Biochemistry 30 **Spring 2009**

Laboratory Experiments

Book B

Experiment #I, Part III - Tripeptide and Protein Modeling

Secondary Structure of Peptides
Tertiary Structure of Dogfish Lactate Dehydrogenase

INTRODUCTION

The function of a biological molecule is intimately linked to its structure. Therefore, one goal of biochemistry has been to develop an understanding of the three-dimensional structure of proteins. Over the past three decades, the combined powers of x-ray crystallography, multinuclear multidimensional NMR, computational chemistry, and an impressive array of physical techniques have resulted in detailed models for the structure of many proteins. Structures have been determined for over 50,000 proteins, primarily by x-ray crystallography or NMR, with the vast majority of these having been determined in the last decade. This work is time consuming and tedious, and x-ray structures of crystallized protein do not always reveal all of the structures which can be adopted by a protein in solution. Given the recent, rapid advances in DNA-sequencing techniques and hence the enormous number of known gene sequences (*cf.*, for example, the human genome project), the ability to predict a protein's tertiary structure directly from its amino-acid sequence would be a monumental advance.

Today, you will use the molecular modeling program HyperChem to build a replica of the tripeptide that you sequenced last week. The tripeptide will be constructed with standard bond lengths and angles, but you will then vary the dihedrals phi (ϕ) and psi (ψ). By calculating the energies of 36 different conformations, you will obtain the data needed to construct a small Ramachandran plot. You will then construct a polypeptide by repeating the basic unit of your tripeptide six times. By selecting the appropriate dihedrals, this octadecamer will be constrained into an α-helical as well as a β-strand conformation. You will obtain a picture of your octadecamer in each of these configurations, reporting molecular dimensions, dihedral angles, and energies.

Finally, using the program RasMol, you will view one subunit of the tetrameric protein lactate dehydrogenase (an enzyme you will purify from chickens later in the semester) and will study various aspects of its tertiary structure.

The write-up for this part of the experiment goes directly in your lab notebook and will comprise answers to all of the questions posed throughout this handout.

About HyperChem

HyperChem is a computer program that displays and manipulates images of three-dimensional molecular models. From these images you can precisely measure bond lengths, bond angles, and interatomic distances. (Although these data may sometimes also be measured on actual models--for example, of the ball and stick variety--the values obtained are only approximate.) HyperChem can also performs complex calculations which are so lengthy as to be impossible without a computer. In order to use the program, you must know the following: what the program can do; what the basis for its calculations is; and how to make the computer do what you want. This introduction contains only enough information to enable you to do the exercises which follow and to learn something from them. More information is contained in the HyperChem manuals.

There are two different approaches to calculating the energy of a molecule. The first approach uses pure quantum mechanics, and is termed the *ab initio* calculation method. The equations associated with this method--originating with the Schrodinger Equation--are complex and time-consuming to solve, and we shall consider this method no further. The second approach focuses on reproducing experimental data and employs many mathematical approximations. These approximations are of two types: semi-empirical quantum mechanical and force field. Semi-empirical quantum mechanical calculations use quantum equations, but tailor them to reproduce chemical data. Both *ab initio* and semi-empirical quantum mechanical calculations are better suited for obtaining molecular orbitals, which is not our interest here. Instead, you will use the force field approach, which has been developed to answer the types of questions you will be addressing today.

Atomic parameters for four different force fields are in the program's memory. Three of these (**BIO+**, **OPAL**, and **AMBER**) were designed for use on large biological molecules such as proteins and DNA. The fourth, which you will use for these exercises, is **MM+**, a force field designed by N.L. Allinger *et al*. to reproduce experimental physical data for smaller molecules, such as your tripeptide.

A force field energy calculation relies on a set of equations which approximate the potential energy of various intramolecular interactions. The potential energy equations contain variable force constants. The value of the force constants in each calculation depends upon the elements involved in the calculation and their bonding arrangements. Not only does the program recognize that the force constants for bonds to carbon and to oxygen differ, but it also assigns different parameters to different bonding arrangements of the same atom. Thus, the atomic force constants differ for an oxygen atom in for example, formaldehyde (**$H_2C=O$**), ethanol (**CH_3CH_2OH**), an water (**H_2O**). Each force field has its own equations and set of atomic parameters.

The parameters of **MM+** are plugged into seven equations which approximate the following interactions: bond stretching (the electrostatic energy associated with the distance between the nuclei); bond dipoles (the energy of charge separation due to differing electron density); angle bending (energy associated with changing the angle between two atoms attached to a central atom); linked angle bending and bond stretching (the two often occur at the same time); out-of-plane bending (energy associated with loss of planarity in sp^2 hybridized atoms); torsional bending (energy of the interaction of dihedral angles); and van der Waals interactions (long-range attraction and short range repulsion of non-bonded atoms).

HyperChem energy calculations are of two types. On the simple side, the program can assign an energy to a geometry. Much more sophisticated is HyperChem's ability to bring the molecule to an optimal, lowest-energy state--to "minimize" the structure. This is done by computationally searching the mathematical surface which the potential energy equation represents, each point of which is the energy of a different geometry. A computational search can be done in four different ways on HyperChem. The essential idea is that the computer explores the geometry and finds the minimum by calculating what sort of changes in geometry (bond lengths, torsion angles, etc.) will lower the energy of the molecule or system of molecules. It then makes those changes, and then calculates what modifications of the new geometry will decrease the energy once again. The minimum is the point at which the computer cannot calculate a change which will further lower the energy. By this method, HyperChem minimizes the energy of a molecule, thus finding a stable conformation, a process called geometry optimization, which for the mathematically literate is discussed below.

WARNING: It is critical to appreciate that the conformation obtained after minimization may be, and typically is, one of a myriad of local minima, rather than the true global minimum. The reason is that the energy-minimization algorithms search, in each step of the calculation, for a similar structure of *lower* energy. When no such lower-energy structure can be found, an energy minimum has been reached, and the calculation ends. Thus, if a molecular conformation must pass through a higher energy state to reach a lower-energy conformation--*i.e.* a transition state--this lower-energy structure will not be found.

The Energy Minimization Procedure

The computer finds a minimum energy by generating an N-dimensional surface. A three dimensional surface is like the surface of the ocean at a given point in time, where every point is described by three coordinates (**x**,**y**,**z**, or latitude, longitude, height above sea level). A point on an N-dimensional surface is described by **N** coordinates. For a molecule of **A** atoms, the energy can be described by a system of **3A** Cartesian coordinates.

The direction of greatest change is what the computer uses to narrow in on the minimum of the surface. This comes from a mathematical entity called the gradient. On a two dimensional surface (for instance, a parabola: $\mathbf{y = x^2 + 2}$), the gradient is simply the derivative of the equation for the curve (**dy/dx = 2x**). With three dimensions or more, partial derivatives are taken and these can become mathematically very ugly. So you have the computer to do it for you.

There are four search methods available on HyperChem, which are, in order of increasing sophistication: **Steepest Descent, Fletcher-Reeves, Polak-Ribiere,** and **Block-Diagonal Newton-Raphson.** Today, we will use the Steepest Descent algorithm, which gives good results relatively quickly.

THE TOOLS

NOTE--in the following instructions, if neither right nor left click is specified, use left click.

The Building Tool - This vital tool has several functions. Left click places atoms in the work space. Left drag places bonds in the work space. Right click erases whatever is under the cursor. Double clicking on the building tool icon in the tool bar at the left of the screen brings up the element palette, from which you choose the element which will be placed on the screen, as described above.

NOTE--if you switch your element choice on the palette and then click on an atom already on screen, it will change to the new type.

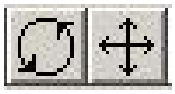
The Select Tool - Anything you left click on will become highlighted and be the subject of any subsequent action (geometry optimization, for instance). To select more than one item, left click on each of your choices in succession. Left click on an empty part of the work space selects everything in the work space.

If one atom is selected, its type is displayed on the line at the bottom of the screen. If one bond is selected, the bond length is displayed. Selection of two non-connected atoms gives the distance between them. Selection of three connected atoms gives the relevant bond angle, while selection of four connected atoms gives the torsion angle.

Right clicking removes from selection whatever is underneath the cursor. Right clicking on the empty work space deselects the entire selection.

Double-clicking on the select tool icon in the tool bar on the left of the screen converts any two-dimensional sketches in the work space (made with the building tool) to three-dimensional models. When you go from 2D to 3D, the program automatically fills the unoccupied valence of any atoms on the screen with hydrogens, so you need not draw them out. HyperChem assumes standard geometries and average bond lengths in creating the models, so it does not matter how crudely you draw the 2D sketch. Once a 2D drawing has been converted to a 3D model, you may further alter it, adding 2D sketches and rebuilding until you have your end product. Note that building a model does not necessarily give the lowest energy conformation, but rather a good guess as to what the geometry may resemble.

