Supplemental Information and Labs
for
Chem 464L
Biochemistry I Lab

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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 bottom portion of this sheet, 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 _____

Changes for Chapter 1 Experiment 1

Since we don't have enough pipets for all groups to do at once

     For experiment 1 part A
          Manual says use P-100
          But you can use any pipet of 200ul or less (ie 200,100,20,10,1)
          Do 10 determinations not 3
          Question 4 report standard deviation not mean deviation

          If using <100 (20,10,1,etc) use analytical balance so have another digit

     For Experiment 1 part B
          Manual says use P-1000
          But you can use 1000 or 5000
               Again use 10 measurements for both 1 and 5
               In part five use 1/10 of total
                    IE is using 5000, use 5000
                    If using 1000 use 100
               Questions 4 and 8 report Standard Deviation
               This Can be done on standard balances

Hand in 1, 3, 6, 9, and 12 from additional problem set

Chapter 2 -Experiment 2

Part A
          I'll assign you two pH's for your buffer
          For each pH
               Hand in a labeled vial containing your sample for me to check.
               Hand in a paper with name of compound and weight used to make 100 mls
               of .10 M buffer

Parts B, C, D as written

Notes on Chapter 4 Enzyme Purification
Experiment 4a Purifying LDH (Comprehensive Version)

1. I prefer the long or comprehensive version of the lab, so we will be doing the experimental procedure given in 4a (pages 109-113).

2. The amount of ammonium sulfate required to get to 40% saturation is wrong in step 4 of comprehensive version. It should be .230g /mL.

3. The tables and questions for the short version of the lab are better, so use the Analysis of Results for the short version of the lab (pages 105-108).

4. While I agree with the author that it is best to do the assay of the protein as quickly as possible, doing the purification and assay on the same day is almost impossible unless you are extremely well organized. Therefore on Day 1, we will only do enzyme purification, and don't worry about the assay.

5. One problem with the assay is that it works best on the Lambda 2 or a spec 20 interfaced into a computer for time acquisition. We may have trouble getting enough machines so everybody can do their assays on day 2 of the lab, so I may hang up sign-up sheets for you to use these machines during the week between day 1 and day 2.

6. A second problem with the assay, is that it works better with a minor modification of making a single solution that contains both CAPS and Lactate.   The modified procedure is given on the next page.

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^+ 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^+ 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^+. 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 μl 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.

Protein Structure Computer Lab

Analysis of protein structures in the Brookhaven Protein Data Bank using the RASMOL graphic program.

Background
Useful references -Leninger Biochemistry - Chapter 4 , Introduction to Protein Structure - Branden & Tooze Garland Publishing 1991

THE RCSB DATA BANK
The RCSB (Research Collaboratory for Structural Bioinformatics) Protein Data bank is a national repository for X-Ray and NMR structures. The data bank currently contains >32,000 different coordinates of ~30,000 proteins, ~1500 nucleic acids and 13 carbohydrates.   Many journals require that the coordinates of a biomolecules be must deposited in the Data Bank before they will publish an article dealing with a new X-ray or NMR structure.

Using the RCSB data bank

1. Use a web browser to access the PDB site
http://www.rcsb.org/pdb/

This will bring up the PDB web site. Explore this site as much as you want. It has lots of links to interesting places. Once you are through exploring, start to look for a protein you want to study. No ideas? Try looking up some proteins given as figures in your textbook, or in the Branden and Tooze text.

Now let's try to get a copy of the X-ray file for your protein. Look for the Search box in the middle of the PDB home page. Plug the name of your protein in and let it search. With luck you will now have a page or two of proteins that fit your search request.

Look at each entry on the search list. You should blue bar, with a line of Title (Title of original publication), Classification (Type of enzyme), and Compound (Specifics on the protein in the structure) below that.   The Blue bar is the most useful. On the blue bar you should see icons that will let you download the file, directly view the data in the file, and view images of the protein in the file. (The key to which icon is which is just above the first blue bar.)

Start with the View icon. You need to have the proper viewer downloaded on you computer before you can use the interactive viewers. There is a blue box on the right of the screen that will help you download a viewer if you want. RASMOL and Chime should be downloaded and installed on all of the chemistry computers, and I had the best luck with the First Glance and Sting Millennium viewer on the computer in my office.

If you don't have any luck with the interactive viewers, you can at least look at the static pictures at the bottom of the page and see if the protein looks like the type you are trying to find.

Be sure to click on some of the links on the left hand side of the page, and try to figure out what information they give you access to before you leave this page. After you are through exploring, hit the back arrow key and return to the main search page.

If this is a set of coordinates you want to look at in more detail, then you need to download it. If you use the download icon, it will download the file in a compressed format. To uncompress the file you will need to have WinZip installed on your computer. If you don't have Winzip, you need to try a different pathway to get the uncompressed file.

Getting an uncompressed file.
Click on the icon for 'View PDB file'. At the top of this page is a link that says 'Save full entry to disk'. Click on this link. One of two things will happen. Either the file will download, or, if you have a computer with a viewer installed, a 3-D image of the protein will come up for you to look at. If this happens to you, hit the back arrow, and try again. Only this time click on the 'Save full entry to disk' with your right mouse button. Now a menu comes up. Find the 'Save Target As' command. This will bypass your built in viewer and let you download the compressed file to your choice of directories.

RASMOL
Simply having the coordinates is not enough. You must be able to examine them in a way that allows you to visualize the structure and understand it on an intuitive level. There are many programs available to do this. RASMOL is such a program. It is Freeware, and may be downloaded from the net (look in the RCBS web site for links to RASMOL or RASMAC). It has already been downloaded onto the computer is Chemistry and is available for your use there.

I think the easiest way to learn how RASMOL works is to experiment. Find the 'Chemistry Programs' directory in the D: drive of one of the Chemistry computers. Load a the protein databank file you want to examine into this directory.
Click on the RASWIN program name or icon
   A black window should come up where your molecule will be displayed.

Click on the 'File' button and 'open' command. If you have loaded a protein data bank file into the directory with RasWin, you should now see it in the window. Click on your file and let it load.

Go ahead and expand the RasWin Window to full screen.
   Hold the left mouse button down and move the mouse, what happens?
   Hold the right mouse button down and move the mouse, what happens?
   Hold the shift key down and the right mouse button, what happens?
   Hold the shift key down and the left mouse button, what happens?

Click on the 'Display' button and try each of the different display modes.
Click on the 'Colour' button and try each of the different color modes.
Click on the 'Options' button and try each of the different options.
Click on the 'Setting' button and try each of the different settings.

At this point you have seen enough that you can play with your protein and display ti to view your structure in many different ways. I will leave a manual for RASWIN in the back room. There are lots of more things you can do with RASWIN by looking in the manual, and using a the separate command line.

EXPERIMENT
In lecture this week you are learning the basics of protein structure, things like α helices, β-sheets, etc. Proteins are commonly grouped into four major categories of structure, all α helix, all β-sheet, α/β (the protein is a mix of the two) and α+β (a two domain protein where 1 domain is primarily α, and the other domain is primarily β.

In this lab you will be working in groups. Each group is to find one protein in the Brookhaven data base to represent each general type of structure. Further, each group will be assigned one particular type of protein to examine more closely.

