Chapter 7

Isocitrate Dehydrogenase Parameters of Enzyme Activity

John H. Williamson and A. Malcolm Campbell

Department of Biology, Davidson College
P. O. 1719, Davidson, NC 28036
Telephone: (704)-892-2184; Fax: (704)-892-2512
e-mail: jowilliamson@davidson.edu; macampbell@davidson.edu

John Williamson is Herman Brown Professor of Biology at Davidson College. He received a B. S. in Agricultural Science from North Carolina State University in 1960, an M. S. in Animal Breeding from Cornell University in 1963 and a Ph. D. in Zoology (Genetics) from the University of Georgia in 1966. He was a USAEC Postdoctoral Fellow at Oak Ridge National Laboratory (1966-67) and a NIH Postdoctoral Fellow at the University of California-Riverside (1967-69) before moving to the Department of Biology at the University of Calgary (1969-1981). He moved to Davidson College as Chair of the Department of Biology (1981-1993) and teaches Introductory Biology; Genetics; Genetics, Society and Decisions; and Research Techniques. His research concerns a battery of $NADP^+$-dependent enzymes in *Drosophila* and *Chlamydomonas*.

Malcolm Campbell is Assistant Professor of Biology at Davidson College. He received his B. S. from Davidson in 1984 and a Ph. D. from Johns Hopkins University in 1992. He was a PEW Teacher-Scholar at Washington University (1992-93) and a PEW Visiting Assistant Professor at Macalester College (1993-94). He teaches Introductory Biology, Molecular Biology, Immunology, and Research Courses. His research concerns the molecular basis of sex determination in *Chlamydomonas*. He established and maintains the Department's Web Site.

Reprinted From: Williamson, J. H. and A. M. Campbell. 1999. Isocitrate dehydrogenase parameters of enzyme activity. Pages 137-163, *in* Tested studies for laboratory teaching, Volume 20 (S. J. Karcher, Editor). Proceedings of the 20th Workshop/Conference of the Association for Biology Laboratory Education (ABLE), 399 pages.

Contents

Introduction

Four Introductory Biology laboratory meetings are devoted to student research on properties of a model enzyme, isocitrate dehydrogenase. During the first laboratory meeting (not presented here), students learn: how to use micropipets, make solutions, about spectrophotometry and absorption spectra, and how to establish and use standard curves. During the second and third laboratory meetings, students perform experiments to test hypotheses, analyze their data, propose new hypotheses and design research protocols to test these new hypotheses. Three-hour laboratory sessions allow adequate time for discussion, independent thought, repeating experiments, and even doing additional experiments, if students so desire. A computer graphing program is used to organize their data for oral presentation of their experiments and interpretations to their peers, during the fourth laboratory meeting.

Equipment

96-Well-Plate Readers (http://www.bio.davidson.edu/Biology/IDH.html; 1-2 for four groups of students) We use BioRad Model 3550UV 96-well plate readers, which have been replaced with a newer model. There are multiple sources of comparable equipment and demonstrations should be requested from several suppliers.

Printers attached to the 96-well plate readers.

We "salvaged" IBM ProPrinters that were "too slow" for other purposes.

Microcentrifuge

To spin down tissue debris in homogenates in Lab 3.

Computers

We use older MacIntosh machines that were recycled from faculty offices.

Graphing program

We use CA Cricket Graph, but many of our students know and use other programs.

Water bath and thermometer

Set at 37°C.

P-20, P-200, P-1000 micropipets

8-Channel Multi-Pipets (one at each plate reader)

Supplies

Yellow Tips and Blue Tips for micropipets		96-Well Plates
Buffer Troughs	1.5 ml Microfuge Tubes	Microfuge Tube Racks
Sharpies	Trash Receptacles	

Solutions

Assay Buffer	Pig Heart IDH	$NADP^+$ Solutions
Isocitrate Solutions	EDTA Solution	Metal Ion Solutions
pH Buffers		

Instructors Notes

An excellent assignment is to have students verify molarities of various solutions. Students will have had some exposure to molar concentrations but, for most, this will be the first time they are expected to determine the concentrations of solutions used in their research projects.

Enzyme and $NADP^+$ solution should be prepared the day of use; enzyme solutions should be kept on ice at all times. All other solutions are stable for months. We prepare stock solutions and provide samples for each group of students. This practice avoids problems of crowding and waiting around a "reagents bench" and contamination of stock solutions.

The current edition of our entire laboratory manual can be found at: http://www.bio.davidson.edu/Biology/Courses/Bio111LabMan/TOC.html. We have developed an IDH web page which is available at: http://www.bio.davidson.edu/Biology/IDH.html.

Assay Buffer, pH 8.5 (0.2 M Tris, 1 mM $MgCl_2$)

Tris-HCl	7.32 g
Tris Base	18.6 g
$MgCl_2$	203 mg
Distilled water to	1 liter

Enzyme Solution

Pig heart $NADP^+$-IDH in 50% glycerol (Sigma # I-2002)

Dilute 60 µl in 1 ml of Assay Buffer (above)

Test for appropriate levels of activity and adjust as needed.

Keep solution on ice at all times.

Isocitrate Stock Solution (4.6 mM)

11.9 mg of DL-isocitrate (Sigma # I-1252) in 10 ml of Assay Buffer (above)

Concentrations of Isocitrate for Experiment 3 (1 ml each; about 100 assays)

Tube	mM*	µl of Stock Isocitrate Sol	µl of Assay Buffer
1	0.23	1000	0
2	0.058	252	748
3	0.029	125	875
4	0.019	83	917
5	0.01	43	957

*Final concentration when 10 µl of solution is diluted to a 200 µl final volume.

$NADP^+$ Stock solution (2.88 mM)

11 mg of $NADP^+$ (Sigma # N-0505) in 5 ml of Assay Buffer

Dilute Concentrations of $NADP^+$ (1 ml each; about 100 assays)

Tube	mM*	µl of $NADP^+$ Stock	µl of Assay Buffer
1	0.144	1000	0
2	0.072	500	500
3	0.036	250	750
4	0.0216	150	850
5	0.0144	100	900

* Final concentration when 10 µl of solution is diluted to a 200 µl final volume.

