

Lab 2A: Sub-Cellular Fractionation

A response is required for each item marked: (#__). Your grade for the lab 2 report (2A and 2B combined) will be the fraction of correct responses on a 50 point scale[(# correct/# total) x 50].

Section 18.6 of your text describes the use of differential centrifugation in fractionating sub-cellular organelles according to their densities. The cells are first broken apart by some type of mechanical disruption device (e.g., the mortar and pestle used in today's exercise) in a buffer designed to maintain integrity of organelle membranes. The cell homogenate is then subjected to a series of centrifugations at sequentially higher gravitational (g) force. Larger organelles of greater densities will pellet at lower g forces than smaller ones of lower densities. The density of an organelle is determined by both its mass and its shape. The sedimentation of an organelle will be dependent on its density relative to the density of the medium surrounding it (homogenization buffer containing 0.25 M sucrose in this experiment).

In today's exercise, you will use differential centrifugation to fractionate organelles from chicken liver cells. The chicken liver homogenate will first be subjected to a low speed centrifugation (1.7 rcf) to produce a **Low Speed Pellet (1.7P)** and **Low Speed Supernatant (1.7S) fraction**. The **1.7P** fraction will be retained for further analysis, while the **1.7S** fraction will be subjected to a second higher speed centrifugation (14 rcf) to produce a **High Speed Pellet (14P)** and **High Speed Supernatant (14S) fraction**. The goal of these centrifugations is to obtain separate fractions for mitochondria and nuclei. The mitochondrial fraction will be used next week in enzyme kinetic assays of a mitochondrial enzyme.

Hypothesis Forming:

Based on the relative sizes of the following organelles, predict whether it will be found in the **1.7P**, **14P** or **14S** fraction. (Ignore the **1.7S** fraction, as it will undergo a second centrifugation to produce the **14P** and **14S** fractions.)

<u>Organelle</u>	<u>Diameter</u>	<u>Predicted Fraction</u>
Nucleus	10 μm	(#1)
Mitochondria	1-4 μm	(#2)
Golgi Cisternae	0.5-1 μm	(#3)
Ribosome	20 nm	(#4)

Now predict the effect of sucrose concentration in the homogenization buffer on this fractionation. Would you expect a higher sucrose concentration to make it more likely or less likely that mitochondria will sediment in the low speed centrifugation? **(#5)** Explain. **(#6)**

You will use your microscope to monitor each of the sub-cellular fractions from your experiments for the presence of nuclei and mitochondria. You will also use a colorimetric assay for the mitochondrial enzyme, Succinate Dehydrogenase, as a second assay for the presence of mitochondria. Predict the degree of enrichment for Orcein-staining and Janus Green-staining organelles in each fraction. Use a scale of +++ to – to indicate quantitative differences between fractions.

<u>Sub-Cellular Fraction</u>	<u>Orcein-staining</u>	<u>Janus Green-staining</u>
1) 1.7P	(#7)	
2) 14P	(#8)	
3) 14S	(#9)	

Protocol

Pre-Fractionation Preparation

Check for the following reagents and supplies on your bench top:

On Ice:

- 1) ~2 g chicken liver in weigh boat
- 2) 50 ml Buffered Sucrose (either 0.25 M or 1.0 M sucrose)
- 3) mortar and pestle
- 4) cheese-cloth lined funnel in 50 ml tube

At RoomTemp:

- 1) 4.0 ml Assay Mix w/ Succinate
- 2) 4.0 ml Assay Mix w/o Succinate
- 3) bag of Eppendorf tubes
- 4) sleeve of 15 ml tubes
- 5) glass test tubes
- 6) yellow and blue tips
- 8) Janus Green stain in dropper bottle
- 9) Orcein stain in Eppendorf tube
- 10) in Lab Bench box:
 - razor blade
 - Sharpie

Pre-protocol Organization

Before you begin your fractionation, always organize your work space.

Label Eppendorf Tubes:

- 1) Label 6 tubes: **1.7P** (your lab group #).
- 2) Label 6 tubes: **14P** (your lab group #).
- 3) Label 1 tube **14S** (your lab group #).