Next week each team will present an oral/multimedia presentation on their chosen protein. I will sit down with each group and have the group show me what they found on a computer of their choice. Things to include in your presentation if you can find them:
Prime reference for the Crystallographic coordinates. What is the enzyme's function? Where does it come from? How was it purified? Look at the format of the file and see what you can understand about the format. What are the salient features of your protein's structure. Where is the active site? How does it work? Are there any other molecules bound to the protein? Are they a necessary part of the structure? Can you pick out any particular secondary or super secondary structures? Are there any related protein files that should be examined? Any features of RASWIN that helped you to visualize the structure? Anything you wanted to do but couldn't?

In evaluating your presentation I will be looking in three major areas. What different things did you find in the Protein Data Bank as a whole, what different things did you find out about your target proteins, and how well did you learn to use the RasWin program.

PROCEDURE
There is no set procedure to this lab. Use the network to get on to the protein data bank. Locate a search engine and start trying to find a likely structure. This may be particularly hard because you don't have a particular target for your search. Try looking in the references for representative proteins that could provide you with a name or reference that you can try searching with. Once you have a structure located see if you can use RASMOL on-line to do a preliminary survey of the protein. If it looks good, then download the complete coordinate file and start digging. Note that once you have a protein identified in the data bank there are several links to other useful information on the protein. .

This is a wide open lab there is no right answer or correct finding. The idea here is to explore as much as you can to get a feel what information is available on a given protein. Keep careful notes on what links are useful and where you find particular pieces of information. In the presentation you may be asked to reproduce a given link so others can see how you did something.

If you want to look at DNA or a carbohydrate go ahead and give that a try as well.

Notes for Experiment 8a

1. We are using the modified assay set up

        2.4 ml CAPS/Lactate

        0.5 ml NAD^+ (for Step 1, lower for Step 3)

2. We only have 3 set of pipets and stock solutions so spectrometers that are close together need to share.

3. LDH sample

        For starters, weigh out 10 mg of your dialyzed protein and dissolve it in 1 ml of deionized water. Do this is an eppendorf tube. Keep on ice for the rest of the period.

4. Step 1.

        Try the following amounts of protein: 50, 20, 10, 5, and 2 µl of the protein stock. After doing all 5 of these experiments, then try to find a volume that gives you a 0.2 OD/minute change by choosing a volume between one of the above fixed volumes. It is easiest to simply use different volumes of protein, but you should consider diluting if you need to deliver <1µl of protein to make the assay work

        For Step 1we won't worry about the few µl's difference in volume in each run.

5. Step 3.

        Once step 1 is accomplished, then you use that 'good' protein volume for all the experiments in Step 3. Here you make the NAD^+ concentration progressively lower, and get a lower rate. Here the volume changes will be significant, so you need to add water to keep the total volume of the NAD^+ aliquot a constant. (For example, if you only use 25 µl of NAD^+, you will need to add 75µl of water so the total volume is still 2.9 ml) I would try cutting the volume of NAD^+ in half each time and see how this works. In 2005 the following volumes of NAD^+ seemed to work pretty well: 500, 250, 125, 60, 30, 15, 10, and 5 µls.

Notes on running the spectrometers for assay

A. Lambda 2 (The good Perkin Elmer)

       1. Check that the time-drive program is set for 340 nm, take data every 5 seconds, for a total time of 1 minute.

       2. Have a water sample in the reference beam

       3. If you have a USB drive, plug it into the machine now so you can record your data directly on the USB drive. If you don't, set up a folder on the D drive to put your data so you can access it later

       4. Run a time experiment as you did before

              A. Insert the mixed buffer&NAD without the protein to run the 'blank'

              B. Add protein, mix, insert cuvet and start the experiment, writing down the name of the file for this data Set (TDxxx.TD)

       5. Once a data set is obtained we will write it to a file you can access with Excel immediately so you can analyze it before you go on to the next experiment.

              A. File the 'File' button - 'Save as' option

              B. When the 'Save as window comes up, highlight the correct file for the original data,

              C. If you have a USB drive set the window to record in Drive G:

              D. Change the data format to ASCII

       6. Once the data had been recorded as an ASCII file, minimize the Lambda 2 window, and start up Excel

              A. Have Excel open the file you just made.

                     Note: the file you just made is NOT in Excel format, so when you are looking for the file, set Excel to look for All files, not just Excel files

              B. As Excel download the data it will show a window asking about the how you want to format the data. Don't try anything fancy, just hit the 'finish' button, and let Excel do what it wants with the data.

              C. Look to the bottom of the Excel page. There should be 2 columns of numbers. The first column should start with a 0 and then have negative numbers . This is actually the time in minutes . I would simply change the negative to positives. The second column had you absorbance data in it. To get your slope you can either plot the data and get a line of best fit (Probably the best for the good final analysis). Or, for experiment 1, where you are trying to get a ΔOD of .2 in 1 minute, simply subtract the first value from the last value, and see what the ΔOD is for the 1 minute interval

              D. Save this data

              E. Minimize the Excel window, maximize the Lambda 2 window and start the next experiment.

B. The Spec 20's with PASCO interface

1. Bring up Pasco system (Double Click on the 'Data Studio' Icon)

       Click on the 'Open Activities' icon
             If there is an activity called 'Spec 20 time'
                   Click on this activity then click on the 'open' button and you are ready to
                   go. If the 'Spec 20 time' activity is not listed then see your instructor

2. It Computer interface should now be functional. Check it out. Hit the start button. With no cuvet in the Spec 20, use the zero knob to set the zero level on the spec 20. The digits window should be displaying a very number >2.   Now put in your solvent blank and set the 100% transmittance on the Spec 20 with the 100% knob. The digits reading on the computer should go down to about .000. Hit the stop button

Go to the 'data' window. Click on both of the data sets that have appeared and hit the delete key to make them disappear.

3. Actual experiment
       A. Set up your experiment on the Spec 20, Set the wavelength to 340 nm.
       B. With no cuvet in the machine set the 0 knob.
       C. Mix the buffer and NAD in a cuvet, insert the cuvet in the machine and set the 100% knob.
       D. Add protein, mix, insert cuvet and hit the 'start' button on the PASCO window
       E. Take data for at least 60 seconds. Once a data set is obtained we will write it to a file you can access with Excel immediately so you can analyze it before you go on to the next experiment.
             1. Do a left hand click on the top of the data table directly on the colored symbol for the data set. The entries in the table should now be outlined in yellow. On the main tool bar click Edit, the copy.

             2. Now minimize the Datastudio window and bring up an Excel window. Click on any cell in the spreadsheet, and hit the paste button. The data from the PASCO program should now be in Excel for you to work with. To get your slope you can either plot the data and get a line of best fit (Probably the best for the good final analysis). Or, for experiment 1, where you are trying to get a ΔOD of .2 in 1 minute, simply subtract the first value from the last value, and see what the ΔOD is for the 1 minute interval
       E. Save this data
       F. Minimize the Excel window, maximize the PASCO window and start the next experiment.

Observing Mutarotation in Glucose

Glucose can be observed in at least 3 different conformations:
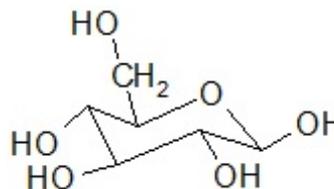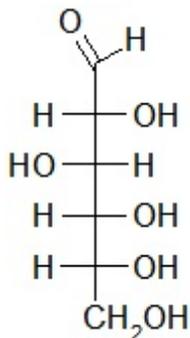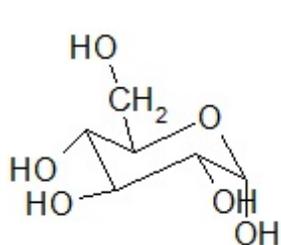
α-D-(+)-Glucopyranose   Open-chain form   β-D-(+)-Glucopyranose
(mp. 146°C. $[\alpha]_D^{25}$= +112°)   D-(-)-glucose   (mp. 150°C. $[\alpha]_D^{25}$= +18.7°)

Glucose is most easily crystallized in the α form, so when your first put glucose into water this is the form you observe. Once in solution, howeverin the glucose freely interconverts between the forms until it reaches in equilibrium distribution of roughly 1/3 α, 2/3 β, and negligible open chain conformation. This process is called mutarotation.