Tris-HCl Buffers for pH Experiment

Stock Solutions	A:	0.2 M Tris Base	24.2 g in one liter
	B:	0.2 M HCl	16.7 ml of 12 N HCl in one liter
	C:	1.0 mM $MgCl_2$	2.03 g in 10 ml

Mix 50 ml of A + X ml of B + distilled water to 200 ml final volume.
Add 20 µl of C to each 200 ml solution.

X	Approx pH*	Determine Actual pH
5.0	9.0	
13.3	8.5	
26.8	8.0	
39.5	7.5	
47.0	7.0	

Divalent Metal Ion Stock Solutions (100 mM)

Metal*	Sigma Cat. #	mg / ml
$MgCl_2$	M-9272	20.3
$MnCl_2$	M-3634	19.8
$CuCl_2$	C-6641	13.5
$ZnCl_2$	Z-3500	13.6

* Final concentration when 10 µl of solution is diluted to a 200 µl final volume: 5 mM.

EDTA Stock Solution (100 mM)

38 mg EDTA (Tetrasodium salt; Sigma # ED4S) in 1 ml of water
Final concentration when 10 µl of solution is diluted to a 200 µl final volume: 5 mM.

NaCl Solution (5 M)

Dissolve 0.293 g in 1 ml of assay buffer.

Survey of IDH in Different Organisms

Obtain fresh materials.
Homogenize 3-5 g in equal volume of Assay Buffer.
Freeze (preferably at -70°C).
Thaw before use.
Centrifuge to remove cellular debris.
Use supernatant as IDH source.

Student Handouts

Introduction

Enzymes are biological catalysts with remarkable power. They increase reaction rates by at least a million-fold by lowering activation energies, allowing chemical reactions to proceed under physiological conditions. Enzymes are highly specific in the substrates used and reactions catalyzed. Enzymes are usually proteins, although some enzymes are other types of biological molecules. Enzymes function best in dilute aqueous solutions under limited conditions of temperature, pH, and salt concentration. Some enzymes require one or more non-protein components called "coenzymes" and "cofactors"; a coenzyme is an organic molecule, while a cofactor may be a metal ion. Some enzymes simultaneously require both a cofactor and a coenzyme. Isocitrate dehydrogenase [IDH] is one of these, requiring both $NADP^+$ as a coenzyme and a divalent metal cofactor, Mg^{2+} or Mn^{2+}.

IDH, a ubiquitous enzyme found in all living organisms, has two catalytic activities [Figure 7.1]. As its name implies, IDH removes hydrogens from its substrate, isocitrate; in addition, it is a decarboxylase, removing a CO_2 from the six-carbon substrate to generate a five-carbon product, α-ketoglutarate .

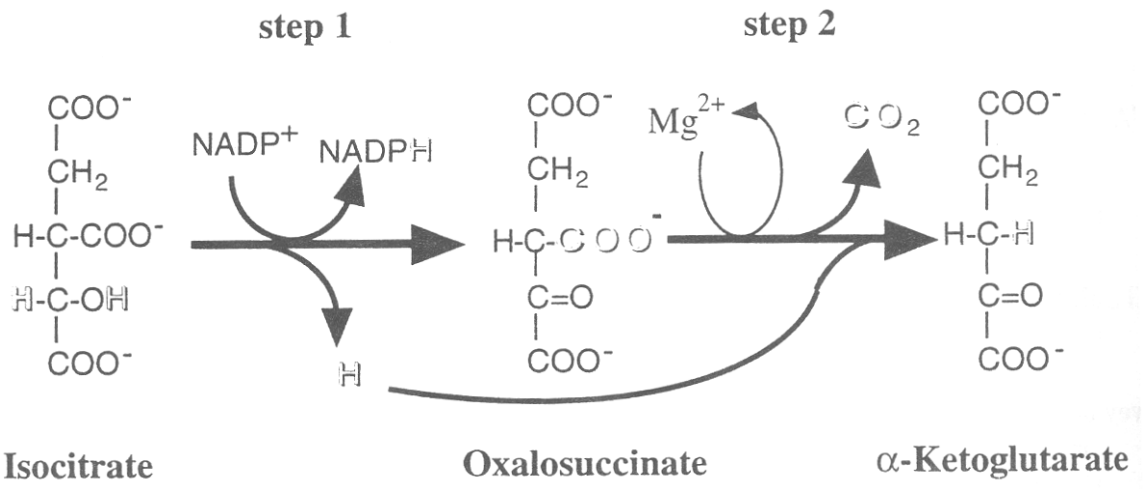

Figure 7.1. IDH catalyzes the sequential dehydrogenation and decarboxylation of isocitrate to α-ketoglutarate. A coenzyme, $NADP^+$, and a cofactor, Mg^{2+} (or Mn^{2+}), are required for activity. Two distinct forms of IDH are found in higher organisms. They differ in their distribution within the cell and in coenzyme requirements. The cytoplasmic form of IDH requires $NADP^+$ as its coenzyme [Figure7.2].