The XY-Rotation Tool - This tool is very useful for changing the view of the molecule. Left drag rotates the molecule around the x and y axes. Dragging horizontally controls the y-axis, and vertically, the x. Use this tool whenever you need to change the view and bring different parts of the molecule clearly into perspective. This is the best tool to have chosen prior to a geometry optimization, as it continues to function while the computer calculates. This allows you to align molecules so that the interesting changes in their geometries during the optimization are visible.



The Z Rotation and XY-Translation Tools - These two tools also change the view of the molecule. The second moves the molecule in the plane of the screen, the first rotates it in the plane of the screen.

The Zoom Tool - Left dragging with this tool increases and decreases the magnification of a molecule.

Space Bar - The space bar centers and fills the screen with the selection (or the whole molecule if there is no selection). It is useful for getting a good look at things.

Other Tools - The other two tools on the screen are also for zooming and are of little use for smaller molecules. Play with them if you like, and if your molecule disappears, you can always hit space bar and have it return.

BEFORE YOU START

HyperChem is a very flexible program, and can be customized to the user's taste and requirements. You must check to make sure that the format you are using is the same as the format of the instructions. Choose **Preferences...** under the **File** menu. Set the **Window** to **Violet**. Set the **Bond** to **By Element**. Set the **Selection** to **Green**. Click on **OK**. Open the **Build** menu, and make sure that there are no checks by any of the options. Under the **Select** menu there should be checks by **Atoms** and **Multiple Selections** only. Make sure the **Display** menu has a check by **Show Hydrogens**, **Show Multiple Bonds**, and **Show Hydrogen Bonds**. Choose **Element Color...** from the **Display** menu. When the dialog box opens, choose **Hydrogen** as **White**, **Carbon** as **Black**, **Nitrogen** as **Blue**, and **Oxygen** as **Red**. Click on **OK**. Finally, choose **Molecular Mechanics...** from the **Setup** menu. When the dialog box opens, choose **MM+** as the force field. Click **OK**, and now you are ready to begin the experiment.

PART I: CONSTRUCTION OF YOUR TRIPEPTIDE

Note: If not specified, use the left mouse button for all clicking! After recording each value in your notebook, DESELECT ALL ATOMS before moving on to the next step. Note further: all HyperChem energies are reported in kcal/mol; also, the minus sign in front of negative energies is very small... don't miss it!

--Open the **Databases** menu and select **Amino Acids**. A conversation window with the three letter names of the 20 amino acids will appear on the screen. Notice that you have a choice of secondary structure (**Alpha helix**, **Beta sheet**, or **Other**). Select **Alpha helix** for starters. Click on the names of the amino acids from N- to C-terminus following the amino acid sequence of the tripeptide you sequenced last week (or your best recreation of that sequence). Close the conversation window by clicking twice on the top left corner. You will then be able to see the tripeptide model on the screen in front of you rendered in colored sticks. Notice that the tripeptide does not contain a carboxylate group at the C-terminus or an ammonium group at the N-terminus. To add these groups, open the **Databases** Menu and select: **Make zwitterions**. Close the Menu and view the Tripeptide in all its glory. Use the XY-Rotation Tool and the Zoom Tool to get a clearer picture of the model. Note the two peptide bonds and the six atoms in the peptide plane. See what the side groups look like, and how they are positioned relative to the peptide bond.

--You should also change the rendering of the model by clicking on the **Display** Menu and choosing **Rendering**. You will have a choice of sticks, discs, spheres, dots, dots and sticks. Try all of them and see what you will get.

--Measure the phi and psi angles. Find the α-carbon of the second amino acid of the

tripeptide. To measure phi, use the selection tool to highlight the following four atoms: the peptide carbonyl carbon of the amino acid number one, the adjacent peptide nitrogen, the α-carbon of amino acid number two, and the peptide carbonyl carbon of amino acid number two. On the bottom of the working space you will see the value of this torsion angle, which is just the phi angle. To measure psi, use the selection tool to highlight the following four atoms: the peptide nitrogen from the second amino acid, the α-carbon and peptide carbonyl carbon of the second amino acid, and the peptide nitrogen derived from the third amino acid. Record these values of phi and psi in your notebook. Remember, your tripeptide is in the alpha-helix conformation: verify that the phi and psi angles you have measured are correct. (That is, phi = -58^{o} and psi = -47^{o}.)

--Now repeat the above exercise, this time building your tripeptide in the beta-strand conformation. Select **New** under **File** Menu., and do not "Save current changes: (untitled)." Your previously constructed tripeptide will disappear. Open the **Databases** window and select **Amino Acid**. Select **Beta sheet**, build your tripeptide, etc., etc. Note, as you will explicitly see in Part III, the structure in front of you is NOT the pleated β-strand--it is the completely extended strand, which does not form stable sheets with any polypeptide sequence save polyglycine.

PART II: CONSTRUCTION OF A RAMACHANDRAN PLOT

--Now, using the computational power of HyperChem, you will construct a low resolution Ramachandran plot. Team up with a neighboring group: following the procedure below, one group should work with glycine, the other with alanine.

--Select **New** under **File** Menu, and do not save the current file. Open the **Databases** window and select **Amino Acids**. When the window opens for choosing secondary structure, choose **Other** for conformation, and set the phi and psi angles each to **180** degrees (note: they may already be set to these values). Build the peptide **Ace Gly Nme** or **Ace Ala Nme**. (Ace is N-acetyl and Nme is N-methyl carboxamide--these groups provide the flanking peptide bonds for the gly or ala α-carbon, as well as neighboring α-carbon equivalents.) Determine the energy for this conformation by pulling down the **Compute** Menu and selecting **Single Point**. (You need not concern yourself with the "Gradient," a measure of the conformational distance between the current geometry and the potential energy minimum.) Now measure the energy of thirty five additional conformations (yes, 35!) by varying phi and psi in 60 degree increments (remember, a dihedral of -180^{o} is the same as +180^{o}), rebuilding your peptide (**Ace Gly Nme** or **Ace Ala Nme**), and determining the **Single Point** energy for the new conformation. To expedite matters, note that you can simply leave the **Amino Acids** window open the entire time, and click on **New** under the **File** menu (do NOT save current changes!) to begin building each new conformation. Once you get the hang of this, the entire process should take approximately 15 minutes. Finally, determine the energy of your derivative with the phi and psi angles for each of the conformations

below:

	ϕ	ψ
β-strand in antiparallel β-sheet	-139^o	+135^o
β-strand in parallel β-sheet	-119^o	+113^o
right-handed α-helix	-58^o	-47^o

--When you are finished determining the energies of all of these conformations, choose the highest-energy one and view it in the spheres mode. Can you see the unfavorable van der Waals contacts?? Sketch this high-energy conformation in your notebook.

--On a piece of graph paper, construct a Ramachandran plot of your data. Note that the two β-strands and the α-helix are obviously allowed conformations--hence, the corresponding energies will give you a range of allowed values with which to interpret the energies of the other 36 conformations. Plot psi on the y-axis, phi on the x with -180^o,-180^o in the lower left corner, +180^o,+180^o in the upper right. From a neighboring group, obtain a copy of the Ramachandran plot for the other amino acid: glycine if you did alanine or *vice versa*. Are the plots as expected? (That is, do they look like those in your handout or in Stryer?) Discuss. Finally, on your Ramachandran plot for glycine, indicate the position and energy for a left-handed α-helix--hint: you needn't use HyperChem to calculate these values!

PART III: PROBING THE SECONDARY STRUCTURE OF AN OCTADECAMER

You will construct a 18 amino acid polymer from six repeats of your tripeptide, in both the α-helix and beta strand conformations.

--Alpha Helix: Select **New** under **File** Menu., and do not "Save current changes: (untitled)." Your previously constructed molecule will disappear. Open the **Databases** window and select **Amino Acids**. Select **alpha helix**, begin with **Ace**, then six repeats of your tripeptide, culminating with **Nme**. (Again, Ace is N-acetyl and Nme is N-methyl carboxamide; these groups provide the flanking peptide bonds for the terminal α-carbons.) Under display menu select **Recompute H bonds**. They should appear as white dashed bonds up and down the helix. Verify that there are 11 main-chain atoms between H-bonding partners--13 atoms counting the carbonyl oxygen and amino hydrogen participating in the H-bond. Measure the distance from the amino-terminus to the carboxy-terminus by choosing the **Select** Tool and clicking on the N-terminal nitrogen atom and the C-terminal capping carboxamide nitrogen atom. (This latter nitrogen atom is chosen, rather than the C-terminal carbonyl carbon, so that the distanced spanned includes 18 amino acids and 18 peptide bonds.) The distance is displayed on the bottom of the working space. Note the distance in your notebook. What is the distance spanned per amino acid? Rotate the image of your helix to view down the helical axis and note the position of the side chains. Notice how the N-

terminal nitrogen atom is directly above the C-terminal capping carboxamide nitrogen atom. Is this merely a coincidence?? Explain.