The physical properties of these conformers are very similar, so few physical techniques can differentiate between them. There are, however three techniques that can do this: Optical Rotation of plane polarized light, Circular Dichroism (differential absorption of circularly polarized light), and NMR. In today's lab we will use these techniques to study Mutarotation.

I. Optical Rotation

In an optical rotation experiment we will measure to rotation of plane polarized light as it passes through a glucose solution. The experimental set up is very simple. We have a source of light, (preferably a sodium lamp vapor lamp) an optical element that polarizes the light coming from the lamp into a single plane, a tube containing our sample, and then a second analyzer crystal, mounted on a vernier scale so we can measure its position or rotation relative to the first crystal. If the sample doesn't polarize light, then the two crystals have a 0° angle between them. If the sample does rotate the light, then we move the analyzer crystal until we see a maximum of light coming through it. The angle between the analyzer crystal and the source crystal is defined as the angle that the light was rotated.

The calculation of a specific rotation looks a bit odd from the modern chemical point of view because the units were created based on measuring concentration in grams /100 ml, and using pathlengths of decimeters (1/10's of a meter). We use units of decimeters for the pathlength because the optical rotation effect is so small you need to have a long pathlength to observe measurable values. The actual equation is:

$$[\alpha]_D^{20} = \frac{\theta \cdot 100}{l \cdot c}$$

where $[\alpha]_D^{20}$ is called the specific rotation, and refers to a measurement taken at 20°C using the sodium vapor D line (588.9nm), θ is the experimental rotation, l is the pathlength in decimeters, and c is the concentration in g/100 ml. Note: this unit makes the effect sound large, the [α] for α glucose is 112°, however, when you plug typical experimental numbers in like c = 5 g/100 ml and the pathlength is 1 dm, the angle you actually measure in the polarimeter is less than 10°!

II. Nuclear Magnetic Resonance (NMR)

NMR or nuclear magnetic resonance is a technique where each nucleus in a compound has a particular resonance frequency. This resonance frequency can be related to the chemical environment around the nucleus. In α- and β- glucose the chemical environment around the anomeric carbon (the 1C in glucose) is different enough for you see different chemical shifts for the C in a C-13 spectrum, or for the H attached to this C in a proton spectrum.

While you are most familiar with proton NMR, obtaining a proton spectrum for glucose in a water solution is difficult because 99% of the signal you observe comes from the protons in the water instead of the protons in the glucose. For this reason we will instead dissolve the sugar in heavy water or D_2O so the background of protons should largely disappear. We will use both ^1H and ^{13}C nuclei to see the effect from two different perspectives

You will notice at least two major differences between C and proton NMR. First, the C chemical shift range is much larger than the chemical shift range of protons, it goes from 0 to roughly 200 ppm. Second, since the more common isotope of C is ^{12}C, and ^{13}C is only present in <1% of the nuclei, so the C signal is much, much weaker, these spectra are much noisier. You will have to take about 100 scans to try to get a reasonable signal, and even then the noise will be 100's of times worse than you will see in a single scan using protons.

III. Circular Dichroism (CD)

Circular dichroism is a technique that it not used too much outside of Biochemistry. Circular dichroism and optical rotation both derive from polarized light interacting in different ways with asymmetric molecules. The major differences are that optical rotation involves the rotation of plane polarized light, and occurs at wavelengths near and far away from absorbance maxima. Circular dichroism involves circularly polarized light, and occurs only at the wavelengths where absorption occurs. When you are measuring circular dichroism you are measuring the very small difference between the absorption of right- and left- circularly polarized light. A positive CD signal corresponds to left circularly polarized light being absorbed more strongly than right circularly polarized light. Again the units measured on the machine, m° are a

bit obscure, but have to do with the rotation of the plane of light due to the differential absorption. In this experiment we won't worry about the exact value of the CD signal, we will instead simply monitor what happens to the signal over time as a method of estimating and comparing the rate of the mutarotation reaction at different pHs.

IV. Prelab questions

1. Calculate the rotation you should expect to observe for a 1.25g/100ml solution of α glucose in a 1 dm cell.

2. Calculate the rotation you should expect to see of a 1.25g/100ml solution of glucose that is 40% α and 60% β.

3. What chemical shift would you expect for C in the following environments

        H-C-O-
        C=O (aldehyde)
        C=O (ester)

(If you haven't had Chem 434, ask Dr. Z. For a table of Chemical Shifts from 'Principles of Instrumental Analysis' - Skoog Holler & Nieman)

4. Calculate the pH of a solution that contains .03 g of $NaH_2PO_4 \cdot H_2O$ and .01 g of $Na_2HPO_4 \cdot 7H_2O$

V. Experiment - Optical Rotation

1. Check the polarimeter's zero setting. Fill the 1 dm tube with deionized water and determine the zero position of the polarimeter. It probably isn't $0.00°$ but is slightly off. Getting a reading using this device takes a little work, both to figure out where the zero point is and how to read the vernier scale. Take time to read the manual and get this measurement right. Note how you need to try the measurement at least 4 times, moving the detecting scope from both clockwise and counter-clockwise directions and then you average these four numbers together to ger your final answer.

2. *Determination specific rotation for four sugars*. Accurately weigh about 1.25 g of α-glucose, fructose, sucrose and maltose and place each in a 25 ml volumetric flask. Fill each flask to the mark deionized water and add one drop of dilute ammonia to catalyze the mutarotation reaction so the different forms come to equilibrium more quickly. Mix the solutions, place in a 1 dm cell and determine the optical rotation for each solution. Use the equation above and your experimental data to determine the [α] of your solutions.

3. *Observation of mutarotation.* Accurately weight about 2.5g of α-glucose and place in a 50 ml volumetric flask. Fill the volumetric to the mark with a designated buffer and take a measurement of the solution's optical rotation as quickly as possible. What is the [α] of this solution? Wait 1 hour, determine the optical rotation for a fresh sample from your volumetric flask. What is the [α] now? Is mutarotation occurring? Save the stock solution do two more determinations of rotation, one after 2 hours and one after 24 hours.

Since the $[\alpha]$ of pure β-glucose is $+18.7°$ and the $[\alpha]$ of pure α-glucose is $+112°$, your experimental $[\alpha]$ value can be used to tell you how much of your glucose is in each form. What are these values after 1 hour? after 2 hours and at 24 hours? Is the $[\alpha]$ of the solution still changing or has it stopped. If it has stopped, determine the % of your sample in the α form and β forms.

Items to hand in for evaluation:

A. Sample calculation going from raw rotation to specific rotation for Glucose

B. Table of results for Experiment 2 listing the four sugars and their specific rotations.

C. Sample calculations showing how to calculate $\%\alpha$ and $\%\beta$ for a given specific rotation in experiment 3.

D. Table of results for experiment 3 showing observed rotation, specific rotations, $\%\alpha$ and $\%\beta$ and time for each different time point observed (0, 1, 24 hrs etc.)