Keep all tubes on ice before use.

Sub-cellular Fractionation Protocol

1. Use a razor blade to finely mince the chicken liver in the weigh boat sitting on ice in your ice pan. (***Keep the weigh boat on ice during this process so that the chicken liver remains cold.***)
2. Use the razor blade to rake the minced chicken liver into your mortar and pour ~5 ml ice cold buffered sucrose over it. Use your pestle to crush the chicken liver into a homogenous mixture (or homogenate) containing as few clumps as possible. Gradually add cold buffered sucrose to a final volume of ~10 ml.
3. Pour the chicken liver homogenate through the cheese cloth-lined funnel sitting in the 50 ml tube in your ice pan. Pour another ~ 5 ml cold buffered sucrose through the cheesecloth in order to recover as much homogenate as possible. Gently wring the cheesecloth and press it against the inside wall of the 50 ml tube.
4. Use your P1000 set on 1000 with a blue tip to transfer the homogenate into the 6 Eppendorf tubes **labeled 1.7P**. Fill each tube to its maximum (~1/4 inch from the top of the tube).
5. **Low Speed Centrifugation:** Place the 6 Eppendorf tubes in the microfuge on your benchtop and centrifuge at the lowest **rcf setting of 1.7 for 5 minutes**. Always place an equal number of tubes on opposite sides of a rotor to ensure that the rotor will remain balanced during centrifugation. Also move the tubes between the ice pan and microcentrifuge as quickly as possible to keep the homogenate cold.
 - a- **Low Speed Supernatant (1.7S):** Use your P1000 set on 1000 with a blue tip to transfer the supernatant from this centrifugation to the 6 Eppendorf tubes **labeled 14P**. Again, fill each tube to its maximum (~1/4 inch from the top of the tube). You will come back to the pellet

fractions in the tubes labeled 1.7P. Leave these tubes in your ice bucket and GO STRAIGHT TO STEP 6 before returning to step 5b.

b- Low Speed Pellet (1.7P): The insoluble pellet fraction from the low speed centrifugation must be resuspended in ice cold buffered sucrose before it can be analyzed. To do so, add 1000 μ l buffered sucrose to the pellet from one tube, gently pipet the liquid up and down to resuspend the pellet before transferring the liquid to the pellet fraction of the next tube. Continue this until all 6 pellets have been resuspended together into a single tube. If necessary, add cold buffered sucrose to this tube to bring the volume level to the tube maximum (~1/4 inch from the top of the tube). **Keep on ice for further analysis as described below. It should be labeled 1.7P (your lab group #)**

6. High Speed Centrifugation: Place the 6 tubes of low speed supernatant from step 5a in your microfuge and centrifuge at **rcf setting of 14 for 10 minutes**. (These tubes should be labeled 14P.) Again take care to balance the rotor and move the tubes between the ice pan and microcentrifuge as quickly as possible. **During the centrifugation RETURN TO STEP 5b.**

a- High Speed (14rcf) Supernatant (14S): Use your P1000 set on 1000 with a blue tip to transfer the high speed supernatant fraction from all 6 tubes to the Eppendorf tube **labeled 14S** and store for further analysis.

b- High Speed (14rcf) Pellet (14P): The insoluble pellet fraction from the high speed centrifugation must also be resuspended in cold buffered sucrose before further analysis. As you did with the low speed pellet, add 1000 μ l buffered sucrose to one pellet, gently pipet up and down to resuspend the pellet before transferring it to the pellet in the next tube until all 6 pellets have been resuspended together into a single tube. Bring the final volume to the tube maximum (~1/4 inch from the top of the tube) **Label this tube 14P (and your lab group #) and keep on ice for further analysis.**

