

Auxiliary Lab Manual

Chem 465L

Biochemistry II Lab

Spring 2012

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## Principles for Safety in the Chemical Laboratory

Safe practices in the chemical laboratory are of prime importance. A student should consider it an essential part of his or her educational experience to develop safe and efficient methods of operation in a lab. To do this, one must acquire a basic knowledge of properties of materials present in the lab, and one should realize the types of hazards that exist and the accidents and injuries that can result from ignorance or irresponsibility on the part of the student or a neighbor.

### Regulations

1. Wear safety goggles at all times while in the laboratory.
2. Report all accidents to the instructor or lab assistant immediately.
3. **NEVER** eat, drink, chew, or smoke in the laboratory.
4. **NEVER** leave an experiment unattended. Inform the lab assistant if you must leave the lab.
5. After the experiment is completed, turn all equipment off, making sure it is properly stored, and clean your area.

Failure to comply with these regulations is cause for immediate dismissal from lab.

### Precautions

1. Approach the laboratory with a serious awareness of personal responsibility and consideration for others in the lab.
2. Become familiar with the location of safety equipment, such as acid-base neutralizing agents, eye wash, fire extinguisher, emergency shower, and fire blanket.
3. Pay strict attention to all instructions presented by the instructor. If something is not clear, do not hesitate to ask the instructor or lab assistant.
4. Clean up all chemical spills immediately.
5. Be aware of all activities occurring within a reasonable proximity of yourself since you are always subject to the actions of others.
6. To avoid contamination of community supplies, do not use personal equipment such as spatulas in shared chemicals and replace all lids after use.

7. Avoid unnecessary physical contact with chemicals; their toxic properties may result in skin irritation.
8. Use all electrical and heating equipment carefully to prevent shocks and burns.
9. **NEVER** handle broken glassware with your hands; use a broom and a dust pan.
10. Wash your hands at the end of the laboratory.

### **Personal Attire**

Choice of clothing for the laboratory is mainly left to the discretion of the student. Because of the corrosive nature of chemicals, it is in your best interest to wear comfortable, practical clothing. Long, floppy sleeves can easily come into contact with chemicals. A lab coat is suggested to help keep clothes protected and close to the body. Accessories also need consideration. Jewelry can be ruined by contact with chemicals. Open toed shoes do not adequately protect one against chemical spills. If hair is long enough to interfere with motion or observation, it should be tied back. Remember that your clothes are worn to protect you.

### **Assembling Equipment**

Equipment should be assembled in the most secure and convenient manner. Utility clamps are provided to fasten flasks, etc., to the metal grid work located at the center of each bench. This keeps top-heavy or bulky equipment away from the edge where it can be knocked easily off the bench. Consider the safe location of the hot plate. Keep it near the grid work to minimize chances of contact with the body. If the aspirator is being used, locate your apparatus near the sink for convenience.

### **Handling Glassware**

Laboratory glassware is usually fragile, and if it is not properly handled, serious injuries may result. Do not force glass tubing or thermometers into a rubber stopper. Lubricate the tubing or thermometer with glycerol or water, wrap it in a towel, and gently insert it into the stopper by using pressure in a lengthwise direction while rotating it. Always grasp the tubing near the stopper. When removing the tubing, remember to protect your hands with a towel. If there are difficulties with this procedure, ask for the instructor's assistance. Apparatus that can roll should be placed between two immobile objects away from the edge of the bench. Chipped or broken glassware cannot be used. There are special receptacles near each bench for these waste materials. After the experiment is completed, all glassware should be emptied, rinsed, and cleaned.

## **Acids and Bases**

In this lab sequence, you will come in contact with several acids and bases. As with all chemicals, caution must be taken to prevent contact with the skin. When handling these chemicals, keep hands away from the eyes and face until they have been thoroughly washed. If an acid or base comes in contact with your skin, flush the area with large quantities of clean, cold water. Eyes are extremely sensitive. Use the eye wash provided in the laboratory, or wash with water for at least 10 minutes. Again, the instructor must be notified immediately. To insure your safety, neutralize acid or base spills before cleaning them up. Boric acid solution is available to neutralize base spills, and carbonate powder is provided to neutralize acids.

## **Attention:**

Students are advised against wearing contact lenses while observing or participating in science laboratory activities. While hard contact lenses do not seem to aggravate chemical splash injuries, soft contact lenses absorb vapors and may aggravate some chemical exposures, particularly if worn for extended periods.

**Please take your contact lenses out prior to entering the laboratory.**

### **Contact Lens Administrative Policy and Waiver Form**

Students are advised against wearing contact lenses while observing or participating in science laboratory activities. While hard contact lenses do not seem to aggravate chemical splash injuries, soft contact lenses absorb vapors and may aggravate some chemical exposures, particularly if worn for extended periods. You are asked to please remove your contact lenses prior to entering the laboratory.

If you do not wish to comply with this recommendation, you must fill out the next page, which is a waiver form.

## **Waiver of Liability, Indemnification and Medical Release**

I am aware of the dangers involved in wearing contact lenses in a science laboratory setting. On behalf of myself, my executors, administrators, heirs, next of kin, successors, and assigns, I hereby:

- a. waive, release and discharge from any and all liability for my personal injury, property damage, or actions of any kind, which may hereafter, accrue to me and my estate, the State of South Dakota, and its officers, agents and employees; and
- b. indemnify and hold harmless the State of South Dakota, and its officers, agents and employees from and against any and all liabilities and claims made by other individuals or entities as a result of any of my actions during this laboratory.

I hereby consent to receive any medical treatment, which may be deemed advisable in the event of injury during this laboratory.

This release and waiver shall be construed broadly to provide a release and waiver to the maximum extent permissible under applicable law.

I, the undersigned participant, acknowledge that I have read and understand the above Release.

Name \_\_\_\_\_ Age \_\_\_\_\_

Signature \_\_\_\_\_ Date \_\_\_\_\_

## Modified Assay Procedure

Spectrophotometer will be set to 340 nm

Spectrophotometer will be in a time acquisition mode, so rather than scanning different wavelengths it will sit at one wavelength and acquire absorbance as a function of time. I will set it up to acquire a data point every 5 seconds for a total time of 5 minutes.

### Assay set up

The book describes an assay where you mix 1.9 ml of CAPS, 0.5 ml of NAD<sup>+</sup> and 0.5ml of Lactate. What I have done is to combine the CAPS and Lactate into a single buffer, so the actual solution you will mix is 2.4ml CAPS/Lactate, 0.5 mL NAD<sup>+</sup> and 0.1ml dilute sample to give you a total of 3.0 ml. Note: for this procedure use only large volume plastic cuvettes. Don't use the small volume ones because there isn't enough room for your sample to mix.

Here is how to perform a single assay.

1. Check that the spectrometer is set up and ready to run. (It should display a white graph with no data on it, and the Start button should be green)
2. Put a cuvette filled with deionized water in the reference side of the spectrometer.
3. Fill a second cuvette with CAPS/Lactate and NAD<sup>+</sup>. Cover cuvette with a piece of parafilm and flip upside down 2-3 times to get it to mix.
4. Click on the green 'Start' button. The computer will tell you to insert a blank
5. Insert your sample. You should have a very low absorbance. If not, then some trace of LDH has already started the reaction. In this case remove the cuvette, wash it, and restart the assay.
6. After the computer is satisfied with the blank, it will tell you to insert your sample.
7. At this point remove take the cuvette out of the machine, add the 100  $\mu$ l of dilute protein, cover with parafilm, flip it upside down 2 or 3 times to get it to mix, insert it into the machine, and tell it to begin acquisition. Make sure you note down the name of the data file it is acquiring the data to (Something like td015). When the lab is over I will print out all the data for all the groups and bring it to class.
8. You should be able to see the absorbance increase with time on the spectrophotometer. If you get a strong rise in absorbance in the first minute, you don't have to wait for the entire 5 minutes. Watch the output on the computer screen. Only stop the run if your changing absorbance curve is displayed on the computer monitor and the absorbance changes by at least .1 absorbance units in the first minute. To stop the run click on the red 'Stop' button then start your next run.
9. When you are through for the day, don't change any of the settings on the spectrophotometer. Leave it just as you found it for the next group.

## Report Sheet for Experiment 7a

1. What is the void volume ( $V_o$ ) of the column?
2. What is the elution volume ( $V_e$ ) for each peak observed for the crude LDH?
3. What is the elution volume ( $V_e$ ) for the peak of the LDH activity? Does this correspond to one of the peaks reported in question 2?
4. What is the elution volume ( $V_e$ ) for the three molecular weight standards?
5. Plot of log molecular weight (Y) and  $V_e/V_o$  (X) for the three standards.  
Calculate a line of best fit for this line
6. Use this line to estimate the molecular weight of all peaks reported in question 2.
7. What is your estimate of the molecular weight of our elk heart LDH?

## *In Vitro* and *in Vivo* $^{13}\text{C}$ Analysis of Anaerobic Yeast Metabolism of 1- $^{13}\text{C}$ - Glucose

In this section you will find:

- 1.) A lab procedure for the *in Vitro* metabolism experiment complete with pre-lab and post-lab questions.
- 2.) A lab procedure for the *in vivo* equivalent *in Vitro* experiment.
- 3.) The papers that describe both methods.

Papers are included for two reasons. The first is that I want you start seeing some real scientific papers and how they are written. The second is that I want you to start to make the connection between what is written, and how that translates into a real lab procedure.

This lab can be performed in a couple of different ways. Sometimes I allocate two weeks to the lab, and have students do both labs. Under this format, Group A students will do the *in vivo* experiment in week one, while group B students will do the *in vitro* experiment. During the following week the roles are reversed, and Group A students do the *in vitro* experiment, while group B students do the *in vivo* work.

In 2010, where we had a dozen students in the lab, it was not possible to have four groups do the *in vivo* experiment in a timely manner in a regular lab period, so we did only the *in vitro* experiment.

I have also tried to use the GC/MS to follow the  $^{13}\text{C}$  label. This has not proven very successful, but I have included the basic procedure, just in case I want to try it again.

## *In Vitro* $^{13}\text{C}$ Analysis of Anaerobic Yeast Metabolism of 1- $^{13}\text{C}$ - Glucose

### **Summary:**

In this lab we will grow up yeast under anaerobic condition, and then give the yeast glucose labeled at the #1 position with  $^{13}\text{C}$ . Under these conditions the yeast will uptake the glucose and begin to metabolize it. As it is metabolized the  $^{13}\text{C}$  of the glucose will get incorporated into other compounds. You should observe the label moving into at least 2 other compounds, glycerol and ethanol.

### **Prelab questions:** (Answers due in box at START of lab)

1. Write the structure of glucose. Identify the #1 C in this compound. From last semester's Biochem lab identify what the C and H chemical shift at this position should be. If you did not do the glucose NMR experiment either ask the instructor or somebody who did the experiment for some spectra to look at.
2. What is the purpose of adding Perchloric acid to the yeast extract?
3. What is the purpose of adding phenolphthalein to the final, killed extract?

### **Experimental Procedure:**

#### *Rehydrating yeast (1 batch does the entire lab):*

Weigh out about 2 g of dehydrated yeast.

Place in a 50 mL Erlenmeyer flask.

Add a small stir bar.

Add about 20 mL of buffer.

Bubble  $\text{N}_2$  gas into flask for 5 minutes to displace  $\text{O}_2$ .

Tightly stopper flask.

Place on magnetic stirrer and let yeast hydrate for 60 minutes:

Loosen stopper every 5 minutes to release pressure

but do not remove stopper so yeast stays under anaerobic conditions.

#### *Preparation of killing solutions:*

Place five Eppendorf tubes on ice.

Add 125 $\mu\text{L}$  of 70% perchloric acid to each Eppendorf tube.

#### *Preparation of yeast:*

Place 4 mL of yeast extract in a 2 dram vial.

Place vial in Pierce reactor set to 25-30 $^\circ\text{C}$ .

Blow  $\text{N}_2$  over vial and rig to keep gentle  $\text{N}_2$  purge going continuously.

*Actual experiment:*

After purging for 5 minutes.

Start stop watch.

Add 125  $\mu\text{L}$  of  $^{13}\text{C}$  glucose to vial.

Immediately remove a 0.8 mL aliquot from vial and place into one of the kill tubes.

At 7.5 minutes, 15 minutes, 22.5 minutes and 30 minutes remove an additional 0.8 mL aliquot and place in a kill tube.

Take all Eppendorf kill tubes and freeze-thaw in liquid  $\text{N}_2$  or dry ice-acetone at least 3 times to rupture the cells.

*Neutralizing acid in kill tube:*

Add 1 drop of phenolphthalein indicator to tube.

Add 50  $\mu\text{L}$  aliquots of 10% KOH to neutralize the perchloric acid.

You can tell the acid has been neutralized because the solution will turn pink!

It will take about 10 'hits' to accomplish this.

The sample can now be left in the refrigerator until you are ready to do your NMR experiment.

You must centrifuge the Eppendorf tube to pellet out the cellular debris before you fill the NMR tube

### **Notes on running the NMR:**

Find the sealed sample labeled 'Sensitivity 5% Ethyl Benzene'.

Place in NMR and make sure it is spinning.

Do the first 6 steps listed on the plastic sheet labeled '<sup>13</sup>C Survey Spectra Guide'.

Remove the 5% Ethyl Benzene sample from the NMR and replace it with your sample. (Again check to make sure the sample is spinning.)

In the PNMR console type 'Shim' so the NMR is shimmed so your sample gives its best results.

Type 'ns'.

For the Perchloric killed experiment, try setting this to 200.

Type 'zg' the computer will ask for the name of the file you want to save this to, give it a name, and let it do its thing.

For each perchloric acid experiment the NMR will take about 25 minutes. When you are through with your first experiment, clean out the NMR tube, load up your next sample and do your next run.

