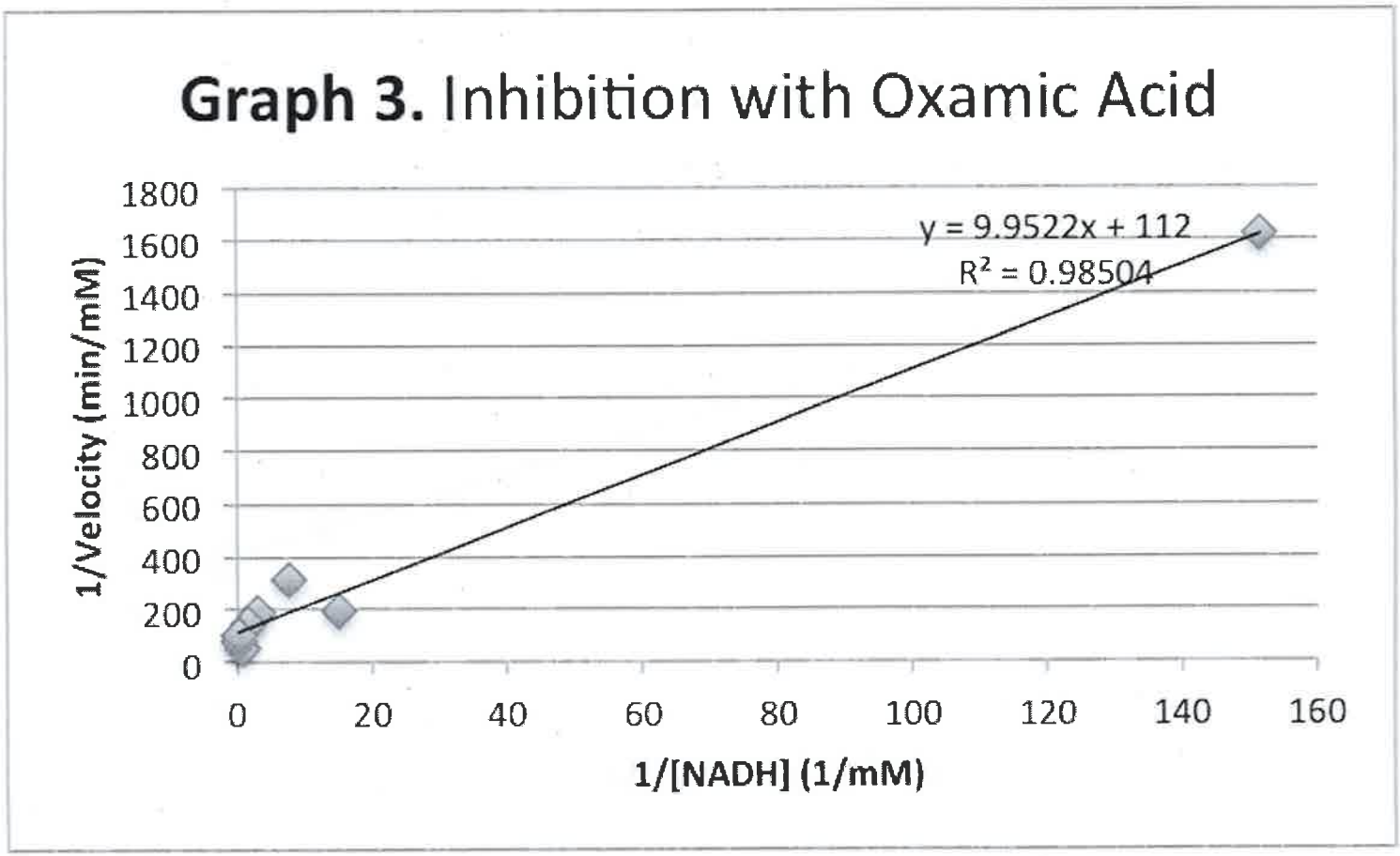
Table 10. Inhibition with Oxamic Acid	
1/[Pyruvate]	**1/[V]**
33.33333333	794.8717949
3.333333333	260.9427609
0.333333333	211.3156101
0.166666667	179.3981481