--You will now partially minimize the energy of your helix to eliminate any particularly bad interactions. First, deselect all atoms. Open the **Compute** Menu and select **Geometry Optimization**. Select **Steepest Descent** algorithm on the left part of the dialogue window. Set the termination conditions for the calculation to be an **RMS gradient** of **0.1** kcal/? mol or **10** maximum cycles, and **Screen refresh** on the bottom to **1** cycle. Click on **OK**. The computer will start the calculation and show the energy in kcal/mole, gradient in kcal/^{o} mol, and whether or not the gradient converges on the bottom of the screen. It will stop the calculation when either of the termination conditions is fulfilled: i.e., the gradient converges or the ten iterations have been performed. Undoubtedly, this calculation will not yet have converged (a complete minimization will take quite a long time), but particularly unfavorable, high-energy van der Waals repulsions will have been relieved. Record the energy in your notebook. Save this file on your U: drive. (You should print a copy of your structure in both the sticks and in the spheres renderings before you leave the lab. You should paste these pictures into your notebook.)

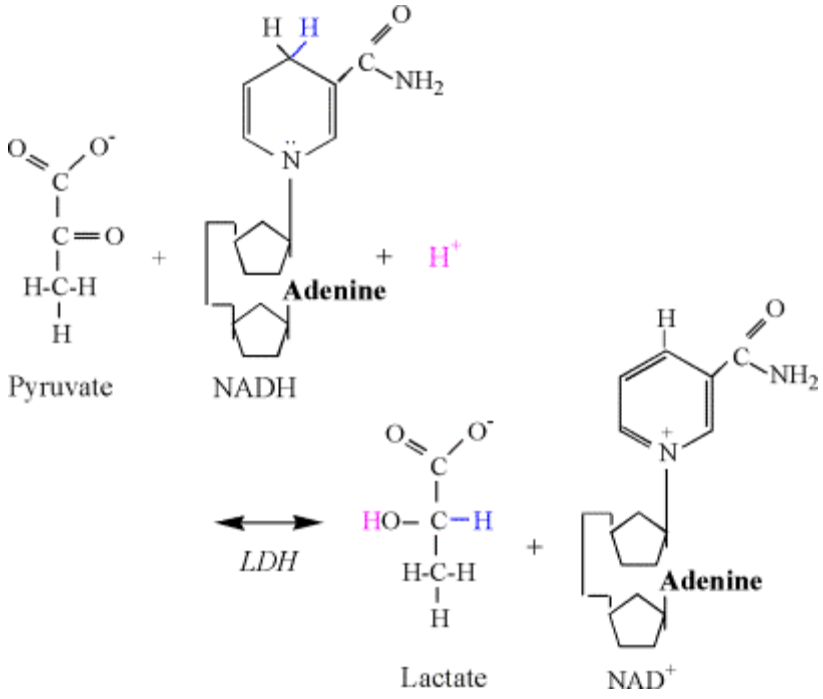
--Beta Strand: Select **New** under **File** Menu, and do not "Save current changes: (untitled)." Your α-helix will disappear. Open the **Databases** window and select **Amino Acid**. Select **beta sheet**, begin with **Ace**, then six repeats of your tripeptide, culminating with **Nme.** Under display menu select Recompute H-bonds. What do you see? Comment. Again measure the distance from the N-terminus to the C-terminus by choosing the **Select** Tool and clicking on the N-terminal nitrogen and the C-terminal capping carboxamide nitrogen atom. Note the distance in your notebook. What is the distance per amino acid in a β-strand? How does this value compare with that for an amino acid in an α-helix? Discuss briefly. Rotate the image of your strand and note the position of the side chains. Can you see the pleats?

--No you can't! So, let's make a pleated β-strand. The appropriate dihedrals are phi = -139^{o} and psi = +135^{o} (for a strand that will participate in a typical anti-parallel β-sheet). Now you should be able to see the pleats. Note that the side chains are perpendicular to the planes of the peptide bonds and that they alternately point in opposite directions--perfect for forming silk! Again, you will minimize the energy of your strand to eliminate any particularly unfavorable interactions. Open the **Compute** Menu and select **Geometry Optimization**. Select **Steepest Descent** algorithm on the left part of the dialogue window. Set the termination conditions for the calculation to be an **RMS gradient** of **0.1** kcal/^{o} mol or **10** maximum cycles, and **Screen refresh** on the bottom to **1** cycle. Click on **OK**. The computer will start the calculation and show the energy in kcal/mole, gradient in kcal/^{o} mol, and whether or not the gradient converges on the bottom of the screen. Again, the calculation won't converge. Record the energy in your notebook. Why is this energy so much higher than that calculated for your α-helix? Save your file on your U: drive; again you should print a copy of your structure

in both the sticks and in the spheres renderings before you leave the lab.

PART IV: DOGFISH LACTATE DEHYDROGENASE

In this section, you will look at aspects of the structure of a protein, namely the enzyme lactate dehydrogenase (LDH). This enzyme catalyzes the reaction:



--Exit from HyperChem. To look at this protein, we are going to use a program which reads **pdb files** containing coordinates of the atoms from a model for the structure determined by X-ray crystallographic analysis. The program translates these coordinates into a visual image of the location of the atoms which can be easily manipulated. There are several such programs available for free on the internet, including DeepView (seen in class) developed by the Swiss Protein Database group, Cn3D developed by the NCBI, and Protein Explorer developed by Eric Martz at UMass using the visualization engine known as RasMol. Of these, Protein Explorer is the easiest to use, and, for many purposes, gives the most useful images. It is a steadily evolving program, and changes without warning – if you discover that some detail in the instructions provided here does not seem to correspond to the screen that you see, congratulate yourself on your modernity, and carry on.

To open Protein Explorer, open the Firefox web browser. A recent modification claims to work in any browser, so pick your poison according to your preferences; instructions here were written while using Mozilla. In the URL window, type www.proteinexplorer.org and hit enter. Notice that you end up somewhere else, namely at a Umass webset, looking at a webpage styled the "FrontDoor" to Protein Explorer. There are a world of treasures accessible from this web page, virtually none of which will be investigated here – that's why you have spare time. To gain real fluency with the program, you should try out the Tutorial, or even the QuickTour. Links to both can be found in the right hand column just below the yellow banner. For this laboratory, however, we will jump right into it, by calling up a structure file by name. We will start with the structur of a single subunit of dogfish lactate dehydrogenase, stored in the protein databases as the file 6ldh.pdb. To open and view this protein, enter "6ldh" (no quotes) in the box provided) in the center column just below the yellow banner) and click the "Go" box. The visualization program will load, fetch the pdb file from a (different) web server, and calculate a visualization.

When it's done, the program will present you with three windows, two stacked on the left side of the screen, and one on the right containing the visualized structure. This structure will appear spinning in the field of view, and obscured by red balls which are actually the oxygen atoms of water molecules present (and resolved) in the crystal. Notice the lower window on the left side of the screen – a few basic characteristics of the structure are noted there. Most of the work we will do will be done using the upper window on the left. In particular, you will see two button in this window, labeled "Toggle spinning" and "Hide/Show water". Click these, and observe the result in the image. Notice that when you click them, a sequence of commands appears in the lower window on the left. These are RasMol commands, and there is a line provided to allow you to enter such commands yourself. Place the mouse on any empty space in the right window, click, and drag. You will see the molecule move in response to your mouse movements. The movement makes it easier to see how the molecule is distributed in three dimensions, and allows you to look on all sides of the structure.

SECONDARY STRUCTURE

Let's examine the structure in more detail. Find the link to "QuickViews" in the upper left hand window, and click on it. This will divide the upper left hand window into two windows. The top contains tools, the middle contains information and options. In the upper window, find the buttons for spin and water, and turn off both if you haven't already. Now look at the structure in the right hand window. This "worm" is the path of the protein backbone. Pull down the "Color" menu by clicking on the little blue box on its right, and select N>C rainbow. Identify the N-terminus.