### **Processing Spectra**

ga (get spectrum a)

When the window comes up, find the spectrum you want to process.

ln (liner prediction)

Usually the first point or two are messed up, so you use the rest of the spectrum to predict what the points should actually be. Use the default values so simply click 'ok'.

bc (baseline correct)

Corrects the baseline of the spectrum if it is offset from y.

lb (line broadening)

Brings up all sorts of options on how to get rid of noise. Set LB to 0.5 and hit 'OK'.

em exponential multiply

Multiplies spectrum by an exponential function. See how it emphasizes the good data at the beginning of the spectrum and eliminates noise at the end of the spectrum?

ft fourier transform

Do a Fourier transform of your data.

ap autophase

Do an automatic phase correction (this will take a few seconds).

You now have a  $^{13}\text{C}$  spectrum to look at. Now let's see what we can do with it. First let's label each peak

dp define peaks

This is a subroutine that let's you manually define each peaks.

c clear

clear any peaks left over from the previous user.

left mouse click on any peak, a label should appear at the nearest peak. If it does not then.

control-P. This toggles the label display on and off. If you don't like a peak, left mouse click near that peak and type:

k kill

to kill that peak on the list.

Once you have finished picking the peaks hit the return button to take you out of the define peaks subroutine.

Next I want to zoom in on the region form 0-100 ppm where all our peaks should be

zo zoom

When you are in zoom mode you should see a cross with the letters zo by it for the mouse pointer. We will stay in this subroutine for the next several steps.

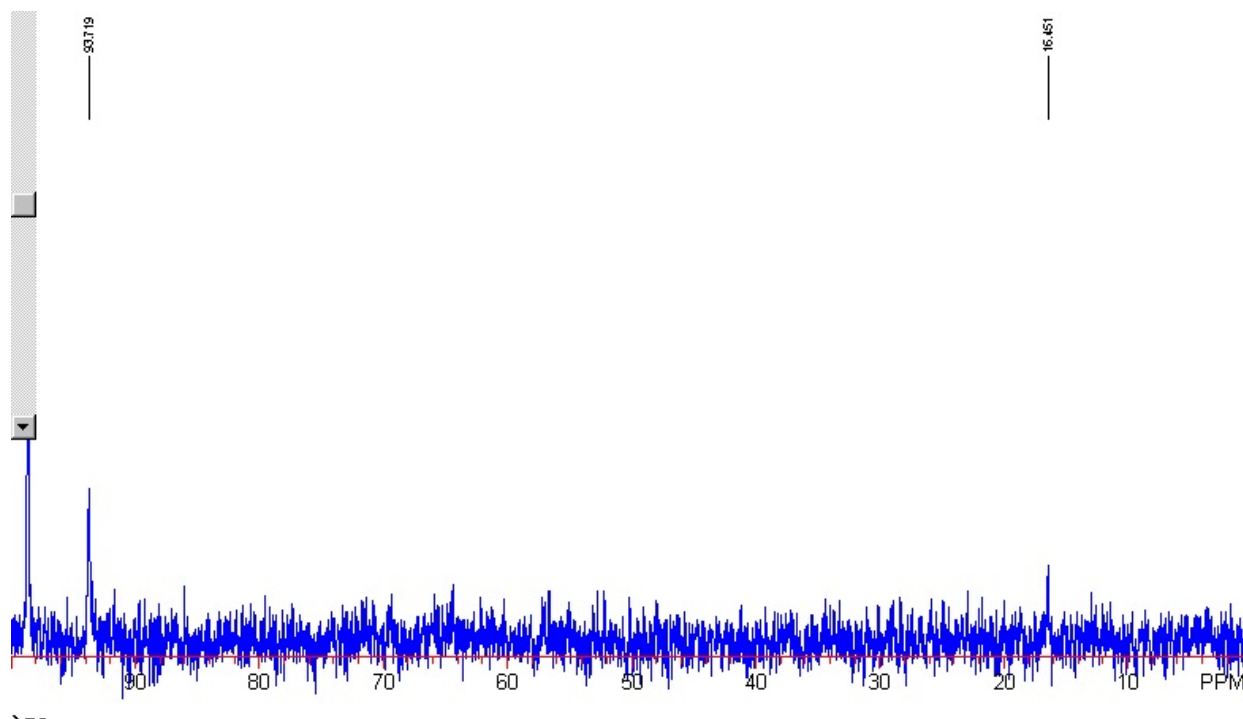
Commands in the subroutine are given by 1 letter codes.

f frequency

Under Horizontal Dimension put 100 ppm for start and 0 ppm for end.

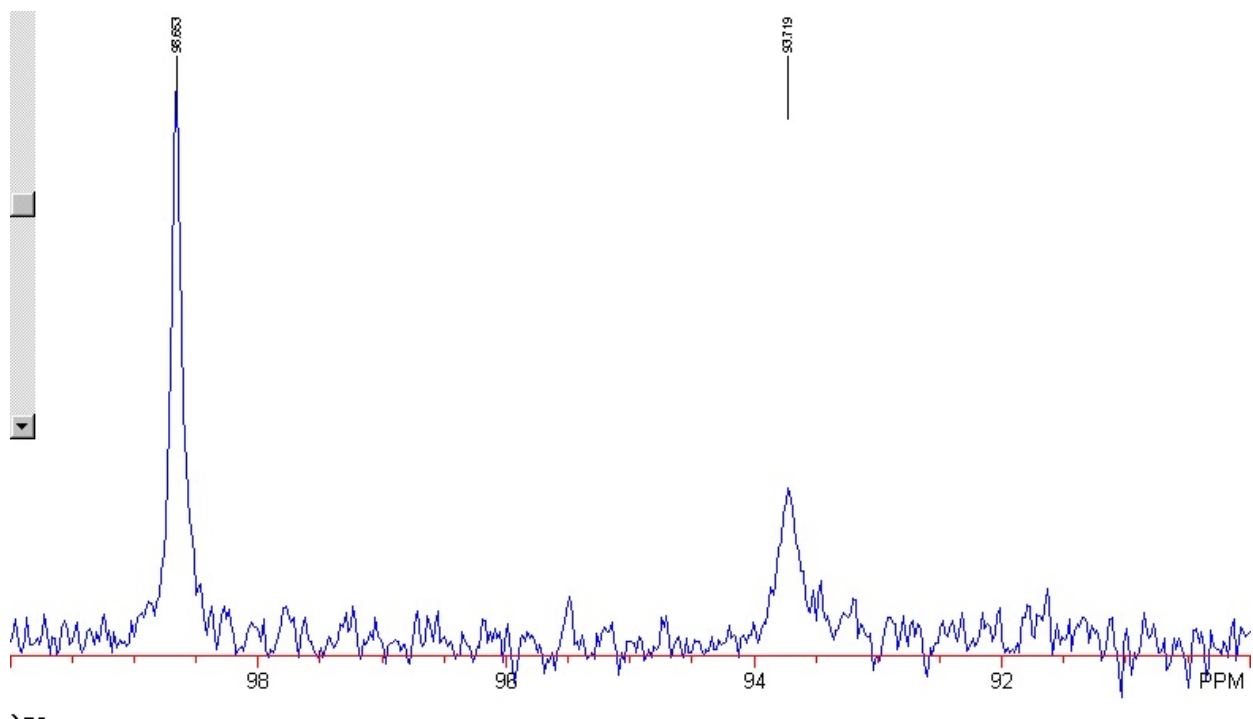
Hit the OK button, you should now see this region of your spectrum colored in red. Do a right mouse click anywhere in the red region, and the spectrum will zoom in to that region. If you want to zoom back out, just click the right mouse button a second time.

At this point I would like you to copy spectrum to a word document to you can record what you have accomplished. Hit the copy button at the top of the page, open Word in another window and past the spectrum into this window as I have done below:



In this spectrum I can clearly see the two glucose peaks in the 90-100 region and the ethanol peak in the 10-20 region. What I want to do next is to integrate the area under these peaks. My experience is that integrating these noisy carbons peaks is a bit difficult unless you really zoom in to see each peak clearly. So I next want to zoom in specifically on the 100-90 region to look at these peaks more closely. So...

- f frequency  
takes you back to the frequency this time set start to 100 and end to 90.  
When you hit the OK button, the spectrum will zoom in on this region  
directly, as shown below.



Now you want to start integrating, Within the Zoom subroutine this is the command to start this sub-subroutine is:

i integrate

Before you do anything, first clear out any previous integrals by typing

c clear

You should now see a continuous line snaking from the lower left toward the upper right.. Next your want to restrict the integral line to the region just before till just after each peak.

Left mouse click twice on one side of a peak, then move the cursor to the other side of the peak and click once. You should now see a single integral with a number beside it located at the peak you are working on, as seen on the next page.

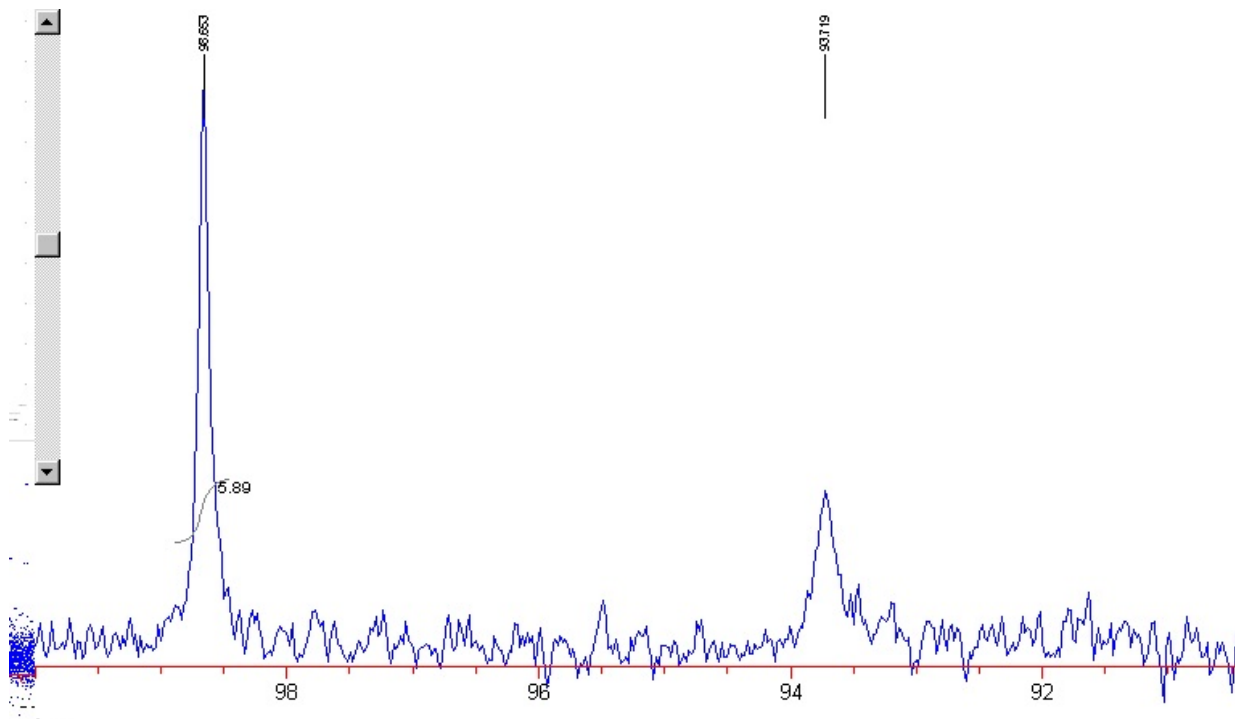
Note: if you make a mistake, and don't like the integral you just made, you can delete two different ways:

Way 1.

type 'l' for delete last integral

Way 2.

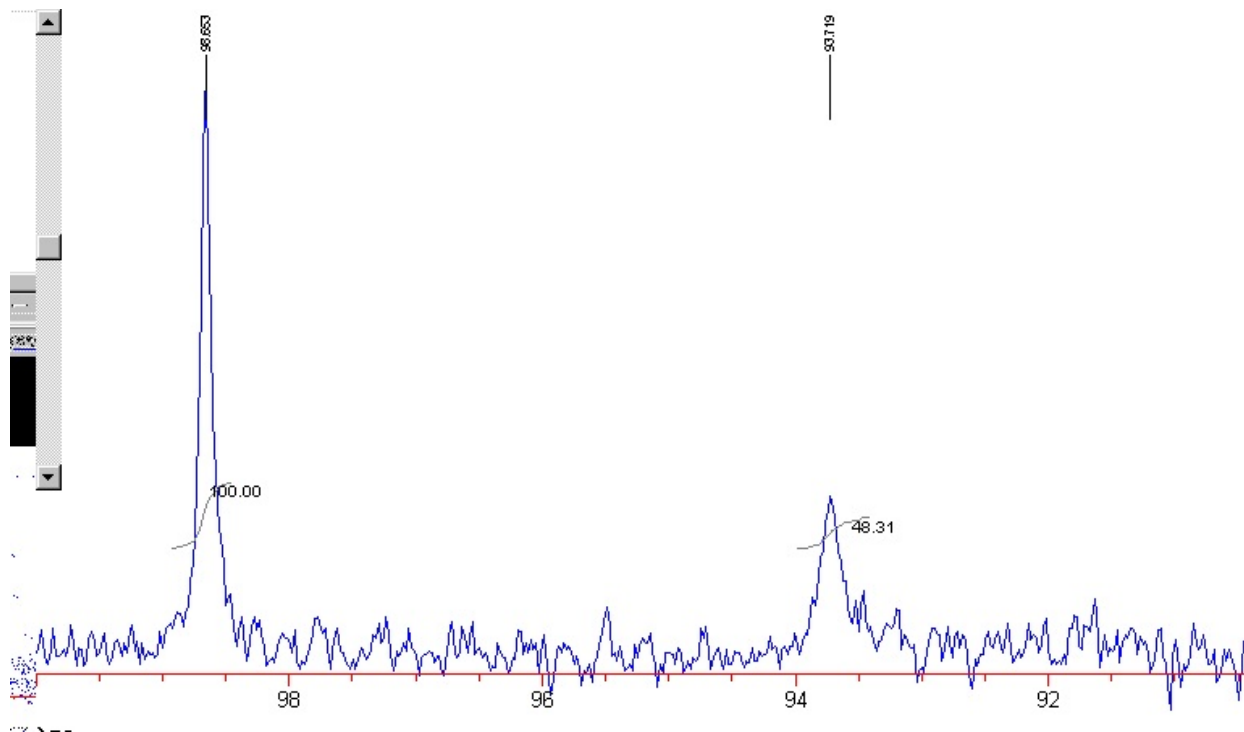
Left mouse click on the integral and type 'd' for delete



