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A rich independent-project-based enzymology lab using plant mitochondrial succinate dehydrogenase

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For well over twenty years, we have successfully run a half-semester inquiry-based enzymology lab at the introductory/intermediate level. In successive weeks, students use black-eyed pea (*Vigna unguiculata*) plants to make a crude mitochondrial purification, hone their pipetting skills with a Bradford protein concentration assay, construct a Lineweaver-Burk plot to determine K_m and V_{max} of succinate dehydrogenase (complex II) in their experiments, consult with their instructor about their independent project proposal, execute their plan, and then write up their results. The literature on succinate dehydrogenase is quite diverse, and accordingly the independent projects are quite diverse in nature. Along the way, students learn about differential centrifugation, accurate pipetting, spectroscopy, systematic errors, outliers, and mining the literature. This lab could be easily scaled back to a two-week lab with simpler learning goals.

Keywords: enzymology, independent projects, organelle isolation, differential centrifugation

Introduction

Enzymology is a workhorse in many cell biology teaching labs due to its reliability and time from start to result. For over twenty years, we have successfully run an independent-project-based lab on succinate dehydrogenase (SDH), an enzyme involved in the both the tricarboxylic acid cycle and the electron transport chain.

Timeline

This six-week project with 3-hour labs can be thought of pedagogically in four phases. In the first week, the students isolate mitochondria from *Vigna unguiculata* (black-eye pea) plants by differential centrifugation, aliquoting, and freezing their samples; the protocol is based loosely on Bonner (1967) and Edwards (1987). This do-as-you're-told lab has the extra benefit of a couple short downtimes, which are valuable for teaching students to plan ahead and write in their lab notebooks and for building community. Additionally, the students isolate mitochondria from a living organism which we find a real plus in a biology class.

In the second week, the students perform a spectrophotometric Bradford protein concentration assay to determine the protein concentration of their isolated mitochondria. This gives a preliminary indication of the success of their mitochondrial isolation and will allow them to compare their results to others. While this lab is optional, it introduces spectrophotometry and standards curves, and does a fabulous job teaching accurate micropipetting.

In the third week, students run a series of SDH assays with the same spectrophotometers. From these, they (1) determine if their isolated mitochondria have enough activity to proceed

fruitfully, (2) learn that when there is no succinate added to their sample, the enzyme is still active, implying that there is succinate in their mitochondrial sample, and (3) construct a Lineweaver-Burk (double-reciprocal) plot to determine K_m and V_{max} of succinate dehydrogenase in their experimental conditions (Lineweaver, 1934). The endogenous succinate in the mitochondria is both an annoyance and learning opportunity since it introduces a systematic error into their Lineweaver-Burk estimations. (For more on this, see Appendix A.)

The fourth and final phase consists of the students' independent projects. Each student group proposes and performs a set of experiments on SDH enzymology based on the primary literature. Many repeat early experiments on SDH, and others propose ideas that (to their knowledge) are novel, though we insist that their logic be based on something that they read in the literature. We set aside a week to discuss their independent project proposals and two more for the execution of them. Ultimately, the students write up their results in a typical IMRaD lab report.

SDH has been studied extensively, and the literature on SDH is quite diverse. Accordingly, the independent projects proposed are quite diverse in nature every year (Appendix B.)

Learning Goals

In addition to reinforcing cell biology concepts including organelles, respiration, and enzyme kinetics, this lab introduces and reinforces many intellectual and lab skills.

The intellectual ones include:
- mining the scientific literature
- designing experiments
- interpreting results including handling outliers
- plotting data
- writing a scientific paper

The lab skills include:
- spectrophotometry
- extensive micropipetting
- dilution
- using large centrifuges including differential centrifugation
- time and space management
- accounting for experimental artifacts
- keeping a lab notebook

Alterations

While we teach this lab at the introductory/intermediate level, this would also make for a fine upper-level lab that would likely progress quicker and need less support.

Regarding equipment, as written the protocol for mitochondrial isolation requires refrigerated centrifuges which will take 250-mL centrifuge bottles. Alterations to the protocol for centrifuges that take only disposable 50-mL tubes can be found in Appendix C.

While we get great satisfaction from the independent project aspect of this course, each of the first three phases of course could be run independently with slight alterations achieving many of the same goals. For example, in this ABLE workshop, we isolated the mitochondria

and ran a quick set of SDH assays with all the necessary components, without individual ones, and with an inhibitor, showing the need and roles of all the individual components. For students, this illustrates differential centrifugation, the use of large centrifuges, and many other lab skills and concepts. Even without a spectrophotometer, the SDH assays show the roles of all the components which could be used for a discussion of the different types of controls and experimental conditions.

Student Outline
Chapter 1: Mitochondrial Isolation

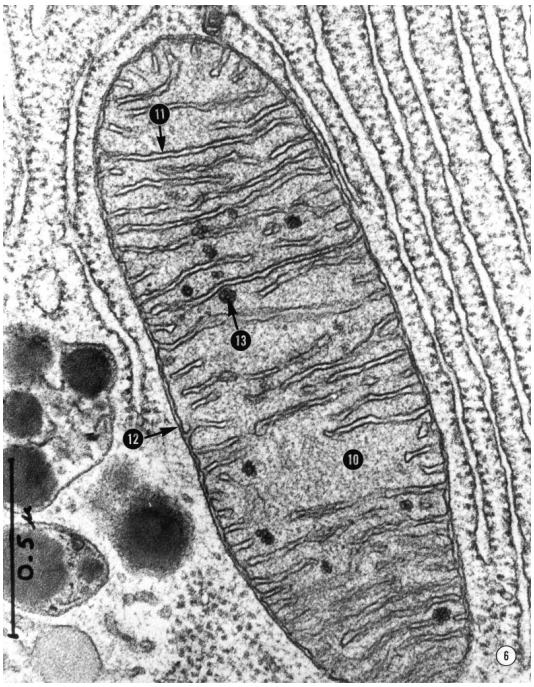

Figure 1. A transmission electron micrograph of a mitochondrion. (Public domain)

A. Project Background

Enzymes act as biological catalysts. They combine with their specific substrates, and through chemical interactions, lower the energy state necessary for the substrate to react with other biological molecules or with water. The substrate molecule binds within the folded protein-enzyme complex in a position called the active site.

The active site performs several functions: (l) it sequesters the molecules destined to interact (molecules which may be at extremely low concentrations); (2) it orients the molecules, bringing the critical chemical groups into proximity; (3) it "activates" the molecules through the interaction of the various amino acid side chains of the protein with components of the reactants—distorting their positions, competing for electrons and ultimately making the electrons that are part of the covalent linkages of the substrate more prone to establish a relationship with the other reactant; (4) it protects the activated substrate from indiscriminate chemical reactions until the correct co-reactant is positioned on the enzyme. In other words, the active site of an enzyme increases the chemical reactivity of the reactants.

In this project, you will study the properties of succinate dehydrogenase (SDH), a central enzyme in the tricarboxylic acid (TCA) cycle. The association of the enzyme and its coenzyme, flavin adenine dinucleotide (FAD), can be represented as E-FAD. This enzyme-coenzyme complex catalyzes the oxidation of succinic acid (succinate) to fumaric acid (fumarate).

As the equation in Figure 2 shows, the hydrogens removed from the succinate are carried by FAD. The hydrogens are then transferred to coenzyme Q in the respiratory electron transport chain. All of this takes place in the mitochondria, so to study succinate dehydrogenase, we must isolate some mitochondria on the first lab day.

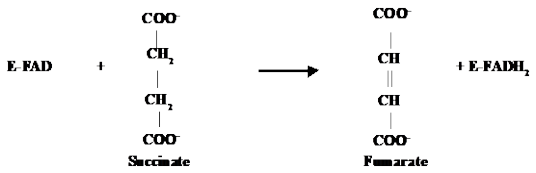

Figure 2. The chemical reaction catalyzed by succinate dehydrogenase.

B. Background on Mitochondrial Isolation

You will isolate mitochondria from black-eyed pea (*Vigna unguiculata*) plants by homogenizing the plant tissue in a blender with a buffered, isotonic sucrose solution. After filtering the homogenate through cheesecloth to remove the largest pieces, you will centrifuge it at a specified centrifugal force to sediment the unbroken cells, cell wall fragments, and cell nuclei. This sediment is called the pellet.

You will centrifuge the supernatant, the fluid that is not part of the pellet, at a higher centrifugal force to sediment (or pellet) the mitochondria. Centrifuging at various speeds for separating biological organelles is *differential centrifugation.* The mitochondria fraction must then be washed once before the final centrifugations. You will freeze the isolated mitochondria in aliquots for subsequent analysis of succinate dehydrogenase.

For all cell fractionation procedures, the solutions and containers must be ice-cold to inhibit the hydrolytic enzymes released when the cells are broken.

The flow chart below (Figure 3) provides an overview of the procedure. For the complete instructions, see the next section.

Each student pair will be assigned a freezer box for storage of mitochondria at -70° C.

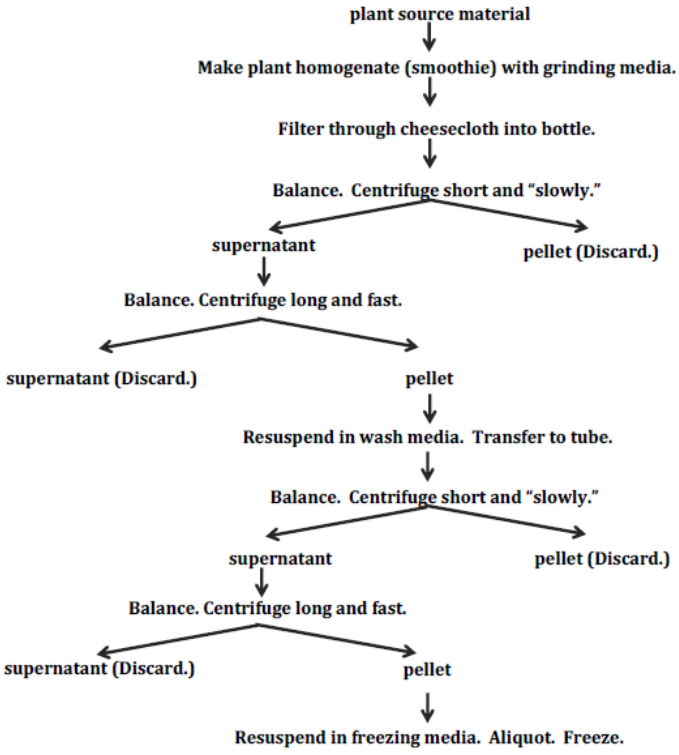

Figure 3. Flow chart of the procedure.

C. Experimental Procedure: Isolating Mitochondria

Make sure that you have pre-chilled all necessary labware by placing them on ice well in advance of their uses. (Figure 4). Except for the blender jar and the solutions, this is your responsibility. You cannot fit everything into your ice bucket.

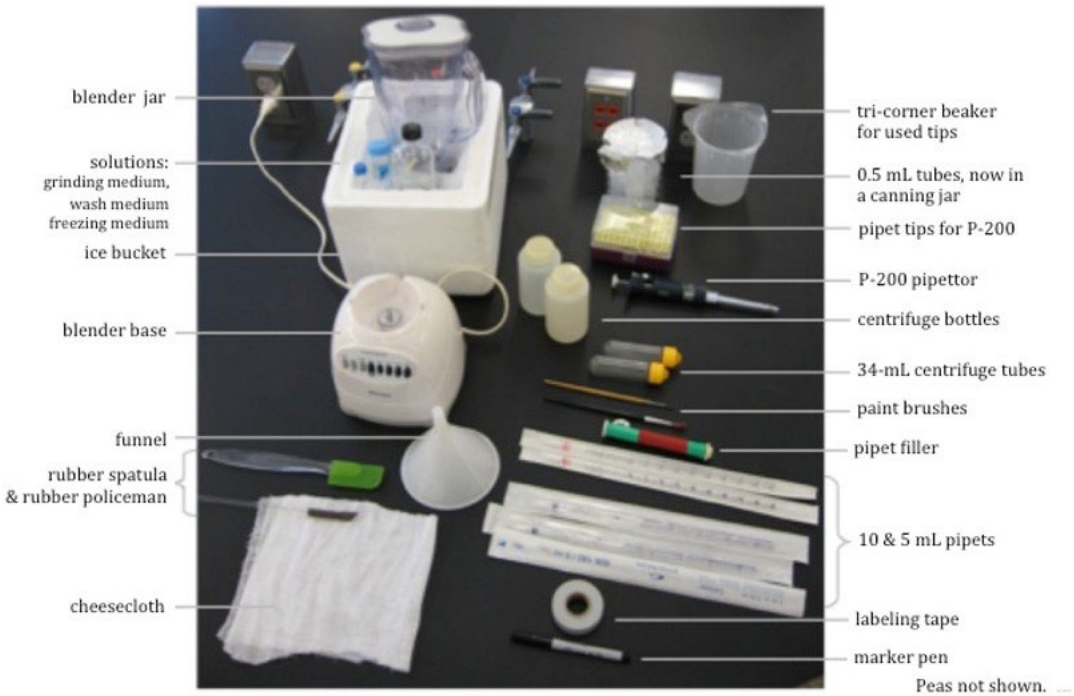

Figure 4. Equipment used in the isolation of mitochondria.