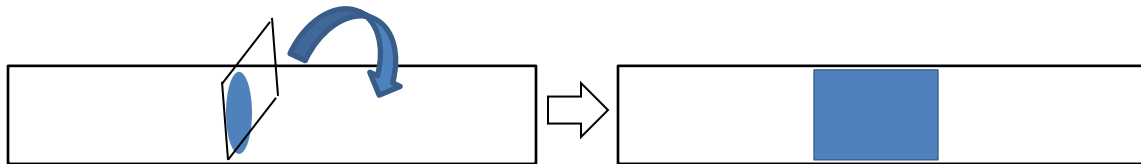
Monitoring Subcellular Fractions for Mitochondria and Nuclei

You will use a combination of microscopic and enzymatic assays to monitor your fractions for the presence of nuclei and mitochondria. You will first use microscopic examination of your fractions that have been stained with specific stains for DNA (Orcein) and for mitochondria (Janus Green). Succinate Dehydrogenase (SDH) activity will be used as a second indicator of mitochondria, as this enzyme is a component of the mitochondrial membrane. In the next lab period, you will use the fraction containing the most mitochondria in quantitative SDH enzymatic assays, allowing you to measure kinetic properties of the enzyme.

Microscopic Examination of Sub-cellular Fractions

- Label 6 Eppendorf tubes and 6 microscope slides as follows:

1.7P-O	1.7P-JG
14P-O	14P-JG
14S-O	14S-JG
- Use your P20 to pipet 10 μ l Orcein stain into the tubes with labels ending with –O and 10 μ l Janus Green stain into the tubes with labels ending in –JG.
- Use your P20 to pipet 20 μ l of each subcellular fraction into its corresponding tube. Mix with the stain by pipetting up and down with the pipette.
- Use your P20 to pipet 20 μ l of each stained subcellular fraction to the microscope slide with the corresponding label. Try to avoid introducing any air bubbles. Place a coverslip over the drop. First draw the liquid against one edge of the coverslip and then gently drop the coverslip onto the surface of the slide as diagrammed below. The stained fraction will spread out evenly between the coverslip and slide.



- Use standard light microscopy to monitor each fraction for the presence of nuclei (round Orcein-positive structures) and mitochondria (Janus Green-positive dot-like structures). Be aware that neither stain is absolutely specific. Why do you think mitochondria might also become Orcein-stained? **(#10)**

Use + and – signs to rate each fraction for enrichment of Orcein-positive and Janus Green-positive organelles. Your results probably will not be all or none. Use a scale of +++ to – to indicate quantitative differences observed between fractions.

<u>Sub-Cellular Fraction</u>	<u>Orcein-staining</u>	<u>Janus Green-staining</u>
1.7P	(#11)	
14P	(#12)	
14S	(#13)	

How close were your predictions to the actual data? How do you account for any discrepancies?
(#14)

Succinate Dehydrogenase Assays

Succinate Dehydrogenase (SDH) activity will be used as a second indicator for the presence of mitochondria in a fraction. SDH is an enzyme of the Tricarboxylic Acid Cycle that is an integral component of the inner mitochondrial membrane. In living cells (*in vivo*), it transfers electrons from succinate to a mobile electron carrier of the mitochondrial electron transfer chain (FAD^+). In our test tube (*in vitro*) assay, we will introduce a poison of the electron transfer chain, forcing electrons to an artificial electron acceptor (DCIP) that undergoes a color transformation after accepting electrons from succinate. This color change will provide a visual indicator of SDH activity in your assays. You will assay SDH activity in each of your subcellular fractions, in the presence (+) and in the absence (-) of the substrate for the enzyme, succinate.

What is the purpose of the assays done in the absence of succinate? What result do you expect to observe in these reaction tubes? (#15)

1. First label glass tubes as follows:

- | | |
|-------------|-------------|
| 1) 1.7P (+) | 4) 1.7P (-) |
| 2) 14P (+) | 5) 14P (-) |
| 3) 14S (+) | 6) 14S (-) |

2. Use your P1000 to pipet 1000 μl of (+Succ) assay buffer into tubes 1-3 and 1000 μl of (-Succ) assay buffer into tubes 4-6. **USE GLOVES WHEN HANDLING THE ASSAY BUFFER. IT CONTAINS AZIDE, A POISON OF THE MITOCHONDRIAL ELECTRON TRANSFER CHAIN!**

3. Use your P200 to pipet 60 μl of *the appropriate fraction to each tube. Gently mix the contents of the tube by tapping the bottom of the tube with your finger.*

4. The reactions will take ~ 5-15 minutes to reach completion. You will visually monitor the reactions in today's lab. A positive reaction is one in which the assay buffer goes from blue to colorless; a negative reaction is one that remains blue. Enter the results of your assays in the chart below, using a scale of ++ to – to reflect quantitative differences between fractions.