Now let's investigate a few aspects of the secondary structure in this protein. Pull down the "Color" menu again and select "Structure". Pull down the "Display" menu and select "Cartoon". Just admire this image for a moment and chat approvingly with your

lab partners. Note the sheets and helices. By now, you and your partners should have noticed that the core of the structure is constructed from three β-sheet domains. Describe each (number of strands, whether parallel or antiparallel, relationship between order in the sequence and order in the sheet domain). Are there any instances of parallel strands which are not linked by the sheet-helix-sheet motif? Are there helices that do not function as simple connectors for sheet strands?

We learned in lecture about specific effects that stabilize or destabilize these secondary structures. Let's have a look at a couple. Pull down the "Display" menu, and select "HBonds". Then go to the central window and select the first box. Note the caveat – not all Hbonds will be visualized. Now examine the structure, where H bonds appear as thin bars. Confirm that the hydrogen bonding properties advertised in theory for sheets and helices are observed in this real structure. In doing so, note the differences in the hydrogen bond pattern between strands at the junction between two β-sheet domains, and between strands in the large sheet domain. In addition, note the backbone hydrogen bonding patterns in the loops connecting helices or sheet strands. Draw three different arrangements in your notebook. Note that one substantial loop seems to largely lack hydrogen bonds. Discuss with your partners and provide an explanation for this situation in your notebook.

Hide the Hbonds by selecting "Hide" under the Display menu, and then checking the Hbond box in the central window on the left. You remember from earlier in the laboratory that proline is incapable of fitting neatly into an α-helix. To see this principle in action, go to the little box at the top of the lowest window, erase the text saying that you can enter commands there, and type in the following "select pro and helix" (not the quotes, of course) and hit enter. This command will find any prolines which the crystallographers assigned to helix regions. The window below the command line should tell you that 21 atoms have been selected (the number of nonhydrogen atoms in 3 proline residues). To see where they are, we only need to make them look different. Go to the Display menu, and select "Spacefill", and then the Color menu to select "Green". Note the significance of the location of these residues in your notebook.

TERTIARY STRUCTURE

Of course, a protein does not look like a collection of curlicues and curvy arrows. To see a more reasonable representation, go to the Select menu, and choose Protein, then to the Display menu and choose Spacefill. Note that the protein now appears to be a relatively compact collection of atoms. By rotating the molecule, you can get a good idea of the surface topology of the protein. Now go to the Color menu and select Polarity2. This coloring scheme colors all the atoms of each amino acid either white, if the amino acid is hydrophobic, or purple if the amino acid is polar. Note the distribution of polar and nonpolar amino acids on the surface. In your notebook, speculate on the reason for the presence of hydrophobic patches.

In this view, the protein interior is invisible. To see into the interior, find the "Slab" button in the QuickViews window at the top of the left side of the screen. Click on this button, and observe the effect on the image of the molecule. Essentially the molecule has been sliced in half vertically, and you are looking at the cut surface, i.e., the protein interior. You can move the plane of slicing forward and back by holding down the Control (Ctrl) key while dragging the mouse up and down in the viewing window. By doing this, you can have a look at all parts of the protein interior. Note the distribution of hydrophilic and hydrophobic amino acids in the cell interior, as well as the degree of close-packing which you find there.

EVERYBODY HAS ONE

If you check, you'll find that the window containing the Front Door to Protein Explorer is still open. Go to that window and load a new structure by entering the code "1LLD" in the little window in the center column. This file contains the structure of an LDH molecule isolated from a bacterium. While we and dogfish are rather close cousins, it's been a long time (2.5-3 billion years or so) since there was an organism which could have counted both us and any bacterium among its descendants. What could we have inherited in common from that old geezer? To investigate one possibility, examine the organization of secondary structure in this bacterial LDH molecule (display as cartoon, colored by structure). To do that, you'll need to look at only one of the two subunits. You can do that by choosing "Chain A" from the Select Menu, followed by "Only" from the Display menu. If you follow this immediately by clicking on the "Center" button, and then hit Cancel, this subunit will be centered in the viewing window for your convenience. By switching between windows. compare this structure with the structure of the dogfish LDH. Comment on the similarities and differences in your notebook.

RESTRICTION NUCLEASE ANALYSIS OF VIRAL DNA

The analysis of large macromolecules, whether proteins or nucleic acids, is considerably facilitated by techniques for subdividing the molecules into sizes which are more tractable for analysis. To be useful, the smaller fragments of the molecules must be specific, i.e., they must be produced in the same way from every molecule of their large precursors, and their relationship to the larger molecules from which they are produced must be known. As we have seen, some proteases provide a basis for such techniques for protein analysis. For nucleic acids, the corresponding agents are the restriction nucleases. These enzymes recognize and cleave DNA at specific, short sequences. Because they are sequence specific, digestion of large DNA molecules gives rise to fragments with defined sizes. Restriction nucleases have revolutionized molecular biology, not only because they permit defined subdivision of large DNA molecules, but also because the sites at which cleavage occurs are used as "mileposts" - location markers in a polymer which is otherwise structurally featureless. The locations of functional positions (genes, operators, recombination sites, etc.) can then be defined with respect to such mileposts.

A first step in the characterization of a large DNA molecule is thus the development of a "restriction map" of the DNA, a linear diagram of the DNA marked with the locations of sites cleaved by various restriction enzymes. An example of such a map is shown below for the DNA from the bacteriophage λ. Generation of the data which give rise to such a map is straightforward: restriction enzymes are commercially available, and the restriction reaction itself and size analysis of its products are technically trivial. Analysis of these products, on the other hand, is a logical challenge.

The DNA fragments produced in these reactions are analyzed by size fractionation on agarose gels. Agarose is a thermotropic medium: dilute solutions (ca 1%) are liquids at temperatures above about 40°C, but become relatively solid at lower temperatures (just like Jello, although the interactions are somewhat different). Once separated, the DNA fragments can be visualized (at sub-microgram levels) by soaking the gels in ethidium bromide. This planar, polycyclic molecule binds to DNA by intercalation between the DNA base pairs; when it is intercalated, the dye fluoresces a bright orange upon excitation with UV light.

In this experiment, we will attempt to generate a restriction map for the DNA molecule which is the entire genome of bacteriophage λ. We will use the restriction sites for the restriction enzyme EcoR1 (from *Escherichia coli*) as our reference point for analysis of the maps for BamH1 (from *Bacillus amyloliquifaciens H*), Hind III (from *Hemophilus influenza D*), and Xho I (from *Xanthomonas holicola*).

PROCEDURES:

Restriction of DNA

The reaction mixtures for restriction of the DNA should each be made up on ice in the following order:

32.5µl	H_2O
5 µl	10x concentrated incubation (not electrophoresis) buffer
2.5µl	DNA (0.2 µg/µl)
5 µl	enzyme #1
5 µl	enzyme #2, if there are two, or H_2O

Be careful to make each addition to the bottom, rather than on the walls, of the tube. Change tips to avoid cross-contaminating reagents! Mix the contents by gently "flicking" the tube with your finger. If droplets are left on the wall, a brief centrifugation will drive them to the bottom of the tube. When all of the samples are ready, and securely labelled, transfer them to the 37° bath and incubate for 60 minutes. At the end of this time, stop the reactions by the addition of 5 µl of "stop" solution (150 mM EDTA; 50% glycerol; 0.1% bromphenol blue). Finally, unless directed otherwise, heat the stopped reactions for 5 minutes at 65°C and quick-cool (plunge the tubes into ice water).

Pouring the Gel

Mix 180 ml of H_2O, 20 mls of 10x concentrated electrophoresis buffer (500 mM Tris-HCl, pH 7.8; 60 mM sodium acetate; 10 mM EDTA) and 1.6 g of agarose. Dissolve the agarose by bringing the solution just to the boiling point, swirling, reheating, swirling, etc., until the solution is completely clarified. Remove from heat and allow the suspension to cool.

While the agarose solution is prepared, the electrophoresis tray should be prepared as well. To this end, tape across the open ends of the "gel trays", folding the corners neatly so that the seal is watertight. Seal the seams by dripping a bead of agarose from your hot agarose solution along the tray/tape seams, using a Pasteur pipette. Place the sealed tray on a flat surface, position the well former so that it is about 1 inch from one end of the tray and raised 1 mm above the glass surface. Pour the moderately cool agarose into the tray and allow it to set.

Prepare 1 liter of electrophoresis buffer (900 ml of water plus 100 ml of the 10x concentrated electrophoresis buffer, well mixed) and pour some of this buffer into the gel apparatus. Remove the support from the wellformer, leaving the wellformer itself sticking into the gel. Remove the tape from the ends of the gel tray, put the gel tray into the electrophoresis tank, and add more buffer until the gel is submerged to a depth

of 2-3 mm. Finally, gently tease out the wellformer.