Preparing the cell lysate

1. Place the stem of your funnel into an iced 250-mL centrifuge bottle. Unfold the cheesecloth and refold it so it is eight layers thick and approximately forms an 8-inch square (The cheesecloth likely starts two layers thick.). Dampen it with tap water and center it inside the funnel; it should not be dripping.

2. Obtain a chilled blender jar and a bag of plant material from the cooler in the front of the classroom. Check that the base is tightly screwed on the jar, and then place 100 mL of cold grinding medium in the blender jar; the grinding medium is pre-measured for you.

3. The weight of the plant material is noted on the bag; record this information in your notebook. Working quickly, tear the plants into small pieces, approximately 1-inch, holding the plants over the jar and letting the pieces fall into the cold grinding medium. The jar should be on ice.

4. Cover the jar, turn the blender control to LOW range and blend on the "Easy Clean" setting for 5 seconds. With the blender OFF remove the lid and use your rubber policeman or rubber spatula to push all the leaves into the Grinding Medium. Replace the lid, and then blend for another 5 seconds. Repeat until you have blended the plant tissue for a total of 15 - 20 seconds. Do not over-blend: excessive blending will destroy the enzyme.

5. Pour the homogenate into the funnel. Using your rubber tool, scrape all the homogenate out of the jar into the funnel. Wearing a pair of latex gloves, gather the ends of the cheesecloth, and twist and squeeze out most of the liquid into the funnel. Discard the cheesecloth and gloves. Label the lid of the bottle with a small piece of tape.

Differential Centrifugation

6. Use grinding medium to balance the centrifuge bottle (including its cap) with that of another group and place the balanced bottles opposite one another in the Fiberlite F14-6X250 rotor (radius=137 mm).

7. With help from an instructor or TA, centrifuge the filtrate at a relative centrifugal force (RCF) of 5,000 times the acceleration due to gravity (5,000 x g) for 2 minutes at 4°C. Use either the table or the last equation in Section E of this chapter to approximate the necessary speed and fill in the blank in the next sentence before you come to class. A speed of _____ rpm is required for an RCF of 5,000 x g. Verify your answer with a TA. During this spin, rinse your blender jar and funnel and hang them on the racks above the sinks (Most of our centrifuges will allow you to set the speed in RCF. Some pieces of equipment will not and learning to use tables like the one in section E can be a useful skill.)

This centrifugation will pellet plastids, as well as large tissue fragments, unbroken cells, cell wall fragments, and cell nuclei.

8. When the spin is complete, retrieve your centrifuge bottle from the rotor and hold the bottle at the same angle as it was in the rotor. Pour out the supernatant *all at once* into a clean, chilled 250-mL centrifuge bottle. The pellet is loose, so be careful not to disturb it when decanting. Discard the pellet down the drain when you rinse the bottle.

9. Centrifuge the 5,000 x g supernatant (containing mitochondria) at 13,000 x g in the Fiberlite F14-6X250 rotor for 15 minutes at 4°C. Again, be sure that the centrifuge bottles are balanced, and the timing starts *after* the rotor achieves the specified speed.

10. Discard all the supernatant from this second spin into your used bottle and ultimately into the sink. The pellet contains "crude" mitochondria which will be washed to remove some of the contamination from broken thylakoid membranes and other cell debris.

11. Use the larger brush to resuspend the pellet now without any supernatant. When the suspension is homogeneous (no lumps), add 2-3 mL of wash medium with a 5-mL pipet, mix again with the brush, and transfer the suspension with the pipet to a 34-mL centrifuge tube. With a clean pipet, …

Wait – we're out of pipets. That likely isn't so, but if it is, visit the "Extra Supplies" bench near the window and take a new one if you need one. Recall, though, that there are islands of plastic in the oceans, so don't take what you don't need.

…rinse the 250-mL centrifuge bottle with about 5-6 mL of wash medium and add it to the 34-mL centrifuge tube with the pipet.

12. Pour in wash medium to the suspension to a level about 1 cm (0.5 inch) below the shoulder (Figure 5a) of the tube. Label the cap with a small piece of tape and use wash medium to balance your tube with that of another group. For this spin, you will use the F15-8x50 rotor. Centrifuge for one minute at 2,000 x g at 4°C.

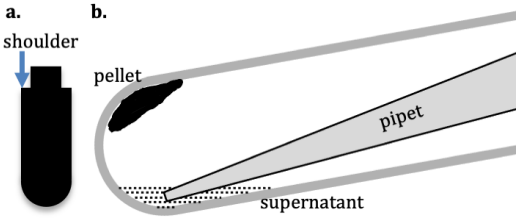

Figure 5. a) 34-mL centrifuge tube with shoulder indicated b) Remove remaining supernatant by holding the tube mostly horizontal, pooling the supernatant away from the pellet.

13. The pellet is very loose, so use a 10-mL pipet to transfer the supernatant to a second chilled 34-mL centrifuge tube. Use care not to mix the waste material in the pellet with the supernatant, which contains mitochondria. Discard the pellet. Label the cap of the centrifuge tube with a small piece of tape and use wash medium to balance it (including the cap) with that of another student. Screw on the cap and centrifuge in the F15-8x50 rotor at 10,000 x g for 15 minutes at 4°C.

Freezing Your Mitochondrial Sample

14. While your sample is in the centrifuge, prepare about thirty-five 0.5-mL tubes for freezing your samples.

Wait – we're out of tubes. If so, visit the "Extra Supplies" bench near the window and refill your tubes jar.

Label each tube lid with a distinctive letter or symbol for your lab group. Place the tubes on ice. Do not close the lids. You will be provided with your own freezer box for storing your samples once the samples are aliquoted.

15. The pellet is loose, so after the high-speed spin carefully remove the supernatant with a 10-mL pipet and discard it. Once most of the supernatant is gone, it is convenient to hold the tube as in Figure 5b to isolate your pellet from the remaining supernatant. The pellet contains your washed mitochondria. Resuspend the pellet first with the smaller brush. It is very important that the clumps be completely dispersed. When the suspension is homogeneous, add 3.0 mL of ice-cold mannitol freezing medium. Keep the tube on ice. Measure the final volume with a 5-mL pipet and record this value in your notebook. Keep the samples on ice.

16. Set your P-200 to 100 µL ("100" on the P-200 display) and distribute 100 µL aliquots of your *entire* sample into your labeled tubes. (An aliquot is a commonly used biology and chemistry term for a portion of a larger whole.)

Don't throw any away.

There are many important things to remember for this step:

-The mitochondria are in suspension; they will settle out to the bottom of the tube when left standing. To ensure accuracy and consistency in subsequent analysis, mix the suspension each time you create an aliquot. Mix by swirling or "finger vortexing," *i.e.*, flicking the bottom of the tube with one finger while securely holding the top with your other hand.

- Remember to also return the suspension to ice periodically to keep it cold.

- Recall to push the plunger of your pipet to the first stop before sucking up your suspension. Dispense by pushing to the second stop.

- This is the first of much pipetting you will do in 41C lab. Trade off with your lab partner at some point. Watch each other and kindly help each other to pipet well.

17. When the tubes are filled, close the lids and keep them on ice until your TA helps you place them in a storage box. The storage boxes are in racks "A - O" in the -70°C freezer in the instrument room, SS 123. The boxes are labeled A-1 to A-7, B-1 to B-7, etc. Sign up for a box on the sheets on top of the freezer. Also record your box number in your lab notebook so you can retrieve it next week.

Cleaning Up

18. Rinse your blender jar, blender lids, and funnels clean, and then hang over the sinks, or put beside the sinks if those racks are full.

19. Place your rubber spatula/policeman, paint brushes, and media and centrifuge bottles, tubes, and caps in their bins next to the sink nearest the classroom.

20. Trash the cheesecloth, pipets, and pipet wrappers.

21. Dump the ice in the sinks and place ice buckets on the cart near the classroom.

22. Wipe up any spills with damp paper towels. Every solution you used today has a high concentration of sugar, so spills will be sticky and attract ants.

D. Reagents

Grinding medium
0.3 M sucrose
30 mM MOPS buffer
5 mM glycine
4 mM cysteine
1 mM EDTA
0.6% w/v PVP-40
(poly(vinylpyrrolidone), average molecular weight 40 kDa)
0.2% w/v BSA
~0.1% v/v Antifoam-A (Sigma)
pH 7.2

Wash medium
0.3 M sucrose
25 mM MOPS buffer
3 mM glycine
1 mM EDTA
0.1% w/v BSA
pH 7.2

(Mannitol) Freezing medium
0.3 M mannitol
10% v/v DMSO (dimethylsulfoxide)
20 mM MOPS buffer
10 mM KCl
5 mM $MgCl_2$
1 mM EDTA
pH 7.2

E. Supplemental Section on Centrifugation

The relationship between RCF, usually measured in g-forces, and rotor speed, measured in revolutions per minute (rpm), can be calculated using a formula you likely learned in physics class, if you have taken one:

$a = r\,\omega^2$

where *a* is acceleration, *r* is the radius of the circular motion, and *omega* is the angular velocity. However, the units we usually use for measuring centrifugation are, without conversion, incompatible with this equation. *a* is measured in *g*'s, which is 9.8 m / s^2. *r* is the radius of the rotor, and is usually measured in mm. Last, ω is measured in revolutions per minute (rpm), and not in radians per second. Putting in the proper conversions, we get

$a\,[\,(\text{m}/\text{s}^2)\,/\,(9.8\ \text{m}/\text{s}^2)\,]$

$= r\,[\,\text{m}/1000\ \text{mm}\,]\,\{\,\omega\ \ (\text{min}/60\ \text{s})\ (\,2\pi/\text{revolution}\,)\,\}^2$

which yields

$a = 1.12\ (10^{-6})\ \text{g/mm/rpm}^2\ r\,\omega^2$

Renaming *a RCF*, for relative centrifugal force, and ω *RPM* and dropping the units, we get the formula $RCF = 1.12\ (10^{-6})\ r\,RPM^2$.

These equations all reveal that RCF depends on the radius, which is usually different for different rotors. It is for this reason that when describing the conditions of a centrifugation, the number of "g" used should always be reported, and not the speed. That way, anyone can still repeat the experiment even if a rotor with a different radius is used.

Today, some machines will detect the type of rotor and allow the user to set either the speed or RCF. When a centrifuge does not do this, one often finds a table near the centrifuge like Table 1, allowing one to set the speed to yield the appropriate RCF. Find the rotor being the used, read the RCF's below it until you find values that bracket your desired force, and then estimate the desired speed from the first column.

Table 1. How to convert between speed and RCF for various rotors.

	RCF (g)		
Speed (RPM)	Fiberlite F14-6X250	SM24 (outer ring)	SS34
500	38	34	30
1000	153	134	121
1500	345	302	272
2000	614	538	484
2500	959	840	756
3000	1381	1210	1089
3500	1880	1646	1482
4000	2455	2150	1935
4500	3107	2722	2449
5000	3836	3360	3024
5500	4642	4066	3659
6000	5524	4838	4355
6500	6483	5678	5111
7000	7519	6586	5927
7500	8631	7560	6804
8000	9820	8602	7741
8500	11086	9710	8739
9000	12429	10886	9798
9500	13848	12130	10917
10000	15344	13440	12096
10500	16917	14818	13336
11000	18566	16262	14636
11500	20292	17774	15997
12000	22095	19354	17418
12500	23975	21000	18900
13000		22714	20442
13500		24494	22045
14000			23708

F. References

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Chapter 2: Bradford Protein Concentration Assay

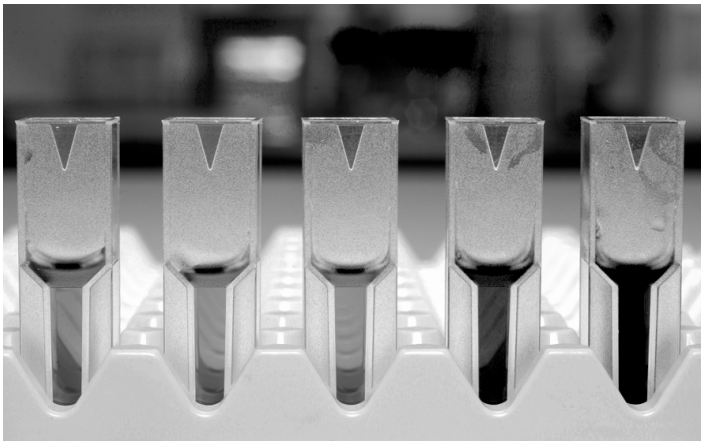

Figure 6. With increasing protein, Bradford assays become bluer. From left to right, increasing amounts of protein have been added to each cuvette resulting in a bluer color. Note that this black-and-white image has been altered to emphasize variation in blue and deemphasize the variation in red and green.