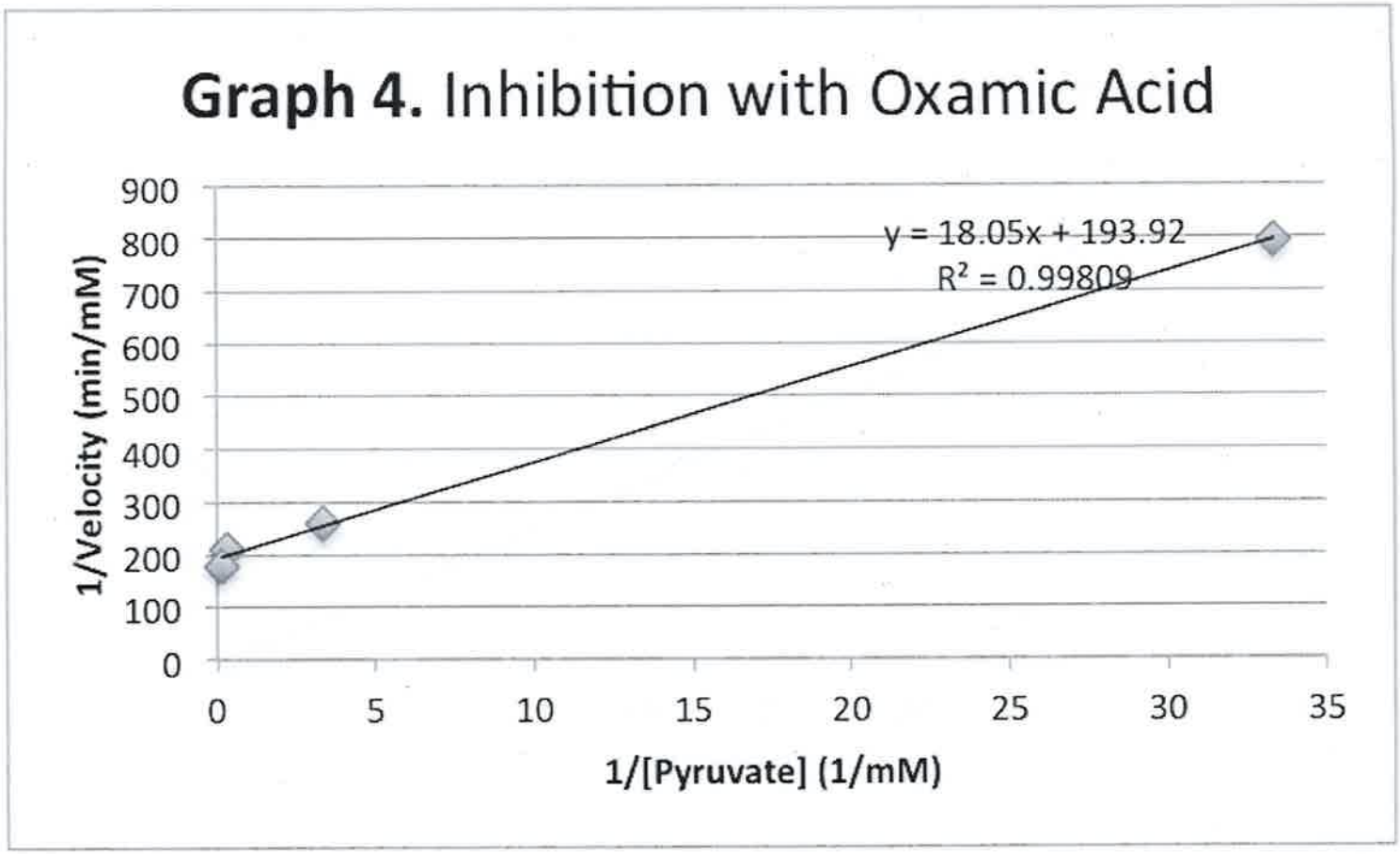
Table 11. Inhibition with Acetamide		
	Inhibition	**No Inhibition**
1/V	**1/S**	**1/S**
151.51515	4200.54201	-----
3.030303	113.678034	-----
1.5151515	52.7210884	-----
0.7575758	44.1595442	-----
0.3030303	42.8769018	-----
0.1515152	32.1910696	-----
151.51515	-----	13281.9195
15.151515	-----	861.111111
7.5757576	-----	319.422978
3.030303	-----	133.591898
1.5151515	-----	74.8792271
0.7575758	-----	59.3869732
0.3030303	-----	50.9031199
0.1515152	-----	41.5828303