Sub-Cellular Fraction**Succinate Dehydrogenase Assay**(+Succ)(- Succ)

- | | |
|---------|--------------|
| 1) 1.7P | (#16) |
| 2) 14P | (#17) |
| 3) 14S | (#18) |

5. After determining which fraction contains SDH activity, discard the other fractions in the lab trash can. **Leave the fraction containing SDH activity in your ice bucket for the TA to freeze. Be sure the tube is marked with your Bench # before you leave the lab.** It will be waiting for you on your bench SDH in the next lab period.

Questions:

How consistent were the results of the two assays you used to monitor your fractions for the presence of mitochondria? **(#19)**

Did your predictions about which fraction would contain mitochondria match your actual results? **(#20)**

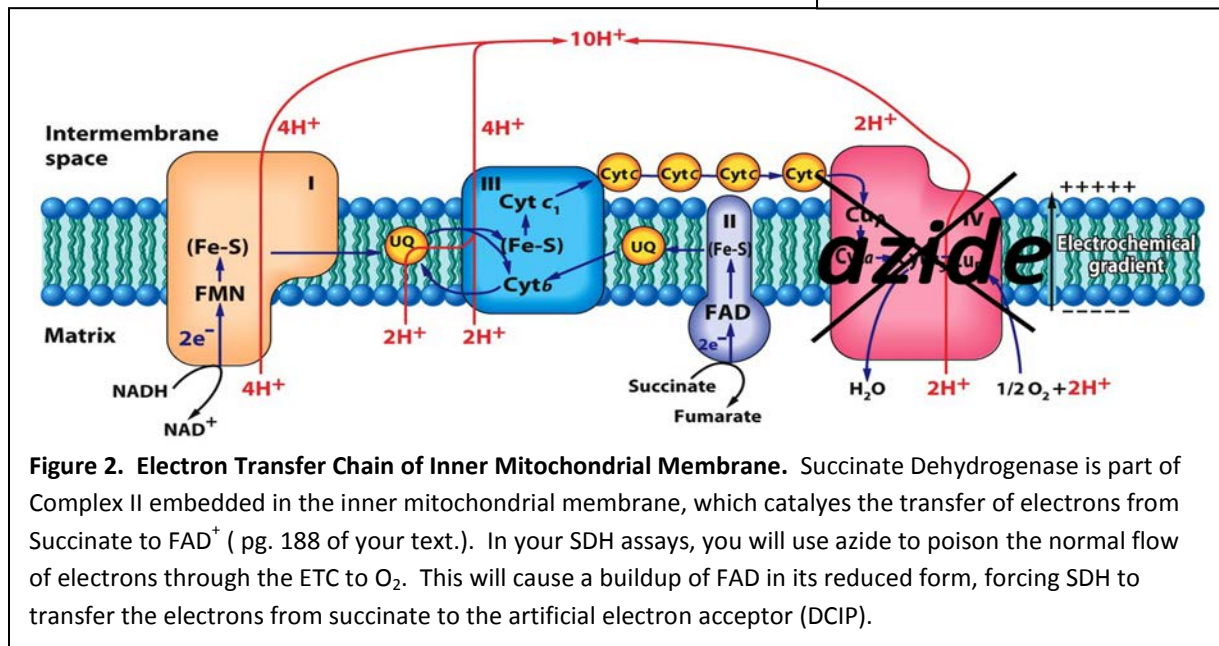
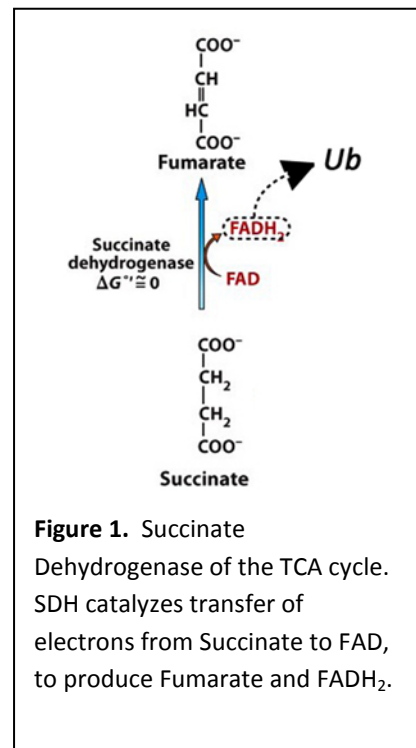
How might you account for any discrepancy? (*Hint: Compare the results of the lab groups using 0.25M buffered sucrose to those of groups using 1.0M buffered sucrose.*) **(#21)**