Electrophoresis

When all of the digested DNA samples are ready for electrophoresis, prepare some blank samples by mixing 90 µl H_2O and 10 µl of "stop" solution. Practice loading 30 µl of this solution into the wells at the edges of the gel. then load your various DNA samples into the remaining wells, avoiding the outermost wells. Note carefully the order in which your samples are loaded, and choose your wells to give an asymmetric pattern of loaded wells (so that you can recognize which sample is which, even if the gel is upside down). Cover the apparatus, connect the leads so that the DNA will migrate through the gel towards the anode (positive) side, and turn on the power, setting the voltage to 30 V. The gels will be run overnight, and the TA will remove them in the morning and stain them by soaking in a solution of 0.5 µg/ml ethidium bromide. Once stained, the gels will be photographed.

Data Analysis

On the day following the laboratory, photos of the gels will be posted on the course website. Using the Eco R1 fragments as molecular weight markers, assign sizes to all of the various visible fragments. Using the double digestion data, and any partial digestion fragments which you can identify, each group should draw a map of the genome, indicating the location of as many sites as can be tentatively assigned. The logic used in making the site assignments should be laid out in your notebook as clearly (and briefly) as you can manage. The lab grade for this exercise will be assigned primarily on the basis of the clarity and brevity of this last item, ***not*** on the accuracy of the map.

Incubation Buffer

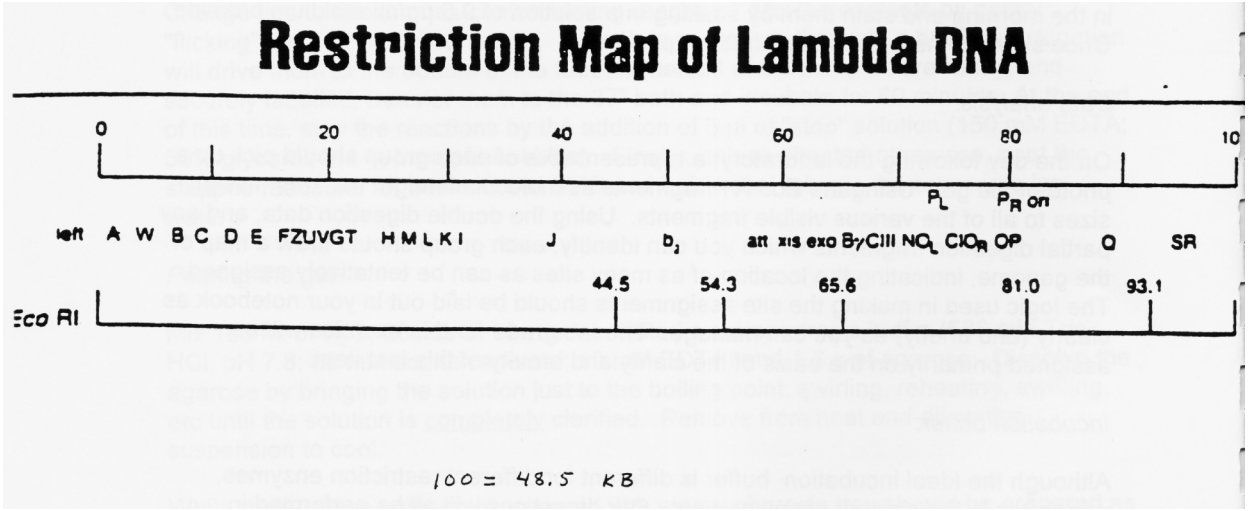
The ideal incubation buffer is different for different restriction enzymes, principally in the amount of salt present. Our digestions will all be performed in: "1" x incubation buffer:

50 mM Tris-HCl pH 8.0
100 mM NaCl
10 mM $MgCl_2$

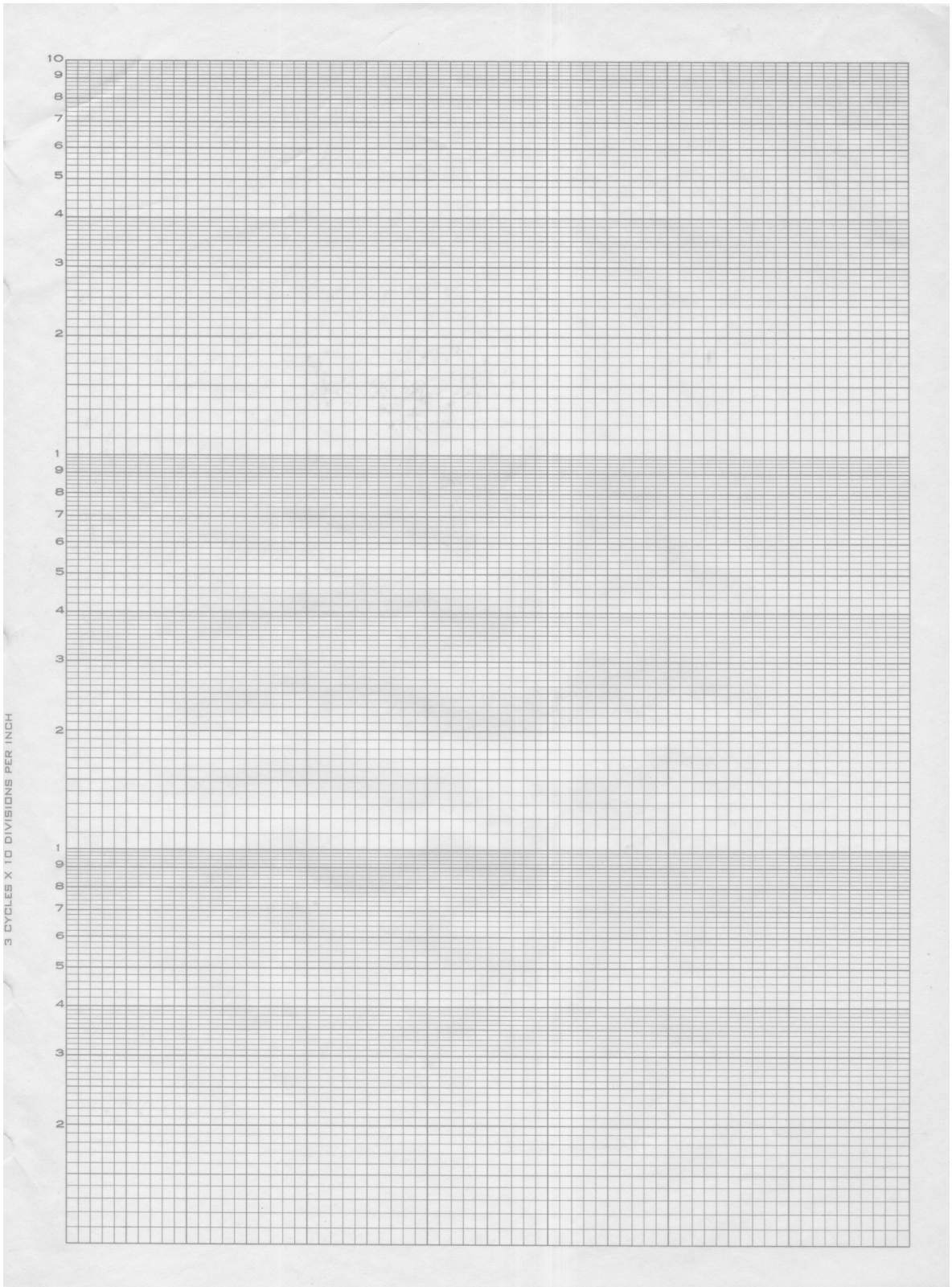
Digestions

Groups should prepare samples of DNA digested as follows:

1. Eco R1
2. BamH1 or Hind III, depending on assignment
3. Xho I
4. Eco R1, no 65° treatment
5. BamH1 (or Hind III), no 65° treatment
6. Xho I, no 65° treatment
7. Eco R1 (once more, for luck)
8. Eco R1 and BamH1 (or Hind III)
9. Eco R1 and Xho I
10. Xho I and BamH1 (or Hind III)
11. Undigested DNA (no enzyme)



A bit of Log scale graph paper for sketching a Log (Mol Wt) vs distance plot:



TRANSPORT LAB

Introduction

The hydrophobic character of the plasma membrane endows it with its essential physiological property, impermeability to the common water soluble, lipid-insoluble molecules. While this is an asset in the retention of the cellular machinery and metabolic intermediates, it offers an obstacle to entry of essential nutrients. In order to overcome this difficulty, cells have evolved special transport mechanisms involving specific protein molecules which recognize and translocate small molecules across the hydrophobic barrier of the plasma membrane. The simplest of these mechanisms involves a type of protein which facilitates the movement of a specific molecule across the otherwise impermeable membrane and allows the equilibration of concentrations on the two sides. No metabolic energy is required for this process; net movement is driven by the energy stored in the concentration gradient across the membrane. this process has been called facilitated diffusion or carrier mediated transport. The best studied example is the glucose transport system of the human erythrocyte. Glucose entry into most animal cells (with the exception of the renal tubule and intestine) occurs via this same mechanism. Examples of facilitated diffusion of carbohydrates have been described in yeast and *E. coli*. glucose uptake by facilitated diffusion is seen in yeast during anaerobic alcoholic fermentation. Efficient flow of glucose into this cell is maintained by the rapid hydrolysis of polysaccharides by externally located enzymes leading to a high local concentration of glucose near the plasma membrane. The great advantage of facilitated diffusion to the cell's economy is that no metabolic energy is required for the operation of the carrier. The primary disadvantage of such a mechanism over active transport is the inability of facilitated diffusion to scavenge nutrients at low concentrations from the environment. Thus, survival by cells possessing this primitive transport mechanism depends on relatively high concentrations of nutrient in the external medium.