E. Record the pH of the buffer you used in the above experiment

VI. Experiment NMR

1. *Initial observation of ^1H and ^{13}C spectra.*

Place **either** about .03 g of $NaH_2PO_4 \cdot H_2O$ and .01 g of $Na_2HPO_4 \cdot 7H_2O$ **- or-** .01 g of $NaH_2PO_4 \cdot H_2O$ and .05 g of $Na_2HPO_4 \cdot 7H_2O$ in a 1 ml Eppendorf centrifuge tube. (See instructor for which buffer you are to use) Now add 0.1 g of α-glucose and 0.02g of TSP to the centrifuge tube. Finally add 1 ml of D_2O to dissolve all of the solids. When this solution is completely dissolved quickly place about .7 ml of this solution in an NMR tube, place the sample in the machine, and determine both the ^1H and the ^{13}C spectra of this sample. As this is being done, one member of the group should determine the pH of the .3ml of solution that remains in the tube with the small volume ISFET pH probe.

Determine the ^1H and ^{13}C spectra of the sample again at 15, 30, and 60 minutes and 24 hours later.

All of the C are attached to alcohols, so they will appear in the chemical shift region between 60 and 100 ppm. The anomeric C signal is between 90 and 100. Do you have one or two peaks in this region? The larger of these peaks would correlate with the α conformer that you are starting with and the smaller peak would be the β conformer. Note that the linear form of glucose would have a carbon in the aldehyde form, and C in an aldehyde has a chemical shift between 175 and 225ppm. If you search your spectrum in this region you won't find any signal indicating that the concentration of the open, linear for of glucose is negligible.

2. *Assignment of chemical shift values (Do after 24 hours).* You now have spectra, and a clue as to where some of the signals are coming from, but no clear assignments of what peaks correspond to what protons or carbons. Now that the sample has been sitting for 24 hours and is closer to equilibrium we will now do some two dimensional NMR to try to make the make these assignments. Following the directions in the NMR manual set up both a COSY and a HETCOR experiments. The COSY experiment will show which protons are within 3 bonds of other protons, while the HETCOR experiment will show which protons are attached to which carbons. Once the experiments are complete, plot the spectra and try to assign all the nuclei.

3. Items to hand in for evaluation
1. Initial ^1H and ^{13}C 1D spectra.
2. ^{13}C spectra at 0 .25, .5, 1.0 and 24 hours. Locate the α and β peaks for the anomoric C in these spectra. Determine the heights of these peaks. Calculate the ratio of the height of the ~98 ppm peak to the ~95 ppm peak . Plot this ration as a function of time. Do you see any evidence that the Glucose has changed form all α, to a mixture of ~1/3α, 2/3 β?
3. COSY and HETCOR Spectra
4. Table showing correlated ^1H and ^{13}C correlations observed in HETCOR. Using the proper sugar numbering system try to identify each correlation with a C in either the α or β form of the sugar.

VII. Experiment Determination of rate of mutatrotation reaction at different pH's using Circular Dichroism.

1. Make 100 mls of a 0.01M phosphate buffer solution. To adjust the pH of 1/3 of this buffer to pH 6, 1/3 to pH 7 and 1/3 to pH 8. Turn on the water bath and set the temperature of the bath to 25°C. (Make sure the water bath is actually hooked up to circulate through the CD cell holder)

Log in and turn the CD machine as given on sheets in the CD lab. Manually adjust the wavelength to 202 nm. Set the sensitivity at 10 m°/cm and the time constant at 4 seconds. Set of the computer to take data at a single wavelength for a fixed time. Set up the interface to reflect the values of wavelength, sensitivity and time constant that the machine has been set to. Now set the interface to record 100 points over a 40 minute interval. When the machine is ready to go, start the final preparation of a sample.

Accurately weigh about .5 g of sample and dissolve in mls of one of the buffers. As soon as the sample is dissolved place 3 mls in a 1 cm cylindrical CD cell. Place the cell in the CD machine and tell the computer to start acquiring data.

Once the data has been acquired, remove the CD cell, rise it with water, and prepare a another sample at a different pH and repeat the experiment, recording the data to a different file.

Repeat with the third buffer.

Plot the three data sets. What was the initial and final reading in each data set? Did any of the sets look like they were leveling off and approaching equilibrium? Estimate the initial rate at each pH. Compare the rate of the mutatoration reaction at the different pH's.

To be handed in for evaluation
1. A.Plots of m° vs time for each pH
   B.Plots of log(m°) vs time for each pH
  C.Plots of 1/m° vs time for each pH
       Are any of the above linear? Go back to a Freshman Chem text section on kinetics and
       find out what these plots mean and what they could tell you about the reaction.
2. Estimate of initial slope of this plot for each pH
3. Assuming the initial slope = initial rate, plot the rate of the reaction vs pH
4. If one of your plots in 1 was linear, what is the rate of the reaction, plot this rate vs pH for your three different pH values.
5. Verbally evaluate the plots 3 and 4. What do they mean?

NMR Cookbook for Biochemistry

Using the NMR is quite computer intensive. You use one program call WinPNMR to actually run the NMR itself and obtain your raw NMR data, and a second program called NUTS to process an manipulate this data into a useable form. Make sure you pay attention to what tasks you are doing with which program.

Preliminaries: Shimming

Shimming refers to making the magnetic field around the sample as homogenous as possible. The reason we do this is that each proton in the magnet resonates at a certain frequency because it is at a certain magnetic field. If the same proton in a different part of your sample 'sees' a different magnetic field, it will resonate at a different frequency, so your peak gets broader when the magnetic field is not uniform across the sample.   In the ancient days (before my time) this was done by literally putting metal shims around the poles of the magnet, hence the name shimming.

On this machine the process has evolved. The computer watches the signal, and the computer adjusts all the shims at once to find the global optimum value. Thus all you have to do is to type 'shim' and walk away for a few minutes, the computer does the rest.

Let's do that now. Put your NMR tube in the white spinner and use the push button on the side of the NMR sample chamber to lower your sample into the magnet. Use the flashlight to look at your sample to make sure it is spinning tell the computer to shim the sample.

Go to the WinPNMR program and type

      shim

This starts a routine to try to homogenize the magnetic field around your sample. This may take a few minutes. Note as this routine starts to run you will have to adjust some parameters. Do you see the information box appear in the upper right hand corner? The only thing you should have to worry about is to use <Ctrl+G> to adjust the receiver gain to the proper value. In 2007 an RG between 10 and 20 seemed to work. Once this is accomplished use <Ctrl+Q> to start the actual shim routine. As it shims watch the display. What you are looking for is to have the yellow display of the data to expand as far across the screen as it can, and to get the largest area (Green letters in bottom left corner) possible. Shimming the magnet is something that should be done **every time** you put a new sample in the machine.

We have found that after this shim routine the spectrometer is left with one of its default parameters dorked. We need to reset this properly so type the following command:
      nu h1

Preliminaries: Acquiring a ^{1}H spectrum and adjusting the field

Acquiring a spectrum

1. Make sure the computer is set on the WinPNMR program. It should have a screen with a blue background and white grid lines. If the blue background is not showing use the mouse to click on the WinPNMR button on the task bar at the bottom of the screen.

2. Type **ns 1** followed by an **enter.**
Ns stands for number of scans, thus you have told the computer to do 1 scan.

3. Type **rg 16** followed by an **enter**.
Rg stands for receiver gain, this command tells the computer how much amplification to put on the signal. Ns=1 means no amplification, ns=100 says amplify the signal 100 times. With this command you are trying to blow up the signal as much as possible, without making the signal so large that the computer can't follow it. By setting ns=1 at this point you are doing no additional amplification, and then you will look at this signal and judge whether or not to change the ns in a later experiment.