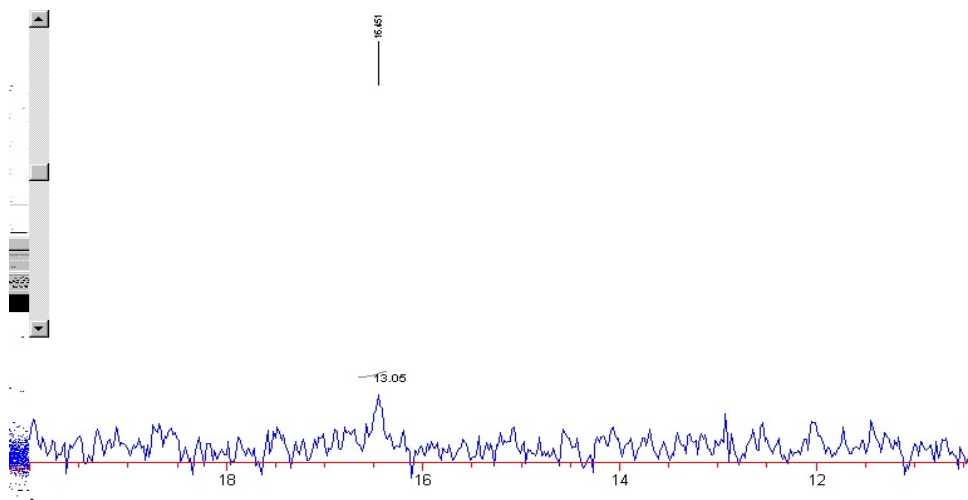
Next you need to set the value of this integral to some arbitrary number say 100. To do this left mouse click on the integral and type 'v' for value, and enter 100 into the window.

Now that this peak is integrated try to integrate the other peak on your own. As I have done on the next page.

In creating the second integral the number associated with this peak is the integral relative to the 100 on your first peak, so do not try to set the value for this peak.



OK, ready for the next step? Hit the return button to take you out of the integration sub-routine and back into the zoom routine. Type f again and set the frequency of the zoom to 20 to 10 so we can work on integrating the other peak. Hit i to continue your integration, and again double left click on one side of the peak and single left click on the other side of the peak to get the other end of the integral.



Continue with this process until you have integrals for all the peaks in your spectrum. Once this is done, the computer can list all of these integrals to the clipboard so you can paste them into a document. To do this type 't' in the integral sub-subroutine. Nothing will happen to the screen, but now when you go to your document and hit the paste button, a table should appear like the one below:

NUMBER	FROM	TO	VALUE
1	98.93 PPM	98.43 PPM	100.00
2	93.99 PPM	93.39 PPM	48.31
3	16.67 PPM	16.35 PPM	13.05

Now how do we use this data to come up with concentrations of glucose, glycol, and ethanol?

Please check me on these calculations!

The glucose solution was 10 mg glucose/50 ul of water

This is equivalent to 100 mg/ 500 ul = 0.1g/0.5 ml = 0.2g/1.0 ml

1000 mls in a liter makes this a concentration of 200g/l

Glucose has a molar mass of 180 g/mole so

$200 \text{ g} \times (1/180) = 1.11\text{M}$  glucose.

To make up your vials you started with 4.0 ml of extract and added .125 ml of glucose so your final volume would be 4.125 ml.

Using good old  $M_1V_1=M_2V_2$   
 $1.11(.125) = X(4.125); X = .0336M$

So the glucose concentration at the start of the experiment is .0336 M. Since each glucose is labeled with  $^{13}C$ , this is also the molar concentration of  $^{13}C$ .

In this spectrum we have 3 peaks with integrals of 100, 48, and 13. So the total integral is  $100+48+13 = 161$   
The total glucose peak is  $100+ 48 = 148$   
And the ethanol peak is 13

Setting up proportions

glucose integral /total integral = moles glucose remaining/total moles  $^{13}C$   
 $148/161 = X/.0336; X = .031M$

Ethanol integral/total integral = moles labeled ethanol/total moles  $^{13}C$   
 $13/161 = X/.0336; X=.0027M$

But remembering only  $\frac{1}{2}$  of the ethanol is labeled, the true ethanol concentration is .0054M

So. By doing this analysis for each time point, you should be able to come up with concentrations of glucose, glycerol and ethanol at each time point, and you should be able to do soe kinetic plots to see if these are zero, first, or second order reactions!  
And maybe get a k??

## In Vivo $^{13}\text{C}$ Analysis of Anaerobic Yeast Metabolism of 1- $^{13}\text{C}$ - Glucose

### **Summary:**

In this lab we will grow up yeast under anaerobic condition, and then give the yeast glucose labeled at the #1 position with  $^{13}\text{C}$ . Under these conditions the yeast will uptake the glucose and begin to metabolize it. As it is metabolized the  $^{13}\text{C}$  of the glucose will get incorporated into other compounds. You should observe the label moving into at least 2 other compounds, glycerol and ethanol.

Included in this manual are two different NMR procedures. In the *Automatic Procedure* you set up a file that runs the NMR at several different times automatically over a 40 minute time period. In the *Manual Procedure* you have to run the NMR yourself every 5 minutes over the same time period.

### **Prelab questions:** (Answers due in box at START of lab)

1. Write the structure of glucose. Identify the #1 C in this compound. From last semesters Biochem lab identify what the C and H chemical shift at this position should be. If you did not do the glucose NMR experiment either ask the instructor or somebody who did the experiment for some spectra to look at.
2. How are you going to change the lab procedure as written to include the 'Yeast Extract Procedure' given in the second paper. Try to write like a lab procedure I would give you.

### **Experimental Procedure:**

**Note:** Only one group can have their sample in the NMR at a time. I anticipate that making the first buffer (Step 1) may take about 20 minutes, Growing the yeast in the buffer (Step 2) should take another 60 minutes, and the actual NMR experiment (step 3) will take about 40 minutes. Coordinate your timing of these steps with other groups so we don't have two groups trying to run the NMR part of the experiment at the same time. If you want to make your buffer, but come in on a different day to do the experiment that is acceptable, but notify Dr. Z. so he can be around to help out.

1. *Prepare Yeast Growth buffer. All groups can do on the first day*

Prepare the following buffer for growing yeast in

0.075 g  $K_2HPO_4$

0.25 g  $MgSO_4$

0.425 g  $KH_2PO_4$

11.15 g  $Na_4P_2O_7$  or 18.7 g of  $Na_4P_2O_7 \cdot 10H_2O$

Bring to about 400 mls, adjust pH to 6.0 then bring to a final volume of 500 ml  
(In 2004 this took about 50 ml of 1M HCl)

**Save this buffer for use in next week's lab as well!**

2. *Should be done about 60 minutes prior NMR time*

Weigh out about 2 g of dehydrated yeast.

Place in a 50 ml Erlenmeyer, add a small stirbar, add 20 ml of buffer

Bubble  $N_2$  gas into buffer and yeast for 5 minutes to remove  $O_2$

Stopper tightly (Rubber stopper, not parafilm- needs to be air tight so no  $O_2$  gets in)

Stir with a magnetic stirrer for 60 minutes

Every 5 minutes loosen the stopper to let  $CO_2$  out of the flask, then reseal so  $O_2$  can't get in

3A *Manual NMR Procedure*

**Should be done just prior to NMR time**

Preparing the NMR

Place 5% Ethyl Benzene reference sample in NMR

Shim

Obtain a proton spectrum for the reference sample

If the TMS peaks is not at 0.0 ppm use the fo command in Pnmr to reset the frequency offset to the correct value.

Set NMR for  $^{13}C$  experiments (nu C13)

Preparing the Sample

-Get an NMR tube ready and purge for 30 second with  $N_2$  gas

-Open your yeast flask

- Remove a **800  $\mu L$  aliquot** and place in an **Eppendorf tube** that has been purged with  $N_2$  Gas

- Place **50  $\mu L$  of Labeled glucose** in **NMR tube** and mix

-Transfer the yeast extract/glucose mix to an NMR tube, and blow  $N_2$  over the top of the mixture before capping

-Place tube in the NMR and get your first spectrum as quickly as possible

## Running the NMR

Shim the sample

Note the time, and Start the first experiment - give your sample a unique name and take at least 24 scans. For the next 30 minutes take a spectrum every 5 minutes with the same number of scans.

(In 2004 the glucose disappeared in the first 15 minutes!)

### 4A. Manual NMR data Analysis

See Processing Spectra, pages 13-20 above.

### 3B. Automatic NMR Procedure

**Should be done just prior to NMR experiment**

Preparing the NMR

Put sample 98% N-propyl Benzoate in spectrometer (and make sure is spinning)

In PNMR

Nu c13

shim

Look at RG third on down in table on left

if not 10 then <ctrl+G> and set to 10

otherwise <ctrl+Q> to let it shim on sample

zgh (acquire a proton Spectrum)

In NUTS

a2 (process spectrum)

qp (quick phase)

move the crosshairs on top of the internal standard (TMS) peak that should be at 0.00

and make a mental note of its positions (like +.006ppm)

In PNMR

fo

Enter the current position of the reference you got from Nuts (above)

Enter 0.00 for 'desired position of reference'

Remove the N-Propyl Benzoate sample from the NMR

Preparing the sample

-Get an NMR tube ready and purge for 30 second with N<sub>2</sub> gas

-Open your yeast flask

- Remove a **800 µL aliquot** and place in an **Eppendorf tube** that has been purged with N<sub>2</sub> Gas

- Place **50 µL of Labeled glucose** in **NMR tube** and mix

-Transfer the yeast extract/glucose mix to an NMR tube, and blow N<sub>2</sub> over the top of the mixture before capping

-Place tube in the NMR and get your first spectrum as quickly as possible

### Running the NMR Experiment

In PNMR

shim

<ctrl+G> and set receiver gain to 1

<ctrl+Q> to let it shim on sample

ns set the number of scans to 20

kms1 This will fire off the new kinetics program we are trying

It will first ask for the name of a data file start the name with  
'data\...'

It will now ask for a relaxation delay, use 2.8

Next it will ask for a file containing delays use the name 'klist2.txt'

You should see a list of the delays load into the window on the  
upper right

Hit the enter key to start the experiment running

When the experiment finishes it will put up a window to tell you it is  
done. Simply hit enter again to finish

### *4B. Automatic NMR Data Analysis*

In NUTS

a1 Bring in the last spectrum for processing

ap Phase the spectrum

<Ctrl +F7> It will now ask for the name of your NMR data file

Once you enter the name it will bring up a window with information  
about the file. If the information looks right, hit the OK button. The  
NUTS program will get all your data and make a stacked plot.

If it looks good, hit the print button.

Now hit the enter key to get out of the stacked plot mode

You are not about to get out of known waters for data processing.

Hit the 'Help' button on the task bar

On the left hand side type in 'GR' under the keyword search button and hit  
return

You will now get help on the GR - get relaxation data command

The right hand window should say 'Relaxation Analysis'

This first line of this window has a link called

'Illustrated Tutorial'

Click on this link

-A tutorial on analyzing kinetic data now appears

You can ignore the first set of commands, and page the window down about 1 screen. It will guide you through the next step or two, but after that, you are on your own to work through the tutorial and figure out your data. I would minimize this window so you can come back to it as needed to see what you are doing.

Back on the main NUTS screen, you should now have a single spectrum displayed

Zoom in on a single peak of the spectrum. Once you have the single peak displayed, hit the enter button and take yourself out of zoom mode.

Type 'GR' Nothing will happen on the screen, but the computer will look up this point in all the other data sets

Type 'DR' the computer will now display the points it has for that peak as a function of time. From this point onward I don't know what to do because nobody had ever generated data like this before at BHSU. Find this point in the tutorial and see how far you can get.

Identify all peaks observed in the spectra. Which ones come from the glucose starting material, which ones come from the glycolytic breakdown of glucose? Make a plot of the intensity of the peaks vs time. What can you say about the rate of degradation of Glucose? The rate of appearance of other peaks?

*Insert* Original papers

J Chem Ed 74 #12 1997 page 1474-1476.

J Chem Ed 76 #11 1999 pages 1564-1566.

*After this page*

## What to hand in

### 1. Answer the following questions:

A. Outline the general steps of glycolysis, identify where glycerol and ethanol would be products of this pathway. Given that the  $^{13}\text{C}$  is on the #1 position in glucose, identify the position that the label should be in, in both the glycerol and ethanol.

B. Right now we are assuming the reference values given in the literature of 18.7 for ethanol and 63.7 for glycerol are correct. Outline additional NMR experiments that we should do to confirm these values.

### 2. For the *in vitro* Yeast experiment

A. All spectra, with peaks identified in at least 1 representative spectrum

B. A plot of the intensity vs time for each major peak in the  $^{13}\text{C}$  spectrum.

C. An analysis of peak intensity vs. time plot.

I. What peaks disappear first or second and why?

II. What peaks appear first or second and does this fit with the model of the glycolytic pathway that is being taught in the lecture?