A. Bradford Assay Background

In the previous lab, you used differential centrifugation to isolate mitochondria. But how do you know SDH is active in the mitochondrial fraction. How much protein do you have? We will answer the latter question this week, and former next week. We will use an indispensable biochemical technique, spectrophotometry, to answer both.

The *spectrophotometer* (colloquially “spec”) quantifies color as an absorbance reading at a particular wavelength of light. (See Supplemental Section D for a greater general discussion of spectroscopy.) The Bradford protein concentration assay is a simple colorimetric method where the amount of color change in the sample is proportional to the concentration of protein present. This color change will be measured as *absorbance* on the spectrophotometer.

In order to know how this color change is proportional to protein concentration, you will create a *standards curve* -- a best-fit curve through a plot of known protein concentrations versus the resulting absorbance measurements. Then you will take measurements of your mitochondrial samples, *interpolate*, and determine the protein concentration of the mitochondrial samples. In subsequent weeks, this concentration will allow you to normalize your enzyme reaction rates so that you can compare your results with those of others. You could express the enzyme activity per milligram of protein used in the assay.

In addition to the important concepts and skills already mentioned above, this is the first lab where *quantitative pipetting* is important. Your instructor will discuss this in prelab.

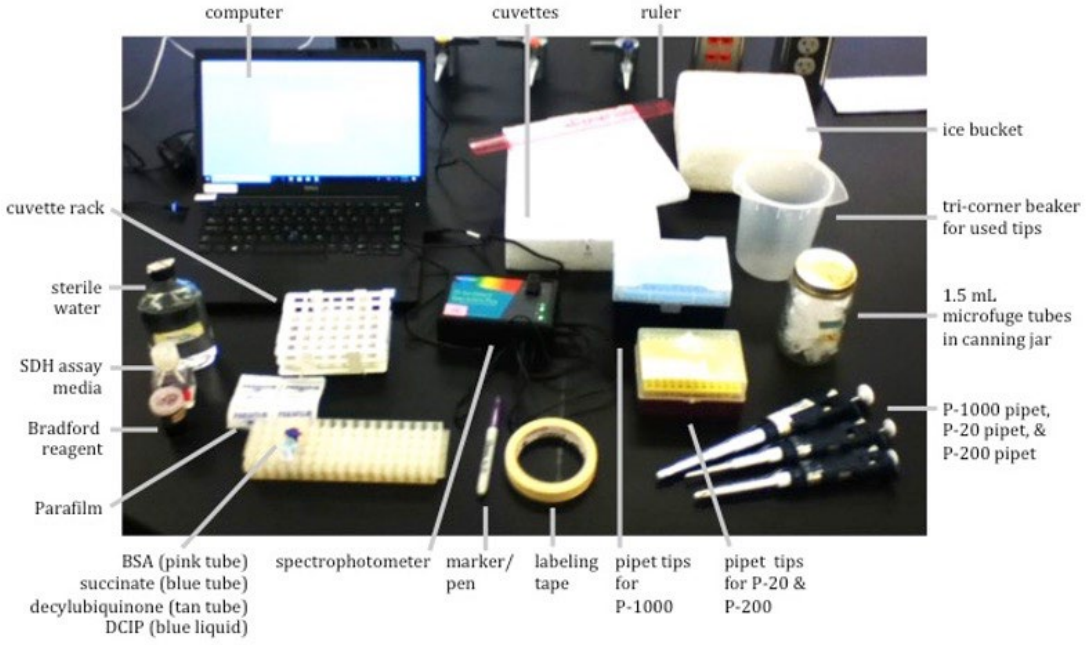

Figure 7. Equipment to be used this week and next.

B. Experimental Procedure: Bradford Assay

Warm up the spectrophotometer

1. Plug in the computer. If the computer is not on, turn it on and log into the account ".\lab" with the password "Biology47".

2. Check the connections to the spec. The power cable should connect an outlet and the small round hole on the right side of the spec. The USB cable should connect a USB port on the computer and the other hole on the right side of the spec.

3. On the computer's desktop, open the folder "41C Logger Pro" files. Open the file "Bradford Assay, Absorbance at 595 nm."

4. Warm up the spec's lamp for 5 minutes. To do so,

- click the "Experiment" menu,
- choose "Calibrate," an
- then choose "Spectrophotometer: 1"

This will begin a 90 second countdown, but wait five minutes total before blanking the spec. Doing so will yield more consistent results.

Preparing the protein standards for the standards curve

5. You are going to measure triplicate sets of standards. Label five sets of three disposable 1.0 mL (semimicro) cuvettes as follows: "2.5"x3, "5.0"x3, "7.5"x3, "10.0"x3, and "12.5"x3. Label one cuvette "0" to use as a blank.

But we ran out of cuvettes! Like last lab, if you run out of something, it is likely on the "Extra Supplies" bench near the window. Help yourself. Cardboard boxes go into the blue recycling bins and not the trash; plastics used in labs are unfortunately not recyclable and need to go in the trash.

More tips can likewise be found there. Take your empty tip box there to be refilled later; take another with tips.

6. Copy Table 2 (below) to your lab notebook and complete it by calculating the volume of stock 100 µg/mL bovine serum albumin (BSA) protein standard and water for each desired standard solution. (Recall that $V_1C_1=V_2C_2$ and 1000µ = 1m.) Do this before class.

Table 2. Increasing volumes of a protein standard solution consisting of 100 µg /mL (C_1) of bovine serum albumin (BSA) is diluted in water in microcuvetes for final concentrations varying from 0 to 12.5 µg/mL (C_2) in a final volume of 800 µL (V_2).

Final concentration of BSA (µg /mL)*	µL of BSA standard	µL of water	Total volume (µL)
0.0	0	800	800
2.5	20	780	800
5.0			800
7.5			800
10.0			800
12.5			800

* in the 800 µL

7. Use the appropriate pipettor (Table 3) to dispense the correct volume of dH2O (Table 2) into the corresponding cuvettes in each set of standards. After the dH2O has been dispensed, dispense the protein standard. Take up the correct volume of standard. Place the pipet tip in the water inside each cuvette or against the inside of the cuvette. Make sure there is no extra protein on the *outside* of the tip before you deliver it to the cuvette. Be accurate! 10% pipetting errors can easily yield 10% errors in your answers.

When you are finished with this part you will have 3 sets of diluted standards.

Table 3. Which pipettors to use for what volumes, and how the displays would read for example volumes. Please ask for assistance if you have any questions about how to use these.

	P-1000	P-200	P-20
Intended volume range in µL	200-1000	20-200	(1-5)-20
Examples of the pipettor display	0 6 2….3 ^	0 8 0	2 0 0
should dispense	624 µL	80 µL	20.0 µL

Bradford assay on standards

8. Use a P-1000 to pipet 200 µL of Bradford reagent into each cuvette of your standards, including the zero standards. The Bradford reagent is viscous, so be sure all of it goes into the cuvette. To mix, place a piece of Parafilm over the cuvette opening and seal it with your finger. Invert the cuvette until the sample is well mixed, but don't shake it: bubbles will interfere with subsequent absorbance measurements. Hint: You can use the same piece of Parafilm several times if you blot it on a Kimwipe between mixings.

Caution: Pipet very carefully. Bradford reagent contains a high concentration of phosphoric acid and is very corrosive. Release the pipettor plunger slowly so the reagent doesn't get into the pipettor barrel and corrode it, destroying its accuracy. Wear gloves and take special care not to spill or splash it. Immediately clean up any spills using lots of water.

Note: that usually we'd use the P-200 set to "200" to dispense 200 µL. However, we choose to use the P-1000 with its larger tips, since the Bradford Reagent's high viscosity impairs accurate pipetting with smaller tips.

Wait five minutes for the color to develop. Meanwhile, construct a data table in your lab notebook for recording these data.

9. Now blank (a.k.a. "zero") the spec: Insert the "0" cuvette into the spec's square hole so that the cuvette's clear sides are parallel to the spec's short sides. Click "Finish calibration," wait a few seconds, and then click on the un-grayed "Ok."

10. Remove your blank and replace it with one of your samples. Record the A_{595} in a table in your notebook. Continue until you have read all of your samples.

Plotting the standards

11. On a full-page in your lab notebook, plot each A_{595} as a function of protein concentration. Use the y-axis for A_{595} values and the x-axis for protein concentration (µg/mL). Be sure to include the graph's origin as a data point in your graph. (Including this point, you should have 16 points.) Label the axes so that you use as much of the grid as possible.

12. If Beer's Law holds true without deviation, your plots should be linear, and you should be able to draw a straight line to "fit" the data. However, in most cases at high concentrations, equilibrium factors cause the function to become nonlinear. It is thus necessary to draw a curve which best fits the data. Even in these cases, the plot is linear at low concentrations, so one can use a straight edge for the first part of the plot.

Draw a best-fit curve for your set of standards data points. A best-fit line is one that minimizes the distances in the horizontal and vertical directions between the line and the data points. However, it does not snake its way through the data to hit every point. As one TA said one year, there should be "no points of inflection."

Prepare mitochondrial samples

In order for your protein assay to be useful, the A_{595} for your sample must fall within the range of values that you measured for the standards. Since you have little idea what the protein concentration is for your suspension of mitochondria, you will make several dilutions of the sample. One of them will probably fall within the range of the standards curve.

Your mitochondria suspension is very concentrated, so you must first dilute by a dilution factor of 100-fold. Remember:

$$\frac{\text{final volume}}{\text{sample vol. used}} = \text{dilution factor}$$

13. Go to the freezer and get the last tube you filled during the last lab, which might contain less than 100 µL. You may let this tube and only this tube thaw in your rack. In subsequent weeks, always thaw your mitochondria on ice so as to retain as much enzymatic activity as possible. This week, this is not important since you are not measuring enzyme activity.

Place 990 µL [P-1000 set to 099] of dH_2O into a 1.5 mL microcentrifuge tube from the jar on your bench. Then add 10 µL of your well-mixed mitochondria suspension, and mix well by flicking and inverting the tube with your finger to be sure all of the suspension is in the dH_2O. Use this 100-fold dilution to dispense your sample into the water in the labeled cuvettes below.

14. Transpose Table 4 to your lab notebook and complete it before class. For example, in the second row, 800 µL / 80 µL = a dilution factor of 10.

15. Make the dilutions of your 100-fold diluted sample.

Table 4. The dilutions of your mitochondrial samples. Finish the table.

1st dil. factor	2nd dil. factor	Final dil. factor	µL of dH_2O	µL 100-fold dil. sample	µL final volume
100	4				800
100	10	1000	720	80	800
100	40				800

Bradford assay on mitochondrial samples

As with the standards curve, we will perform the measurements in triplicate. Then we will plot the mean values.

16. Set up 3 sets of 3 cuvettes. Label one set "400," one set "1000," and one set "4000." These are the dilution factors that we will use for our mitochondrial suspension. Dispense the correct volumes of H_2O and 100X

dilution of your mitochondria sample into the cuvettes following your chart. Add 200 µL of Bradford Reagent to each cuvette [P-1000 set to 020]. Mix, wait 5 minutes, and read A_{595} as before.

Interpolate the protein concentration of your sample

17. Calculate the mean A_{595} value for each of your diluted samples. Interpolate from the standards curve to determine the protein concentration. In other words, find the A_{595} value on the y-axis, move horizontally until you intersect the standards curve, and then go down from there to the x-axis and record the protein concentration at that point on the x-axis.

Calculate the protein concentration

18. Decide which value or values from your plot are more trustworthy. Use this/these concentration(s) of the diluted sample(s) you assayed by multiplying the interpolated concentration(s) by the final dilution factor(s) to determine the protein concentration of your mitochondria.

Cleaning Up

19. Place the BSA protein standard in the appropriate rack on the front table.
20. Pour the contents of your cuvettes down the sink. Wash it down with some water.
21. Trash used tubes, used cuvettes, Parafilm, and the contents of your tip bucket.
22. Shut down the computer. Unplug the spec and the computer at the outlets.

C. Reagents

Bradford reagent

Coomassie Brilliant Blue G-250 dissolved in acidic conditions. We typically buy it from Bio-Rad Laboratories, and they do not divulge their exact recipe.

BSA

0.1 mg/mL bovine serum albumin dissolved in distilled water.