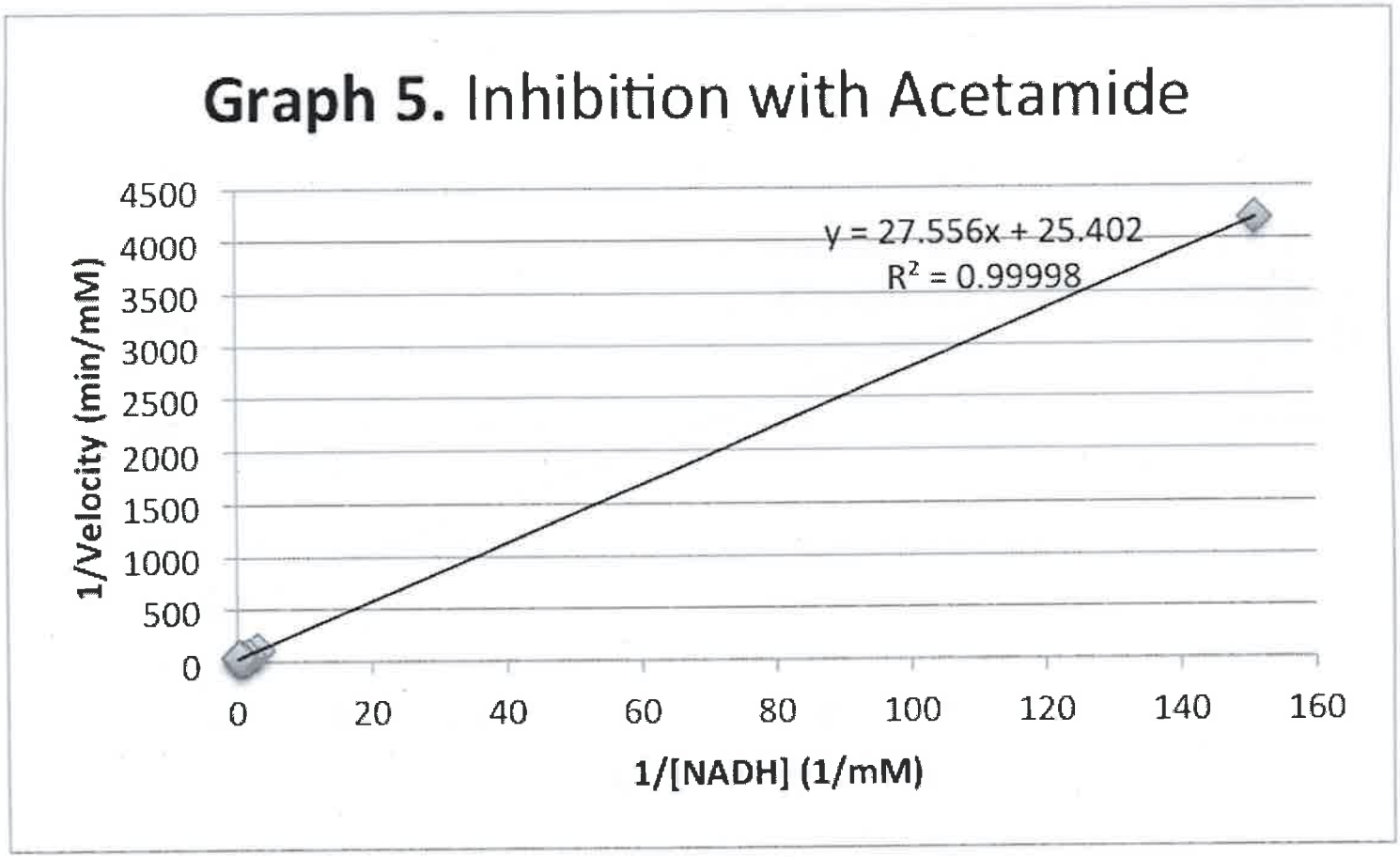
Table 12. Inhibition with Acetamide	
1/[Pyruvate]	**1/[V]**
33.33333333	152.6341704
0.666666667	24.6618934
0.333333333	21.84637068
0.166666667	21.4384509
0.066666667	28.23315118