III. Assume that the data follows first order kinetics, determine the  $t_{1/2}$  and  $k$  for the disappearance of glucose and the appearance of glycerol and ethanol. (Ask if you need a review of 1<sup>st</sup> order kinetic analysis from Gen Chem). From your plots does the first order kinetics seem like a reasonable order parameter?

### 3. For the *in vivo* yeast experiment:

A. All spectra, with peaks identified in at least 1 representative spectrum

B. A plot of the intensity vs time for each major peak in the  $^{13}\text{C}$  spectrum.

C. An analysis of peak intensity vs. time plot.

I. What peaks disappear first or second and why?

II. What peaks appear first or second and does this fit with the model of the glycolytic pathway that is being taught in the lecture?

III. Assume that the data follows first order kinetics, determine the  $t_{1/2}$  and  $k$  for the disappearance of glucose and the appearance of glycerol and ethanol. (Ask if you need a review of 1<sup>st</sup> order kinetic analysis from Gen Chem). From your plots does the first order kinetics seem like a reasonable order parameter?

4. Compare and contrast the answers you got for 2 and 3.

Note for the people who used the kms routine: While I think the NMR processing is doing a first order fit, the times that were feed into the program by the klist.txt file were wrong, so the parameters you get from the live experiment probably won't match with the killed experiment.

5. For the group that used the Mass Spec

A. Make the following tables:

Control (unlabeled Glucose)

Time	Signal mass 28	Signal Mass 44	Signal Mass 45	$(44/28) \times 100\%$	$(45/44) \times 100\%$
0					
5					
Etc					

Experiment (labeled Glucose)

Time	Signal mass 28	Signal Mass 44	Signal Mass 45	$(44/28) \times 100\%$	$(45/44) \times 100\%$
0					
5					
Etc					

Plot the data in the last two columns from the above tables in a single graph vs time. The 44/28 number represents the relative amount of  $\text{CO}_2$  in the sample. Is the %  $\text{CO}_2$  constant in this experiment? The 45/44 number represents the % of  $^{13}\text{C}$  labeled  $\text{CO}_2$  relative to the natural  $^{12}\text{C}$   $\text{CO}_2$ . Is this constant in the experiment, or do you see evidence of the  $^{13}\text{C}$  label in the glucose getting oxidized all the way to  $\text{CO}_2$ ?

5. For the group that did the NMR kinetic analysis

Make a processing 'Crib Sheet' that another group could use to both visualize and process their NMR data. Include all pertinent commands, and a few appropriate plots to illustrate what you should see.

## A Reminder of Kinetics Principles

### Zero Order kinetics

Rate Law:	Rate = k
Integrated Rate Law:	$[A] = -kt + [A]_0$
Straight line Plot:	$[A]$ vs t
Rate Constant from Above Plot:	Slope = -k
Half time:	$t_{1/2} = [A]_0/2k$

### First Order kinetics

Rate Law:	Rate = k[A]
Integrated Rate Law:	$\ln[A] = -kt + \ln[A]_0$
Straight line Plot:	$\ln[A]$ vs t
Rate Constant from Above Plot:	Slope = -k:
Half time:	$t_{1/2} = .693/k$

### Second Order kinetics

Rate Law:	Rate = k[A] <sup>2</sup>
Integrated Rate Law:	$1/[A] = kt + 1/[A]_0$
Straight line Plot:	$1/[A]$ vs t
Rate Constant from Above Plot:	Slope = -k:
Half time:	$t_{1/2} = 1/k[A]_0$

## A $^{31}\text{P}$ NMR study of a Kinase Reaction

### Prelab Questions:

1. What is the natural abundance of  $^1\text{H}$ ,  $^{13}\text{C}$ , and  $^{31}\text{P}$ ?
2. What is the  $\Delta G^\circ$  of the Hexokinase and Phosphofructokinase reactions?
3. Given the above  $\Delta G^\circ$  values, calculate the  $K_{\text{eq}}$  for the above reactions.
4. If you start the above reactions with .1M ATP and .1M sugar, what should the equilibrium concentrations of ATP, ADP, sugar and phosphorylated sugar be?

### Experimental outline

Each group will:

1. Run  $\text{H}_3\text{PO}_4$  Standard (rg 50, 8 scans)  
use fo command in WinPNMR set peak to 0.00 ppm
2. Next run appropriate Buffer tube (rg 50, 8 scans)  
Record the chemical shift (in ppm) of the phosphate buffer.  
This will be your reference for all other  $^{31}\text{P}$  spectra that include this buffer.  
(2010 Hex buffer was~ 2.4, PFK buffer was ~2.9)

### Hexokinase Reaction

If necessary, mix up 2X glucose and 2X ATP in Hexokinase buffer.

Mix .3mL of 2X glucose with .3 mL of 2X ATP.

Fill NMR tube with this solution.

Shim.

Obtain and save spectrum (rg 100, 32 scans).

Add 5  $\mu\text{L}$  of Hexokinase to top of NMR tube.

Mix in tube.

Wait 5 minutes. Obtain and save 5 minute spectrum (rg 100, 32 scans).

Wait additional 5 minutes. Obtain and save 10 minute spectrum.

Remove tube from NMR and replace with ADP standard.

Obtain and save ADP spectrum.

Remove ADP standard and replace with your sample.

Wait at least 24 hr

Shim

Obtain and save 24 hour spectrum.

### Phosphofructokinase reaction

If necessary mix up 2X F-1-P and 2X ATP in PFK buffer.

Mix .3 mL of 2X F-1-P with .3 mL of 2X ATP.

Place in NMR tube.

Shim.

Obtain and save spectrum (rg 100, 32 scans).

Add 5 uL of Phosphofructokinase to top of NMR tube.

Mix in tube.

Wait 5 minutes. Obtain and save 5 minute spectrum (rg 100, 32 scans).

Wait additional 5 minutes. Obtain and save 10 minute spectrum.

Remove tube from NMR and replace with ADP standard.

Obtain and save ADP spectrum.

Remove ADP standard and replace with your sample.

Wait at least 24 hr

Shim

Obtain and save 24 hour spectrum.

### **Processing Spectra**

ga get the spectrum

lb .5 Hz

em exponential multiply (to reduce noise)

ft Fourier Transform

qp quick phase (Ap autophase does not work!)

Use zoom to zoom in on the large phosphate peak

Adjust spectrum so this peak is at the appropriate ppm

Hold down right mouse button and move mouse till crosshairs are in middle of peak

DO NOT RELEASE button

type 'o' on keyboard

Tell the computer what this chemical shift should be

zo zoom

f tell it the frequency of zoom

start frequency 10 ppm

Stop frequency -30 ppm

pp pick peaks

(You may have to use the mh or minimum height to get this properly adjusted)

(You should clear before you go on to your next spectrum)

Print in landscape

Try to assign all the Phosphorous peaks

If you can find 1 phosphorous peak that is unique for ATP and the product, integrate these two peaks to come up with a ratio of Product:ATP and use this to come up with a  $\Delta G$  for your reaction.

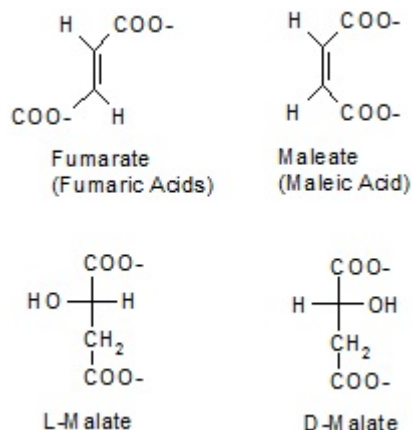
### What to hand in

1. If  $\text{H}_3\text{PO}_4$  has a chemical shift of 0.00 ppm, what is the chemical shift for the phosphate in your buffer?
2. Print the spectrum of your starting material. On the spectrum draw the chemical structures of your starting materials. Identify each resonance on your spectrum with a phosphorous in your structure.
3. Print the spectrum of your final product. On the spectrum draw the chemical structures of your final products. Identify each resonance on your spectrum with a phosphorous in your structure.
4. The peak for the terminal phosphorous in both ADP and ATP is split into a doublet. In some of your intermediate spectra this looks more like a triplet. Why?
5. It looks like we cannot determine the  $\Delta G$  for the Hexokinase reaction because the  $K$  for this reaction is so large that there is not enough ATP left for us to measure.
  - A. Can you think of another reason that ATP might disappear from your NMR tube?
  - B. Look over the glycolytic pathway and suggest some other step that we might follow using a  $^{31}\text{P}$  experiment. Would this reaction be better or worse than the hexokinase reaction? Why?

## NMR Analysis of the Fumarase Reaction

### Background:

The 7<sup>th</sup> reaction in the TCA cycle is the hydration of Fumarate to form L-malate. This reaction is catalyzed by the enzyme fumarase (or fumarate hydratase). This reaction is highly stereo-specific: it will work only with the trans double bond found in fumarate, but not the cis bond found in maleate, and in the reverse direction it will work only with L-malate, not D-malate.



Based on standard state conditions, the  $\Delta G$  of the reaction is  $-3.8$  kJ/mole so the reaction is slightly favorable, and should come to an equilibrium that has measurable concentrations of both products and reactants. That is what we will try to do in today's lab. Let this reaction run to equilibrium, and measure the concentration of reactants and products, from there calculate  $K_{eq}$  of the reaction, and from that calculate  $\Delta G$  for the reaction, to see how it compares to the 'book' value.

The experiment itself is almost too simple. One group will be given a 0.2M solution of Fumarate, the other group will be given a 0.2M solution of L-Malate. After you have obtained  $^1\text{H}$  NMR spectra of these solutions, you will add a bit of fumarase to catalyze the interconversion of fumarate and malate. Once you have added the fumarase you will put the NMR tube back in the NMR and obtain a spectrum after 1 hour and 24 hours of reaction. The 1 hour spectrum should give you an interesting intermediate state to look at, and the 24 hour spectrum should give you a final, equilibrium mixture to analyze. The equilibrium mixture will contain both Fumarate and Malate peaks. By analyzing the intensity of these peaks you will be able to quantify the amount of each material, and use these concentrations to calculate  $K_{eq}$  and  $\Delta G$  of this reaction.

### **Prelab Questions:**

1. Predict the  $^1\text{H}$  NMR spectrum of malate.
2. Predict the  $^1\text{H}$  NMR spectrum of fumarate.

### **Experimental Procedure:**

1. Prepare fumarase solution by diluting 10 $\mu\text{L}$  of stock enzyme into 200  $\mu\text{L}$  of  $\text{D}_2\text{O}$ , then place on ice until you are ready to use.
2. Tune the NMR with the 5% Ethylbenzene standard sample.
3. Obtain 1 ml of either the 0.2M malate solution or the 0.2M fumarate solution. Place the solution in the NMR tube and obtain and save a good proton spectrum. (Don't forget to shim first)
4. When you are ready, add 50 $\mu\text{L}$  of enzyme to the top of the NMR tube and mix the solutions in the NMR tube together. Wait 1 hour, and obtain a proton spectrum of this mixture.
5. Remove your tube from the NMR. Keep your NMR tube for at least 24 hours, then run another spectrum, to see if you have reached equilibrium.

### **Analysis**

As you might guess with an experiment this simple, the real work is in the analysis. Both groups will exchange data so everybody has access to both starting, ending, and kinetic data sets.

A. Assign all the peaks in the pure malate and pure fumarate spectra.

B. The assignments for the malate are going to be a little funny, because the spectrum for malate at the start of the experiment is going to be different than the spectrum at the end of the experiment. How and why does the spectrum for malate change in this experiment?

Equilibria data - looking only at spectra of the solution after 24 hours

C. Find out how to integrate the peaks in your spectra.

Compare the integrals of the Malate peaks to the integrals of the fumarate peaks

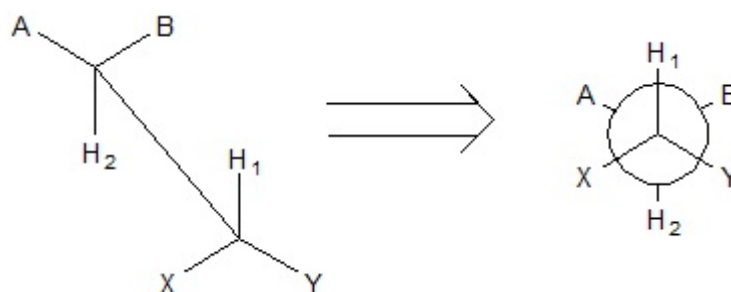
If the ratio of integrals of (Malate peaks):(Fumarate peaks) is the same for both spectra than you were truly at equilibrium, because you got to the same ratio regardless of starting with malate or fumarate.

1. Were you at equilibrium?
2. From your ratios calculate the relative concentrations of malate and fumarate.
3. Knowing that the total concentration of malate+ fumarate was .2M, what were the concentrations of Malate and fumarate in the equilibrium mixture?
4. What is the equilibrium constant for the reaction Fumarate  $\rightarrow$  Malate?
5. What is the  $\Delta\text{G}$  for the reaction Fumarate  $\rightarrow$  Malate?

#### D. Determination of configuration of proton and deuterium in Malate.

In Organic chemistry you were introduced to the spin-spin coupling that leads to the multiplet splitting between adjacent protons. The coupling between adjacent protons is actually very geometry dependent. In most organic molecules you never see this because there is free rotation between adjacent protons so the coupling you observe reflects an average of all the rotational conformers. The one place you do sometimes see geometry in spin-spin coupling is when protons are adjacent to each other in a ring system that restricts their rotational freedom.

Examine the following system:



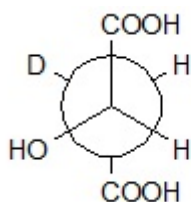
In the above diagram the H's are at  $180^\circ$  apart and you have a maximum coupling of almost 15 Hz. As the angle decreases to  $90^\circ$ , the coupling drops to zero. Then as the angle drops to  $0^\circ$  the coupling goes back up to about 12 Hz.