D. Supplemental Section on Spectrophotometry

The Nature of Light

Electromagnetic radiation occurs over a very broad spectrum of wavelengths. Radiation from particular parts of this spectrum is given various titles such as radio waves, visible light, X- and gamma rays, and ultraviolet and infrared. The portion of radiation which is classified as visible light extends over a small part of the electromagnetic spectrum within the wavelengths from 6×10^{-6} meters to 1×10^{-7} meters. Between these limits there lies a still smaller region that is visible to our eyes: within the wavelength range from 4×10^{-7} meters to 7.5×10^{-7} meters (400 nm to 750 nm). For reasons of convenience the wavelength of light is usually expressed in nanometers (nm); 1 nm = 10^{-9} meter.

In the visible region of the electromagnetic spectrum particular wavelengths are perceived as different colors. The term monochromatic, meaning “of single color,” is therefore used to describe wavelengths selected from a very small part, or band, of the spectrum.

There is a reciprocal relationship between the wavelength (λ) and the frequency (ν) of light such that their product is equal to the speed of light (c), which is a constant (3×10^{8} m sec^{-1}).

$$\lambda \nu = c$$

Thus, light of a short wavelength will have a high frequency, and light of a long wavelength a low frequency. Table 5 shows the names given to the various wavelengths commonly classified as light.

Table 5. Types of electromagnetic radiation in and near those visible.

Wavelength in nm	Band or *color*
100-250	far ultraviolet
250-400	near ultraviolet
400-750	visible
400	*violet*
550	*green*
700	*red*
750-2000	near infrared
2000-6000	far infrared

Light is emitted in minute, discrete quantities; a quantum of light is a photon. The energy, E, (in ergs) contained in one quantum of light radiation is directly proportional to frequency and inversely proportional to wavelength. The factor of proportionality is Planck's constant, h (6.554 x 10^{-27} erg second). The relationship between light energy and frequency or wavelength are given by the following equations, respectively:

$$E = h \nu \qquad \text{or } E = h c / \lambda$$

A beam of monochromatic light can be thought of as a stream of photons, all having closely similar energies.

Absorption of Light by Molecules

A beam of light passing through a substance can be absorbed if the energy of the photons happens to coincide with a particular energy transition available in the absorbing substance. So, if light of changing wavelengths is passed through a substance, the absorption of that light will rise to a peak value and then fall again as the wavelength coinciding with an energy transition is reached and passed. In some substances, several such peaks may be found and may even superimpose to form a broad peak.

If the amount of absorption is measured and plotted against wavelength, the resulting curve is called an *absorption spectrum* and is characteristic of the absorbing substance. The absorption at a wavelength where absorption is greatest is termed the peak extinction, extinction maximum, or absorption maximum (λ_{max}).

If absorption is measured at a fixed wavelength, the value obtained will depend on three factors. First is the thickness of absorbing matter through which the light passes. The thicker it is, the more absorption can take place because there are more absorbing molecules in the light path. Second is the concentration of absorbing matter present. If there are more absorbing molecules per unit volume [higher concentration], then more light will be absorbed. Third is the efficiency with which the matter can absorb light. If one absorbing molecule is more efficient than another at absorbing the light, then at equal concentrations and thicknesses, a sample of the first molecule will absorb more light than one of the second. In the ultraviolet and visible regions of the spectrum, measurements are normally made on solutions, whereas infrared spectra are often determined on solid samples because solvents tend to absorb in this part of the spectrum.

Measurement of Light Absorption

A spectrophotometer is a widely used and indispensable instrument for the study of cell and molecular biology as well as many other disciplines. Therefore, it is important that you learn how it works.

A spectrophotometer measures the intensity of monochromatic light that has passed through a sample, with the objective of determining how much light was absorbed (or scattered) by the sample.

Because absorption varies with wavelength, it is essential to have a light source and wavelength selector (monochromator) that together produce a beam of light with a narrow band of wavelengths. The *amount* of light in this beam will vary at different wavelengths because the spectral distribution of energy from the source lamp is uneven. The efficiency of the photo detector is also wavelength dependent. In addition, since the sample is a solution that must be contained in a suitable vessel (the cuvette), a reference (blank) measurement is necessary to compensate for absorption by the solvent and cuvette.

In practice two identical cuvettes are used: the reference or "blank" cuvette, containing only the solvent, and the sample cuvette, containing the test solution. At a given wavelength the photo detector will respond linearly to the amount of light falling on it, so when the reference cuvette and the sample cuvette are placed into the beam one after another, the ratio of the amount of light transmitted by the two of them can be determined. This ratio is called the *fractional transmittance* of the sample (or when multiplied by 100, the *percentage transmittance*). The concentration of absorbing substance is *not* directly proportional to the fractional transmittance, however. The reason for this becomes clear if absorption is considered at a molecular level.

Consider the case in which the wavelength of a parallel monochromatic beam of light passing through a solution is chosen to be such that the solute can absorb light. The chance that a given solute molecule will absorb a photon will be proportional to light intensity. Similarly, the chance that a photon will collide with a solute molecule will depend on the solute concentration. From these facts follow the laws of light absorption.

Laws of Light Absorption

Lambert's Law: For a thin layer of a given sample, the same proportion of the incident light is absorbed whatever the incident intensity.

A sample cell can be thought of as being divided into a number of thin layers, each successive layer absorbing the same proportion of light falling on it. Thus, the first layer absorbs half the light, the second layer will absorb half the remaining light, (*i.e.*, a quarter of the total initial light) and so on. Extrapolating this argument to layers of infinite thinness, the light intensity will decrease exponentially through the cell as successively more of it is absorbed. This may be expressed mathematically as

$$\ln (I_0 / I) = a L$$

where I_0 is the incident light striking the cell, I is the emergent light, a is a constant, and L is the path length of the cell (Figure 8).

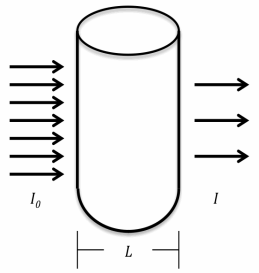

Figure 8. Pictorial definitions of the variables discussed.

Beer's Law: Light absorption is proportional to the number of absorbing molecules. Using an argument like before:

$$\ln (I / I_0) \propto c$$

where c is the concentration of the solute molecules and $\propto$ is the proportionality symbol.

Combining the two laws above, we obtain

$$\ln (I_0 / I) = a' c L$$

or

$$\log_{10} (I_0 / I) = 2.303\, a' c L$$

where the constant $a' = ac$.

If c is the molar concentration of the solute and L is in cm, then 2.303a' is a constant called the *molar extinction coefficient*, normally given by the symbol ε. (Other extinction coefficients may be in other units of concentration, *e.g.*, mg/mL or %.)

Log_{10} (I_0/I) is termed the *absorbance* (A) or *optical density* (OD) of the solution and is directly proportional to the concentration of solute if the path length is kept constant. Thus, the expression above becomes the *Beer-Lambert Equation:*

$$A = \varepsilon c L$$

Using this extremely useful expression it is possible to calculate the concentration of a solution if the extinction coefficient of the solute and the path length of the cell are known, simply by measuring absorbance. The extinction coefficient is given by:

$$\varepsilon = A / (c L)$$

A is a ratio and therefore unitless, c is often expressed as molar (moles/liter), and L is often expressed as cm. Using these ε would have units of M^{-1} cm^{-1}. Since ε varies with wavelength it is usually quoted at the peak extinction wavelength – where the solute absorbs maximally. For example, for chlorophyll *a* dissolved in 80% acetone (and 20% water), ε_{663} = 82.04 mL/mg cm^{-1}. (Yes, those units are not M^{-1} cm^{-1}. If this troubles you now, just wait.) This means that if one measures the absorbance of chlorophyll *a* at a wavelength of 663 nm in a solvent of 80% acetone, one can substitute 82.04 into the Beer-Lambert equation, use the measured absorbance value, and solve for c, concentration. In this case, what will be the units of concentration? ……. Yep, mg/mL.

Chapter 3: SDH Enzymatic Assays & K_m and V_{max} Determination

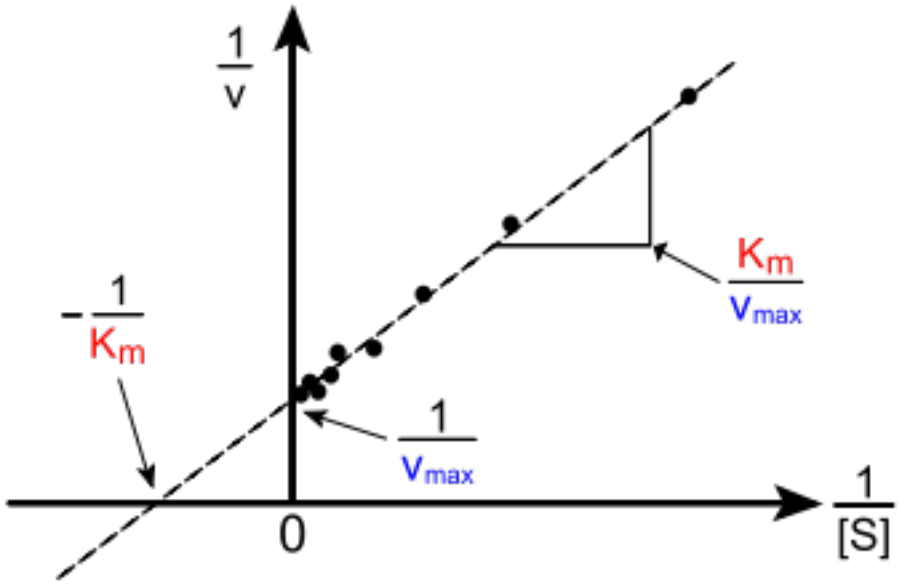

Figure 9. The Lineweaver-Burk Double-Reciprocal Plot. The reciprocal of the initial enzymatic velocity, 1/*v*, is plotted as a function of the reciprocal of the substrate concentration, 1/[S]. K_m can be calculated from the *x*-intercept and V_{max} from the *y*-intercept.

A. SDH Assay Background

In the previous lab, you determined the protein concentration of your mitochondria. This week you will learn if the SDH in your mitochondria are active, and how active they are. Additionally, you will determine the enzyme's K_m – the substrate concentration at which the reaction rate is half maximum – and the V_{max} – the theoretical maximum rate of reaction for a given amount of enzyme. Much of the equipment you will use is the same as last week (Fig. 7), but you will use it in very different ways.

Succinate dehydrogenase catalyzes the oxidation of succinate to fumarate during the TCA cycle in the mitochondria. *In vivo*, this reaction requires an electron acceptor: the co-enzyme flavin adenine dinucleotide (FAD). To monitor the succinate to fumarate reaction *in vitro* requires the chemical reduction of an *artificial electron acceptor*. To use an artificial electron acceptor, the normal path of electrons in the electron transport chain must be interrupted, which is accomplished by adding a quinone to the reaction mixture. The quinone we will use is *decylubiquinone,* (duq) and we will use it at a high enough concentration such that it will out-compete Coenzyme Q (ubiquinone), the endogenous electron acceptor for succinate dehydrogenase in the mitochondrial inner membrane. Instead of following the normal pathway to cytochrome oxidase, electrons are intercepted and delivered to the artificial electron acceptor, *2,6-dichloroindophenol (DCIP).* When electrons are accepted by DCIP it turns from blue to colorless. The reaction can be summarized as follows:

$$E\text{-}FADH_2 + DCIP_{oxidized}\,(blue) \rightarrow E\text{-}FAD + DCIPH_{2\ reduced}\,(colorless)$$

Since the oxidized (without added electrons) form of DCIP absorbs light maximally at 600 nm (λ_{max} = 600 nm), the change in absorbance, measured at 600 nm, can be used to measure the extent to which the reaction has taken place. In other words, the rate of change of the absorbance can be used to directly measure the rate of the reaction. We will monitor the absorbance continuously and collect the data on a computer. Then we will use software to determine the initial slope of the change in absorbance as a function of time to give the initial rate of the reaction.

Consider how you will assay for succinate dehydrogenase (SDH) activity. In any assay, the more enzyme present, the higher the rate of the reaction. The rate should increase proportionally to the amount of enzyme,

provided that substrate is not limiting. Hence it is important to add the same amount of enzyme each time, by pipetting accurately and by mixing the mitochondria before adding it to each reaction.

Also, the longer the mitochondria are thawed, the more the enzyme has denatured. Hence, only thaw one tube at a time and treat any tube thawed for more than 20 minutes as "bad." You should get two to four assays per tube of mitochondria. Therefore, it is important that you have all of the components of the assay assembled, and have the computer and spectrophotometer ready for the reaction to start *before* you thaw your first frozen sample.

Section C contains background on LB plots.

B. Experimental Procedure: First SDH Assays

Calculations

1. Copy Table 6 to your lab notebook and complete it. First, calculate the volume of your mitochondrial suspension that contains 100 µg of protein using the concentration you arrived at last week. Check this calculation with your lab partner. Do these before coming to class. Now, calculate the volume of Assay Medium that will make the volumes of all the components in a row add up to 1.0 mL. (How many µL are in a mL? If in doubt, check chapter 2, section D.)