4. Type **zg** followed by an **enter**.
Zg stands for zero go. In other words, zero out the computers memory and go ahead with the experiment. .

5. A window will appear in the middle of the screen asking for where you want to store your data. Since you don't need to save this data simply **hit OK** to accept the default.

6 . It will take the computer about 10 seconds to set up the instrument and start the experiment. As the experiment runs you should see a yellow noisy trace in the middle of the screen that is your actual data. A window with instructions will also appear in the upper right hand corner of the screen.
*If you get **RED** noise in the middle of the screen your receiver gain was too high **go back to step 5 and make rg about half of what it was.**

*If you get **YELLOW** noise look at the start of the yellow pattern (the left hand side) does it go from about ½ way down to about ½ way up on the screen?

Yes - great- its all working, so go **back to step 4, set ns = 4 and run a set of 4 scans.**

No - you need to set your gain a bit higher. **Go back to step 5 and set rg to be about double what it was.**

When the window at the upper right disappears your data is complete and ready for processing.

Adjusting the spectrometer frequency

1. It is now time do use the NUTS program to process this data into a form that you can understand. **Use the mouse to click on the WinNuts button** on the taskbar at the bottom of the screen

2. You should now have a mostly WHITE screen in front of you. If you don't, try the WinNuts button again or call for help.

3. Type **a2 (no return)**

4. Type **qp (no return)**
qp stands for quick phase. The phase parameters recorded in the computer are probably a bit off so the peak shapes are not symmetric. This command automatically adjusts the spectrum to give a nice symmetric peak shape.

5.You now have a nice looking spectrum, but your TSP internal standard probably isn't at 0.0 ppm where it should be. There are two ways to fix this. One is to adjust the display in the Nuts routine, the other is to adjust the frequency on the NMR transmitter. This is what we will do now.

6. Hold the left mouse button down and move the crosshairs on top of the TMS peak. Mark down the exact position of this peak (to three decimal places) including the sign.

7. Go back to the WinPNMR program

Type the command **fo** (for frequency offset)
The computer will ask where the current position of the reference is, give it the position
        you just found in NUTS
It will then ask for where the peak is supposed to be. Enter 0.00
The computer will then reset itself. You are now ready to acquire your NMR data.

Part I: Acquiring and processing your ^{1}H proton spectrum

Acquistion -^{1}H

1. Make sure the computer is set on the WinPNMR program. It should have a screen with a blue background and white grid lines. If the blue background is not showing use the mouse to click on the WinPNMR button on the task bar at the bottom of the screen.

2. Since we are alternating between ^{1}H and ^{13}C mode make sure you are in ^{1}H mode. They way you can tell is if the computer prompt says H1>. If it doesn't execute the command **nu h1**

3. Type **ns 1** followed by an **enter.**
Ns stands for number of scans, thus you have told the computer to do 1 scan.

4. Type **rg 16** followed by an **enter**.
Rg stands for receiver gain, this command tells the computer how much amplification to put on the signal. Ns=1 means no amplification, ns=100 says amplify the signal 100 times. With this command you are trying to blow up the signal as much as possible, without making the signal so large that the computer can't follow it. By setting ns=1 at this point you are doing no additional amplification, and then you will look at this signal and judge whether or not to change the ns in a later experiment.

5. Type **zg** followed by an **enter**.
Zg stands for zero go. In other words, zero out the computers memory and go ahead with the experiment. .

6. A window will appear in the middle of the screen asking for where you want to store your data. If you don't want to save this data simply **hit OK** to accept the default. If you do want to save the data, give it a file name you can remember. Start the name with the path 'data\chem 464L\yourname' so all of the data files obtained in this class go to the same place.

7 . It will take the computer about 10 seconds to set up the instrument and start the experiment. As the experiment runs you should see a yellow noisy trace in the middle of the screen that is your actual data. A window with instructions will also appear in the upper right hand corner of the screen.
*If you get **RED** noise in the middle of the screen your receiver gain was too high **go back to step 5 and make rg about half of what it was.**

*If you get **YELLOW** noise look at the start of the yellow pattern (the left hand side) does it go from about ½ way down to about ½ way up on the screen?

> Yes - great- its all working, so go **back to step 4, set ns = 12 and run a set of 12 scans.**

> No - you need to set your gain a bit higher. **Go back to step 5 and set rg to be about double what it was.**

When the window at the upper right disappears your data is complete and ready for processing.

Once you have acquired your first ^{1}H spectrum we need to immediately acquire your first ^{13}C Spectrum, so jump ahead to part II acquiring your ^{13}C Spectrum. For completeness, however I will include the directions for processing your ^{1}H Spectrum now

Processing -^{1}H

1. It is now time do use the NUTS program to process this data into a form that you can understand. **Use the mouse to click on the WinNuts button** on the taskbar at the bottom of the screen

2. You should now have a mostly WHITE screen in front of you. If you don't, try the WinNuts button again or call for help.

3. Type **Ctrl +F2**

4. Type **qp (no return)**
qp stands for quick phase. The phase parameters recorded in the computer are probably a bit off, so you need try again to try to get the spectrum to look right.

5.You now have a nice looking spectrum, but your TSP internal standard probably isn't at 0.0 ppm where it should be, let's fix that.

6. **Hold down the LEFT mouse button, and move the crosshairs** you get so it is aligned right on top of the right most peak in your spectrum. This is a peak corresponding to a standard called TMS that is supposed to be located at 0.0ppm on the X axis.

While still holding the left mouse button down type o with your other hand
This brings up the OFFSET INFORMATION window that lets you set the offset.
        Locate the bottom box on the left side of this window
        This is the Horizontal dimension in PPM. **Set this to 0.0** and hit **OK**.

You should now have your spectrum back, and the right most peak should be located right above 0.0. If not, try again.

7. Type **pp** (pick peaks)
This command will label all peaks in your spectrum over a certain threshold.
Print this spectrum with the peaks if you want. On the other hand, if this looks like too much clutter, you can clear the picked peaks from the top of the spectrum with the following steps. Click on **'view'** then click on **'show peak labels'**

Part II. Acquiring your ^{13}C Spectrum

The second most useful nucleus for NMR is the ^{13}C nucleus. It is useful because all organic compounds have C in them, and the ppm range for C goes from 0 to about 200, so there is a much larger range of chemical shifts to work with. C is a bit of a pain, however for two reasons; first its magnetogyric ratio is lower than a proton, so its internal 'magnet' is only about 1/4 the strength of the proton, so its signal is about 1/4 that of a proton. Second, the natural abundance of the ^{13}C is less than 1%, so your signal is an additional 100X smaller. The net result is that Carbon NMR is inherently a lot noisier, and you need to have lots more sample to get an experiment to work.

Let's start with a simple 1D carbon spectrum

Instructions for the ^{13}C experiment are found in the EFT manual. In the section marked 'Guides' on page 8. Follow the instructions as given, with the following notes:

Step 3 - Nu c13- changes all the spectrometer electronics so it will look at C instead of protons. You can see that all the parameters on the page change when you execute this command.

Optional step after 3 until step 7 you can skip!.

Step 7 - verify that the default parameters are OK. Every time I have done this neither the SI nor the RD are set right. To change SI simply type 'si' a window will come up, type the correct value in the window. Similarly to reset RD simply type 'rd' and put the correct value in the window that appears. Also set the number of scans to 48. Type ns 48.