Lab 2B: Kinetic Assays of SDH Activity in Mitochondrial Fraction

In last week's lab exercise, you used the mitochondrial stain Janus Green and Succinate Dehydrogenase (SDH) activity assays to identify which of your sub-cellular fractions was enriched with mitochondria. This week you will use that fraction in quantitative assays of SDH activity that will allow you to measure kinetic properties of the enzyme. SDH catalyzes the sixth step of the Tricarboxylic Acid (TCA) cycle (Figure 1 and pg. 179 of your text). This is one of four reactions of the TCA cycle in which electrons are transferred from a carbon substrate to an electron carrier.

These electrons will ultimately be transferred to electron carriers in the electron transfer chain (ETC) situated in the inner mitochondrial membrane (Figure 2). SDH is unique among the enzymes of the TCA cycle, in that it is a direct participant in the ETC. Electrons entering the ETC through SDH (Complex II) are passed from succinate to FAD^+ , then to Ubiquinone, and ultimately to O_2 (Figure 2 and pg. 188 of your text). The energy released in the electron transfer reactions of the ETC is harnessed to pump protons (H^+) across the inner mitochondrial membrane and establish a proton motive force needed to drive ATP synthase activity in the production of ATP.

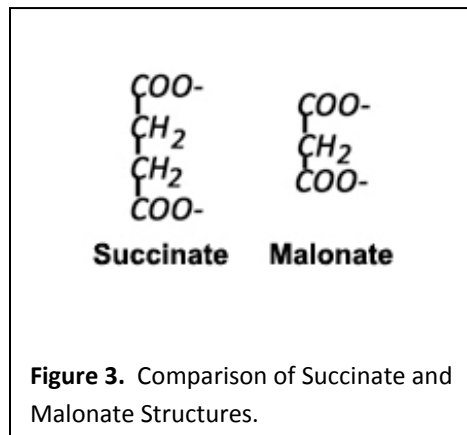
In order to provide visual evidence of SDH activity in your assays, an electron carrier that changes from blue to colorless after accepting electrons (2,6 dichloroindophenol or DCIP) will be used as an indicator. To force SDH to transfer electrons to this artificial electron carrier, it is necessary to poison transfer to its



natural electron acceptor in the ETC (FAD^+). This is done by poisoning the terminal step of the ETC, which is catalyzed by cytochrome oxidase. This causes all electron carriers of the ETC to accumulate in reduced form. An irreversible inhibitor of cytochrome oxidase, Na-azide, will be added to your reactions to this end. **Because of its inhibitory effect on cytochrome oxidase, it is poisonous to all aerobic cells (including yours) and should be used with great caution. Gloves should be worn while assembling and handling the SDH assay reactions, and keep your hands away from your mouth while wearing the gloves!**