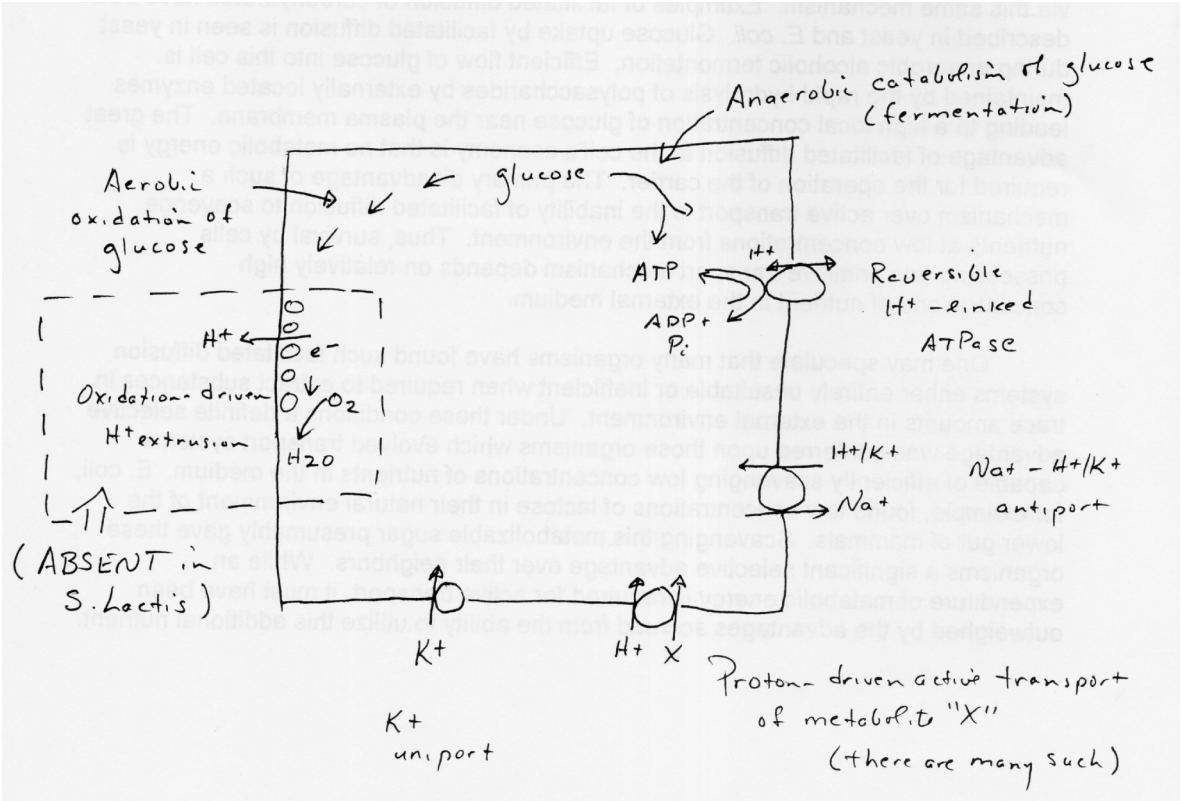
One may speculate that many organisms have found such facilitated diffusion systems either entirely unsuitable or inefficient when required to extract substances in trace amounts in the external environment. Under these conditions a definite selective advantage was conferred upon those organisms which evolved transport systems capable of efficiently scavenging low concentrations of nutrients in the medium. *E. coli*, for example, found low concentrations of lactose in their natural environment of the lower gut of mammals. Scavenging this metabolizable sugar presumably gave these organisms a significant selective advantage over their neighbors. While an expenditure of metabolic energy is required for active transport, it must have been outweighed by the advantages accrued from the ability to utilize this additional nutrient.

Bacterial Coupled Transport

In 1963 Mitchell[1] proposed that microorganisms couple the movement of protons down their electrochemical gradient with the uphill membrane transport of various substrates (substrate-proton symport). According to the chemiosmotic hypothesis, cells extrude protons either as a result of oxidative electron transport (aerobically) or as a result of the activity of a membrane-bound ATPase (anaerobically). Such proton extrusion results in both a membrane potential (inside negative) and a pH gradient (inside alkaline). The energy stored in this electrochemical gradient of hydrogen ions is converted into active transport of the substrate by coupling of the flows of the two substances. According to Mitchell's nomenclature, the electrochemical force or protonmotive force (Δp) is equal to the sum of the electrical potential across the membrane (Δψ) and the potential due to the pH gradient (expressed in electrical units). At 25° and with ΔpH equal to the outside pH minus the inside pH, the relationship would take the following form:

$$\Delta p_{mv} = \Delta \psi_{mv} - 59_{mv} \Delta pH$$

A still-current model for the transport processes in bacteria like *E. coli*, *S. lactis*, and *S. faecalis* is diagrammed below.[2-4]



In bacteria that can function aerobically, oxidation is thought to drive a pump which pushes H^+ to the outside of the cell. Thus, metabolic energy is used to establish a pH-gradient and most likely a membrane potential ($\Delta\psi$) as well. Glucose, for example, can ultimately provide the energy to run the proton pump during oxidation. In turn, the protonmotive "force" (Δp) generated as a result of oxidation can be used for the phosphorylation of ADP, or can be used to drive the transport of metabolites into the cell against concentration gradients. (Notice the similarity to the oxidative phosphorylation process in the mitochondrion.)

Under anaerobic conditions glycolytic degradation of glucose forms ATP which can drive the membrane-bound ATPase in the reverse direction to produce a pH gradient and a $\Delta\psi$ (ATP hydrolysis makes the inside of the cells alkaline and negative in potential relative to the medium). This membrane energy or protonmotive energy can be used for the active transport of metabolites into the cell.

References

**Abstracted from the following papers:

T. H. Wilson, E. R. Kashet and M. Kusch. (1972). The Molecular Basis of Biological Transport. ***Miami Winter Symp. 3*** (eds. J. F. Woesner and F. Huijing), Academic Press.

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1. Mitchell, P. (1963). Molecule, Group and Electron Translocation Through Natural Membranes. ***Biochem. Soc. Symp. 22:***142-168.
2. Maloney, P. C., E. R. Kashket and T. H. Hastings. (1974). A Protonmotive Force Drives ATP Synthesis in Bacteria. ***Proc. Natl. Acad. Sci. 71:***3896.
3. Kaback, H. R. (1986). Active Transport in *E. coli*: Passage to Permease. ***Ann. Rev. Biophys. Biophys. Chem 15:***279-319.
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Experimental

In this experiment you will study the energy-dependent uptake (active transport) of radioactive methyl thiogalactoside (^{14}C-TMG), a nonmetabolizable substrate. Since you will be attempting to produce accumulation of TMG against a concentration gradient, it is clear that an energy source is needed to drive the uptake. Yet the anaerobe, *S. lactis 7962* cannot undertake oxidative phosphorylation. And the sample of cells that we prepare for you will have been washed free of all metabolizable substrates by several centrifugations in either distilled water or tris buffer. Thus the only way you can establish the protonmotive force necessary to drive uptake of TMG is by (1) changing the pH of the medium containing the cells, (2) artificially inducing a membrane potential (e.g., a K^+-diffusion potential), or (3) adding a metabolizable substrate such as glucose, arginine, or galactose, which provide energy for anaerobic metabolism leading to the synthesis of ATP and the pH gradient and the membrane potential that result from ATP hydrolysis.

You will measure the active transport of ^{14}C-TMG by *S. lactis* cells driven by energy sources (1), (2), and (3). Therefore, in one experiment you will artificially generate a pH gradient across the cell membrane by lowering the pH of the medium containing the cells. In another experiment, you will artificially induce a membrane potential by using the K^+-selective ionophore, valinomycin. Glucose will provide the energy source in the third experiment.