Step 8 - Actually acquire the spectrum. As usual the computer will ask for a file name to keep the data in. Again use the data\chem 464L\yourname format. Notice what the first scan looks like. Lots of noise. Also watch what happens as each additional scan gets added in. Do you see how a pattern that is large on the left and tapers to the right, emerges, as well as some regular modulations within this signal seem to appear out of the noise? This is your actual data. The overall process here will take about 5 minutes.

Steps 9 & 10 - *You don't have to do this now, but you can come back and process the data at any time, provided it was properly saved.*
Transfer to NUTS and process the data. Because the C has so much noise, an extra process step is added to remove noise using the LB function. The 0.5 value given in the text works well. If you want to find out how this gets rid of noise see Dr. Z. It is a math transform that involves the convolution of your data with an exponential function, and draws on concepts that you had in that first FT computer lab.

Do the optional step after 10 to pick the peaks, then plot the spectrum (Step 11)

Do the step 12 to save this transformed data as well.

Part III Acquiring 2D NMR data (after 24 hours to come to equilibrium)

Great, you have a C-13 spectrum, what does it mean. First let's try to relate the ^{13}C to the ^{1}H spectrum. The experiment that does this is called the HETCOR for Heteronuclear Correlation. This experiment is a 2-dimensional experiment with ^{1}H in one dimension and ^{13}C in the other dimension. Any peak then tell you what ^{1}H nucli are attached to which ^{13}C nuclei.

Instructions for the HETCOR experiment are found in the EFT manual. In the section marked 'Guides' on page 11. Follow the instructions as given, with the following notes:

> Step 3 - You should still be in C13 mode, so the prompt in the WinPNMR should be C13>. If this is what you have, type het to do the hetcor experiment As usual, the first step in the process is to give the computer a file name to save the data under. Again the 2 second relaxation delay works pretty well. 16 scans per experiment should also work for this sample. Once the experiment starts it will tell you how long you have to wait. Go ahead and get another soda while you wait, but don't bring it in the lab.
>
> Step 10 - Go to NUTS and run the command <Ctrl+F6> for the hetcor.
>
> Step 12 - Remember that you click the border command from the buttons on the upper right of the screen. For the top border use the PROCESSED C-13 spectrum, for the right border use the PROCESSED H-1 spectrum.

In this 2D experiment the horizontal dimension is C while the vertical is Proton. Each peak in this spectrum represents a C and its attached proton. Thus you should be able to pick out the C and assign all of the C that were attached to the hydrogens

Make a table that gives the chemical shifts of all C and H atoms in this compound and identify where each of these atoms is in the molecules structure if possible.

At this point you really the above table doesn't help too much, because you can identify the anomeric C, but nothing more. So let's try another NMR experiment called the COSY for Correlated Spectroscopy.

This is another 2D experiment, but in this experiment one proton is correlated to the proton that is either 2 or three bond away.

Instructions for the COSY experiment are found in the EFT manual. In the section marked 'Guides' on page 11. Follow the directions as given, with the following notes:

> Give this a name so you can find it later.

> Just as the instructions say, relaxation delay of 2 works well.
> This sample has a great signal to noise ratio so set number of scans to 4
> > (With a poorer sample you can use 8 or some multiple of 4, but the experiment takes longer)

When the experiment starts your signal will be red, indicating the receiver gain is too high. This is because the default receiver gain written into this experiment is 40 and is about 4 times too high for this sample. Hit <Ctrl+G> and adjust the gain until the signal is showing yellow. Once the signal is good, then hit <Ctrl +Q> to actually start the experiment. The window in the upper right hand corner will tell you how long the experiment will take. Sit back and get a coffee or a soda and wait until you get a message that the experiment is finished.

After the experiment has finished enter the NUTS program (Instruction 9) When you hit <Ctrl +F5> NUTS asks you what file you want to process. Once you have found the file, it will then open a window that displays the acquisition information about the experiment, and you can go in and add comments to be recorded with the raw data. If you don't have any comments you can simply hit OK at the bottom of the window. Once the window clears, the computer does the 2D Fourier transform and plots your data.

You should now have a square in front of you. Black marks on the square indicate where peaks are.

> Note Instruction 12, where you add borders. You do not type in the border command, it is one of the buttons on the upper left hand corner of the screen. When is asks to pick the top spectrum, use the processed 1D spectrum that you did in the previous section.

> Finish with all of the instructions and print your COSY spectrum.
> When you have finished plotting, make sure you go to the **file** button and click on the **exit ip** command. This takes you out of the 2-D mode. If you don't do this, the next person who tries to process a 1-D spectrum in Nuts will get all sorts of error messages and things won't work.

Now what does the COSY mean? Well locate your diagonal that runs from the bottom left to the upper right. This diagonal contains all the peaks that are in your 1D spectrum. Can you see mark on the diagonal that corresponds to each peak on the 1D spectrum?

The off-diagonal peak represents proton peaks that are coupled to each other. Can you see an off diagonal peak that correlates with your anomeric H? Since the anomeric H is C #1, this correlated proton must be on C #2. Try to see how many protons and C you can now assign.

Isolation of Genomic DNA from Wheat Germ

Purpose: In this lab you will extract DNA from wheat germ, and the use the UV absorbtion spectrum of this material to roughly estimate the purity of the DNA.

Background: In theory this procedure can be used to isolate DNA from any source, and for the past 6 years we have used bison liver as the source, so you are isolating DNA from the same organism as you isolated your LDH. However, we have never gotten really good yields from liver tissue so in 2011 we will try using wheat germ as our source instead of liver.

First, what is wheat germ? Wheat germ is the part of the wheat kernel that contains the embryonic plant, so it should contain cells that are rich in DNA. In contrast, the bulk of the wheat kernel (the part we make flour from) is mostly starch that is used as a source of energy for the growing embryo, so it will have very little DNA.

Like many procedures used to isolate DNA, the first step is to homogenize the source tissue in a buffer that contains EDTA and NaCl. Tris is often used for this buffer because it buffers well at pH 8, and this is a pH where DNA is very stable. EDTA is a chelating agent, that is, it binds divalent cations. It is included in the homogenation buffer because many of the enzymes that degrade DNA require a divalent cation for their activity, and so, binding the divalent cations with EDTA we destroy the ability of these enzymes to degrade DNA. If we were isolated DNA from a bacterial culture, the EDTA would serve a second role because binding divalent cations weakens the outer membrane of gram negative bacteria. NaCl is included in the buffer to provide a nice moderate ionic strength so the charge-charge interactions between DNA, and the protein nucleosome is moderated, so the DNA is not so tightly bound to the protein.

In the next step of the procedure you add SDS (sodium duodecylsulfate) You should remember this compound as a detergent that is used to denature proteins to make SDS gels. It serves the same purpose here; it denatures proteins, and also helps to disrupt the membrane around the cells and the nucleus, to help release the DNA from the cell and the proteins inside the cell.

Next you add 5M NaCl to the solution. This makes the ionic strength of the solution much higher that it was. What will happen now is that some of the proteins will begin to precipitate. This high ionic strength also screens out the charge-charge interactions between DNA molecules making it easier to get the DNA to aggregate into long strands when you add ice cold ethanol in the last step of the purifiaction.

Other steps frequently seen in DNA purification procedures are adding proteinase K, and doing a phenol extraction. Proteinase K is a proteolytic enzyme that helps to destroy proteins in the homogenate. This is a step I probably should add to make this procedure work for liver tissue, but this enzyme is extremely expensive. Phenol extraction is a step that helps to dissociate the DNA from the nuclear proteins. I haven't included this step in this week's procedure because phenol

can give you a nasty chemical burn, but you won't feel it happening because it is also a local anesthetic.