Warm up the spectrophotometer

In this experiment, the spectrophotometer will send data to your computer via the USB port allowing you to measure the progress of the reaction in real time.

2. Follow steps 1 to 4 of the previous chapter except in step 3 open the file "SDH assay." In addition to the application menu, you should see a table, an incomplete graph, and "Absorbance at 599.9 nm."

Blanking the spectrophotometer & running the first assay

3. The first step in making a measurement is to zero the spectrophotometer with the blank. (Each day you should make one new blank.) This will correct for any lights scattering by the mitochondria themselves. Label one cuvette tube "B" (for blank) on the top half of the cuvette. Add the room-temperature components, except for the mitochondria, from left to right as indicated in the top row of Table 6. (The mitochondria will be added later.) Cover the cuvette with parafilm, and invert twice to mix the contents. Why is there no DCIP in the blank? If you're not sure discuss this with your lab partner, a TA, and/or your instructor.

Table 6. First SDH assay components. Note that the 20 mM succinate sample will be performed in triplicate. Duq is always in brown tubes at 3.0 mM.

Sample	Assay medium	Duq	1.00 M Succinate	DCIP	Mitochondrial suspension
Blank	____ µL	10 µL	20 µL	0 µL	____ µL
20 mM succ.	____ µL	10 µL	20 µL	20 µL	____ µL
0 mM succ.	____ µL	10 µL	0 µL	20 µL	____ µL

4. Prepare the cuvettes for your 20 mM assays. Label 3 cuvettes "20" as before. Add the components as shown in the table, again leaving out the mitochondria, and mix.

Which pipet should be used for which volume? How do we set the volumes properly? Table 3 in the previous chapter will help.

Some tire of changing the volumes of the pipets. Many things like this are part of learning to work effectively in the lab. Pipet the same volume repeatedly when you can. Trade off with your lab partner. Do not take a pipet from another bench; this causes problems later on.

5. Thaw one aliquot of frozen mitochondria on ice. While waiting, set your pipettors to the correct volume for 100 µg protein of your mitochondria suspension. Check the mitochondria to see if it has thawed by flicking the tube. Once thawed, keep it on ice. Remember, mitochondria are in suspension; mix well before taking out a sample.

6. As soon as the mitochondria sample has thawed, thoroughly mix it, add the correct volume of mitochondria to the blank cuvette, invert the cuvette with Parafilm twice to mix, and place it in the spectrophotometer so that the clear sides are parallel to the short sides of the spectrophotometer. As in the last chapter, click "Finish calibration," wait a few seconds, and then click on the un-grayed "Ok."

7. Mix the mitochondrial suspension thoroughly, and add the correct volume to one of the cuvettes labeled "20." Mix by inverting twice more, and place it in the spectrophotometer. Click on "COLLECT" (Figure 10). Allow the reaction to proceed for about 2 minutes, then click on "STOP."

Note the shape of the curve. Where does it seem the most linear? In subsequent runs, you will likely not need to run the assays for 2 minutes. The time will depend on where it is linear.

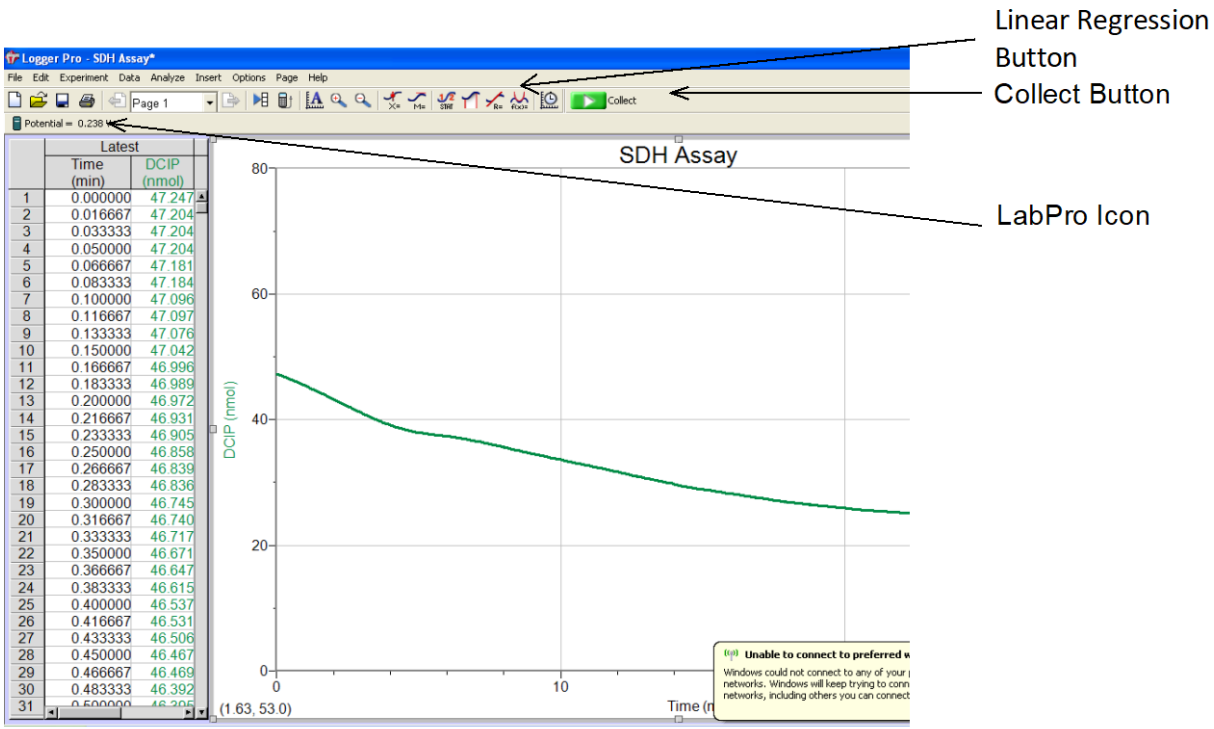

Figure 10. A LoggerPro screen with key buttons pointed out.

Determining the rates of reaction from the slopes of the plots

8. The plot is typically quite linear, particularly after running for 10-30 seconds. Position the cursor at the left-most edge of the linear region click-and-hold while you move the cursor to the right-most edge of your region of interest, and then release. The selected portion of the data will be outlined by two vertical lines. Choose a region at least 20 seconds wide.

For a linear regression, click on "Analyze" from the menu bar, and select "Linear Fit" from the Analyze menu, or alternatively use the "Linear Regression" button, "R=" on the tool bar (Figure 10). A line will be fitted to the region of the plot you have selected, and a box will appear indicating the "m" and "b" values for the equation of the line. Record the "m" values (slopes) in your notebook. Include units for the slopes. Why is the slope negative? Again, if you are unsure, discuss this.

Save each run with an informative name in the folder within "41C Logger Pro Files" corresponding to your lab day.

9. Repeat steps 7 & 8 for the second and third "20" cuvette. Likely, running the assay for one minute should be sufficient now. If your current tube of mitochondria does not contain enough, throw away the remainder and thaw another.

Important notes: Mitochondria thawed for 0 to 20 minutes show approximately the same activity, but after that the activity begins to drop. Plan to use as much of a tube of mitochondria as you can within 20 minutes of thawing.

10. When done with these assays, check with your instructor or TA. These results can reveal when SDH activity is too weak or inconsistent to proceed effectively with the remainder of this project. Also, she might suggest

that you alter the volume of mitochondria so as conserve them for future assays. If there are changes to the procedure, you will need to rerun these assays.

11. Prepare the cuvette for your 0 mM assay. Label 1 cuvette "0." Add the components as shown in the table, again leaving out the mitochondria, and mix. What would you predict the rate to be with no substrate added?

Add your mitochondria and mix and run the assay as before.

What could account for any enzyme activity in this sample? Is this troubling? This is another potential moment for discussion.

C. Lineweaver-Burk Background

You will now do more SDH assays, plot their rates of reaction, and construct a Lineweaver-Burk (LB) plot to estimate K_m and V_{max}. These values might well be useful for your independent projects. Additionally, in your independent projects, you likely will make several LB plots under different conditions, so the practice this week will be valuable.

Some of the SDH assays you will need to perform will involve succinate concentrations below 5 mM. For this, you would need to add less than 5 µL of a 1 M stock. Your P-20 pipet will pipet volumes between 0.5 and 5 µL, but in order to increase the accuracy of your additions, you will need to perform a dilution of your 1 M succinate stock and add more than 5 µL of this more dilute, secondary stock. This, and other dilution complications are covered in section E.

Stepping back, some students are startled by the terse nature of some of the instructions in the next section. This is a steppingstone for you. In two weeks, you will be performing experiments of your own design, are you will make all the detailed instructions. In the next section, you will need to fill in the details of the procedure.

D. Experimental Procedure: Lineweaver-Burk plot

Equipment set up

12. Your spectrophotometer and computer are already on, and the spec is blanked. If it had not been, you would need to perform many of the steps in section B.

SDH assays

When choosing *x*-values – succinate concentrations – for your LB plot you want at least one point at a fairly high substrate concentration. The 20 mM samples you have already run will serve as that high substrate concentration.

When estimating a line from a plot of points, it is best to have the values along the x-axis evenly spaced. To do otherwise creates *leverage points*, or points have undue influence on the estimated line. Since the LB plot is a *double-reciprocal plot*, you want to choose substrate concentrations whose reciprocals are evenly spaced. If you chose substrate concentrations that were themselves evenly spaced, like 5, 10, 15, and 20 mM, that would yield a plot like in Figure 11.

Figure 11. Four evenly spaced values do not have evenly spaced reciprocals.

These are clearly not evenly spaced, and 1/5 would be a leverage point. A better set of concentrations would be 2, 3, 5, and 20 mM. Plot the reciprocals of these for yourself and see.

13. Now perform SDH assays for 2, 3, and 5 mM in duplicate (2 cuvettes for each concentration.)

14. Plot the reciprocals of the succinate concentrations versus the reciprocals of the absolute values of the rates. You should have 9 points. Is this linear? It is never perfect. What might account for the non-linearity? Hint: what happened when you added no succinate back in section B. Yet again, this could be another moment for discussion.

15. Calculate K_m and V_{max}. Figure 9 at the beginning of this chapter provides a reminder as to how to do this from your plot.

Cleaning Up

16. Keep the provided 1 M succinate stock, duq, and DCIP in the rack on your bench.

17. Pour the contents of your cuvettes down the sink. Wash it down with some water.

18. Trash used tubes, used cuvettes, used mitochondria, parafilm, and the contents of your used tip buckets.

19. Dump the ice in the sinks and place ice buckets on the cart near the east sink.

20. Shut down the computer, and unplug the spectrophotometer and the computer at their outlets.

21. Generally tidy up.

E. Reagents

(SDH) assay medium
50 mM MOPS
1.0 mM potassium EDTA
0.01% w/v Triton X-100
KOH to bring the solution to pH 7.2

Duq (decylubiquinone)
3.0 mM in DMSO
(This has an absorbance of about 0.90 at 450 nm.)

Succinate stock
1.0 M succinic acid in assay medium, pH'd to pH 7.2 with potassium hydroxide

DCIP (2,6-dichloroindophenol)
2.0 mM in assay medium

F. References

Hardin J, Gertoni G, Kleinsmith LJ. 2012. Becker's The World of the Cell, Eighth Edition, Pearson Education, Inc., San Francisco.

Chapter 4: Proposals, Independent Project Execution, and Paper

A. Introduction

In Chapter 3 you measured the K_m and V_{max} catalyzed by succinate dehydrogenase (SDH) in isolated mitochondria. Now that you are familiar with the equipment and methods of carrying out the SDH assay you should able to formulate a hypothesis about some aspect of SDH that interests you, and conduct an experiment of your own design. Your assignments regarding searching and reading scientific literature should be helpful in determining this aspect.

Your hypothesis must be testable using the available equipment and supplies. You will have two weeks to complete your experiments and interpret the results. You will present your data and findings in a written lab report.

B. Experimental Design

First, decide what aspect of succinate dehydrogenase activity you wish to study. For example, you may want to examine some property generally applied to enzymes like inhibition. You should use your knowledge from the lectures and reading material, but the primary sources for your inspiration should be scientific papers and your own curiosity. Use the literature to find out what other work has been done and published in journals on the topic. Express your question or hypothesis concisely and demonstrate the line of reasoning you followed in formulating your hypothesis. Also, state the importance and relevance of your particular question or hypothesis.

Next, you and your partner are to design an experiment to answer your question or test your hypothesis. Be sure to include the controls necessary for you to interpret your results unambiguously. Make specific predictions for the outcome of your experiment based on your hypothesis.