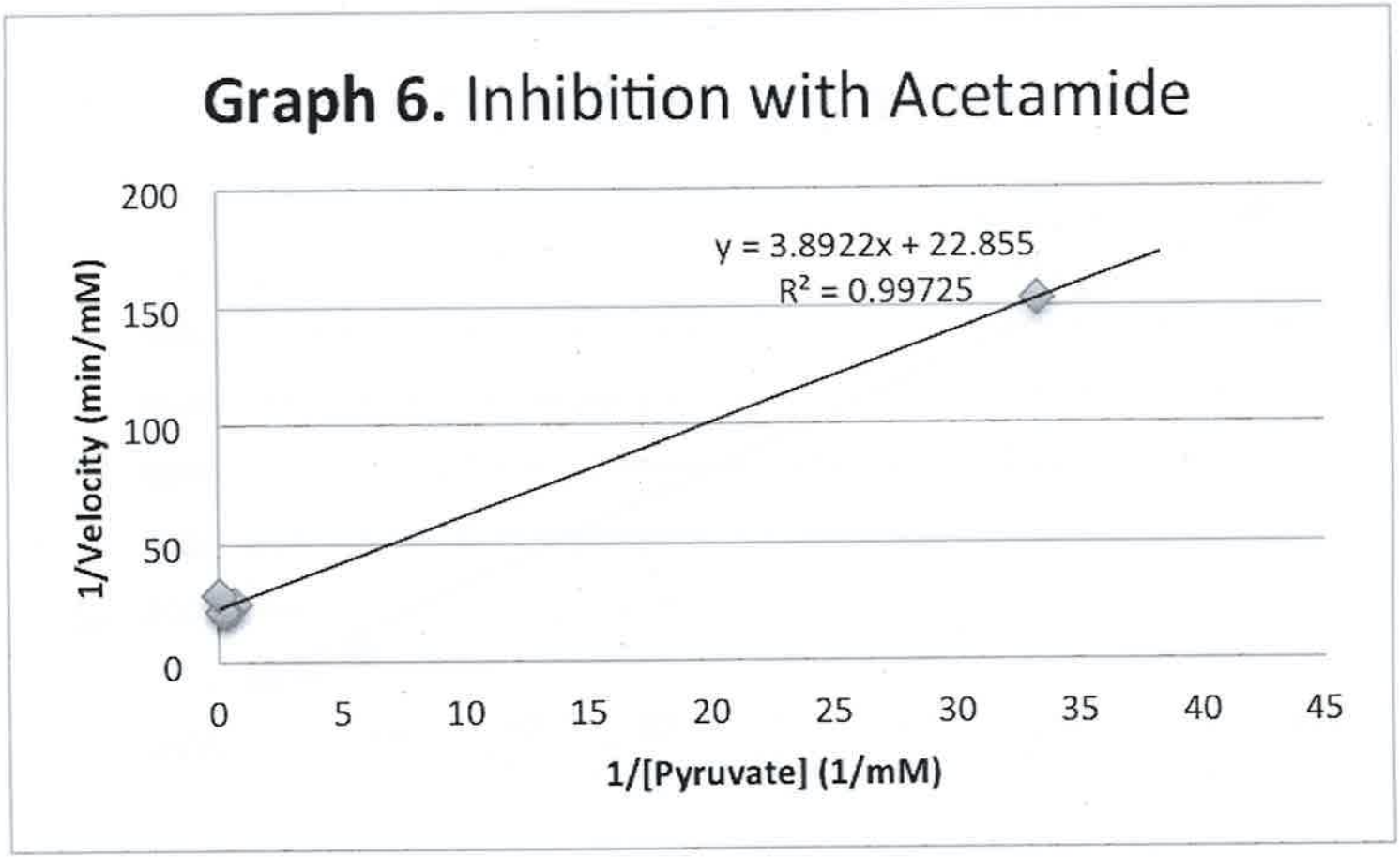
APPENDIX C

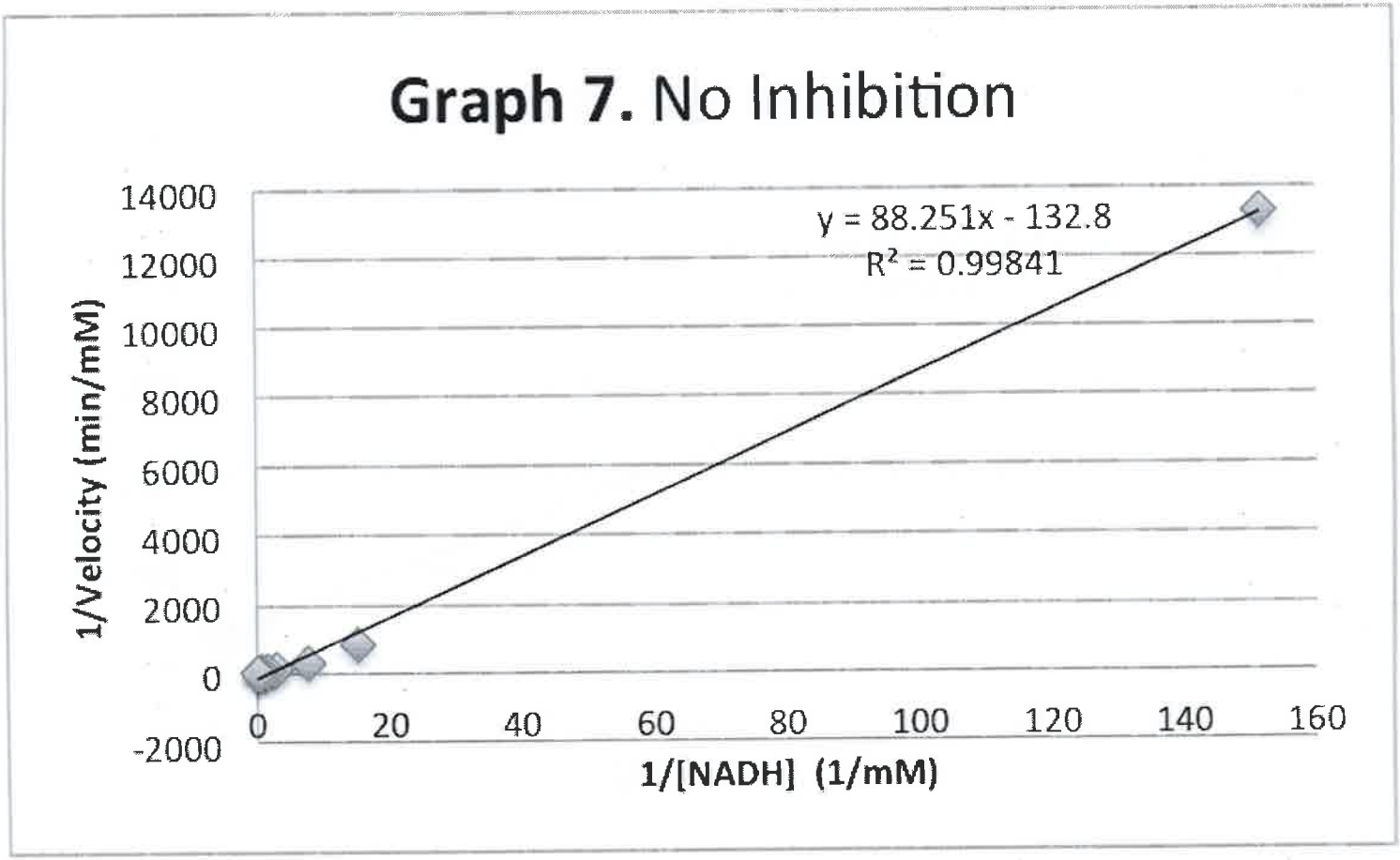
GRAPHS

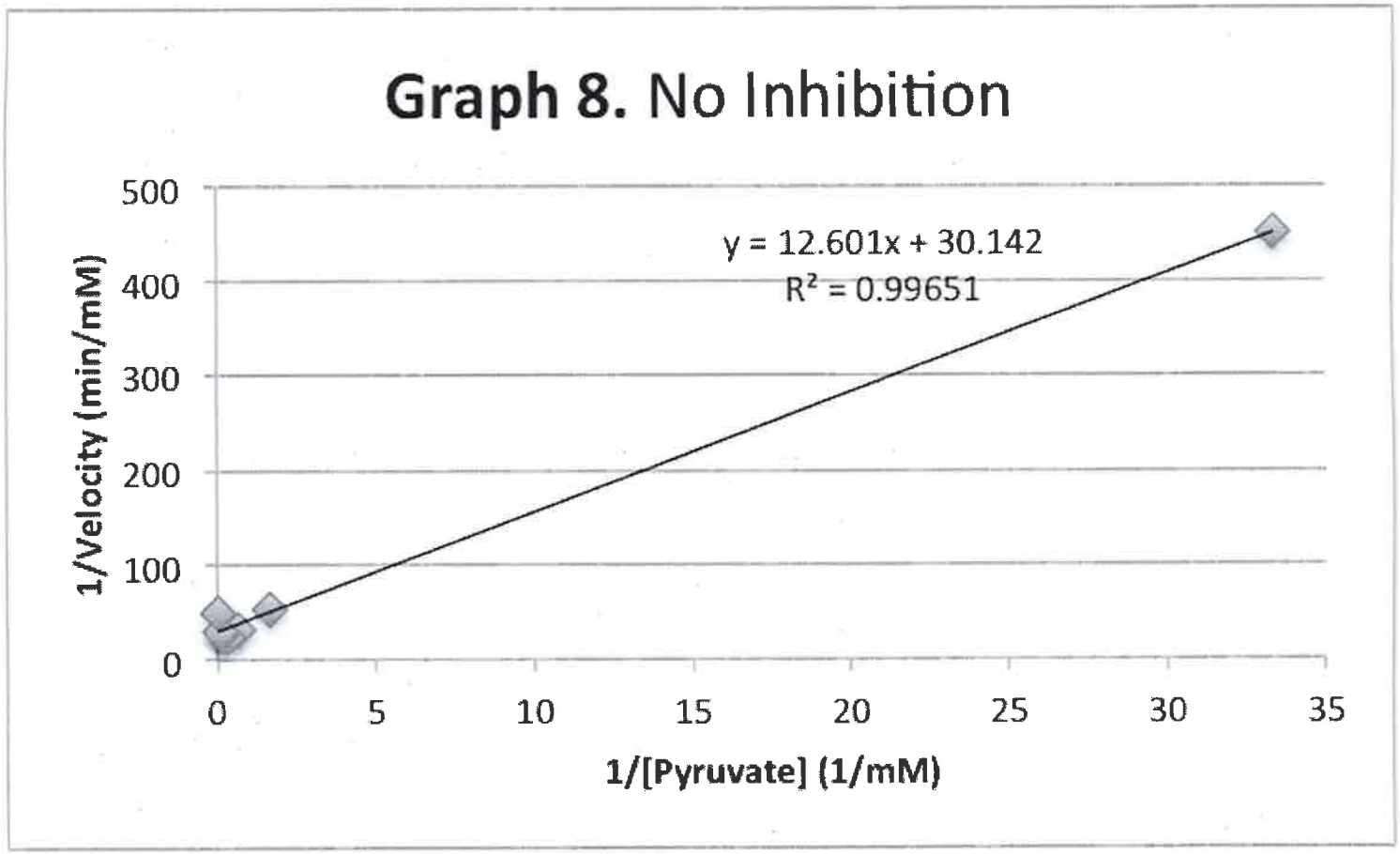

Graph 3. Inhibition with Oxamic Acid
1/Velocity (min/mM)
1800
1600
1400
1200
1000
800
600
400
200
0
0
20
40
60
80
100
120
140
160
1/[NADH] (1/mM)
y = 9.9522x + 112
R² = 0.98504


Graph 4. Inhibition with Oxamic Acid
y = 18.05x + 193.92
R² = 0.99809
1/Velocity (min/mM)
900
800
700
600
500
400
300
200
100
0
0
5
10
15
20
25
30
35
1/[Pyruvate] (1/mM)


Graph 5. Inhibition with Acetamide