Procedure:
1. Make 100 mls of TEN (Tris-EDTA-NaCl) buffer
   50 mM Tris Base
   100mM EDTA
   100mM NaCl

   Check with the instructor that you have calculated the correct amount of each material
   You will have to heat this up on a hotplate to dissolve the SDS, then cool the solution back
      down to RT before you adjust the pH.
   Adjust pH of buffer to 8.0 with NaOH

2. Get about 15 g of wheat germ.

3. Place tissue and 100ml of TEN solution in blender.

4. Blend briefly until you have a homogenous suspension.

5. Add 1/10th volume of 10% SDS and gently mix the solution.

6. Place the homegenate/SDS solution in a 60º C water bath for 30 minutes. Gently stir the solution occasionally during this time period.

7. Pour into two Oak Ridge centrifuge tubes, balance the tubes and centrifuge for 10 minutes at 8,000 x g at 10ºC.

8. Discard the pellet of cellular debris.

9. Pour the supernatant into a beaker and add 1/10 volume of 5M NaCl and mix.

10. Place in a refrigerator for 24 hours so protein will precipitate.

11. Place in two 55 ml Oak Ridge tubes, balance, and centrifuge at 8,000 x g for 10 minutes at 10ºC.

12. Again discard the pellet

13. Transfer the supernatant to a pre-chilled beaker (probably use two 50 ml beakers).

15. Slowly add about 10 ml of 100% ice-cold ethanol by pouring gently down the side of each beaker so it layers on top of the water with minimal mixing.

16. The DNA will begin to form a glop at the ethanol-water interface. Let this solution sit for a few minutes, then carefully spool the DNA onto a modified Pasteur pipette.

17. Wash the DNA on the pipette with 70% ethanol. Then allow to dry at RT for about 1 hour.

18. Place 3 ml of TE buffer in a test tube, and place the Pasteur pipette with the DNA into the test tube so the DNA dissolves into the buffer. This make take several minutes or you can let it sit in the refrigerator overnight. You may gently swirl the DNA during this process.

19. After the DNA has re-dissolved in the TE buffer, obtain a UV spectrum of the material between 350 and 220 nm using quartz cuvettes. Before you actually run the spectrometer, take a look at your sample. Is it cloudy? If it is cloudy, place it in an Eppendorf tube and centrifuge in a microfuge at high speed for 2 minutes to clarify the solution. You will have to use trial and error to find the right dilution so that the 260nm peak has an absorbance of about 1.0. Analyze the purity of your DNA using the information given below.

Spectrophotometric Analysis :
Both DNA and RNA have an absorbance maximum at 260 nm. If you have a pure DNA sample, a solution that has a concentration of 50 μg/mL will have an A_{260} of 1.0. This will vary slightly depending on the %GC in the DNA, but not enough to make a difference in to day's analysis.

The most common problem observed in DNA isolated by this procedure is that some particulate matter is suspended in the final DNA sample. This can either be DNA that has not fully dissolved in the TE buffer, or it might be protein precipitate that has not been completely removed from the sample. The easiest way to check for this problem is to look at your absorbance at 330 nm. With a properly dissolved DNA sample that does not contain any protein precipitate, the A_{330} should be less than 5% of the A_{260} value. If it is higher, then you have a problem.   The problem may not be the sample either. One other factor that can cause a high reading at 330 nm is a dirty cuvette. So if you have an anomalously high reading at 330 nm check that your cuvette is clean before you give up on your sample.

Proteins have an absorbance peak at 280 nm, so this is another wavelength we can examine. A pure DNA sample should have an A_{260}:A_{280} ratio between 1.8 - 1.9. If the ratio is higher than 1.9, then you probably have RNA as a contaminant. If the ratio is lower than 1.8 then you probably have a protein contaminant.

The final wavelength to examine is 234 nm. This is a wavelength where DNA should have an absorbance minimum, and the A_{234}: A_{260} ratio for DNA should be about .5, if it is higher, that would indicate either phenol or protein contamination.

Fill in the following table:

Dilution	A_{330}	A_{280}	A_{260}	A_{234}	A_{260}/A_{280}	A_{234}/A_{260}	[DNA] (Dilute solution)	[DNA] (Original)

Use the above data to evaluate the purity of your DNA

Prelab Questions:

        1. The TES extraction buffer contains EDTA. Why?

        2. In step 5 you add SDS to the cell extract. Why?

        3. In step 9 you add ice cold NaCl. Why?

        4. In step 12 you add ethanol. Why?

Lipid Lab

Prelab questions:
Among the compounds you will isolate today should be Palmitic, Stearic and Oleic acids, cholesterol, and stigmasterol.   Give the structure of these compounds

Synopsis:
Common fats or oils will be subjected to basic conditions in a methanol solvent. These conditions will serve to cleave fatty acid-glycerol ester bonds, but will leave other lipids like cholesterol intact. Since the fatty acids are now in the acid form, they are slightly soluble in water and so they can be separated from other lipids by solvent extraction. The fatty acids are then derivatized to make then non-polar and they will be injected on the GC/MS to try to identify the different fatty acids found in the sample, and get a rough estimate of the amount of each fatty acid found in the sample. The non-hydrolyzable lipid sample may also be subject to GC/MS to try to identify other lipid components.

Day 1 Isolation of lipid components and derivitization

      Obtain 1.0 g of fat or oil and place in a 50 ml beaker on a hotplate in the hood. Add 25 ml of 10% methanolic KOH. Heat this solution to boiling and keep it simmering for about 30 minutes. Add additional methanol as needed to keep the volume constant. Record any observations.
      At this point the fatty acids have been converted to their sodium salts and are no longer soluble in the cold methanol, while other components like cholesterol are still soluble in the solvent. Allow the solution to cool and note if any precipitate forms on cooling. Gravity filter the solution using a stemless funnel and filter paper. The liquid will contain cholesterol or stigmasterol and other non-hydrolyzable lipids, while the solid contains the sodium salts of the fatty acids. SAVE BOTH.

Liquid:
      Put a 100 mL round bottom flask, place in a rotovap and try to take the sample to dryness. If this takes more than 15-20 minutes, see your instructor. Is there anything there? Scrape a single spatula full of this solid and attempt to dissolve it in 75 mL of Dichloroethane. After it is looks to be as dissolved as it is going to, place in a 250 mL separatory funnel and add 75 mL of water. Gently swirl these two liquids together. If it looks like no emulsion is going to form mix the solvents more rigorously to get a good extraction. Remove the organic layer, and discard the aqueous layer. Check with your instructor to make sure you are saving the correct liquid. Place the organic layer in a small beaker and allow the solvent to evaporate overnight. If there is anything there, it is probably cholesterol.

Solid:

Heat the filter paper until it is completely dry (moderate heat). Make notes on the sample's color, consistency, smell, etc. Note this material will melt and burn if it gets too hot, so be gentle.

Place about .1-.2 g of this material in a 4 dram sample vial. Add 3 mL of sodium methoxide solution, cap and place in the reactor at 70° for 3 minutes.

Remove the vial from the water bath and cool with either water or ice. If fatty acids are present a precipitate will form on cooling.

IN THE HOOD

Carefully open the vial and add 3 mL of BF_3 solution to complete the reaction. Recap vial and put in the reactor for an additional 3 minutes.