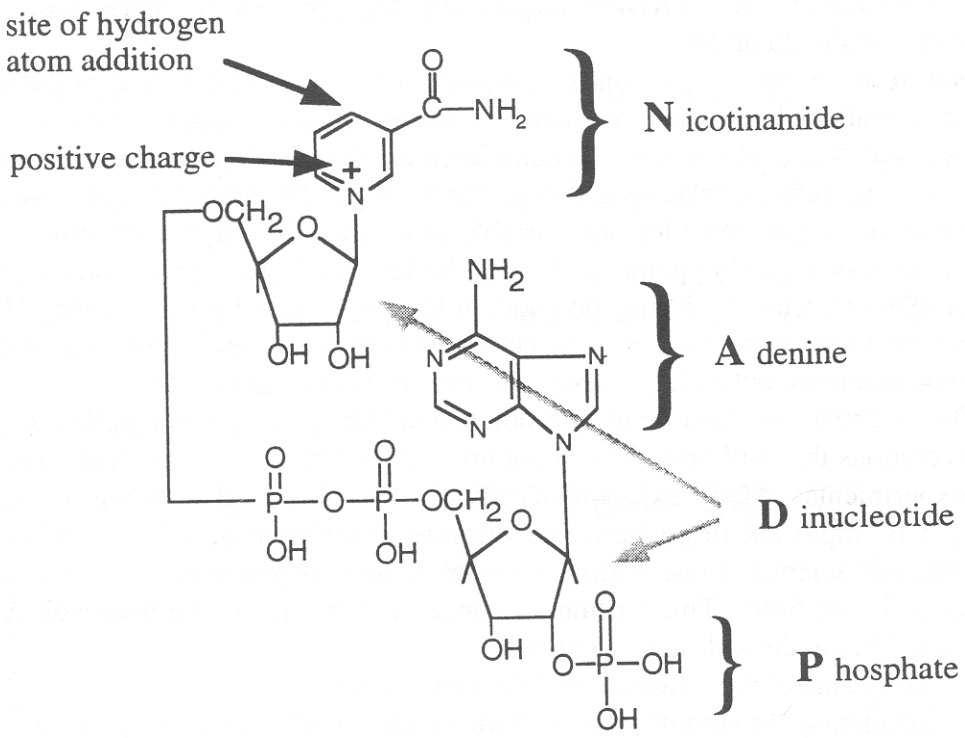

Figure 7.2. The molecular structure of $NADP^+/NAD^+$. The active site is the same in both compounds. In NAD^+ , the phosphate group is replaced with an H^+. This diagram illustrates what the letters N-A-D-P represent.

The $NADP^+$-dependent form of IDH is considered to be the only IDH in bacteria and cyanobacteria and is the most prevalent form of IDH in most plants and animals. In higher organisms, this form appears to be found in all organs and tissues. The NAD^+-dependent form of IDH is limited to eukaryotic organisms and is localized in mitochondria. You may already know this form of IDH from previous study of the Krebs cycle. Both forms of IDH require a divalent metal ion.

Points of Interest: (i) $NADP^+$-dependent IDH activity is especially high in cardiac tissue and is often monitored in the blood of heart attack patients. Detectable IDH activity in the arterial blood suggests severe tissue damage with leakage of the soluble (cytosolic) IDH into the blood system. (ii) In 1996, a team of researchers found a species of vole, living in the area contaminated by the Chernobyl nuclear disaster, that is resistant to mutations caused by radiation. They found that the voles had elevated levels of IDH, which they believe is protecting them from radiation-induced mutations (*Science*, Vol 273, 19 July, 1996, p. 313).

Protocols

IDH activity is routinely measured using a spectrophotometer to monitor the reduction of $NADP^+$ to NADPH. While performing assays, the spectrophotometer is set at 340 nm, the absorption maximum of NADPH (Appendix A). Assays are performed at a standard temperature, usually 25 or 30°C.

Before a scientist begins an experiment, she must first define a problem and suggest possible explanations based upon previous knowledge or observations. In other words, she must

develop an hypothesis, which might be considered an "educated guess" or a tentative explanation as to the cause and effects relating to that problem. A good hypothesis is one that is testable and fosters predictions that consider one variable at a time. The hypothesis may turn out to be incorrect, but it is a good hypothesis if it can be tested. In fact, an hypothesis that cannot be tested is useless to science - it may be good philosophy, but not good science. Hypotheses can not be proven to be correct - they may be tested extensively and rigorously and they may be proven to be incorrect, but an hypothesis can never be proven to be true.

Our scientist must first define a problem and then develop her hypothesis. She must next devise predictions that will hold, or will not hold, if the hypothesis were true. These predictions lead to experiments. Many experiments may be possible, and may all be tried eventually; however, it is important to perform one discrete experiment at a time. After designing an experiment, our scientist must outline a series of logical procedures to be completed in the laboratory or in the field. This written sequence of steps is called a **protocol**. A well-planned protocol will include the following elements:

1. An outline of the sequence of detailed procedures.
2. Calculations of volumes, concentrations, *etc*., of all reagents to be used.
3. Tables constructed for recording data.
4. Procedures for testing and organizing data for presentation.

Lab 1

In their first laboratory meeting, our students learn how to use micropipets, how to prepare solutions, about spectrophometry and absortion spectra and how to establish and use standard curves. Our approach can be found at:
http://www.bio.davidson.edu/Biology/Courses/ Bio111LabMan/TOC.html.

Lab 2

Objectives

With this laboratory, we begin a three week study and discussion of some of the properties of $NADP^+$-dependent isocitrate dehydrogenase [IDH]. The objectives of these laboratory sessions are to:

1. Learn spectrophotometric analyses of enzyme activity.
2. Determine how the amount of enzyme in the assay affects the rate of activity.
3. Determine how the amount of substrate in the assay mixture affects rates of activity of an enzyme.
4. Determine the effects of environmental conditions on enzyme activity.
5. Learn how to organize data into tabular and graphic form.

Experiment 1. How To Perform IDH Assays

Hypothesis 1: A successful assay for IDH activity simultaneously requires enzyme, isocitrate and $NADP^+$.
Hypothesis 2: Under ideal conditions, IDH activity will be linear for at least three minutes.

To test your hypotheses, you will need to set up assays as in **Table 7.1**. You should ask yourself "What is the purpose of each assay?" You should also ask why Assays 5 - 7 are identical.

Table 7.1: How to perform IDH assays.

Wells	Buffer	$NADP^+$	IDH	Isocitrate
A 1	190	10	0	0
A 2	180	10	0	10
A 3	180	10	10	0
A 4	180	0	10	10
A 5	170	10	10	10
A 6	170	10	10	10
A 7	170	10	10	10

All volumes are in µl. In this experiment, you will initiate the reactions by adding 10 µl of substrate solution as the last step. You will use a multi-tip pipet, at the plate reader, to add substrate to all wells.

Procedure

1. Use the P-200 micropipet to add Assay Buffer to the indicated wells.
2. Use the P-20 micropipet to add 10 µl of $NADP^+$ to all wells, except A 4.
3. Use the P-20 micropipet to add 10 µl of IDH to all wells, except A 1 and A 2.
4. Place the microplate in chamber of the plate reader.
5. Use the Multi-8 micropipet to add 10 µl of isocitrate to all wells, except A 1 and A 3.
6. Activate the plate reader.
7. After printing, remove your plate from the plate reader.
8. Retrieve your data from the printer.
9. Return to your station and organize your data in the **Table 7.1a**.
10. Prepare a graph of your data.