While malate does not have a ring structure, the two carboxylic acid functions are strongly repelled from each other and will align at  $180^\circ$  from each other, effectively restraining the free rotation in this molecule.

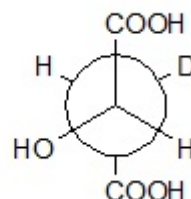
As the malate reaches equilibrium, one of the protons on the  $\text{CH}_2$  group will be stereospecifically replaced with a deuterium, and this is why the malate proton spectrum simplifies and loses intensity at the  $\text{CH}_2$  frequency.

Once the malate has reached equilibrium we can use the coupling constant between the vicinal protons to decide which proton was replaced.

Look at the two diagrams below for deuterated malate:



Case I (2S, 3S)-3-<sup>2</sup>H-malate



Case II (2S,3R)-3-<sup>2</sup>H-malate

In case I the vicinal protons are nearly 90° apart so the coupling constant will be small. In case II the protons are 180° apart so the coupling should be about 15 Hz.

Look at your final equilibrated spectrum and locate the doublets around the peaks at ~4 and ~2. Use the cursor to find the frequency of the peaks of these doublets in Hertz, not ppm. The  $\Delta f$  between the splitting is your coupling constant for this interaction. In theory the coupling for the peak at ~4 should be the same as that of the peak at ~2 since it is the same coupling interaction, but you might measure slightly different values experimentally.

Questions:

- D-1. What is the average coupling constant you observed in Malate?
- D-2. Do you have the (2S,3S)-3-<sup>2</sup>H-malate structure or the (2S,3R)-3-<sup>2</sup>H-malate?

## NMR directions

First put in tube with either Malate or Fumarate

Shim

Hit <Cntrl+G> and adjust the receiver gain so you have a reasonable signal

Hit <Cntrl+Q> to start shimming

ns 1 Set number of scans to 1

zg zero memory and go with experiment

Ignore the question about saving this to a file

Look at the Signal. At the start does it go from about  $\frac{1}{2}$  way below the center to about  $\frac{1}{2}$  way above the center? If it is smaller change rg to a large number and try again. If it is large, or it is red, change rg to a smaller number. Keep trying until you get it right. Once you have the gain adjusted correctly, you can proceed.

Switch to Nuts

a2 process the NMR spectrum.

qp quick phase to make it look nice.

zo zoom in on the rightmost peak. Use the cross hairs to see what its value is.

Switch back to PNMR

fo Field offset (the command to get the zero properly aligned).

Give the current position of the reference that you just got from NUTS.

Give the value it should be 0.00

Now you have a good signal, and a properly adjusted field, you can obtain your spectrum.

ns 8 Take 8 scans so you get a good clean spectrum.

zg zero and go. This time when it asks a file name, give it something you can remember because you will need this spectrum.

For your 1 hour spectrum you can do just the zg command, but for your 24 hour spectrum you should start at the top of the page and do everything over.

See instructor if you need additional crib sheets for processing  $^1\text{H}$  NMR spectra.

## Using the integral command to analyze the equilibrium fumarate/malate mixture

**Start Nuts program.** (Note if Nuts is already running, kill it and start over so you don't have to deal with somebody else's parameters fouling up your analysis.)

-hit **OK**

type **a3** to run the macro to process a spectrum from a data file  
Find the file corresponding to your spectrum from the open file menu  
will bring up the 'Data Acquisition Parameters' window  
either hit enter, or **click on OK** to exit this window

Should now have a spectrum in front of you, probably out of phase so

type **qp** quick phase, should make peaks nice and symmetric

### *Getting the ppm properly aligned*

Hold left mouse button and place it directly on top of the TSP peak (furthest right hand peak) that should be at 0.00 ppm. While still holding the mouse button down, type **o** on the keyboard. The offset information window comes up. Type 0.00 PPM in under Horizontal dimension column, and either hit enter or click the mouse on the OK button to shift the scale to match the scale to the peak position

### *Zooming in on the useful part of the spectrum*

Now zoom in on the region of interest, for us it is from 2 to 7 ppm

type **zo**

type **f**

Set 'Start of Zoom' to 7 ppm

Set 'End of Zoom' to 2 ppm

**click on OK**

You will now see a this region with a red background on your display.

- **Right mouse click** anywhere in this red region, and the display will zoom in on that region

- Either click on the zoom button at the top, or simply **hit the enter key** to get out of zoom mode.

### *Making the peaks bigger*

Use the up arrow key or the slider on the right to increase the size of your peaks. It is OK to have the water peak (~4.8) go off scale, but you want to get the peaks ~6.5, ~4.3 and ~2.4 expanded to almost full scale.

### *Integration*

type **id** starts integral display subroutine

You should have a new slider on the left hand side of the screen, and a black line starting near the bottom left of your screen that ascends to the upper right.

This line represents the total integration of all peak in this region

Click on the left hand slider with the mouse and move it up and down. See how the black line moves up and down? Adjust the slider until the black line flows

from lower left to upper right without dropping down to zero anywhere in the middle of the spectrum

Right now the water peak at ~4.8 is so massive that it makes the integrals of you smaller malate and fumarate peaks minuscule. You also may have some other spurious peaks do to impurities that you don't want to integrate. So now we need to tell the integration routine which peaks we really want to concentrate on.

Position your **mouse on one side of the fumarate peak** at ~6.5 ppm. **Double left click** and move the mouse slightly you should see a green vertical line where your mouse was. Now **move the mouse to the other side of the peak and left click a single time**. The integral line should disappear from the entire spectrum, and you should see a small black line that represents just the integral of this one peak.

Do this process again to define integrals for the malate peaks from ~4.1-4.5 ppm and 2.2-2.9 ppm.

Now that the integrals you are interested in are defined, you now want to set their values. If this was a single compound you would know how many protons should be in each peak, so you could set your integral value to this value. In our case, with peaks from two different concentrations of two different compounds, we will just set one integral to some arbitrary value. Let's set the fumarate peak at 6.5 ppm to an integral of 1. To do this **move the mouse to the middle** of the integral, **hold down the left mouse button**, and type '**v**' on the keyboard. The 'Integrate Relative Values' should come up; type 1.0 for current relative value and click on the 'OK' button. This integral gets assigned a value of 1. And the integrals on your other 2 peaks should now have value assigned to them as well.

Grab the slider on the left and move the integrals up and down until you get a pleasing display, and print your spectrum. If you want to put these integral values into a word document or a spreadsheet, so you can calculate with them, **Click on 'edit' - 'Type integral list to clipboard'** then bring up the program you want and paste the values into your program.

### Analysis

The fumarate integral at ~ 6.5 is now set to 1

Let's call the malate integral at ~4.3 ppm  $X$  and the malate integral at ~2.5 ppm  $Y$

There should be 2 protons in the fumarate peak and 2 protons total in the malate peaks, so if we had a 1:1 ratio of fumarate : malate then the total  $X+Y$  should also equal 1. However we know that the  $\Delta G$  for this reaction is negative, so the equilibrium should favor more malate, so the total malate integral ( $X+Y$ ) should be greater than 1.

$$\begin{aligned} K &= \text{products/reactants} \\ &= [\text{malate}] / [\text{fumarate}] \\ &= (X+Y)/1.0. \end{aligned}$$

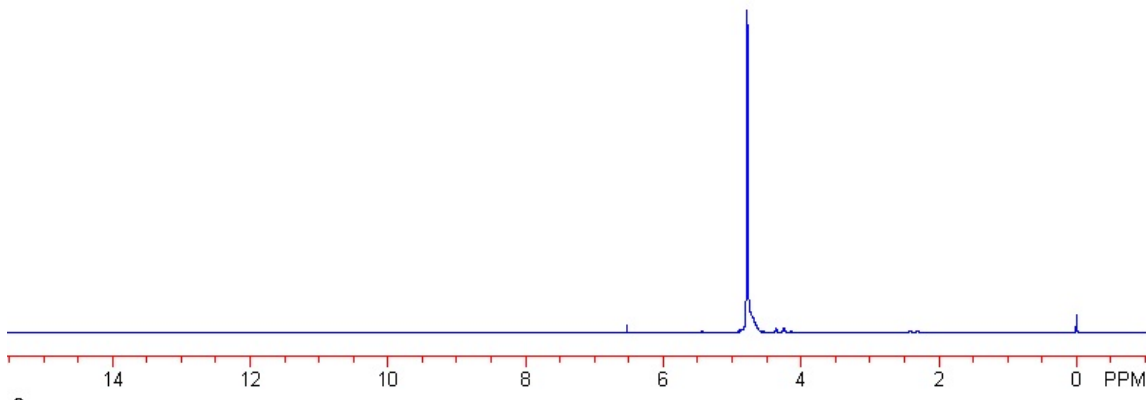
You should be able to take it from here.

### Additional Notes on NMR analysis of Fumarate Malate experiment.

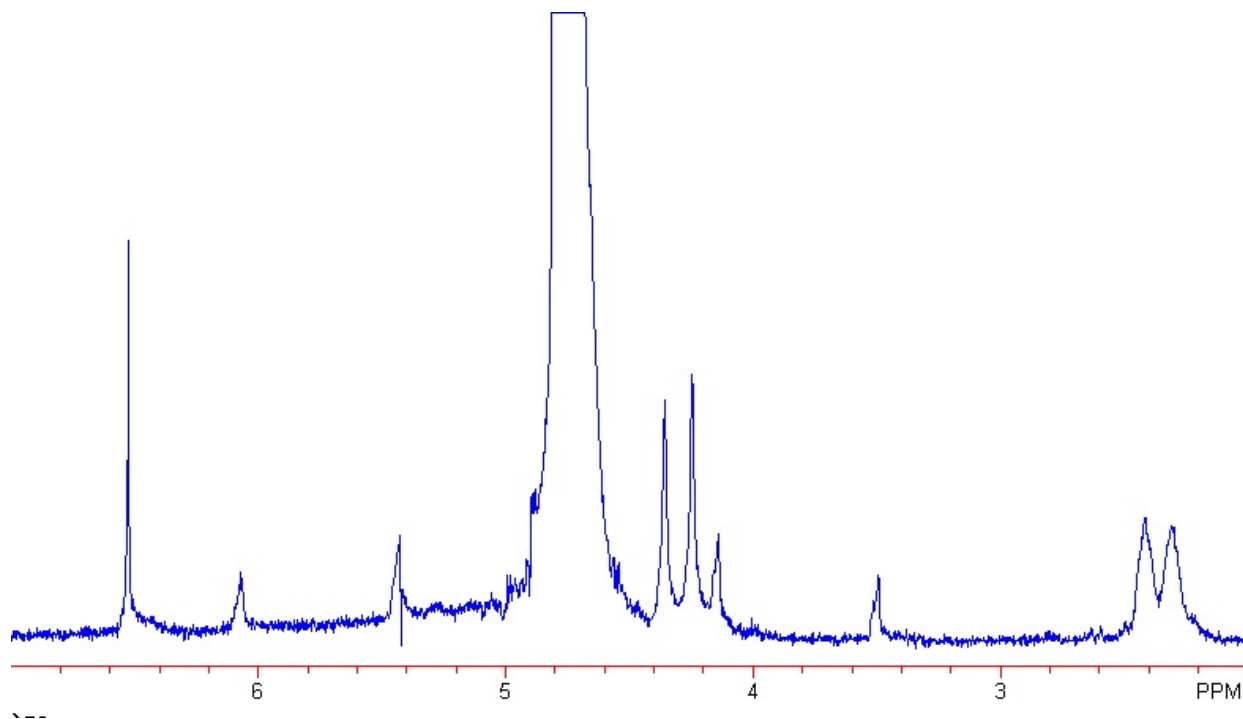
A. Analysis for  $\Delta G$ . This will work as outlined above, however, I have observed one artifact called a spinning side band that you need to be aware of so you don't mistake it for a malate peak. Here is what to look out for.

Spinning side bands are artifact that appear symmetrically on either side of a strong peak. Look at the spectrum below. This is one group's final spectrum with the TSP adjusted to zero.

Note that water is dominate peak. At this scale you don't see any spinning side band because they are so small that you can't see them.



In this spectrum I now zoom in on 2-7 region and blowing up the peak height so you can see fumarate and malate peaks. Now you can see something fishy. There are some peaks here that look to be symmetric about the water peak. Can you spot them?



They are at  $\sim 6.1, 5.4, 4.08$  and  $3.5$ . They are an artifact of an imperfectly shimmed sample. You can tell they are spinning side bands because there are at the same  $\pm$  distance from the strongest peak in the spectrum, the water peak. Let me prove that to you.

I will put the cursor on each of these peaks and get their frequency in Hertz

Frequency (Hz)	546.8	489.3	430.6	373.6	315.7
$\Delta F$	57.5	58.7	56.9	57.9	

These are called spinning side bands because their appearance is related to the speed at which the tube spins. You speed up the spinning rate the peaks will shift outward, you slow the spinning rate the band will move inward.

Bottom line in this spectrum you can ignore the peak at  $4.145$  ppm. The real peak you are doing your analysis with are at  $6.53, 4.356, 4.247, 2.414$  and  $2.306$

These are the peaks you should concentrate on for your analysis.

## B. Determination of coupling constants in Malate

Put the cursor on each of the two peaks around 2.34 and record the positions of these peaks in Hertz. What is the difference, in Hz, of these two peaks? That is your coupling constant.

Next put your cursor on each of the two peaks around 4.3, and record these positions in hertz. What is the difference, in Hz, of these two peaks? That is your coupling constant.

Since these two peaks are coupled to each other, they share the same coupling constant. Average these two values together to get an average coupling constant.

Is your coupling constant high ( $>10$ ) or low ( $<5$ )? Do you have the (2S,3S)-3-<sup>2</sup>H-malate structure or the (2S,3R)-3-<sup>2</sup>H-malate?