Evaluation of your project will include an assessment of the quality and sophistication of your project, the hypothesis, as well as the experimental approach and design.

C. Feasibility

Keep in mind that the experiment you design must be feasible. Think about the reagents which must be prepared and whether your experiment can be carried out given the facilities at your disposal. Look around the lab and see what is available, and/or ask your laboratory instructor if certain equipment can be used or whether special chemicals can be purchased.

1. Some reagents may be extremely toxic and special precautions must be taken for their use. Others are too toxic to permit in the lab.

2. Radioactive, explosive, federally illegal drugs, or extremely expensive materials are not allowed.

3. If using a special reagent, is it soluble in water? (Google it and compare the concentration you might find to those you might want to use.) If not, will the solvent of choice be compatible with SDH activity?

4. What concentration of the reagent will you want? Though you do not need to keep rigidly to your plans, you need some idea of a concentration not pulled out of thin air.

5. The temperature of the reaction can be controlled by fitting the spectrophotometers with cuvette holders attached to a circulating water bath. (We have a limited number of these.)

Do not consider temperatures over 70 C, since that is the limit of tolerance of the tubing used to connect the devices. Also, do not propose an experiment to "see how temperature affects activity" or to see at what temperature the enzyme denatures. These experiments typically lead to trivial interpretations of the data.

6. pHs below pH 6.2 and above pH 8.2 change the indicator dye color and therefore cannot provide information about the reaction. If changing pH from the "normal" 7.2, do not plan on going above or below this range.

7. Nitric oxide (NO) emitting compounds are expensive and tend not to have effects in our assay.

If you have concerns about the feasibility of your experiment, ask your instructor **early** for advice before proceeding so you don't waste time considering an experiment that cannot be done.

Always start with a "pilot" experiment to make sure your system is working as expected. Don't waste time and materials setting up hundreds of cuvettes only to find a flaw in the experimental design.

D. Approval

Construct an outline of your experiment, including the necessary controls. This outline should include all of the **specific** details of the experiment. Referring to it, make a list of all materials, chemicals and equipment **in addition to any you used in Project I-B** that you will need. You may assume that all materials used in Chapter 3 will be available. Bring this "outline" to lab for discussion of your project on the specified date.

You must submit a final written proposal, both hard and electronic copies, on the due date (see lab schedule). The proposal must include the following:

1. background information, including citations to your references
2. your question and hypothesis,
3. the rationale for your hypothesis (the information and thought processes that led you to it),
4. the significance of your question,
5. your experimental approach,

including

6. your flow diagram, and,
7. your predictions, based on your hypothesis.

The proposal must cite at least 5 references from primary literature in a "Literature Cited" section (see Section G). Approval will be based on two primary criteria: (1) whether or not the experiment will unambiguously test your hypothesis or answer your question, and (2) whether or not the experiment is feasible (materials needed are readily available, and the experiment can be carried out in two lab periods.)

E. Execution

Again, you have two lab periods to execute your experiment. Come prepared to work each day with a basic outline of what you plan to do, and all the detailed calculations of volumes and concentrations you can.

In addition to the clean-up procedures from the last chapter, please return your special reagents as directed by your instructor.

F. Interpretation & Presentation of Results

Following your experiment, you and your lab partner will give a written report of your results. Specific instructions will be given by your instructor. Ask yourself:

What is the significance of the answer?
Was your hypothesis supported or not?
If not, what is your alternative hypothesis, based on your results?
What conclusions can you draw from your results?
What would your next experiment be? Why?

Materials

Mitochondrial isolation

For 24 students working in pairs, one would need the items listed below for the mitochondrial isolation. *'d items will be used in the remaining weeks of this lab.

Items needed for each student group

(1) Approximately 720 grams of black eye pea sprouts, divided into twelve for the student groups. Detailed directions for their growth are given in Appendix D. To reliably grow this quantity of sprouts, one needs

- (a) 1020 trays or flats. Count 12.
- (b) 1020 non-vented humidity domes. Count 12.
- (c) Vermiculite to half to 2/3 fill said trays or about two cubic feet
- (d) about 450 g of seeds
- (e) Sufficient dark and warm space to grow them.
- (f) dishpans or large buckets for rinsing the pea sprouts
- (g) a salad spinner.
- (h) plastic bags. Count 12.

(2) Conventional kitchen blenders. Count 12.
(3) Round paint brushes for each student group. Twelve large (approximately size 4) and twelve small (approximately size 1).
(4) Rubber spatulas or rubber policemen. Count 12.
(5) Funnels approximately 150 mm across. Count: 12.
(6) Labeling tape*. Count: 12.
(7) Lab markers*. Count: 12.
(8) Pipet fillers. Count: 12.
(9) 5-mL disposable pipets, three per group. Count: 36.
(10) 10-mL disposable pipets, 2 per group. Count: 24.
(11) Cheese cloth. We use grade 50, though I am not sure how big a difference different grades will make. 8 layers of an approximately 20 cm square per group. 0.5 square-meter per group is generous. 6 square yards or meters.
(12) 0.5-mL microcentrifuge tubes. Count: 500 divided for the twelve groups.
(13) 250-mL centrifuge bottles, two per group. Count: 24.
(14) 34-mL Oakridge-style centrifuge tubes, two per group. Count: 24.
(15) Pipettors*: P200. Count: 12.*
(16) Tips* for pipettors.
(17) Ice buckets*. One for each student group and one for each balance. The ones for the student group should be large enough to generously hold at least a blender jar, item 2.
(18) Bottles for grinding medium holding at least 100 mL. One for each student group and one for each balance.
(19) Tubes for wash medium holding at least 40 mL. One for each student group and one for each balance.
(20) Tubes for freezing medium holding at least 3 mL. Count: 12.
(21) Grinding medium: 1.5 L.

Recipe:

Sucrose, 0.3 M
MOPS, 30 mM, pH 7.2
EDTA, 1 mM
PVP-40 (poly(vinylpyrrolidone), average molecular weight: 40 kDa), 0.6% w/v

This base solution can be stored at 4°C prepared weeks ahead of time. The day of lab, dissolve the following into the solution:

- Glycine, 5 mM
- Cysteine, 4 mM
- BSA, 0.2% w/v

Grinding media saved from one day to the next should be treated as if it does not contain these last three

ingredients.
To each 100-mL aliquot for each student group, add one or two drops anti-foam, antifoam-A from Sigma. Place the aliquots in their ice buckets.
Place the leftover grinding media in the ice buckets near the balances for balancing the centrifuge bottles.
(22) Wash medium: 500 mL
Recipe:
Sucrose, 0.3 M
MOPS buffer, 25 mM, pH 7.2
EDTA, 1 mM
This base solution can be stored at 4°C prepared weeks ahead of time. The day of lab, dissolve the following into the solution
Glycine, 3 mM
BSA, 0.1% w/v
Wash media saved from one day to the next should be treated as if it does not contain these last three ingredients.
For each student group, aliquot 35-40 mL and place in their ice buckets. Place the leftover wash media in the ice buckets near the balances for balancing the centrifuge bottles.
(23) Freezing medium: 50 mL
Recipe:
Mannitol, 0.3 M
DMSO (dimethylsulfoxide), 10% v/v
MOPS buffer, 20 mM, pH 7.2
KCl, 10 mM
$MgCl_2$, 5 mM
EDTA, 1 mM
Make 3-mL aliquots for students and place in their ice buckets.
(24) Teeter-totter balances for balancing their centrifuge bottles and tubes. Two or three.
Cups to be placed on the balances with the centrifuge tubes makes the much easier.
(25) Transfer pipets. At least two for each balance.
(26) Refrigerated centrifuges and rotors for the spins. The maximum speed with the centrifuge bottles in 13,000 x g and 10,000 x g with the centrifuge tubes. With so much use of the centrifuges, it is very helpful to use each spot within the rotors only once for each of the four spins. When one needs to reuse a spot for the same spin, the lab can become longer and labored with the waiting. The centrifuges and rotors should be chilled to 4°C before lab.
We have been using Thermo Scientific's Sorvall Legend series with fixed Fiberlite rotors. Compared to older, stronger Sorvall centrifuges, these have many advantages. They are about half the price or less, the floor models are on casters and so can be moved fairly easily from lab to lab, and the rotor installation is nearly foolproof so that students do not damage the centrifuges or the rotors during that process. The only disadvantage is they do not spin as fast.
(27) Easily accessible -70°C or -80°C freezer space for the 350 to 500 0.5 mL-tubes and associated freezer boxes.
We do not know how well -20°C works.

Bradford and SDH assays

For 24 students working in pairs, one would need the items listed below for the Bradford assay and the SDH assays including 2 weeks of independent projects. Items used for just the Bradford are marked with a "B" and those used for just the SDH portion of the lab with a "S."
(1) Semi-micro visible-wavelength disposable cuvettes. 100 per group is generous for the four weeks of lab.
(2) 1.5 mL microcentrifuge tubes.
(3) Pipet tips, P200 and P1000. This is a very pipetting intensive lab, and student groups can consume a box of P200 tips each lab day. They use considerably fewer P1000 tips.
(4) Pipettors: P20, P200, P1000. Count: 12 of each.
(5) Ice buckets. Count: 12.
(6) Labeling tape. Count: 12.
(7) Lab marker. Count: 12.

(8) Cuvette rack or empty cuvette boxes. Count: 12.
(9) Parafilm for mixing cuvettes. Likely no more than 0.5 meters per student group.
(10) Scissors for Parafilm. Count: one.
(11) Spectrophotometers and computers to run them with LoggerPro or similar software. Count: 12.
We started using Vernier's Go Direct SpectroVis Plus Spectrophotometers a few years ago to great success. We have small LoggerPro programs for the collections of Bradford and SDH assay data that we can share
(12) Distilled or RO water, 20 mL per student group.[B]
(13) Bradford reagent. Coomassie Brilliant Blue G-250 dissolved in acidic conditions.[B] ~5 mL total aliquoted for each group.
We typically buy it from Bio-Rad Laboratories (Item number: 5000006. Note that BioRad does provide educational use discounts.) They do not divulge their exact recipe; while I have made it before, it is messy and complicated, and Biorad's doesn't go bad as quickly. Note that Bradford reagent stains glass and plastics. Do not place this in anything you want to reuse for another solution.
(14) Bovine serum albumin (BSA) standard solution, 0.1 mg/mL dissolved in sterile distilled water.[B] 12-15 mL. 1 mL aliquots are provided for each group.
We also typically buy this from Bio-Rad Laboratories (Item number: 5000007. Again, BioRad does provide educational use discounts.)
(15) SDH assay medium, 1.5 L.[S] 50 mM MOPS, 1.0 mM potassium EDTA, 0.01% w/v Triton X-100. Use KOH to bring the solution to pH 7.2. Provide each student group a small bottle and provide access to more with a funnel.
(16) Decylubiquinone, ~3.0 mM dissolved in DMSO, ~10 mL.[S] Aliquot into small brown microcentrifuge tubes. Store at -20C when not in use. We typically call this "duq" and pronounce it "duck" though some like "duke."
This is likely the most expensive part of the non-independent project lab. Instead of trying to weigh out the small portions needed for the lab, I use a spec to make a solution with an absorbance of 0.90 at 450 nm, which is approximately 3.0 mM. (Note that this is nowhere near the absorbance maximum, but it works.)
Also, do not dissolve this in ethanol unless you plan to use it all in one day.
Last, some SDH assays use phenazine methosulfate in place of the duq. This works and is dirt cheap. However, the apparent rates of reaction are considerably lower and less consistent. If you just want a demonstration lab and do not care about quantitation, this could work well.
(17) 1.0 M succinic acid in assay medium, pH'd to pH 7.2 with potassium hydroxide, ~20 mL.[S] Aliquot into microcentrifuge tubes and provide students access to more.
This is a dicarboxylic acid, whose pKa is about 5. It takes a lot of KOH to reach pH 7.2, and it is easy to overshoot pH 7.2 I will usually make a little too much and around pH 6.5 or 7.0 reserve some of it to add back to main solution if I overshoot pH 7.2. Also, many of the solutions chosen for the independent projects are also dicarboxylic acids, and a therefore equally annoying to pH.
Seven years ago, we made a liter of this one year and froze 200 mL aliquots. Thus far we have seen no evidence of degradation.
(18) 2,6-dichloroindophenol (DCIP), 2.0 mM in assay medium, ~20 mL. Aliquot into microcentrifuge tubes and provide students access to more.
It is important that there is enough of this that the decline in absorbance is linear with time for a several minutes and little enough of this that the spectrophotometer is not pinned at its upper limit, even with sometimes poor student pipetting. With our specs and our students, the 2.0 mM seems to be the sweet spot.
In order to make this, mix vigorously 10-40 grains DCIP in ~25 mL of SDH assay medium; filter. Blank a spec set to 600 nm with SDH assay medium. Then mix 20 µL of the likely too concentrated DCIP solution with 980 µL SDH assay medium. The target absorbance is 0.88 to 0.92. Mix SDH assay media with the too concentrated to DCIP solution to achieve this.