y = 27.556x + 25.402
R² = 0.99998
1/Velocity (min/mM)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
0
20
40
60
80
100
120
140
160
1/[NADH] (1/mM)


Graph 6. Inhibition with Acetamide
y = 3.8922x + 22.855
R² = 0.99725
1/Velocity (min/mM)
200
150
100
50
0
0
5
10
15
20
25
30
35
40
45
1/[Pyruvate] (1/mM)


Graph 7. No Inhibition
y = 88.251x - 132.8
R² = 0.99841
14000
12000
10000
8000
6000
4000
2000
0
-2000
1/Velocity (min/mM)
0
20
40
60
80
100
120
140
160
1/[NADH] (1/mM)


Graph 8. No Inhibition
y = 12.601x + 30.142
R² = 0.99651
1/Velocity (min/mM)
500
400
300
200
100
0
0
5
10
15
20
25
30
35
1/[Pyruvate] (1/mM)

APPENDIX D

EQUATIONS

Table 13. Equations Used	
Lineweaver-Burk Plot	$y = mx + b$
Michealis-Menten Constant	$K_M = m/b$
Rate of Reaction	$V_{Max} = 1/b$
Alpha	$\alpha = m'/ m$
m' and m relationship	$m' > m$
Inhibition Constant	$K_I = [S]/(\alpha - 1)$
Competitive Inhibition	$V_{Max} \approx V_{Max}$
Noncompetitive Inhibition	$K_M \approx K_M$
Specific Activity	Sp. Act. = 60 X m/[6.2 X mg_{LDH}]