# DNA Denaturation Lab I - UV

## Purpose

- Demonstrate the hyperchromicity that occurs DNA is denatured.
- Demonstrate the dependence of DNA melting temperature with base content.
- Demonstrate the hysteresis in the DNA melting curve.

## Background

Read text Lehninger pages 291-293

Just like proteins, DNA can be denatured by either raising the temperature or by changing solvent conditions. The primary change that occurs in DNA denaturation is that the hydrogen bonds holding the two strands of DNA together are disrupted, and the strands separate. This denaturation can be followed using several different physical techniques.

The most direct physical technique is Infrared Spectroscopy, since using this technique you can identify the absorbance bands associated with the vibrations of the NH and CO bonds involved in the hydrogen bonds, and you directly see if these bonds are present or absent. Unfortunately the experiment requires the use of a temperature controlled IR cell and we do not have one at the present time.

The second most direct techniques is transmission electron microscopy, since you can use this technique to visualize the DNA and, under the right conditions, you can see DNA with open loops in it, showing regions of the DNA that have begun to denature. BHSU has an electron microscope, but it is a scanning instrument, rather than a transmission instrument, so it doesn't have the power to do this kind of experiment. Thus we are left with more indirect techniques.

The UV spectrum of DNA is also sensitive to whether the DNA is in the native or denatured form. This is because when DNA is in the native form, all the bases in the DNA are stacked on top of each other in the helix so closely that they touch, and this close, intimate contact, makes the electronic changes that accompany the absorption of light are different than they would be if the base was isolated in solution with no other bases nearby.

Because of these different interactions, DNA in the native form absorbs less light at 260nm (and more light at 200 nm) than it does when it is in the denatured form. If we watch the 260 nm absorbance of DNA we can see that it increases in intensity as the DNA denatures, and we call this a hyperchromic shift. This is a very dramatic effect, with the absorbance increasing by as much as 50%. Thus the easiest experiment to follow DNA denaturation is to simply put a cuvet containing DNA in a UV spectrophotometer, and then to follow the absorbance of the sample as you heat the DNA to denature it.

This denaturation curve follows a typical sigmoid shape, and we call the point in the middle of the transition between native and denatured the melting temperature,  $t_m$ .

It was discovered many years ago that the  $t_m$  of DNA correlates with the % composition of the DNA. That is, DNA that had a higher percentage of GC had higher  $t_m$ 's than DNA with lower GC content. Your first instinct is to say that this makes sense, the GC pairs have 3 hydrogen bonds while AT pairs only have 2, so the more GC there is the more hydrogen bonds you have to break, and therefore the  $t_m$  should be higher. The actual reason for the higher  $t_m$  is a little more complicated, and has to do with the GC base pair having a stronger base stacking interaction with the bases around it, so

the hydrogen bonding is only part of the story.

In today's lab you will be using several different DNA's. DNA from *Clostridium perfringens* has 26.5% GC, that from *Micrococcus luteus* has 76% GC, and *E. coli* DNA is 50% GC. There will also be an unknown DNA sample for which you will determine the GC content. When you plot  $t_m$  vs %CG (Figure 8-30b from Lehninger) you should obtain a straight line. Use your data plot this line, and then use this line to estimate the GC content in the unknown DNA sample.

Once the DNA is denatured it can, in theory, be renatured or reannealed. However, depending on the properties of the DNA this can be difficult. If you start with a small plasmid DNA that is formed into a covalently closed loop, renaturation is easy because the sequence is small, and the two strands of the DNA are still physically associated with each other through the covalently closed loops, so the two strands of the DNA find each other quickly and get properly aligned. On the other hand when you try to reanneal a large, complex DNA like that from a mammalian cell, it is virtually impossible for each strand to find its proper mate and to get aligned. As a result the DNA stays somewhat denatured, even when you return it to its original temperature. The inability of the DNA UV curve to trace itself in both heating and cooling curves is called hysteresis, and is commonly seen in large, complicated DNA molecules.

### Experimental Procedure

We only have one water bath for temperature control, and I estimate that this experiment will take most of one afternoon. Thus only one group will be able to do the lab in the regularly scheduled lab period, and the other groups will have to set up a time when they can come in and do their work.

Each group will be provided with a DNA sample at an appropriate concentration for UV analysis and a sample of the buffer to be used as a blank. First obtain a full spectrum of your sample at room temperature between 330 and 220 nm. Confirm that you have an absorbance at 330 nm of .05 or less, and an absorbance at 260 nm somewhere between .4 and .8.

Find the button that has 3  $\lambda$ 's on it. This is a program to run the spectrophotometer at particular wavelengths for several samples. Click on the  $\lambda$  and it puts you into the wavelength scan program. Fill in the blanks for 2 wavelengths, 330 and 260 nm. Now find the tab in this window that let's you fill out the sample table. Depending on your sample you will obtain spectral measurements at the following temperatures:

*C perfringens*

25, 50, 53, 54, 55, 56, 58, 60, 65, 50, 25 (total of 12)

*L. luteus*

25, 70, 75, 78, 79, 81, 90, 80, 77.5, 72, 25 (total of 11)

*E coli*

25, 55, 62, 66, 68.5, 70, 75, 70, 66, 62, 55, 25 (total of 12)

Unknown

25, 55, 58, 60, 62, 63, 65, 67.5, 70, 60, 50, 24 (total of 12)

The above temperatures are the SET temperatures on the water bath, the actual temperature in the sample will be significantly lower (about 10%) Set the water bath to these temperatures but record the actual temperature in the sample. How do you get the actual temperature? The Lambda 2 will be set up with a temperature sensor sitting underneath the sample cuvet. Make sure the wire from this sensor does not get in the light beam and that you know how to get temperature readings with this sensor.

Find the appropriate tab in this window to set up a data table with the correct number of spectra. You can change the name of each run to match the temperature you want. When you are ready, hit the start button. The machine will ask for you to put in a blank, and it will record the absorbances of the blank at 330 and 260. It will then ask you for each sample. Replace the blank with your sample. You will need to cap the cuvet with parafilm to keep sample evaporation to a minimum during the experiment.

Record the temperature of the cell and allow the spectrometer to take your RT temperature data. After this is recorded, set the water bath to the next temperature. After the water batch reaches this temperature, let the system come to equilibrium for at least five minutes before you record the next spectral data point. There will probably be a difference in temperature between what the water bath is set for and what the sensor records. Always use the temperature sensor readings, since these reading reflect the actual sample conditions in the cuvet.

## Data Analysis

In past years bubbles have formed on the inside of the cells during the heating phase of the reaction. These bubbles raise the overall absorbance of the solution. Since the increase in absorption due to bubbles is the same at both wavelengths, the simple way to remove this error is to simply subtract the OD<sub>330</sub> value from the OD<sub>260</sub> value for each of your readings.

We will assume that your initial value for OD<sub>260</sub> at RT is the absorbance of 100% native DNA and is the lowest absorbance in your set of data. We will further assume that the OD<sub>260</sub> at the highest temperature represents the absorbance of 100% denatured DNA and is the highest absorbance in your data set.

The % denaturation of your DNA, then is calculated with the following equation:

$$\% \text{ Denaturation} = \frac{(OD_{260} \text{ at a given } T) - (OD_{260} \text{ at RT})}{(OD_{260} \text{ at highest } T) - (OD_{260} \text{ at RT})} \times 100\%$$

You can see that the numerator represents change in OD of the given sample, and the denominator represents total change in OD that occurs when the sample is completely denatured, so one over the other represents fraction of denaturation.

### I. Melting Curves

Calculate this value for each of your points, then plot % denaturation vs temperature, as shown in figure 10-30 from your text. You should either plot the heating curve and the cooling curve on different plots, or use different colors on the same plot to help differentiate these two curves. Do the heating and cooling curves superimpose on each other, or is there a hysteresis effect? Can you think of any way that you could minimize the hysteresis experimentally?

### II. $T_m$

On your heating curve, estimate the point at which you have 50% denaturation. This is your  $t_m$  or melting point. Post this point in the lab or outside Dr. Z's office, along with the name of your sample so all groups have access to this information.

*M. Luteus* is 76% GC, and *E. Coli* is 50% GC, Calf Thymus is 42% GC, and *C. Perfringes* is 26.5% GC,

### III. Dependence of $T_m$ on GC content

Once all groups have posted their  $T_m$  values, plot %GC vs  $t_m$  for the set of known DNA samples. Is it a reasonable straight line? From where the  $t_m$  value of the unknown fall on this line, determine the % GC for the unknown sample.

## DNA Denaturation Lab II - IR

### Introduction

Last week you found that you could denature DNA by heating it up, and detected this denaturation by watching the hypochromism of the DNA in the ultraviolet region. Today we will try something new. We will denature the DNA by changing the pH of the solution, and will try to follow the denaturation by directly observing the vibrations of the C=O and NH bonds of the bases that are involved in the hydrogen bond using IR spectroscopy.

### Background

You should already have been exposed to infrared spectroscopy in organic chemistry. Absorptions in the infrared region of the electromagnetic spectrum correspond to changes of vibrations of atoms within a molecule. Absorption bands in the  $\sim 2500\text{ cm}^{-1}$  to  $\sim 4000\text{ cm}^{-1}$  can usually be assigned to specific functional in an organic molecule, while bands in the  $<2000\text{ cm}^{-1}$  range are a complex mix of atoms vibrating against each other, so are harder to assign to a given functional group, but the overall spectrum in this region is called the 'fingerprint' region because it can be used to uniquely identify most organic molecules.

The best picture to explain infrared absorption, is to think of all the atoms in a molecule connected by springs. Each absorbance band in the infrared corresponds to an energy that kicks a spring and its attached atoms into motion. This motion can be a simple back and forth motion, or a wig-wag, or a few other kinds of motions.

In theory infrared spectroscopy is the ideal way to detect denaturation in DNA because the vibrations of the NH's or CO's involved in hydrogen bonds will be very different when the DNA is in a native form where the hydrogen bonds are formed between the bases, and the denatured form, when the hydrogen bonds are made to water instead.

While the theory is good, the experiment is difficult. First of all, if you remember your IR from Organic Chemistry, you'll remember that IR cells typically use NaCl windows because they are cheap and transparent in the IR. But if we are working in water, our windows will literally dissolve as we do the experiment!

Our first task is to find a different window material. We will use  $\text{CaF}_2$  windows. These windows are insoluble in water, so they won't dissolve, and they are transparent for most of the IR region. Figure 1. shows the IR spectrum of the  $\text{CaF}_2$  windows you will be using. You can see that they absorb IR radiation strongly below  $1000\text{ cm}^{-1}$ , but since that is not a region we are interested in, that is no problem.

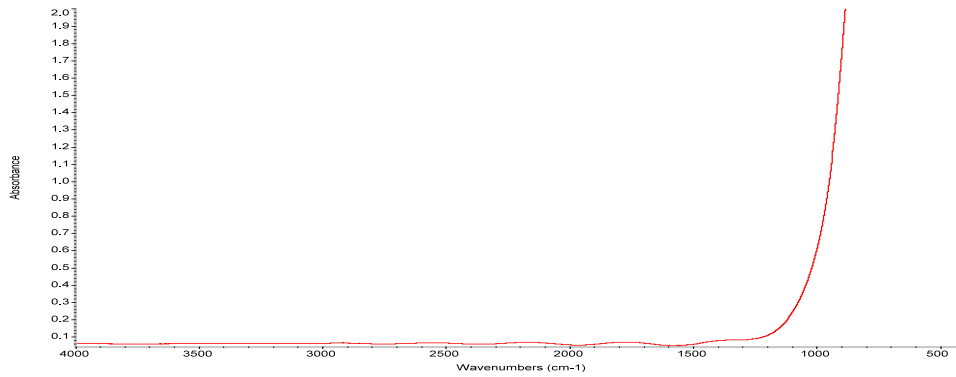


Figure 1. IR absorption of CaF<sub>2</sub>

The region of the IR spectrum that contains the vibrations of the NH's or CO's involved in hydrogen bonds is from about 1550-1750 cm<sup>-1</sup>. Unfortunately, as shown in Figure 2, pure water has an absorbance right in the middle of this region at about 1600 cm<sup>-1</sup>.

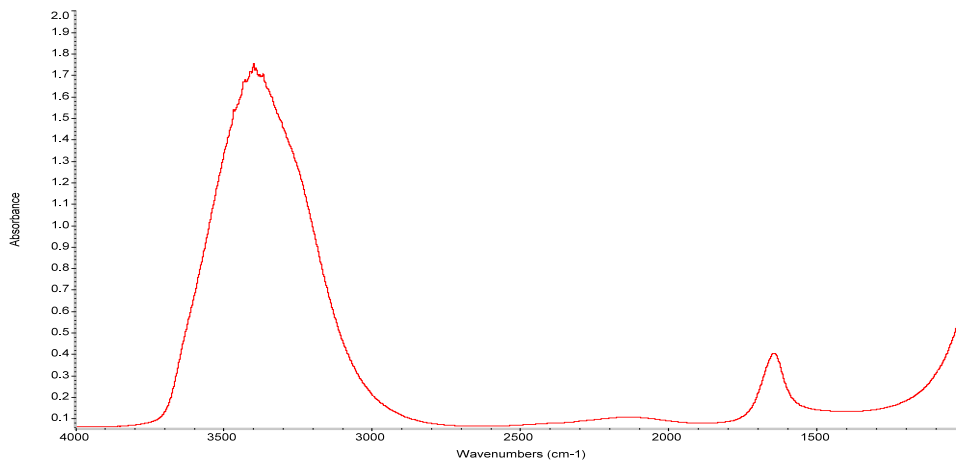


Figure 2. IR absorption of H<sub>2</sub>O film.

Then you also have the problem that the solvent water concentration will be about 55 M, while the concentration of the DNA you are looking for will be a fraction of a millimolar, so this peak that looks fairly small in Figure 2, will be humongous compared to your DNA absorbance.