Table 7.1a: Data from triplicate IDH assays.

Time, min	Well A 5	Well A 6	Well A 7
0			
0.5			
1			
1.5			
2			
2.5			
3			

Considerations - Experiment 1

Compare your data from Wells A 1 through A 7. Was there activity in Wells A 1 - A 4? Was there activity in Wells A 5 - A 7? Was the activity the same in Wells A 5 - A 7? Was activity linear for three minutes? If not, explain your observations. Do your data support your hypotheses? If not, how will you change the protocol?

Determine the "corrected" reading for each assay by subtracting the reading from the "control", Well A 1. (Would Well A 2, A 3, or A 4 provide better "control" data?)

Construct a graph that visually portrays your data from Wells A 5 - A 7 by plotting absorbance as a function of time (in minutes). The initial rate of a reaction may be determined from the slope of the line joining each successive point.

Experiment 2. Effects Of Varying Enzyme Concentration

Problem: What is the relationship between the rate of a reaction and the amount of enzyme in the assay solution when substrate and coenzyme are abundant (non-limiting)? This question might become "In subsequent experiments, how much enzyme solution should I use in each assay?"

Hypothesis: IDH activity will vary directly with the amount of enzyme in each assay.

To test this hypothesis, you will need to follow a protocol that holds all conditions constant except the amount of enzyme added to each assay. All tests should be run more than once; routinely, enzyme assays are run "in triplicate". For example, Wells B 1, B 2, and B 3 in Table 7.2 are triplicate assays containing 5 µl of IDH. Set up reactions as per **Table 7.2**.

Table 7.2: The effects of varying enzyme amounts.

Wells	Buffer	$NADP^+$	IDH	Isocitrate
B 1-3	175	10	5	10
B 4-6	170	10	10	10
C 1-3	165	10	15	10
C 4-6	160	10	20	10
D 1-3	180	10	0	10

Procedure

1. Use the P-200 micropipet to add Assay Buffer to the indicated wells.
2. Use the P-20 micropipet to add 10 µl of $NADP^+$ to all wells.
3. Use the P-20 micropipet to add the indicated volume of IDH to each well.
4. Place the microplate in chamber of the plate reader.
5. Use the Multi-8 micropipet to add 10 µl of isocitrate to all wells.
6. Activate the plate reader.
7. After printing, remove your plate from the plate reader.
8. Retrieve your data from the printer.
9. Return to your station and organize your data in **Table7.2a**.
10. Prepare a graph of your data.

Table 7.2a: Data from varied enzyme amounts.

Time, min	5 µl of IDH	10 µl of IDH	15 µl of IDH	20 µl of IDH
0				
0.5				
1				
1.5				
2				
2.5				
3				

Considerations - Experiment 2

Compare the data from Wells B 1 through C 6. Was there activity in all wells? Did activity vary with the amount of enzyme in each assay? Was the activity the same in the three wells with the same amount of enzyme? Was the activity linear for the first three minutes for each volume of enzyme? If not, explain your observations. Do your data support your hypothesis?

Determine the mean activity for each set of triplicate assays. Construct a graph to portray your data. Compare the activity with the volume of enzyme in the assay solution. [Hint - take advantage of the formula: $y = mx + b$.]

Construct another graph that compares the volume of enzyme versus absorbance at a specific time, *eg.*, the three-minute reading, or the slope of the three lines from your previous graph. What conclusions can you reach from your results?

Experiment 3. Effects Of Varying Isocitrate Concentration

Problem: What is the relationship between the rate of a reaction and the amount of isocitrate in the assay solution when the amounts of IDH and $NADP^+$ in the assay are held constant? Before you start this experiment, develop an hypothesis and sketch a graph predicting the relationship of activity *vs* isocitrate concentration.

Procedure: To test your hypothesis, you will need to follow a protocol that holds all conditions constant except the amount of isocitrate added to each assay. **Table 7.3** outlines such a protocol using five concentrations of isocitrate. Each concentration is tested in triplicate. Add reagents to your wells as listed from left to right.

Table 7.3: The effects of varying isocitrate concentration.

		Isocitrate			
Wells	**Buffer**	**Sol***	**Vol**	**IDH**	**$NADP^+$**
E 1-3	170	1	10	10	10
E 4-6	170	2	10	10	10
F 1-3	170	3	10	10	10
F 4-6	170	4	10	10	10
G 1-3	170	5	10	10	10
G 4-6	180	Blank		10	10

*****The concentration of these isocitrate solutions will be provided by the Instructor. The second number refers to the volume [µl] to be used. **NOTE:** In these assays we will initiate reactions with $NADP^+$. Do not add $NADP^+$ until your plate is in the plate reader.

Procedure

1. Use the P-200 micropipet to add Assay Buffer to the indicated wells.
2. Use the P-20 micropipet to add 10 µl of isocitrate to all wells, as indicated.
3. Use the P-20 micropipet to add 10 µl of IDH to all wells.
4. Place the microplate in chamber of the plate reader.
5. Use the Multi-8 micropipet to add 10 µl of $NADP^+$ to all wells.
6. Activate the plate reader.
7. After printing, remove your plate from the plate reader.
8. Retrieve your data from the printer.
9. Return to your station and organize your data in **Table 7.3a**.
10. Prepare a graph of your data.

Table 7.3a: Data from varied isocitrate concentrations.

Time, min	0.23 mM	0.058 mM	0.029 mM	0.019 mM	0.01 mM
0					
0.5					
1					
1.5					
2					
2.5					
3					

Considerations - Experiment 3

Analyze the data from your experiment. Determine the mean activity for each concentration of isocitrate. Construct a graph that compares activity as a function of isocitrate concentration. Do your data support your hypothesis? Is the relationship between activity and concentration of substrate linear? Explain this relationship, referring to Figure 7.1.

Preparation For Next Week's Lab:

In next week's lab, you will study the effects of environmental conditions on enzyme activity. Each team of students will design an experimental protocol to study one of the following:

1. What are the effects of 37°C on the stability of IDH?
2. What are the effects of pH of the assay solution?
3. What are the effects of $NADP^+$ concentration?
4. What are the effects of different divalent metal ions?
5. What are the effects of varying salt concentrations?
6. Which species or tissues have the most activity?