The second experiment is based on the following considerations. Cells that were grown in a high potassium concentration medium can be considered to be K^+-rich inside (even after washing, since the cell membrane is relatively impermeable to K^+). If the cells are suspended in a K^+-free medium (sodium phosphate buffer) there is a large K^+gradient across the membrane, but the K^+ diffusion potential is still zero because no K^+ can move across the membrane. If valinomycin is added to the medium, a diffusion potential is immediately established that has a magnitude given approximately by the Nernst potential.

$$V_M = 59mV \log (K^+_{in} / K^+_{out})$$

Since $K^+_{out} \sim 0$ in your experiment, you will be generating the maximum V_M possible when you add valinomycin.

Preparation, Filtration, and Timing

Have 10 filters ready for each experiment and a place to put each of them after the sample has been taken. Before taking a sample, place a filter on the suction apparatus, fasten the chimney, and pass 5 ml of the appropriate buffer through the filter under suction. Keep the vacuum on until the sample has been taken and washed. When applying the cell sample to the filter, put it dead-center on the filter and then rapidly wash with 5 ml buffer. Cut the vacuum, remove the filter, and set up for another sample. The wet filters containing the cell samples should be placed at a labeled and

PROTECTED location to dry for at least half an hour (a gust of wind as a student walks by will scatter and mix the dried filters). After drying the filters, place each in a separate scintillation counting vial which has been appropriately labeled with 1/4-inch letters just below the threads for the lid. (Remember scintillations of light must be able to get out of the counting vial to reach the photomultiplier tube during the counting process.) the label on the scintillation vial should include your lab day, group symbol, experiment number, and sample number.

A short time is required for the TMG concentration to come to equilibrium once the cells have been added to the TMG-containing buffer. If t = 0 is defined as the time at which you add the valinomycin, glucose, or acid to begin the uptake reaction, i.e., to energize the cells, then you should take samples at approximately -5, -3, and -1 minute in order to determine the equilibrium TMG distribution before cell energization. After energization, the first 3 samples should be taken as rapidly as possible. The last 4 samples can then be stretched out so that the last one is taken at about 10 minutes after energization. Before you begin each experiment I suggest making a table that has the sample number, anticipated time of sampling, and blanks for the actual time of sampling.

In a day or two we will have counted your samples. The results will be posted in the course web site. In addition we will post the concentration of the stock cell suspension (mg dry cell weight per ml stock suspension). From this value you may calculate the total internal cell volume in your "uptake-reaction" mixture, since it has been determined that the internal volume of *S. lactis* is 1.83 µl cell water per mg dry cell weight. We will also provide you with the specific activity of the ^{14}C-TMG and with the counts per minute of the TMG-containing buffer solution to which you added cells to begin an uptake experiment.

pH Experiment

You will be provided with a "pH" buffer tube. this tube contains ^{14}C-TMG and 0.1 M KCl in 1 ml of <u>dilute</u> (5 mM) KPO_4 buffer at pH <u>7.5</u>. Allow this tube to come to room temperature, add 0.2 ml of the stock cell suspension and begin taking samples. Wash these samples with the <u>dilute</u> pH <u>7.5</u> buffer (labeled "Dilute pH-wash"). Energize the cells at t = 0 by adding 0.2 ml of the 0.5 M KPO_4 <u>acidic</u> (pH <u>4.7</u>) buffer. The addition of this acidic buffer should bring the cell incubation medium pH down to about 5.5. Wash cell samples taken after energization with the pH 4.5 buffer (labeled "Acid pH wash").

Valinomycin Experiment

You will be given a "VAL" buffer tube. This tube contains ^{14}C-TMG in 1 ml 0.1 M $NaPO_4$ buffer at pH 7.5. Allow this tube to come to room temperature, add 0.2 ml of the stock cell suspension, and begin taking samples. Wash the samples with $NaPO_4$ pH 7.5 buffer (labeled "Val Wash"). Have the lab instructor add a few µl of valinomycin solution when you want to energize the cells (make sure the instructor is nearby and has VAL before you begin the experiment). The same wash buffer can be used after energization.

Glucose Experiment

This experiment will be run exactly as the VAL experiment, except that you will energize with 40 uL of 1 M glucose rather than with valinomycin.

Write-Up

1. Plot counts/min vs. time for the three experiments. On the right hand vertical axis plot $[TMG]_{in}/[TMG]_{out}$.
2. Discuss whether your results support the Mitchell Theory for the active uptake of nutrients by bacterial cells.
3. Speculate about why some of your curves for ^{14}C-TMG uptake rapidly decrease after several minutes.
4. Why isn't the membrane potential $V_M = \infty$ when K^+-containing cells are suspended in K^+-free medium in the presence of valinomycin?

MITOCHONDRIA: OXIDATION AND PHOSPHORYLATION

Introduction

In this experiment we will measure mitochondrial electron transport and the mechanism and efficiency of its coupling to the synthesis of ATP. Mitochondrial electron transport will be measured by measuring the consumption of O_2 using the Clark polarographic electrode (whose operation is described in the Appendix).

The tools for analyzing the system will be various substrates and inhibitors; the method for application of these tools will be measurement of their effect on the rate of ADT-dependent oxygen consumption. *You are not provided with specific protocols for these experiments.* Rather, you are in the position of those scientists who first developed the schemes for oxidative phosphorylation that we have studied and discussed outside the laboratory. To illuminate your path, you should consult the text and lecture notes for basic information about the mechanism of electron transport and oxidative phosphorylation.

Biochemicals

The solutions of substrates and inhibitors which will be provided in the laboratory are detailed in TABLE I Reagents. Many of these are expensive and should not be wasted. The amounts to be added can be calculated from the suggested dilutions from stock and a measurement of the volume of your incubation chamber.

All acids have been adjusted to pH 7.2 with NaOH.

These solutions may be added to the reaction chambers with Pipetmen micropipets. When making these additions, exercise care to minimize the introduction of bubbles.

ADP (50 µl) should be addeded **accurately**. Interpretation of your experiments will require knowing how much ADP has been added. Every effort will be made to provide an ADP solution whose A_{260} (1 cm) is 0.502 when diluted 1000-fold. The Merck Index gives ϵ_{260} for ADP as 15.4 x 10^3 liters/mole cm. If the actual value for the A_{260} is different, you will be informed at the beginning of the laboratory.

Mitochondrial Preparations

Rat-liver mitochondria will be prepared by the instructors before you arrive, by the following procedure. The liver is homogenized in a medium containing 0.25 M sucrose, 0.003 M EDTA, and 0.005 M Tris-Cl, pH 7.4. A teflon-pestle Potter-Elvenhjem homogenizer (clearance, about 0.006 inches) operating at about 300 RPM is used and care is taken to keep the tube immersed in an ice bath during homogenization. After removal of nuclei and unbroken cells by centrifugation at 600 g for 10 minutes, the mitochondria are pelleted at 10,000 g for 15 minutes. In order to remove endogenous substrates, the mitochondria are washed twice with 0.25 M sucrose, 0.005 M Tris Cl, pH 7.4 by centrifugation at 10,000 g for 15 minutes. After the final wash, the

mitochondrial preparation is suspended in the sucrose-Tris solution to give about 25 mg protein/ml and stored on ice. Addition of about 0.3 ml of this suspension to incubation medium in the reaction chamber should result in a satisfactory rate of O_2 consumption. If not, the amount of mitochondrial suspension added should be adjusted to give a rate (in the presence of substrate alone) which will result in the exhaustion of the O_2 in the chamber in no more than 5 minutes.

The incubation medium contains:

Sucrose	0.10 M	
KCl	0.020 M	
Potassium Phosphate	0.003 M	pH 7.5
Tris-Cl	0.005 M	

The function of the sucrose in all of the above solutions is to maintain isotonicity without increasing ionic strength; Tris-Cl is a buffer, and phosphate a requirement for oxidative phosphorylation. The EDTA in the homogenization medium serves to remove Ca^{++}, which can act as an uncoupling agent. *It is of the utmost importance that the mitochondria be kept cold until they are used*; deterioration and uncoupling can result if they are warmed.

Experimental: Oxygen Uptake

An example of a typical experiment is attached, to give you an idea of how the data from your experiments might look. Consider this diagram carefully, so that you understand what each of the parts measures, and how each of the parts might vary given the presence of different substrates and/or inhibitors. Your expectations in this regard are the hypotheses which your experiments should test.