The way we get rid of this is by using D<sub>2</sub>O instead of H<sub>2</sub>O. How does this work? The mass of the deuterium atom is twice the mass of the hydrogen atom. With twice the mass these vibrations slow down and shift to a lower frequency. Figure 3, shows the spectrum of a thin film of D<sub>2</sub>O. You can see that the main frequencies have shifted down so the range we want to look at between 1550 and 1750 now appears to be open for our experiment.

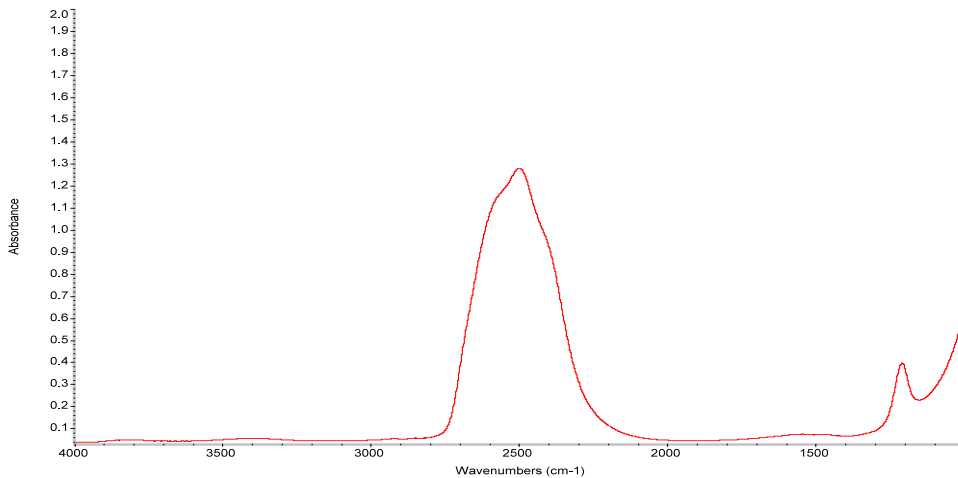


Figure 3. IR absorption of D<sub>2</sub>O.

We aren't home free yet. Remember what I said about the DNA concentration being in the mmolar range? To get an IR signal for DNA at this concentration we will have to use a pathlength of 200 $\mu$ m or 0.2 mm. Figure 4 shows the spectrum of D<sub>2</sub>O in a cell of this pathlength. This pathlength is literally 1000's of times larger than the thin film I showed in Figure 3, thus all the tiny rolls and swells in the baseline in Figure 3 get magnified a thousand times to get Figure 4. You can see that we should still be able to do our experiment, but it won't be pretty.

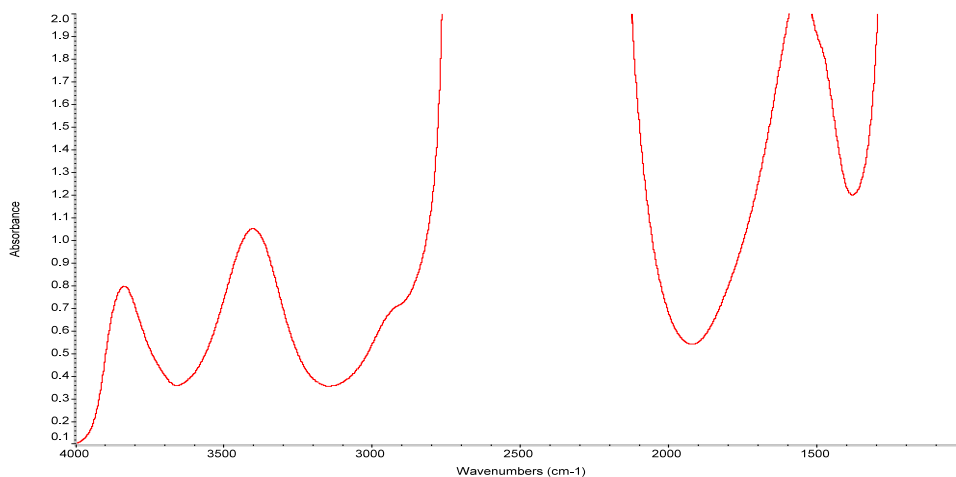


Figure 4. IR absorption of D<sub>2</sub>O @ 200  $\mu$ m pathlength.

One added bit of fun. I don't have a reference IR spectra for either native or denatured DNA. Thus we will have to get a spectrum under conditions we assume are native, and then simply watch for it to change as we change conditions, and that will be our test for denaturation.

## Sample Preparation

You will be given two or three 1.0 ml samples of DNA that have a concentration of 2 mg/ml in D<sub>2</sub>O. You will also be given 1M KOD. For this lab each group will be told to add different specific amounts of KOD to their samples. Find out which samples you have to make up. After you have made these samples use the small pH electrode to determine the pH (Technically pD) of your samples. As you manipulate these DNA solutions pay attention to their physical properties. When DNA denatures the solution should become less viscous because the DNA rods become loose and floppy. Do you see any evidence of denaturation before you get the sample into the IR machine?

## IR Experiments

*Part I. Determining the pathlength of your cells.*

1. Assemble and label both demountable IR cells.
2. Start the IR machine.
3. Set the machine to scan background first and to work in transmittance mode.
4. Scan a sample.

The computer will ask you to place a background sample in the machine.

-Place nothing in the machine as this first background.

The computer will now ask for your sample.

- Place one of your empty IR in the IR machine and take its spectrum.

- You should now obtain a pretty sawtooth pattern that represents

interference fringes of the IR light as it passes through the cell. We will use these fringes to determine the pathlength of this cell.

5. Use the **View** - display limits command to set the displayed plot limits. Try the following parameters first

X-Axis Limits

Start: 3500 End: 2000

Don't worry about the Y axis limits, we'll set those in the next step.

Hit OK

6. Look at your spectrum. If the spectrum is off scale click on the **View** - Full Scale

This will set the Y limits so the spectrum is properly displayed.

7. Move the cursor over the left most peak, and note down the frequency displayed in the lower left hand corner of the screen. We'll call this F<sub>1</sub>

8. Move the cursor over the rightmost peak and note down the frequency of that peak as well. We'll call this F<sub>2</sub>

9. Now count the total number of peaks in the interference pattern. This is N

10. The pathlength of this cell can now be determined from the equation:

$$\text{Pathlength}(cm) = \frac{\# \text{ of fringes}}{2(\nu_{\text{High}} - \nu_{\text{Low}})}$$

11. Repeat steps 4-10 to find the pathlength of your other cell.

*Part II. Obtaining IR spectra of your DNA samples.*

1. Set the IR machine to work in absorbance mode.
2. Scan a sample, you should reset the machine to 16 scans for a better signal. The computer asks you to place a background sample in the machine.
  - Fill one of your labeled cells with D<sub>2</sub>O and run this as a background.The computer now asks to run a sample.

-Take the cell containing D<sub>2</sub>O out of the IR machine. Place about .5 ml of your DNA in a syringe. Place an empty syringe on one port of the cell and your syringe with DNA on the other port. Gently flush the D<sub>2</sub>O out of the cell and replace it with the DNA solution. Replace the cell in the IR and take the spectrum of the DNA sample. When this run is complete save this spectrum on the computer for future processing.

If you have a second DNA sample, use the second IR cell to obtain your reference and sample spectra of the second sample.

**Notes on the Omnic Program that runs the IR**

Make sure the IR is turned on (there is an idiot light in the front lower left hand side). If the OMNIC program is not running, click on the OMNIC icon on the main computer screen.

Once the Omnic program is running, find the Col Smp icon. Click on this icon. The computer will ask you to run a background spectrum. Mount the cell that contains D<sub>2</sub>O only and collect the background. The computer will now ask if you are ready to run a sample. Remove the D<sub>2</sub>O cell and replace it with your sample cell, and hit the yes button.

After a minute or two the computer will display the final spectrum and a window that asks if you want to add the spectrum to window 1. Click Yes. Your spectrum is now displayed on the computer screen. At this point you can print the raw spectrum if want. Before you go any further save this spectrum (file - Save). First save the spectrum using the default \*.SPA format so the computer has it in a format that it can use. Now use the file - save as command and also save the file in \*.CSV format. In this format the spectrum is simple of list of X and Y values, and you can use excel to clip out bits and pieces so you can make your own plots of the data with excel if you want.

After the data is safely recorded to a file, feel free to play with it a bit. You should be able to manipulate the X and Y coordinates to display only the 1550-1750  $\text{cm}^{-1}$  we are interested in, and make the Y scale best fit the data in this region. You may also want to try the autobaseline button, and see if you can remove any baseline role.

After you are finished playing, hit the Clear icon. This will remove the spectrum from the computer's screen. If you don't do this, you have this spectrum showing up underneath your next spectrum.

### **Processing IR spectra (2008 instructions may not be needed?)**

1. Start the ER Omnic Program
2. Use the **File** -open command to get your spectrum displayed.  
Use the **View** - display limits command to set the displayed plot limits. Try the following parameters first  
X-Axis Limits  
Start: 2000 End: 1500  
Don't worry about the Y axis limits, we'll set those in the next step.  
Hit OK
3. Look at your spectrum. If the spectrum is off scale click on the **View** - Full Scale.
4. Once this region is displayed, click the Autobaseline (Aut Bsln) button along the top button bar. This will try to remove the role that we have in our baseline.
5. Now click on the original spectrum and it will turn red. Hit the clear button and it will be cleared from the screen.  
Now let's zoom in on the actual area of interest  
Use the **View** - display limits command to set the displayed plot limits. Try the following parameters first  
X-Axis Limits  
Start: 1750 End: 1550  
Again don't worry about the Y axis limits, we'll set those in the next step.  
Hit OK
6. Look at your spectrum. If the spectrum is off scale click on the **View** - Full Scale. Plot this spectrum. Also save it as a .csv file so you can output the data in excel to look as actual absorbance values.
7. Obtain IR spectra for all samples, 0, 2, 4, 6, 8, and 10  $\mu\text{l}$  of KOD. Because the baseline changes from spectrum to spectrum this data will be a little hard to interpret. Start with the 8 and 10  $\mu\text{l}$  samples and identify peaks that correspond to denatured DNA. Now look at the other samples. Which samples contain these denatured DNA IR peaks, which ones don't seem to match?

## Analysis

All groups pool their data. Choose one spectra for your native DNA spectrum and one for your denatured spectrum. Hand in an a spectrum for both native and denatured forms in the region of  $1750\text{-}1550\text{ cm}^{-1}$ . Also look at the other regions of these spectra. Are there any other IR frequencies that you could use to detect DNA denaturation?

Pick out one peak in both the native and denatured spectrum. Knowing that the DNA was at a concentration of  $2\text{ mg/ml}$ , and that the molecular weight of a DNA base pair is  $660\text{g/base pair}$ , calculate the  $\epsilon$  of this peak. In the UV range, the  $\epsilon$  of a base pair is  $6,600\text{ liter}\cdot(\text{mol base pair})^{-1}\cdot\text{cm}^{-1}$ . How does this compare to the  $\epsilon$  in the IR region?

Comment on the denaturation process. Did you see a gradual denaturation of the DNA as the pH increased, or an abrupt change? Did you observe any change in viscosity of the DNA solution before or after the denaturation?

Assuming you have successfully denatured the DNA by raising the pH, Predict what you would observe if you took the sample and adjusted its pH back to pH 7.

# DNA Supercoiling Lab

## Purpose

To demonstrate the topological problems that occur in covalently closed-circular DNA.

## Background

When a linear piece of DNA is formed into a closed loop, the number of twists of one strand around the other becomes fixed, and a new level of structural isomers appears called topoisomers.

Topoisomers of DNA are DNA molecules that vary in overall structural topology, or, more simply, vary in how one strand is wrapped around the other strand.

Topological problems were heavily investigated in the 1970's, and in today's lab we will be trying to reproduce some classical experiments done by Paul Anderson and William Bauer (Anderson & Bauer 'Supercoiling in Closed Circular DNA: Dependence upon Ion Type and Concentration' *Biochemistry* 17 594-601, 1978)

In today's experiment you will be working with a piece of DNA called pBR 322. This is a small artificial loop of DNA that has a molecular weight of  $3.1 \times 10^6$  and contains exactly 4,365 base pairs, of a known sequence.

If you perform electrophoresis on pBR322 in a standard agarose gel, you will typically see two bands. The slowest moving band is pBR322 that has a nick in it so it is relaxed and penetrates the gel slowly because it is a big floppy loop. The faster moving band represents ccc pBR322 that contains supercoils and the supercoils make it twist up on itself, so it penetrates the gel more quickly.

Let's do some topology math. The field of topology has been around for many years in Mathematics so the math to handle these problems was already well understood long before Chemists and Biologists even knew there were topological problems in Biochemistry.

For a closed system like ccc DNA we have:

$$Lk = Tw + Wr$$

Lk is the linking number, and describes the total number of times one strand is wrapped around the other.

Tw is the Twist number, it describes a local twist of one strand around the other.

Wr is the Writhe number and describes a non-local twist of one strand around the other

Lk is constrained to be an integer, but Tw and Wr do not have to be integers. The description of Lk, Tw, and Wr is very clear to a mathematician in this field, but it is not as obvious to us chemists and biologists. Since Tw is a local wrapping, it corresponds to the twist of one strand around the other, or the wrapping that develops from the fact that B-form DNA has 1 turn for every 10.5 base pairs. Since Wr is a more global wrapping, it corresponds most closely with the overall supercoils observed in native DNA. Lk is then the sum of the wrapping from these two sources. The thing they don't tell you about in our textbook is that B-form DNA with 10.5 bp/turn occurs under very specific conditions of ionic strength. If you change the ionic strength, you change the bp/turn. Why? The 10.5 bp/turn structure of DNA comes about from two opposing forces. One force is the hydrophobic force trying to pull the hydrophobic bases as close together as possible, trying to shorten the helix. Opposing this you have the charge-charge repulsion of all the negative phosphates trying to get away from each other, and trying to stretch out the helix as much as possible.