Before leaving lab today, each group will complete the following:

1. Develop a clear, concise, and simple hypothesis about the effects of one of the above environmental conditions upon enzyme activity.
2. Design an experiment to test that hypothesis.
3. Prepare a protocol to carry out that experiment.

Lab 3

Environmental Parameters of Enzyme Activity

I. Introduction

Last week you learned:

1. How to perform isocitrate dehydrogenase assays.
2. The relationship between activity and amount of enzyme in an assay.
3. The relationship between activity and substrate concentration.
4. How to present experimental data in graphic form.

In this week's lab you will determine the effects of environmental perturbations of our standard assay conditions. Each group of students will have:

i. Chosen one of the following experiments to complete.
ii. Designed an experimental protocol for that experiment.
iii. Reviewed the protocol with the Instructor.

II. Methods and Materials

You will use the same general methods that you used in Lab 2. All equipment, solutions, and supplies required to carry out your experiments have been prepared and ready for your use. You may wish to review your protocol again and assign specific tasks before you start your experiments.

Experiment 4: The Effects of pH on IDH Activity

Hypothesis: pH of the assay buffer will have no effect on IDH activity.

To test this hypothesis, you will need to follow a protocol that holds all conditions constant except the pH of the assay buffer.

Table 7.4: The effects of varying pH of the assay buffer.

Wells	**pH**	Buffer	$NADP^+$	IDH	Isocitrate
A 1-3	7.0	170	10	10	10
A 4-6	7.5	170	10	10	10
B 1-3	8.0	170	10	10	10
B 4-6	8.5	170	10	10	10
C 1-3	9	170	10	10	10
C 4-6	Blank	180	10	0	10

Procedure

1. Use the P-200 micropipet to add Assay Buffer to the indicated wells.
2. Use the P-20 micropipet to add 10 µl of $NADP^+$ to all wells.
3. Use the P-20 micropipet to add 10 µl of IDH to all wells, except C 4-6.
4. Place the microplate in the chamber of the plate reader.
5. Use the Multi-8 micropipet to add 10 µl of isocitrate to all wells.
6. Activate the plate reader.
7. After printing, remove your plate from the plate reader.
8. Retrieve your data from the printer.
9. Return to your station and organize your data in the table below.
10. Prepare a graph of your data.

Table 7.4a: Data from varying pH of the assay buffer.

Time	pH 7	pH 7.5	pH 8	pH 8.5	pH 9
0					
0 .5					
1					
1.5					
2					
2.5					
3					

Considerations - Experiment 4

Does IDH activity vary when the pH of the assay mixture varies, or are levels of activity the same regardless of pH? Do you need to test activity other pH values? Explain how the pH of the assay mixture might affect activity of an enzyme.

Experiment 5: Does IDH Have a Metal Ion Requirement?

Hypothesis: IDH activity does not require a divalent metal in the assay solution.

To test this hypothesis, you will need to follow a protocol that holds all conditions constant except the presence or absence of divalent metal ions in the assay solution.

Table 7.5: Does IDH Have a Metal Ion Requirement?

Wells	Metal, µl	Buffer	$NADP^+$	IDH	Isocitrate
D 1-3	None	170	10	10	10
D 4-6	EDTA 10	160	10	10	10
E 1-3	Mg^{2+} 10	160	10	10	10
E 4-6	Mn^{2+} 10	160	10	10	10
F 1-3	Cu^{2+} 10	160	10	10	10
F 4-6	Zn^{2+} 10	160	10	10	10
G 1-3	Blank	180	10	0	10

Procedure

1. Use the P-200 micropipet to add Assay Buffer to the wells, as indicated.
2. Use the P-20 micropipet to add metal solutions to wells, as indicated.
3. Use the P-20 micropipet to add 10 µl of $NADP^+$ to all wells.
4. Use the P-20 micropipet to add IDH to all wells, except G 1-3.
5. Place the microplate in the chamber of the plate reader.
6. Use the Multi-8 micropipet to add 10 µl of isocitrate to all wells.
7. Activate the plate reader.
8. After printing, remove your plate from the plate reader.
9. Retrieve your data from the printer.

10. Return to your station and organize your data in the table below.
    [Subtract the mean control value (G 1-3) from other mean values.]
11. Prepare a graph of your data.

Table 7.5a: Data from metal ion experiment.

Time	None	EDTA	Mg^{2+}	Mn^{2+}	Cu^{2+}	Zn^{2+}
0						
0.5						
1						
1.5						
2						
2.5						
3						

Considerations - Experiment 5

Does IDH require a divalent metal ion for activity? Does additional Mg^{2+} added to the standard assay buffer increase activity? What does this observation mean? Does the addition of Mn^{2+} added to the standard assay buffer increase activity? What does this observation mean? How do you explain the effects of Cu^{2+} and Zn^{2+} on IDH activity?

Experiment 6: The Effects of Temperature on IDH Stability

Hypothesis: Exposure to 37°C will have no effect on IDH stability.

To test this hypothesis, you will need to follow a protocol that holds all conditions constant except temperature. You will incubate samples of IDH at 37°C for 0, 2, 4, 6, 8, 10 min prior to assaying activity. Hold all samples on ice until incubations are complete and assay all the samples at the same time.

Heat treatment

1. Label six 1.5 ml microfuge tubes: 0, 2, 4, 6, 8, 10. Place the tubes on ice.
2. Use the P-200 micropipet to add 100 µl of IDH to each tube.
3. Place Tubes 2 - 10 in the water bath at 37°C.
4. Remove each tube at the appropriate time and place the tube on ice.
5. Assay IDH activity as in **Table 7.6** (all volumes are microliters):

Table 7.6: Does exposure to 37°C affect IDH stability?