Over the course of a day, its absorbance will typically drop about 0.01 to 0.02. Each day recheck the absorbance to see if it is within tolerance and add more concentrated DCIP to achieve the appropriate absorbance.

Notes for the Instructor

Mitochondrial isolation lab

While the mitochondrial isolation lab is a relatively low stress, do-as-you're-told lab with just enough downtime to help students and faculty get to know one another, there are many possible pitfalls that should be pointed out during prelab which this writer calls "Keys to success." Starred points are particularly important.

(1*) Keep things cold and think ahead. You cannot fit everything in your ice bucket. What are the next things that you want cold?

(2) Be sure the blender bottom is screwed on well.

(3) Don't over-blend the peas.

(4) Balance your tubes all four times being sure to balance the cap with your tubes.

(5) Be sure to know whether you should be keeping the supernatant or the pellet. Partners, help with this.

(6) When pouring off the supernatant, follow the directions closely.

(7*) When resuspending the pellets with the paintbrushes, be sure there is no extra fluid in the tube. Especially at the last step, to do otherwise leads to lumps of mitochondria, which leads to nonuniformity in otherwise identical SDH assays later.

Bradford Assay Lab

There are three main focuses for the instructor before this lab: helping students to understand spectrophotometry and the concept of a blank, conveying how to pipette accurately, and illustrating the use of the standards curve. While the first two are relatively common experiences for many instructors, the way we handle the third is likely of greater interest. We have our students plot the standards curve by hand and choose which data from the dilutions of the unknowns to use. When we have allowed students to use spreadsheet software like Excel or Google Sheets to plot their data, as a rule they simply do not look at their data for outliers or problematic data points. While many of them are concerned by the subjectivity, we tell them that it is OK to be concerned and that one often needs to reconcile inconsistencies in data. Regarding the choosing of a dilution from which to infer the concentration of protein in their mitochondria, this, too, turns into a conversation about the reliability of data and errors.

SDH Lab with Lineweaver-Burk Plots

It should be emphasized to the students that they should have only one tube of mitochondria thawed and in use at a time. Once thawed, one should treat it as if it expires after 20 minutes. At 20 minutes, the SDH activity has fallen by about 10-15%, and while this rule might not be perfect, in practice it works well.

The previous week, they determined a protein concentration for their mitochondria, and they would start this week by testing the rate of reaction of 100 µg of protein with 20 mM of succinate. However, to best manage their mitochondrial supplies for the rest of the lab, we provide the following rules:

(1) If the calculated volume for 100 µg is between 28 and 35 µL, try just 28 µL to fairly reliably get three reactions per 100 µL aliquot.

(2) If that calculated volume is more than 40 µL, try just 40 µL to fairly reliably get two reactions per 100 µL aliquot.

(3) If the rate of reaction is less than 2.5 nmol DCIP reduced per minute, then raise the amount of mitochondria used. If the rate is this low and 40 µL of mitochondria are already being used, other interventions are likely necessary, like replacing their mitochondria with another preparation of mitochondria. (We often have the student TAs make these as part of their training.)

(4) If the rate of reaction with no added succinate is more than a third of the value with 20 mM succinate, then that is often problematic in future experiments.

The succinate endogenous to the mitochondria causes additional activity, and the points plotted in a Lineweaver-Burk plot subsequently drift downward especially at low substrate concentrations. While one can estimate the endogenous succinate concentration and then correct for it, this is too complicated for our introductory

students. We instead explain the systematic error and ask our students to determine the region where the Lineweaver-Burk plot is linear and use just those concentrations. Intriguingly, while this systematic error does alter appreciably their estimates of the K_m and V_{max}, it does not alter the direction of changes in these values. For example, when students add a competitive inhibitor to their reactions, they typically see K_m increase and V_{max} stay relatively the same as expected despite the systematic error. For a more detailed explanation of how the endogenous succinate impacts the analysis, see Appendix A.

Independent Projects

In the week before they execute their independent projects, students meet with an instructor for 15 to 20 minutes to discuss their proposed project. Proposing such a sophisticated experiment is usually new to them, and while they typically have made admirable progress on their proposal, most often have many gaps in their logic. For example, many who propose using an inhibitor have not determined what concentration of inhibitor to use or how to determine that. "We were just going to use 1 molar," has been heard many times. Solubility issues are typically not on their minds, either. Because of this, we have developed a very loose script that can be found in Appendix E.

In the weeks prior to their independent projects, they have slowly become acquainted with the primary literature, how to search it, and how to glean information from it through a series of scaffolded assignments. Those have not been included here because the complexity and length of this project is already rather substantial.

Chemicals and conditions used in the independent projects in 2019 are listed in Appendix B. Some of these require different solvents and conditions to control pH; those are listed in the appendix as well.

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Bonner WD. 1967. A general method for the preparation of plant mitochondria. Meth. Enz. 10:126-133.

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About the Authors

Having been at Pomona College since 2009, Jonathan Moore is now associate professor of biology and teaches genetics and cell biology and coordinates introductory lab courses. Ross Pringle has been a lab coordinator and instructor for introductory genetics and ecology labs, as well as the greenhouse manager at Pomona College since 2019. David Becker joined Pomona College in 1986 and is now an emeritus professor; his research addressed how extreme temperatures affect the light reactions of photosynthesis and mechanisms plants employ to tolerate them; he and several of his students were heavily involved in the initial development of this course lab project.

Appendix A: About the endogenous succinate and how it alters a Lineweaver-Burk plot

First, some grounding: in our assays, the K_m is between 1.0 and 2.0 mM, and V_{max} can vary significantly, depending on the quality of the peas, the mitochondrial preparation, and the volume of mitochondria used. Still, V_{max} varies between 2.5 and 12.0 nmol of DCIP per minute. The succinate endogenous to the mitochondria has been about 0.5 to 1.0 mM on the few occasions we have estimated it. However, when we have mold growing extensively with the peas, those values were likely more like 2.0 to 3.0 mM. In the theoretical examples below, we have set K_m = 2.0, V_{max} = 1.0, and the endogenous substrate in the mitochondria and unaccounted for is 0.5.

Let's first consider how the endogenous substrate affects a Michaelis-Menten plot. (Figure 12)

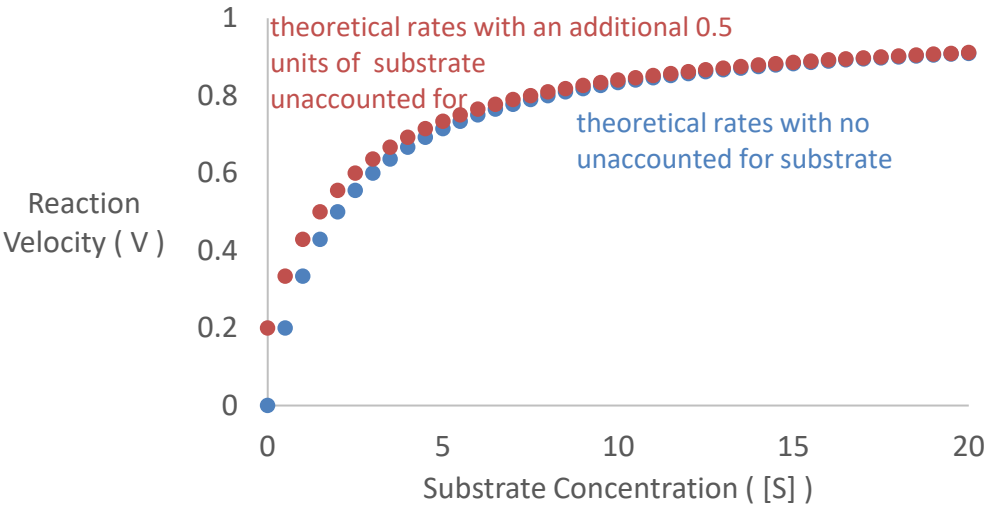

Figure 12. How the unaccounted-for substrate in the mitochondria influence a Michaelis-Menten plot.

The red plot, including endogenous substrate, is the same as the blue plot, not including extra substrate, but shifted leftward by the amount of extra substrate. This results in a large error in the reaction velocity at low values of substrate and a very modest error in for high concentrations.

We will now consider what happens when we plot these as Lineweaver-Burk (double-reciprocal) plots. (Figure 13)

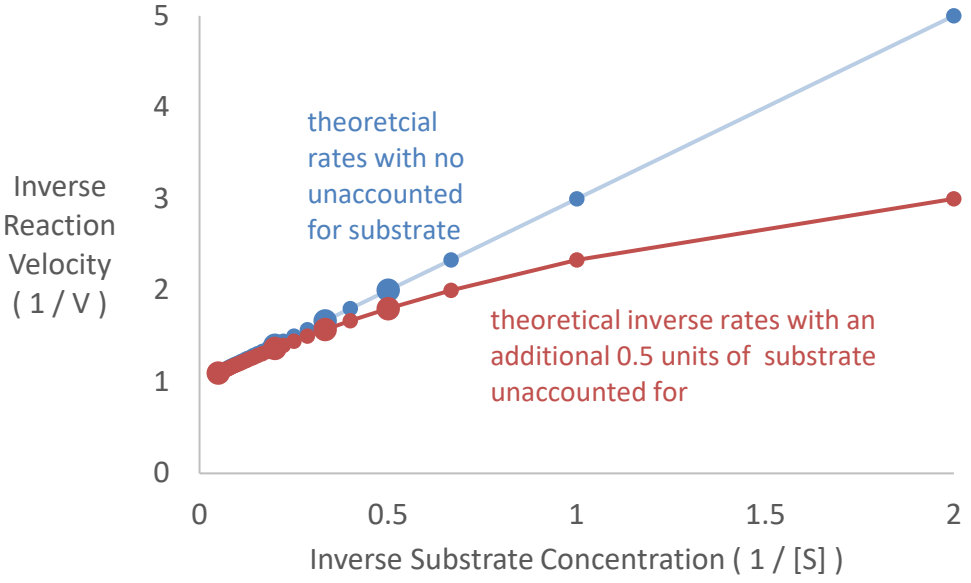

Figure 13. How the unaccounted-for substrate in the mitochondria influence a Lineweaver-Burk plot. The extra-large points are at 2, 3, 5, & 20, the values in mM that we ask our students to first use.

The succinate endogenous to the mitochondria causes the points plotted in a Lineweaver-Burk plot to drift appreciably downward at small substrate concentrations. (As one goes rightward in in this plot, the red curve approaches a horizontal asymptote with a *y*-intercept equal to the inverse of the rate from the endogenous substrate.) When our students plot their values and make a line of best fit, this line has a smaller slope. (Figure 14) Also, the *y*-intercept is slightly higher, so the estimate of V_{max} is slightly lower. Because the extrapolation to the *x*-axis is so much further, the estimate of K_m is even more greatly influenced while still being lower.

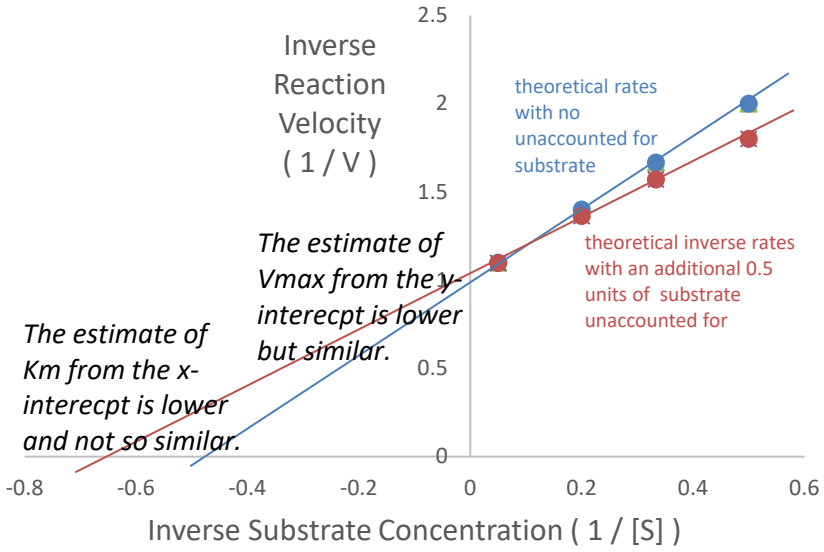

Figure 14. How the extra substrate will influence the estimates of K_m and V_{max}.