Your quantitative assays of SDH will allow you to determine the K_M and v_{\max} of the enzyme in your mitochondrial-enriched fraction. These will be determined from Michaelis-Menten and Lineweaver Burke plots of your data for the velocity (speed) of the SDH reaction over a range of succinate concentrations. SDH activity will be monitored through the change in color of DCIP as measured with the spectrophotometer on your bench top. DCIP is blue and strongly absorbs light in the 600 nm range when oxidized and becomes colorless in its reduced form. The velocity of the enzyme can then be measured as the decrease in A_{600} absorbance over time over a range of substrate (succinate) concentrations. The maximal velocity of the enzyme (V_{\max}) and the substrate concentration giving $\frac{1}{2}$ the maximal velocity (K_M) can then be determined from plots of your data. Two types of plots (Michaelis-Menten and Lineweaver-Burke, described on pg. 100-103 of your text) will be used to determine these values. These parameters give important insight into the strength of an enzyme's catalytic activity (from V_{\max}) and affinity for its substrate (from K_M) and are useful in the design of drugs that alter enzyme activity in the treatment of human disease.

You will carry out these SDH activity assays in the presence and absence of a known inhibitor of SDH activity (malonate). The velocity data under each condition will be plotted against substrate (succinate) concentration on both types of plots, allowing you to determine the V_{\max} and K_M of SDH in your fractions from each type of plot. This will allow you to see the advantages and disadvantages of both plotting methods. You will also compare the K_M and V_{\max} for the enzyme in the presence and absence of malonate, allowing you to determine whether it acts as a competitive or non-competitive inhibitor. As you may recall from lecture, competitive inhibitors compete for the substrate-binding site on the enzyme, and, therefore, bring about an apparent **increase** in the K_M of the enzyme (apparent **decrease** in affinity). This type of inhibition can be overcome by raising the substrate concentration. Although the concentration of substrate required to saturate the enzyme will be increased in the presence of a competitive inhibitor, the maximal velocity (V_{\max}) the enzyme can reach at that saturating concentration is not affected. Non-competitive inhibitors bind at sites other than the substrate-binding site and, therefore, do not affect the K_M of the enzyme. The V_{\max} of the enzyme, however, is reduced in the presence of this type of inhibitor. Based on the structures of the malonate and succinate shown in Figure 3, which type of inhibitor do you predict malonate to be?

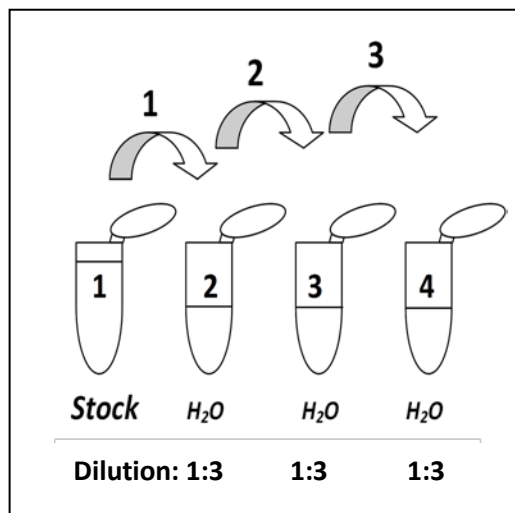


Lab Exercise

Reagents/Supplies at Your Bench:

- 1) your mitochondrial fraction (on ice)
 - 2) 5 ml Assay Buffer in tube labeled MASTER (**labeled MASTER**)
 - 3) 1 ml DCIP (**labeled DCIP**)
 - 4) 1 ml Na-Azide (**POISONOUS**) (**labeled Azide**)
 - 5) 0.5 ml Malonate (**labeled Mal**)
 - 6) 1 ml Succinate (0.2 M) (**labeled Succ**)
 - 7) 50 ml H₂O (**labeled H₂O**)
 - 8) Spectrophotometer (one per bench pair)
 - 9) sleeve of glass test tubes (one per bench)
 - 10) bag of Eppendorf tubes (one per bench)
 - 11) bag of 15 ml plastic tubes
 - 12) Eppendorf tube rack (one per bench pair)
 - 13) rack for glass test tubes (one per bench)
 - 14) ruler
 - 15) in Lab Bench Box: timer, calculator, pencil
1. Before you begin, locate each item in the list above on your bench.
 2. Turn on your spectrophotometer and set the λ at 600 nm by **first pushing the A/T/C button to put the instrument in Absorbance (A) mode and adjusting the nm setting up or down.**
 3. Blank the instrument by placing a glass test tube fill with ~ 3ml H₂O in the cuvette holder of the spectrophotometer. **Push the 0 ABS button.** The instrument should now read 0.000.
 4. You will use a timer to monitor how long the SDH reactions take to complete. This time will be used to calculate the velocity of your reactions (ΔA_{600} reading/min.) **Set your timer to 60 min. but do not push Start until the reactions have been completely assembled.**