Wells	Min, 37°	Buffer	$NADP^+$	IDH	Isocitrate
D 1-3	0	170	10	10	10
D 4-6	2	170	10	10	10
E 1-3	4	170	10	10	10
E 3-4	6	170	10	10	10
F 1-3	8	170	10	10	10
F 4-6	10	170	10	10	10
G 1-3	Blank	180	10	0	10

Procedure

1. Use the P-200 micropipet to add Assay Buffer to wells, as indicated.
2. Use the P-20 micropipet to add 10 µl of $NADP^+$ to all wells.
3. Use the P-20 micropipet to add 10 µl of IDH to all wells, except G 1-3.
4. Place the microplate in the chamber of the plate reader.
5. Use the Multi-8 micropipet to add 10 µl of isocitrate to all wells.
6. Activate the plate reader.
7. After printing, remove your plate from the plate reader.
8. Retrieve your data from the printer.
9. Return to your station and organize your data in the table below.
   [Subtract the mean control value (G 1-3) from other mean values.]
10. Prepare a graph of your data.

Table 7.6a: Data from temperature experiment.

Time, min	0 Min	2 Min	4 Min	6 Min	8 Min	10 Min
0						
0.5						
1						
1.5						
2						
2.5						
3						

Considerations - Experiment 6

Is IDH stable at 37°C? How can your results be explained? How would you design this experiment to test your hypothesis further?

Experiment 7: Does enzyme activity vary with concentration of $NADP^+$?

Problem: What is the relationship between the rate of a reaction and the amount of coenzyme in the assay solution when the amount of enzyme is held constant? Before you start this experiment, develop an hypothesis and sketch a graph predicting the relationship of activity *vs* substrate concentration.

Procedure: To test your hypothesis, you will need to follow a protocol that holds all conditions constant except the amount of coenzyme added to each assay. **Table 7.7** outlines such a protocol using six concentrations of $NADP^+$. Each concentration is tested in triplicate. Add reagents to your wells as listed from left to right.

Table 7.7: The effects of varying the concentration of $NADP^+$.

		$NADP^+$			
Wells	**Buffer**	**Sol***	**Vol**	**IDH**	**Isocitrate**
D 1-3	170	1	10	10	10
D 4-6	170	2	10	10	10
E 1-3	170	3	10	10	10
E 4-6	170	4	10	10	10
F 1-3	170	5	10	10	10
F 4-6	180	Blank		10	10

The concentration of these $NADP^+$ solutions will be provided by the Instructor. The second number refers to the volume [µl] to be used. NOTE: In these assays we will initiate reactions with isocitrate. Do not add isocitrate until your plate is in the plate reader.

Procedure

1. Use the P-200 micropipet to add Assay Buffer to the wells, as indicated.
2. Use the P-20 micropipet to add 10 µl of $NADP^+$ solutions to indicated wells.
3. Use the P-20 micropipet to add IDH to all wells.
4. Place the microplate in chamber of the plate reader.
5. Use the Multi-8 micropipet to add 10 µl of isocitrate to all wells.
6. Activate the plate reader.
7. After printing, remove your plate from the plate reader.
8. Retrieve your data from the printer.
9. Return to your station and organize your data in the table below.
   [Subtract the mean control value (F 4 - 6) from other mean values.]
10. Prepare a graph of your data.

Table 7.7a: Data from varying the concentration of $NADP^+$.

Time, min	0.144 mM	0.072 mM	0.036 mM	0.0216 mM	0.0144 mM
0					
0.5					
1					
1.5					
2					
2.5					
3					

Considerations - Experiment 7

Organize the data from your experiment. Determine the mean activity for each concentration of $NADP^+$. Construct a graph that compares activity as a function of $NADP^+$ concentration. Do your data support your hypothesis? Is the relationship between activity and concentration of $NADP^+$ linear? Explain this relationship, referring to the model in Figure 1.

Experiment 8: The Effects of NaCl on IDH Activity

Hypothesis: Sodium chloride will have no effect on IDH activity.

To test this hypothesis, you will need to follow a protocol that holds all conditions constant except concentration of NaCl in the assay solution.

Table 7.8: The effects of NaCl on IDH activity.

Wells	[NaCl] M	Buffer	5M NaCl	$NADP^+$	IDH	Isocitrate
D 1-3	0.0	170	0	10	10	10
D 4-6	0.1	166	4	10	10	10
E 1-3	0.2	162	8	10	10	10
E 4-6	0.3	158	12	10	10	10
F 1-3	0.4	154	16	10	10	10
F 4-6	0.5	150	20	10	10	10
G 1-3	Blank	180	0	10	0	10

Procedure

1. Use the P-200 micropipet to add Assay Buffer to the wells, as indicated.
2. Use the P-20 micropipet to add NaCl to the wells, as indicated.
3. Use the P-20 micropipet to add 10 µl of $NADP^+$ to all wells.
4. Use the P-20 micropipet to add IDH to all wells, except G 1-3.
5. Place the microplate in the chamber of the plate reader.
6. Use the Multi-8 micropipet to add 10 µl of isocitrate to all wells.
7. Activate the plate reader.
8. After printing, remove your plate from the plate reader.
9. Retrieve your data from the printer.
10. Return to your station and organize your data in the table below.
11. Prepare a graph of your data.

Table 7.8a: Data from the effects of NaCl on IDH activity.

Time, min	0 M	0.1 M	0.2 M	0.3 M	0.4 M	0.5 M
0						
0.5						
1						
1.5						
2						
2.5						
3						

Considerations - Experiment 8

Did you observe differences in activity between the treatments? What was the relationship between the concentration of NaCl and activity? Explain how salt might affect enzyme activity.

Experiment 9: Does IDH activity vary among different organisms or tissues?

This is an "open ended" experiment; you may design a single, additional experiment, comparable to the ones listed above, or expand these topics into a research project of wider magnitude.

1. You may chose to survey IDH activity:
    i. In a wide variety of related species [think salad bar].
    ii. In different tissues of a single species [think chicken giblets].
2. Homogenize your samples in cold Assay Buffer, using a kitchen blender.
3. Filter the homogenate through two layers of cheesecloth into a small beaker that is on ice.
4. Transfer 1 ml samples to 1.5 ml microfuge tubes, spin for 5 minutes.
5. Transfer the supernatant to clean 1.5 ml microfuge tubes, kept on ice.
6. Use standard conditions to assay IDH activity.

Before You Leave Lab

1. Be certain that you have collected all of the data you need to make your experiment complete.
2. Be certain that each member of the group understands fully what was done and has a copy of all of your data.
3. Schedule a meeting of your group [why not now?] to analyze your results and prepare slides for your group's oral presentation.