While one can estimate the endogenous succinate concentration by the method of standard additions and then correct for it, this is too complicated for our introductory students. (In brief, to perform the method of standard additions, one takes very good measurements of rates at a series of low substate concentrations, assume that these are still in the linear-range of a Michaelis-Menten plot, and then extrapolate a Michaelis-Menten plot leftward. Where this line intercepts the x-axis is the negative of the substrate concentration added by the mitochondria.) We instead explain the systematic error and ask our students to determine the region where the Lineweaver-Burk plot is linear and use just those concentrations. Intriguingly, while this systematic error does alter appreciably their estimates of the K_m and V_{max}, it does not alter the direction of changes in these values. For example, when students add a competitive inhibitor to their reactions, they typically see that K_m increases, and V_{max} stays relatively the same as expected despite the systematic error.

Appendix B: Chemicals and Conditions Used in Independent Projects

Below are the 27 different chemicals and conditions requested by 43 student groups in spring of 2019. The ideas represented by this list were arrived at by students looking at the primary literature.
Many of these reagents are quite benign, and others are rather toxic. However, we consider most reasonably safe when provided to the students (a) pre-dissolved, (b) in the quantities they need which are often tens of microliters, and (c) with appropriate precautions and personal protective equipment.

If not stated, the solvent is SDH assay media which is aqueous.

malonate, 1 M
2-methyl succinate, 1 M
itaconate, 200 mM
oxaloacetic acid, dry aliquots, and basic assay media (pH 13.0) to dissolve it.
Ultimately at 100 mM, and pH 7.2.
isocitrate, 100 mM
salicylate, 10 mM
chloramphenicol succinate, 100 mM
$CaCl_2$, 1 M
$MgCl_2$, 1 M
NaCl, 1 M
KF, 1 M
KCl, 1 M
KBr, 1 M
$ZnSO_4$, 1 M
K_2SO_4, 1 M
ATP, 0.1 M
siccanin, 10 mM in DMSO
3-nitropropionic acid, 1 M. We have been told that according to the literature, this difficult to adjust the pH without altering the covalent structure of the molecule. We suggest that students do not add more than 20 µL of this to an assay, which does not lower the pH much.
diazoxide, 100 mM in DMSO
carboxin, 250 µM
thenoyltrifluoroacetone, 100 mM in DMSO
boscalid, 10 mg/mL in DMSO
rhein, 2 mg/mL in DMSO
guanidine HCl, 2 M
phenazine methosulfate, 0.5 M
a series of assay buffers to control pH between 6.2 & 8.2. Below 6.2 the DCIP is no longer reports the rate of reaction as it turns pink. We no longer recall why above 8.2 is problematic.
temperature, which we do with older specs that can be temperature controlled.

Appendix C: Alterations to the protocol for centrifugations with only 50-mL tubes

In "*Experimental Procedure: Isolating Mitochondria,*" the following alterations should be made.

In step 1, the "250-mL centrifuge bottle" would be replaced with a 250 bottle or flask. Be sure the stems of the funnels fit into the neck of your bottles or flasks.

Replace step 6 with "Pour the supernatant roughly evenly into four pre-chilled 50-mL centrifuge tubes. Use grinding medium to balance two of these (including their caps). Do the same with the other pair. Place one set of balanced tubes opposite one another in the centrifuge rotor. Do the same with the other pair. (Don't mix up which is which. You should write on the tubes to help with this.)"

Replace step 8 with "When the spin is complete, retrieve your centrifuge tubes from the rotor and keep the tubes at the same angle as they were in the rotor. Pour out the supernatant from each tube *all at once* into clean, chilled 50-mL centrifuge tubes. The pellet is loose, so be careful not to disturb it when decanting. Discard the pellet down the drain when you rinse the tubes."

In step 9, you will need to centrifuge for 20 minutes at 10,000 x g, unless your tubes and centrifuge can handle a harder spin. "Bottles" need to be again replaced with "tubes."

In step 10, "pellet" should be replaced with "pellets."

Replace step 11 with "Use the larger brush to resuspend one of the four pellets, now without any supernatant. When the suspension is homogeneous (no lumps), add 2-3 mL of wash medium with a 5-mL pipet to each tube, and mix again with the brush.

"Repeat the resuspension with the other three pellet, likewise, adding 2-3 mL of wash media to the pellets. Transfer these suspensions with a pipet to first tube, thereby combining the resuspensions. With a clean pipet, …

"Wait – we're out of pipets. That likely isn't so, but if it is, visit the "Extra Supplies" bench near the window and take a new one if you need one. Recall, though that there are islands of plastic in the oceans, so don't take what you don't need.

"…rinse the last three tubes with about 4-5 mL of wash medium each and add this medium to first tube with the pipet."

In step 12 replace "a level about 1 cm (0.5 inch) below the shoulder (Figure 5a) of the tube" with "about the 35-mL mark on the tube."

In step 13 replace "a second chilled 34-mL centrifuge tube" with "another chilled 50-mL centrifuge tube."

In "Materials" under "Mitochondrial Isolation," items 13 & 14 are replaced with nine 50-mL centrifuge tubes per group, or 108 for twelve groups.

Obviously, the centrifuges in item 26 would be replaced with yours that would take 50-mL tubes.

Appendix D: Growing Pea Sprouts

Cowpeas (*Vigna unguiculata*), a group that includes black eyed peas and crowder peas, are germinated and grown in flats of vermiculite in dark/very low light conditions for 12-14 days and then harvested to be used in this SDH enzymology lab.

Pomona College has used California Black Eyed Pea #5 and other Black Eyed Pea varieties, the identities of which are lost institutional memory, as well as the cowpea variety "Red Ripper." Growing cowpeas to be used in this lab requires the following steps:

Sowing cowpeas in vermiculite

1. Measure out the desired number of dry cowpeas.
    a. Use ~50 mL of cowpeas per 10 x 20 in. (25 x 50 cm) tray to be planted.
    b. One tray usually provides enough cowpea sprouts for one student group for the entire project
2. Soak the measured dry cowpeas in water in a suitable container (a plastic beaker works well) overnight (>8 hrs) to stimulate germination.
    a. Be sure that the water level is well above the tops of the dry cowpeas, as they will absorb some water.
3. The next day, fill 1020 trays 1/2 to 2/3 full with vermiculite.
4. Distribute ~50 mL soaked cowpeas per 1020 tray on top of the vermiculite. (Figure 15)
    a. These do not have to be evenly spaced, but its better if the peas are not touching one another.
    b. If some peas are too close together, now is the time to space them out.
5. Use a hand to pat the cowpeas into the vermiculite.
6. Give each tray a gentle side-to-side shake to help the vermiculite cover the cowpeas a bit more. (Fig. 15)
    a. Ideally, cowpeas should be more than halfway covered to almost completely covered by vermiculite.
7. Water trays of sown cowpeas until vermiculite is thoroughly wet, however, there should be no standing water.

Figure 15: Cowpeas after sowing, after patting into vermiculite, and after shaking.

Growing cowpea sprouts

8. Transfer watered trays to either…
    a. growth chambers set to 27°C (81°F) with lights off OR
    b. a DARK greenhouse with a 24°C heating & 28°C cooling band. If growth chambers are available, they seem to give the peas a better start.

The dark environment minimizes chlorophyll production which interferes with the spectrophotometry.

9. Place a 1020 non-vented humidity dome on each tray to maintain elevated moisture levels to promote germination and sprout growth.
10. Check moisture level in trays once or twice a day, water as necessary to keep vermiculite thoroughly wet.
    a. There should be no standing water in the trays.
    b. This is a balancing act between too much and too little water.
    c. It takes a bit of patience and practice to get right.
    d. Overwatering, poor airflow, and low germination rates can lead to mold. (Figure 16) Mold itself does not seem to impair the growth of peas, nor does it lower the succinate dehydrogenase activity. It does, however, tend to correlate with high levels of succinate in the harvested mitochondrial samples, which is discussed in Appendix A.

Figure 16. Sprouts that have fallen over. Also, pictured are white spots of mold, which are suboptimal.

11. Monitor cowpea sprout growth. (Figure 17)

Figure 17. Different stages of sprouting or growth.

12. Once sprouts are close to touching the top of the humidity dome, remove the dome and either
    a. transfer to DARK greenhouse (24°C heating/28°C cooling) from growth chambers OR
    b. continue growing in dark greenhouse uncovered.
13. Continue to check moisture levels and water trays as needed.
    a. If weather conditions are arid (e.g. US Southwest) and the vermiculite dries out quickly, it may be necessary to add humidity to the greenhouse, using either empty 1020 trays filled partially with water for passive evaporation or with a dedicated humidifier (e.g. a Hydrofogger).
14. Grow cowpea sprouts for 12-14 days.
    a. The exact temperature and humidity in your growth chamber/greenhouse can either speed up or slow down growth.
    b. Sprouts should be approximately 15-25 cm tall at time of harvest.
    c. They should appear milky white or light brown/gray.
    d. Note: Once sprouts reach a certain height they may fall over if not anchored well enough in the vermiculite, if the roots are still able to absorb water they should continue to grow. (Figure 16).

Harvesting cowpea sprouts

15. Wiggle your fingers under the vermiculite to loosen it. Pull the sprouts out of vermiculite by the base and shake over the tray to remove the bulk of the stuck-on vermiculite. Try to get the roots as much as conveniently possible since the greatest mitochondrial activity gram-for-gram is there.
16. Agitate the peas in standing water and transfer to a new vessel. Repeat this process twice more to remove most of the remaining vermiculite.
17. Run the sprouts through a salad spinner to remove excess water.
18. Give each lab group about 60 grams of pea sprouts in a plastic bag with the weight of the peas written on it. (More than 70 g overwhelms the amount of grinding buffer provided.) It is OK if you break the sprouts. Unsprouted peas will not yield mitochondria.
19. Pea sprouts can be stored at 4°C for at least two days in plastic bags with many holes cut in the side.

Appendix E: One way to approach SDH independent project proposal discussions

(1) "What do you want to do?"

This is really a way to hopefully get them talking about their experiments. The more they say, the less we might need to ask about later.

It is good at this point before things might go too far to determine how feasible an idea is. Recall that pH is restricted between 6.2 & 8.2, temperatures need to stop at 65°C, and compounds may be too toxic, expensive, or just not available.

(2) "So if you … what do you think might happen and why?"

This is to hopefully get them to talk about hypothesis, prediction, and rationale from the literature. If they start discussing papers, this can be the inroads to specifics that will be useful in #4…

(3) "Of what you just said, what is the hypothesis, and what is the prediction?"

Often at this point, this distinction is lost on them.

If they say the right things, but with the wrong term, just restate what they said calling the appropriate parts the hypothesis & predictions. Sometimes, the depth of the hypothesis is just that *x* is a non-competitive inhibitor because my paper says so, I'm guessing that mammalian and pea SDH will work the same, and it doesn't structurally look like succinate. That's fine.

If they don't say the right things, asking why's and giving them some time can be especially valuable. This is the point when you might need to send them away for a few minutes to contemplate their papers and then move on to the next group if you're already behind. This often happens with pH or temperature as a topic.

(4) "What are the first assays you'd run?" or "Walk me through your experimental design."

Sometimes students will launch into this after question 1, but rarely. What you want to know is how much they've thought about their experimental design. This standard spiel needs to be modified depending on what they tell you:

(4a) "So what concentration of inhibitor were you considering?"

"Do you get a sense of that from your papers?"

If not, then encourage them to flip through it. Often, they have not bothered to look at the figures or looked at them and decided they were too hard to consider at that moment.

If they still cannot find it and you want to dive in and help them, great.

If you don't want to, or you cannot find it well, here are guidelines:

- If it is small like malonate or a small ion, try 1 mM as a starting point.
- If it is big and organic (think ring structures), try 0.1 mM.

Regardless, encourage them to do a pilot run testing different inhibitor concentrations. If one sees between 1/3 and 2/3 inhibition and that repeats, that's marvelous.

(4b) "If you see less inhibition, what might you do?"

Try more inhibitor. If they see too much inhibition, try less. Two oddities:

Counter-intuitively, sometimes you see more inhibition with less inhibitor. Don't bring this up during the discussions.

100 mM anything will inhibit this assay. Concentrations above 20 mM rarely have a specific effect.

(4c) "So what concentration of succinate do you want to run these experiments at?"

K_m or a bit above is good. The amount of inhibitor you'd need to add to see an effect at 20 mM is a lot, if your inhibitor is competitive.

(4d) "Once you have determined the concentration of the inhibitor that you want to use, then run your assays for your experimental & control LB plots."

This is a good point to discuss how their LB plots looked last week and what succinate concentrations they might want to exclude next time and which they might want to add.

At some point, discuss the number of replicates they will want to do at each stage.

(5) Concentrations and solvent controls

At some point you will need to tell them what their stock concentration of inhibitor is, how they will change pH, or change temperature. This is the moment that solvent controls may become necessary to discuss.

(6) "Do you have other ideas in case this doesn't go as well as you expect, or as a follow-up?"

Just encourage them to think about it. It is not a terribly satisfying project when you spend two lab periods determining your compound has no effect.

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