As you change the ionic strength of the solution you change the number of ions that surround the helix and that, in turn, changes the phosphate-phosphate repulsion. Overall then you should have maximum repulsion, most stretched out helix and most base pair/turn at low ionic strength (low ion concentration), and least repulsion, most condensed helix least base pair/turn at high ionic strength (high ion concentration).

In today's lab then, we will put DNA into solution containing different concentrations of  $\text{NH}_4\text{Cl}$ . At the highest concentration the DNA will be the most twisted, and at the lowest concentration the DNA will be the least twisted. But how will we observe how twisted the DNA is?

For this we will use the enzyme topoisomerase I from Vaccinia virus. A topo I enzyme relaxes DNA by cutting one strand of the DNA, allowing the strand to unwind as needed, and then reattaching the two strands. This topo I specifically looks for sites (C/T)CCTT, then clips to the right of the T.

The experiment, then, is to put the DNA into different concentrations of  $\text{NH}_4\text{Cl}$ , and then treat it with topo I for one hour at  $30^\circ\text{C}$  to allow it to relax. We then kill the enzyme by adding 1% SDS (sodium dodecylsulfate) a detergent. At this point the DNA now becomes locked at whatever Lk it was. We can now analyze the DNA using agarose gel electrophoresis.

I started this write up by telling you that native pBR 322 should run as 2 bands on this gel. What you will see with these topoisomers of pBR322 and that you should now see several bands. The uppermost band will still be the completely relaxed DNA. The next fastest band will be DNA that has a single supercoil in it because the supercoil makes the DNA kink up slightly and that lets it penetrate the gel more quickly. Since all the DNA in the gel will have the same Tw (now determined by ionic strength of the gel) if a band has a Wr that is different from relaxed by 1, then its Lk is also different by 1. Thus each band in the gel represents a DNA with a different  $\Delta\text{Lk}$ . By counting the number of bands, you should be able to find the average  $\Delta\text{Lk}$  from the fully relaxed state.

### Experiment

1. Obtain six Eppendorf tubes and labels them 1- 6.
2. Fill the tubes according to the following table:

	Tube	1	2	3	4	5	6
DNA stock		1µl	1	1	1	1	1
50 mM Tris		1µl	1	1	1	1	1
1M NH <sub>4</sub> Cl		4µl	1	0.5	0	0	4
Water		3µl	6	6.5	7	8	4
Topo I		1µl	1	1	1	0	0

Add reagents in order on the table. I.E. add the topo I last

3. When all tubes are filled, mix and let react at 30°C for 1 hour.
4. After one hour add 1µl of 1% SDS to each tube and mix. This should kill the enzyme and stop the reaction.

During this incubation time you can prepare your agarose gel.

#### Preparation of 1% agarose gel

Mix the following in an 125 ml Erlenmeyer Flask

27 ml of water

3 ml 10X TAE buffer

.30 g of Agarose

heat to boiling on a hot plate

swirl until all of the agarose dissolves (Keep hot)

Cool to 55°C (Just cool enough that you can hold it in your hand)

Pour agarose into a gel mold that has been prepared by sealing the ends and insert a 6 tooth comb.

Allow to cool completely (or at least 15 minutes after it starts to gel)

#### Gel Run

Add 1X TAE buffer until it is about 0.5 cm above the center support (This will take about 300 ml)

Remove comb from your gel and the seal on the ends of the gel

Place the gel in the electrophoresis chamber with the well side toward the black electrode (the buffer should cover the surface of the gel)

Add 2 µl of loading dye to each of your tubes

Carefully place 10µl of each sample in different wells on your gel

Connect the apparatus to the power supply and run the gel at between 95-100V until the bromophenol blue dye in the loading buffer is close to the end of the gel. (About 1.25 hour)

## Developing Gel

Carefully remove gel from the electrophoresis apparatus and transfer to a developing tray

Flood the gel with the ethidium bromide staining solution. **Make sure you are wearing gloves whenever you touch with the ethidium bromide staining solution, or the gel containing ethidium bromide because this compound is a potential mutagen and carcinogen.**

Allow the gel to stain for 10 minutes

Carefully pour the staining solution back into the stock bottle.

Rinse the gel a few times with deionized water to remove any excess stain.

Obtain pictures of your gel under UV light, where the binding of ethidium bromide to the DNA will make a bright band appear where ever there is DNA.

## Questions to turn in

1. What is the  $\Delta Lk$  from the relaxed state for pBR322 for each concentration of  $NH_4Cl$  used?

2. In the above answer did you get a single  $\Delta Lk$ , or a distribution of values? If you got a distribution, explain why. If you got a distribution, then your answer for #1 should reflect your estimate of the center of the distribution.

3. Make a plot of  $\Delta Lk$  vs  $[NH_4Cl]$  and  $\Delta Lk$  vs  $pNH_4Cl$  ( $-\log[NH_4Cl]$ )

4. In setting up your gel assay you had 6 different tubes with 6 different assay conditions. The reason for tubes 1- 4 is obvious, in these assays you varied the concentration of  $NH_4Cl$ . What is the purpose of tubes 5 and 6?

## DNA/RNA sequence analysis - Computer Lab

In this lab you will be given a sequence of a piece of RNA or DNA. You are to locate the open reading frame on this DNA/RNA and translate the DNA/RNA into a protein sequence. The protein should not correspond to any currently known sequence, but to a new protein. As a new protein you are to characterize the protein as extensively as possible, using the tools available to you on the net. This can include proposing a possible 3-D structure as well as a possible activity.

Finally since you are now moving into a section of the text dealing with control, your final task will be to modify your sequence to it could be properly transcribed and translated in an *E coli* cell.

### Background

NCBI is part of the National Library of Medicine (NLM) which is a part of National Institutes of Health (NIH). Let's start at their website <http://www.ncbi.nlm.nih.gov>. Read a little background about this site. Now look on the left hand column on this page. Where we are going to be working is primarily the different data bases

**GeneBank** - <http://www.ncbi.nlm.nih.gov/GeneBank/index.html> - the data base for DNA/RNA sequences

**Literature Databases** - <http://www.ncbi.nlm.nih.gov/Literature> -the literature database you can search for articles for free! This data base can be incredibly useful for research, but we won't use it in this exercise.

**Molecular databases** - <http://www.ncbi.nlm.nih.gov/Database/>

This is the entry point for Entrez

This is the key to the entire system because it is a grand program that is designed to let you navigate between all of these different databases with little or no knowledge of computers or databases. Thus you can 'mine' all the information you want with just a little intuition and a mouse click. The only down side is that *Entrez* is so flexible and intuitive, it is easy to get lost following one line of thought and you might not get back to where you started. Make sure you keep track of paths and questions that you use along the way to you can reproduce your results.

And finally

**Tools** - <http://www.ncbi.nlm.nih.gov/Tools/index>) the site you will be working in

You might want to bookmark these sites to you can get to them more easily the next time.

**Procedure:**

Find the location of the file that contains your unknown DNA sequence. It is most likely a text file located on the L:drive under the Handouts - MichealZehfus-Biochemistry - Chem 465L- Database Mining directory

Go to **Tools** link as described on the previous page.

Find the ORF Finder tool. (ORF = Open Reading Frame)

Click on the name or icon to start it running

Cut and paste you gene sequence into the box titled  
'or sequence in FASTA format'

Don't fill in anything else

Hit the 'Orf find' button

You should now have a display with long rectangles on the left and numbers on the right

Each long box is the sequence with the orf shown in color.

Rectangle on the first line represent reading frames that start at position 1,4,7, etc. The second line has reading frames that start at 2,5,8, etc. The fourth line has reading frames that start at the Last codon an read to the left, etc.

The number on the right list the orf's by size and psotion

Click on any of the colored orf's (either on the rectangle or in the listing by size)

The orf and the sequence of the protein coded by the orf will appear at the bottom of the display

Find the longest orf that reads right to left in your sequence.

Get the display of the sequence for this orf

Cut and paste this display into a file on your computer so you can report this in your final write-up.

Find the longest ORF that reads form left to right in your sequence.

Get the display of the sequence for this orf

Cut and paste this display into a file on your computer so you can report this in your final write-up.

Note that this orf is actually on the complementary strand of DNA, so when you locate it on your DNA sequence, not only will the AUG (RNA start codon) be turned around, so it reads right-to-left, but you will have to figure out the compliment of the reversed sequence to find it on this strand.

From this point on we will focus only on the longest ORF in your DNA.

Click on this longest ORF so it is displayed on the screen.

Note the area above the ORF display that is in pink. The program should read 'blastp' and the Database should read 'nr' Blast P is a program that will compare your protein sequence with the sequence of all other proteins that are available. Hit the BLAST button to see if your protein has a match. (This will take a few seconds)

A new screen that will show the parameters that you used in your BlastP search. To actually see the results of this search click on the 'View Report' button.

You should now get a window that shows any matches to your protein. From this point follow the direction on the page, click on the image and it will show you the details of the matches you find. Note that the upper part of the screen shows where the matches are, but as you move down the screen, you can see the actual sequences of your protein and the proposed match protein. Cut and paste the best match to your local computer for your lab report.

Now that you have a protein, let's see how much different information you can squeeze out of the sequence. Bring up the site '<http://ca.expasy.org/>' This is a site that is built around the protein rather than the DNA, with lots of tools to try to figure out what is built into the protein's sequence. Notice all the tools in the right hand column. At this point I will turn you loose. Try every tool you can and see what these tools tell you about your protein. At the minimum you should be able to get an exact molecular weight, a pI, a proposed secondary structure, knowing a closely related protein you might even be able to propose a 3D structure. The list is endless. The more things you can tell me about your proposed protein, the more points.

Once you feel you have dredged up as much information on your protein as you can, I have one final task for you. The sequence you were given came from a eukaryotic DNA. Suppose as a research project you wanted to clone this DNA into *E coli* so you could over express it and purify the protein. I now want you to take your sequence and propose how it would be modified to include certain *E coli* control sequences as described in the write-up section.

**Write-up** (you can submit electronically to save trees!)

1. Print your original DNA sequence with the longest ORF that reads from left-to-right, colored or bolded to show where it is.
2. Print the protein sequence of the longest ORF that read from left-to-right.
3. Print your original DNA sequence with the longest ORF that reads from right-to-left, colored or bolded to show where it is.
4. Print the protein sequence of the longest ORF that read from right to left
5. What is the name of the protein your sequence appears to be most similar to.
6. Print the sequence of your protein and the most similar protein in a manner that shows how these protein are the same and how they are different.

For this protein now find at least:

7. Exact molecular weight,
8. pI,
9. Proposed secondary structure
10. + Other. For each other fact you dig up on this protein, describe the steps you used to get this fact.

For sequence modification

11. Print the DNA sequence for your protein plus 200 nucleotides on each end
12. Insert a Shine-Dalgarno sequence so this will work on an *E coli* ribosome. Highlight this sequence in red, and give a brief justification for sequence and location.
13. Insert a promoter sequence into the DNA so RNA polymerase with the  $\sigma^{70}$  will bind and make many copies of this RNA. Highlight in Blue, and give a brief justification for sequence and location.
14. Insert a sequence so the RNA polymerase will fall off this gene via  $\rho$ -independent termination. Highlight this sequence in Green, and give a brief justification for sequence and location.

Additional notes:

The websites you are visiting change yearly, so the directions I have given above will not work exactly as written. Below is some added directions I gave in 2010. By the time you do this lab, the site will have changed again, so be flexible, and explore on your own.

As usual, in the two years since I last wrote how to access this information, the organization and content of the sites has changed, so what I have for written directions no longer matches what you will see on your screen. Here are some newer directions to get you started.

The main address is still good: [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)

For background information click on the links for 'More about the NCBI' and 'Mission' in the text near the top center of the page.

**I would definitely check out each of the links on the right hand side of the page called 'Popular resources'. For each resource write yourself a sentence or two about what the resource can be used for. Some of these resources like 'structure' give you access to lots of other resources, like the protein databank, so feel free to work you way around and access some sub-categories of resources and write them up too!**

Now look at the resources table on the left hand side

Find 'DNA & RNA' and click on it. Look at all the different DNA and RNA databases you have access to at the top of this page, then all the tools and downloads. For now find the GenBank database, click on it and read about it. **How many bases are currently in the data base?**

For literature data bases, to get free access to lots of literature, you can either use the literature resource on the left, and page through all the different literature databases, or you can use the 'Popular resources' on the right and get into PubMed or Bookshelf directly.

To get to the 'Entrez' tool for searching all the data banks for all the information about a protein, you can either go to Resources - Proteins - Protein on the left, or Popular Resources - Protein on the right. **Just for fun, see how many reference you can get to either Elk LDH or Bison LDH through Entrez**

Okay enough playing around, on to the problem at hand, searching all these data banks for the DNA sequence I have generated for you. Find the sequence I have given yo on the L: drive.

First we need to find the open reading frames in your sequence. In the NCBI site under 'Resources -Sequence Analysis' under tools, find 'Open Reading Frame Finder' Follow directions on page 42 of your text onward.

I ask you to go to the 'ca.expasy.org' site to see what information you can dredge up about the sequence I have given you. You should still do this, but this site is now oriented toward proteomics and mass spec analysis of proteins, so the programs I want you to run are buried. Find the Tools and Software and click on 'Full list'. The first two sets of tools are for mass spec, but from that point onward there are many different tools sets you can use. For sure try the third set 'Identification with isoelectric point...' And you might try the 'Prediction or characterization' tools as well. As you go further down this list there are all sorts of things you can try, up to, and including, predicting secondary and tertiary structure! Have fun.

Finish the last paragraph on page 60 of your text and the write up on page 61 as written, but add answers to anything in **bold print** from above!