5. Make a 1:3 dilution series of the 0.3 M Succinate Stock (**Succ**) as shown in the panel on the right.
 - A) Begin by labeling 4 eppendorf tubes (1, 2, 3, and 4, reflecting their position in the dilution series).
 - B) Transfer 1 ml of the Succinate Stock into the tube labeled 1 and 0.6 ml (600 μ l) H₂O into the tubes labeled 2, 3, and 4.
 - C) Then transfer 0.3 ml (300 μ l) of the Stock from tube 1 into tube 2 containing 0.6 ml H₂O. Cap and mix, before transferring 0.3 ml of the 1:3 diluted



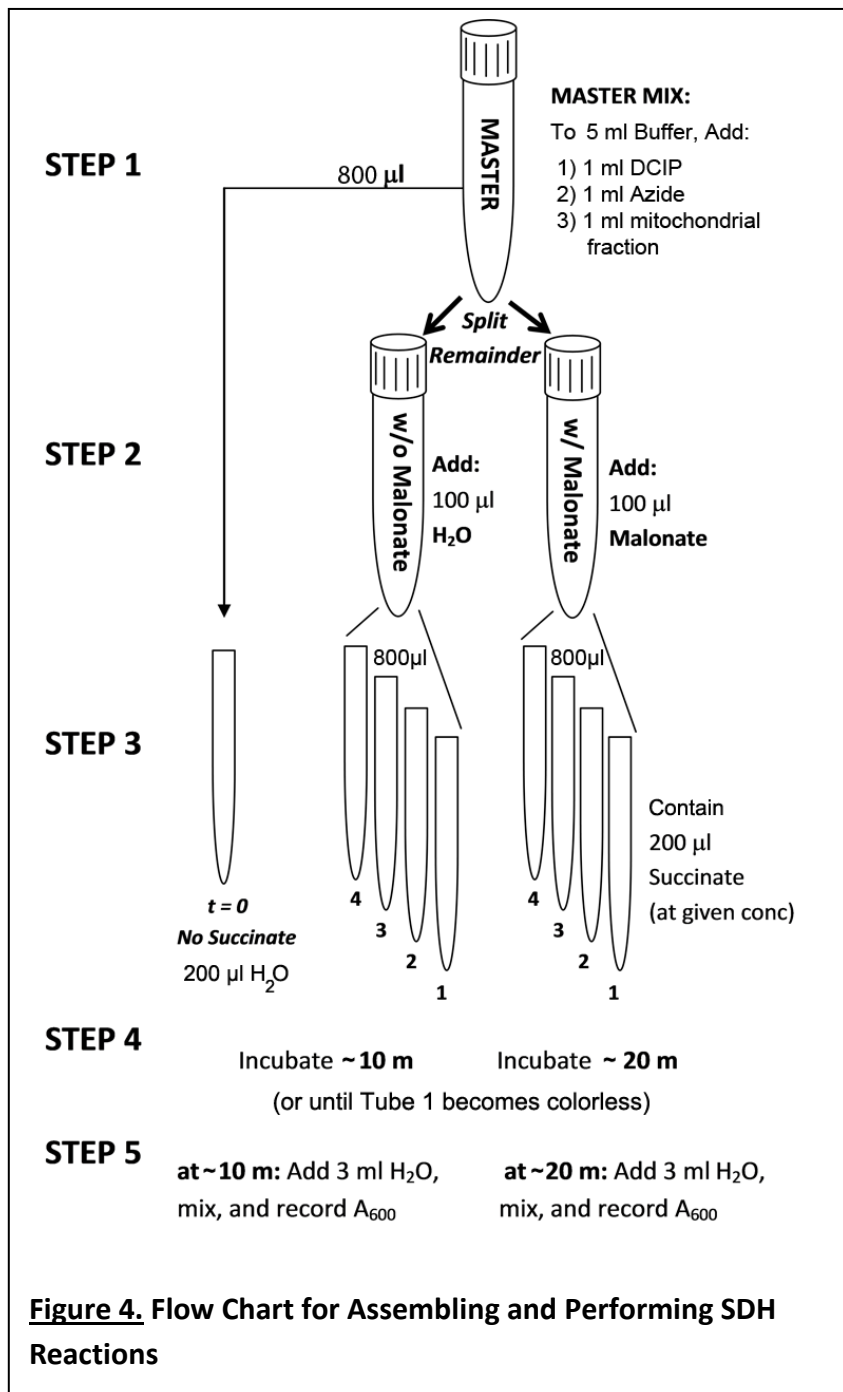
stock from tube 2 to tube 3 (also containing 0.6 ml H₂O). Repeat with the contents of tube 3 into tube 4.