Lab 4

During laboratory meeting number 4, students present their work in the form of a research symposium: http://www.bio.davidson.edu/Biology/Courses/Bio111LabMan/TOC.html

Appendix A

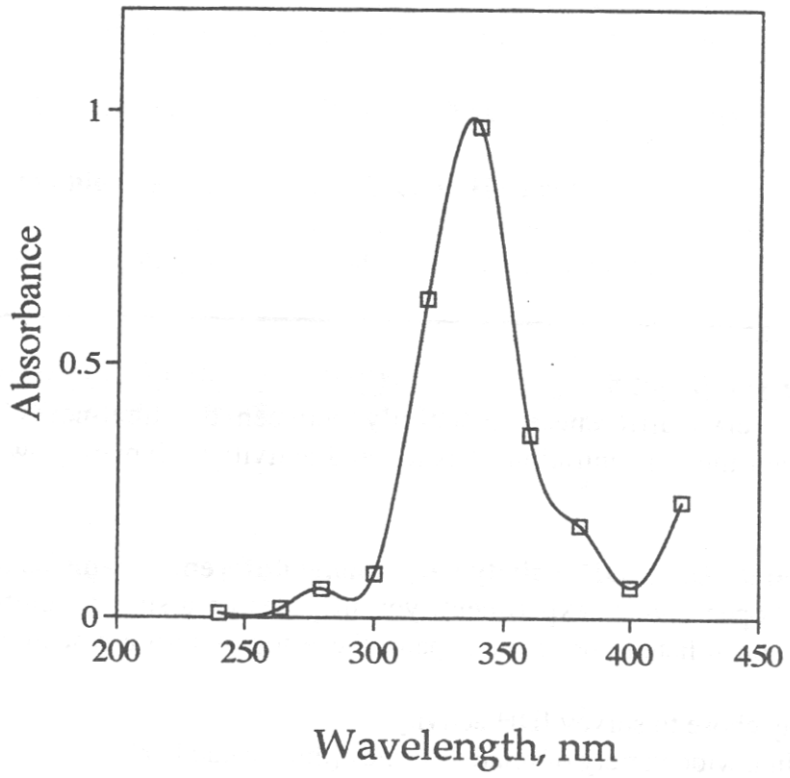

Figure7A-1. An absorption profile for NADPH.

Appendix B

Representative Results from Lab #2

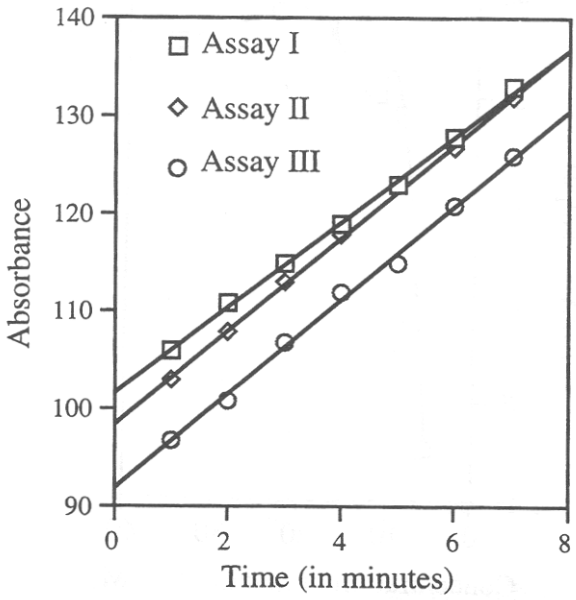

Figure 7B-1. IDH assays are repeatable and are linear for at least three minutes.

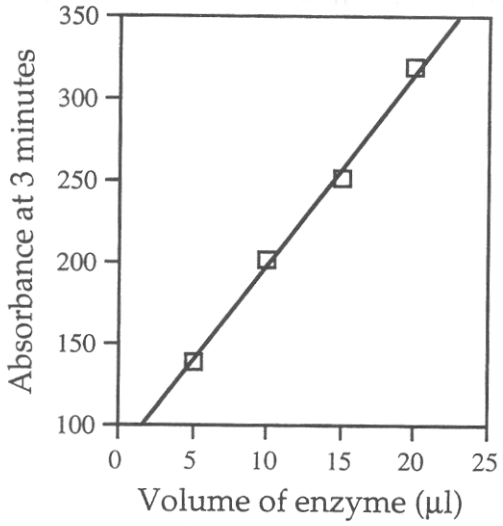

Figure 7B-2. IDH activity is directly proportional to the amount of enzyme added to the reaction mixture.

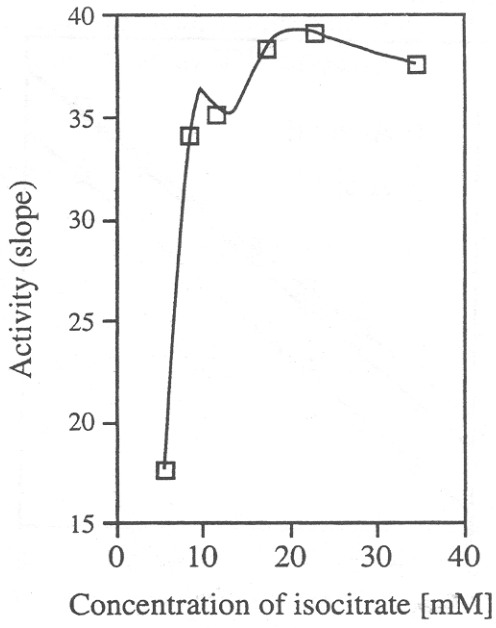

Figure 7B-3. IDH activity is not directly proportional to the amount of substrate added to the reaction mixture.