Allow to cool

Carefully pour the contents into a 125 mL separatory funnel. Wash vial with 25 ml of water and place washing into separatory funnel. Wash vial with 25 ml of pentane or hexane, and place washing into the separatory funnel. Now mix the contents of the sep funnel well and isolate the organic layer. (Again check with the instructor to make sure you wave the correct layer!) Make sure the organic layer has no water in it by adding some anhydrous sodium sulfate, then pour it into a small labeled beaker to evaporate overnight.

Day 2 GC/MS analysis of above sample

Dissolve the sample that contained the derivatized fatty acids (the solid) in about 5 mL of hexane. Dissolve the sample that should contain the cholesterol and other neutral lipids (the filtrate) in about 5 mL of dichloromethane.

Transfer an aliquot of both samples to 1.5 mL centrifuge vial

Spin both samples for 1 minute at high speed

Go to GC/Mass spec

Click on shortcut to class logbook and bring up log

enter your names, date, sample and any other data you wish into the log

Minimize the logbook

click on MS 5973 icon

program to operate the Mass Spec should come up

it should be running a method called lipid1

This run will take 19.0 minutes

When Mass spec is ready (Green box in the upper right corner of the oven icon)

Click on green arrow

This will bring up a Data acquisition window

Fill in your name under operator

give the file name as a name you will remember (Keep the *.D end of the file name)

Fill in any sample details that you want included in the data on the computer

Once you have filled in the window, click on the 'Start Run' Button at the bottom of the window. The window will disappear and in a few about a minute a window will appear on the computer with your next instructions.

Before you follow these instructions, maximize the class logbook, write your file name in class logbook , and minimuze the logbook window again.

The first instruction is to press the prep run button on the GC. Do this now.

Once the pre-run light is on steady you can inject your sample. Only inject 1 ul of your lipid sample. The usual technique is to suck about 10 ul of sample into the syringe then spray it out into a waste container 2 or 3 times to clean out the needle and the syringe. Then take in your sample. Inject the sample into the GC by inserting the needle completely into the injection port, press the start button on the GC to tell the computer to acquire data, then remove the needle from the injection port. Immediately wash the needle and syringe with pure pentane by sucking pentane into they syringe and squirting it out into a waste container at least five times.

By now you should have noticed that the computer is displaying a message about overriding the solvent delay. Essentially the solvent delay tell the computer not to acquire any data in the first 2.5 minutes, becasue this is when the solvent blows through the column, and when the mass of solvent hits the detector it can gunk it up. NEVER OVERIDE the solvent delay. So Hit the NO button, (Or don't do anything and all, that is just as good)

The computer is now acquiring your data, and there is not much you can do. If this is your second run, you can start to analyze your first run, but other than that, the machine is now on autopilot. At the end of the run, the machine will automatically cycle itself back to the ready state. You can tell when it is ready for your next injection by watching the little box on the upper right hand corner of the oven icon. If it is red the machine isn't ready, if it is green - you are good to go.

Use of MS5973 Data Analysis program for analysis of GC/MS data

Double Click on MS5973 Data Analysis icon

What comes up is the last chromatogram analyzed, so you must first load your chromatogram

Click on File – Load data file – Find your file on the list and hit OK

You will have 2 data sets displayed.

The upper set is the TIC or Total Ion Chromatogram or the sum of all the mass spec signals as a function of time

The Lower is the FID or Flame Ionization Detector signal. Since this detector was turned off during your run, all it shows is a lot of garbage, so you may close that window.

The TIC window shows where the different compounds come off during your run, but it doesn't show the mass spectrum of any particular peak. To get the mass spectrum of a component peak you need to tell the computer which peak to use.

Move the cursor to one edge of a peak. Now press the right mouse button and hold it down while you move the cursor to the other side of the peak. When you release the mouse button the mass spectra will of that peak will appear in the lower window labeled average of … where the two numbers listed are the start and end times of the peak you are analyzing.

The mass spectrum itself is not terribly useful because in the ionization process the molecule was decomposed into anywhere between 10 and 100 fragments, and the mass of each different fragment appears in the spectrum. However, because the ionization conditions are fairly well standardized, each compound usually yields the same pattern of fragments when it is ionized, so the overall pattern you see can be matched into a library of compounds and their fragments. The Analysis routine can access the NIST library and can math the pattern of fragments in your compound to 1000's of other compounds.

To do this

Click on Spectrum – NIST Search

The NIST search window that comes up is pretty busy

Top center is your spectrum

Bottom Center is the best match that the computer could find for your spectrum

In between is a figure that is supposed to show the differences between the two.

Right bottom is a screen that shows the general information about the match, including such useful items as the name (or names) and the molecular weight

Left bottom is a window that shows the different matches and how good they are

Move your cursor over to that screen and click on a few different matches. See how the other screens change as a different spectrum and compound is matched to your sample? Also note that some of the entries are labels M, which means the mean or average of several different spectra form different sources, while others are labeled with an R (?original) for a single spectrum

The best match found by the computer may NOT by your compound. Go up and down the list of matches and look for names that look familiar. Use your own judgment to see if you think some of these other compounds look better or worse than the 'best' match. Also use some chemical logic, should the proposed match be in your sample?

Once you have identified all of the peaks in the chromatogram, next try to quantitate how much is in each peak. This is done by going to the upper bar and finding the chromatogram tab. This opens a short menu and the bottom part of this menu deals with integrating the area in each peak and reporting it. If you simply click on the %Report it will give you a table with the % of total of each peak. If you look carefully you will probably see come blue lines on each peak showing how the computer chose the peaks and the baselines used for each area. You can play with some of the other choices under the chromatogram tab to see if you can improve your results.

There are a lot of options from this window, and I haven't figured them out, feel free to play around a bit. The biggest thing that I haven't found is some way to print out the match! If you can figure this out tell me, it might be worth some bonus points. If you can't figure this out, simply write down your matches in your notebook for future reference. One you are tired of this window simply hit the X in the upper right corner to kill the window. You should now be back on your chromatogram. Find another peak and investigate it. Have fun. When you are finished you can either load another data file, or kill the window by hitting the X. At his point a bell will chime and you will be told to save the changes. Ignore this – hit Yes for exit and you are done

Post lab analysis:
For this lab I have decided to have you submit a formal lab report. See the website:
www.mhhe.com/biosci/genbio/maderinquiry/writing.html

For a nice concise description of the standard format for a lab report. These lab reports are to be *individual efforts* not group efforts. I want to see your writing, not your group's writing

More information on GC/MS

Running the GC/Mass spec gets you exposed to two instruments, the Gas Chromatograph and the Mass Spectrometer. Since this is a Biochemistry Class, I can't spend time describing the details of these instruments other, than the brief overview that I give you in the lab. I'm sure, however, that some of you are curious as to how these thing work, so I want to give you some alternate resources to look at if you are interested. For an explanation of Gas Chromotagraphy try the following web site

http://www.shu.ac.uk/schools/sci/chem/tutorials/

Go to the lower part of the page, 'Resources for Analytical Science' and under chromatography hit the gas chromatography link

For more information on the mass spec part of the machine go the shelves in by the door, and find a rack of CD's. Find the CD entitled 'MSD Reference Collection'. Put it in the CD ROM of the GC/MS computer, then double click on the HP5973 MSD Reference Icon. This will bring up an HP instruments page on the computer. On the left side click on the MS Fundamentals link. You now will have a page with several additional links. The one you want to look at is 'MS Hardware' This has information and several short movies in it that will take you through the MS instrument part-by-part. Read as much as you like. When you are finished , get out of the window and put the CD back where you found it