Note that the graph contains a critical number: the concentration of O_2 which is present at the beginning of the experiment. This number incorporates a conversion factor between voltage (what your recorder actually measures) and O_2 concentration. *This factor is only valid if the buffer is O_2-saturated at the beginning of the experiment* (before addition of mitochondria). To be sure that this conversion factor applies, it is essential that your buffer be well aerated, and temperature equilibrated (at room temperature) before beginning the experiment. Conversion of this concentration to moles of oxygen requires, in turn, that *you know (and record) the total volume in the reaction chamber*.

Other hints:

Once mitochondria have been added to the chamber, carry out the experiment without delay.
After making an addition, it is necessary to observe the recorder tracing until the full effects of the addition have been displayed, after which further additions should be made quickly. For example, after addition of substrate, allow the reaction to proceed far enough to establish a straight

line on the recorder before making the next addition. After adding ADP, observe the stimulation of O_2 uptake (if any); when the rate returns to a steady state at or near its pre-ADP slope, a further addition may be made if desired.

Be sure to wash the chamber and electrode *thoroughly* after using an inhibitor.
The first run with any new mitochondrial preparation should include a test for stimulation by ADP and note should be made of the respiratory control ratio (see below). A preparation which is not stimulated by ADP to give a control ratio of at least 3 is damaged and uncoupled and useless for many of the experiments described below.

Suggested Experiments

You should devise and carry out any experiments desired. Some suggestions:

1. Determine P:O ratios for several substrates and thereby deduce the relative contribution to phosphorylation within the electron transport chain. NOTE: It is very difficult to determine P:O ratios for ascorbate-TMPD, as the latter can act as an uncoupling agent. Addition of Mg^{++} (5-10 mM) has been reported to prevent this action.

2. Using inhibitors, establish the order of entry of various substrates into the electron transport chain. Where does the endogenous substrate enter?

3. Investigate whether the different uncouplers such as dinitrophenol, valinomycin, Ca^{++}, or the electron shunt TMPD, all operate in the same way.

References

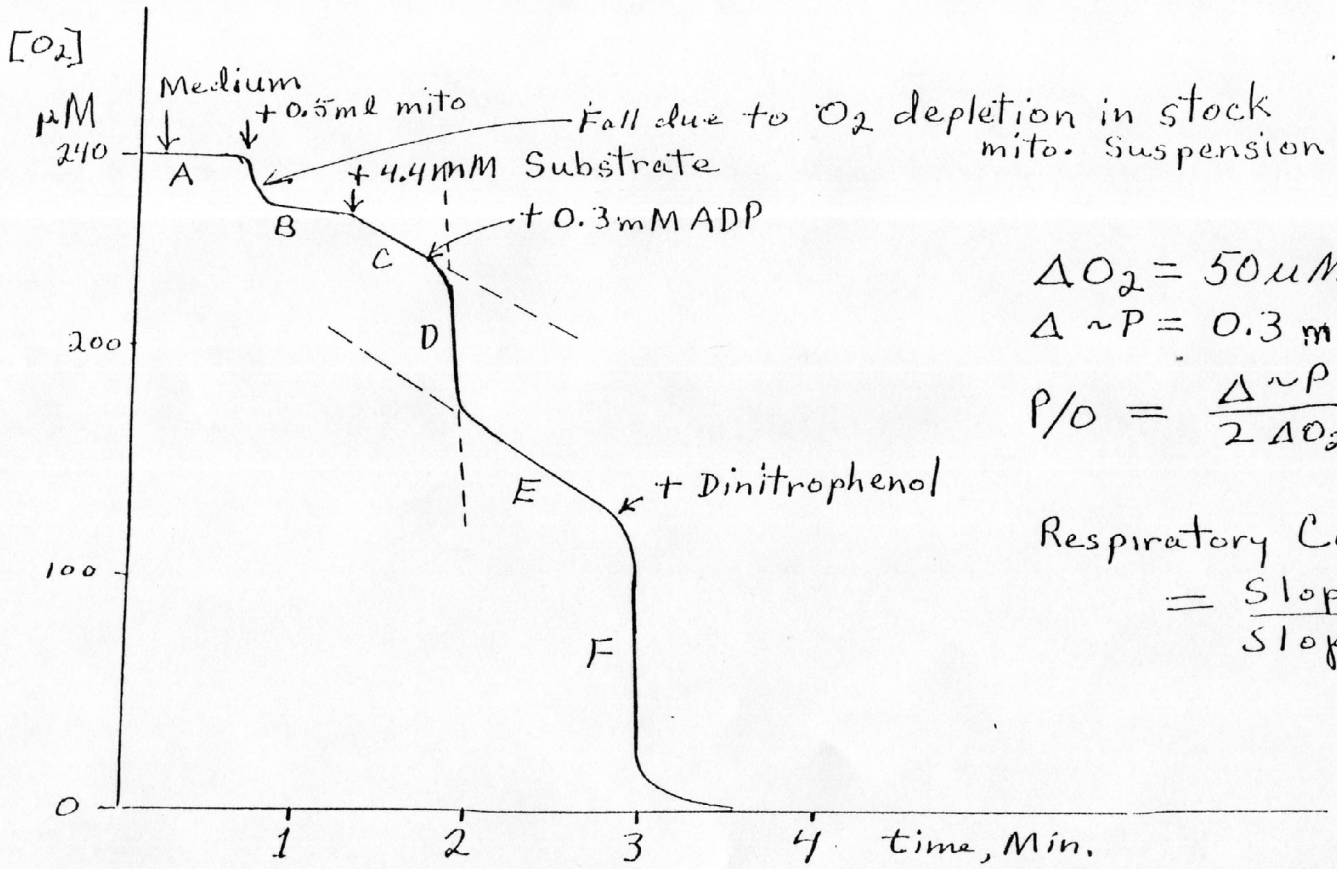
Packer, L. (1967). Experiments in Cell Physiology, Academic Press, NY. See experiments 14, 15, and 19.

Estabrook, R. W. and M. E. Pullman (eds.). (1967). Methods in Enzymology, Vol. X, academic Press, NY. See articles 6 and 8.

TABLE I: Reagents

		Stock Concentration	**Suggested Final Concentration for Trial**
Item	**M**	**Solvent**	**M**
Sodium Succinate	1	H_2O	$5x10^{-3}$
Sodium α-Ketoglutarate	1	H_2O	$5x10^{-3}$
Sodium β-Hydroxybutyrate	1	H_2O	$5x10^{-3}$
Na ADP	0.03	H_2O	$3x10^{-4}$
2,4-Dinitrophenol	0.01	EtOH	10^{-4*}
Na Azide	1	H_2O	10^{-2*}
Na Malonate	1	H_2O	$2x10^{-2*}$
Oligomycin	$1x10^{-3}$M	EtOH	$2x10^{-6*}$, (or 5x more)
Na Ascorbate	1	H_2O	$5x10^{-3}$
Rotenone	$1x10^{-4}$	EtOH	10^{-6*}
Antimycin	$1x10^{-3}$	EtOH	10^{-6}-10^{-5*}
TMPD	0.05	H_2O	$1x10^{-4}$, (or <u>less</u>)
$MgCl_2$	1	H_2O	5 to $10x10^{-3*}$
$CaCl_2$	1	H_2O	5 to $10x10^{-3*}$
Valinomycin	$1x10^{-4}$M	EtOH	$1x10^{-6*}$

* concentrations should be increased if necessary



$\Delta O_2 = 50 \mu M$

$\Delta \sim P = 0.3\ mM$

$$P/O = \frac{\Delta \sim P}{2\,\Delta O_2} = \frac{300 \mu M}{100 \mu M} = 3$$

Respiratory Control Ratio $= \frac{\text{Slope D}}{\text{Slope E}}$

Figure 1. A typical O_2 electrode trace.

APPENDIX

THE CLARK OXYGEN ELECTRODE

The oxygen electrode (illustrated below) consists of a platinum cathode and a silver anode immersed in a KCl solution. this assembly is separated from the solution to be tested by a thin teflon membrane which is freely permeable to O_2 but not to water, salts, and most other solutes. A potential of 0.6-0.8 volts is maintained across the electrode. In the presence of O_2, the following reactions take place:

Anode: $2Ag + 2Cl^- \rightarrow \underline{2AgCl} + 2e^-$

Cathode: $2H^+ + 1/2\ O_2 + 2e^- \rightarrow H_2O$

Because these reactions depend on the availability of oxygen, the <u>current</u> which flows through the electrode is stoichiometrically proportional to the O_2 concentration. This current may be carried through a standard resistance and the voltage across the resistance recorded with a strip-chart recorder, or the current may be passed into an operational amplifier current to voltage converter. With either circuit the output voltage (recorder deflection) is proportional to O_2 concentration at the electrode membrane.