Appendix C

Representative Results from Lab #3

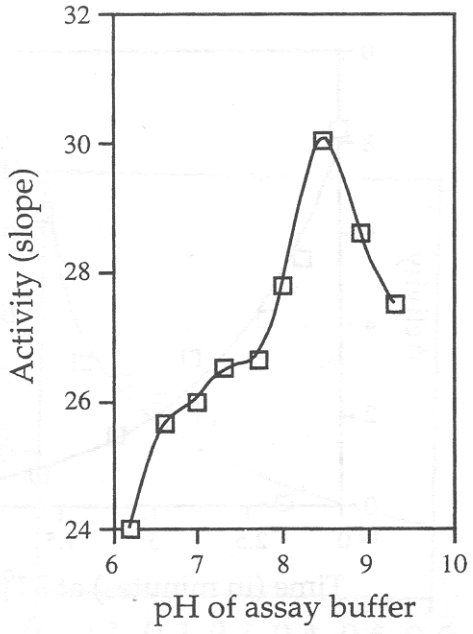

Figure 7C-1. Maximum IDH activity depends on the pH of the assay mixture.

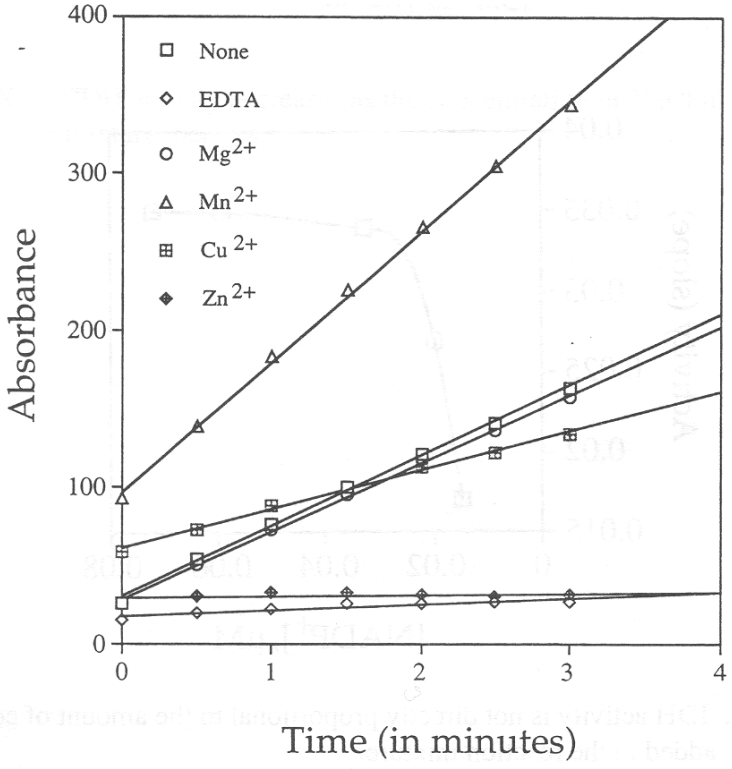

Figure 7C-2. IDH activity is dependent upon the presence of a divalent metal ion. However, some metal ions are toxic to IDH activity.

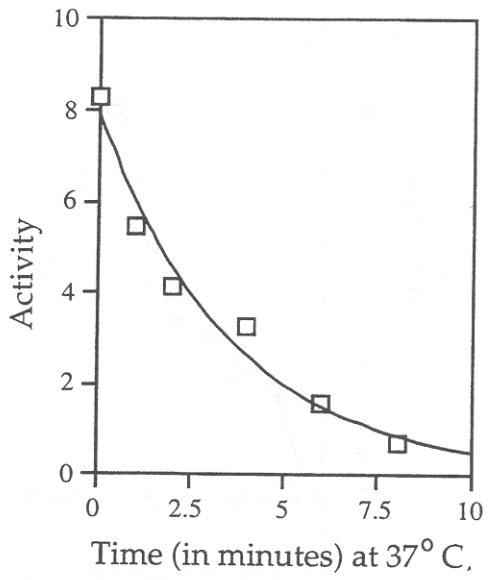

Figure 7C-3. IDH activity decreases when the enzyme is incubated at 37°C.

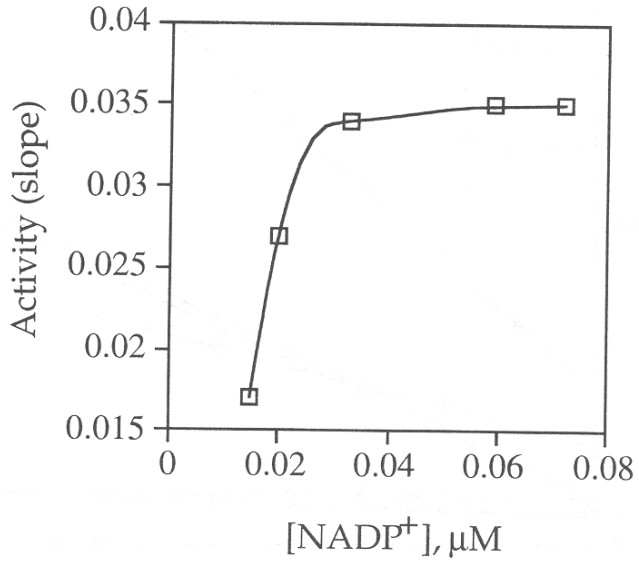

Figure 7C-4. IDH activity is not directly proportional to the amount of coenzyme added to the reaction mixture.

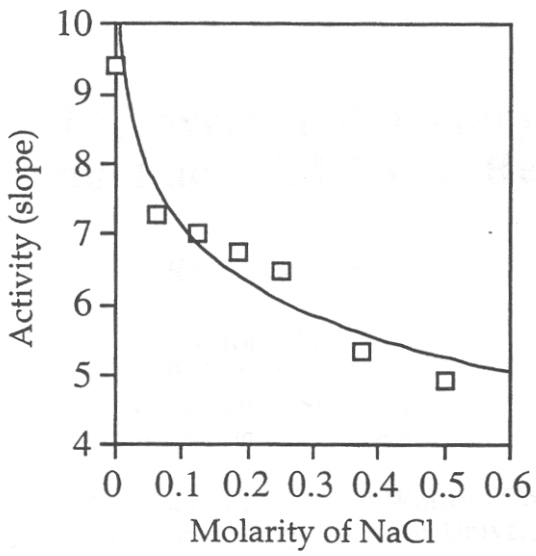

Figure 7C-5. IDH activity decreases as the concentration of NaCl in the assay mixture increases.