6. Label a series of glass test tubes for the SDH reactions as follows:

- t = 0
- w/o-1 w/-1
- w/o-2 w/-2
- w/o-3 w/-3
- w/o-4 w/-4

Then transfer 200 µl Succinate from each dilution series tube into the glass reaction tubes of corresponding number (from dilution series tube 1 to reaction tubes w/o-1 and w/-1, from dilution series tube 2 to reaction tubes w/o-2 and w/-2, etc.) Transfer 200 µl H₂O into the t=0 glass reaction tube.

7. The SDH reaction assays will be assembled in the reaction tubes from step 6 above as outlined in the flow chart of Figure 4. A MASTER mix of all other components of the reaction will be added to these tubes, such that each tube of a reaction series differs only in the concentration of succinate it contains. The reactions will begin with the addition of MASTER mix to each reaction tube.



Step 1: To assemble the MASTER mix, add 1 ml (1000 µl) Na-azide (**Azide**), 1 ml DCIP (**DCIP**), and 1 ml of your mitochondrial fraction to the plastic tube **labeled MASTER** at your bench. This contains 5 ml Assay Buffer. Cap the tube and invert several times to mix.

Step 2: Transfer 800 μl of the assembled MASTER MIX to the glass tube labeled $t=0$. This tube will provide the A_{600} reading for your reactions at the beginning of the reaction (at time 0), as it lacks succinate (contains H_2O in its place). DCIP will remain blue in it. Then use a Pasteur pipette to split the remainder of the MASTER MIX between it and a second 15 ml plastic tube. Label the original tube “w/o Malonate” and the second one “w/ Malonate”. The graduated markings on the plastic tubes can be used to estimate volume equivalence. Then add 100 μl Malonate (**Mal**) to the tube labeled w/ Malonate and 100 μl H_2O to the tube labeled w/o Malonate. Cap the tubes and invert several times to mix.

Step 3: The addition of the two master mixes to their respective series of reaction tubes will initiate the reactions. Transfer 800 μl of the w/o Malonate Master Mix to each tube of the w/o reaction series and 800 μl of the w/ Malonate Master Mix to each tube of the w/ reaction series. Vigorously flick the bottom of each tube to mix and allow the tubes to incubate on your benchtop at room temperature. **Press Start on your timer to begin timing of the reactions.** The reactions should be allowed to proceed until tube 1 of each series, (containing the highest concentration of succinate) becomes colorless. This will take ~ 10 minutes for the reactions lacking Malonate (w/o Malonate series) and ~ 20 minutes for those containing Malonate (w/ series) but will vary between mitochondrial preps. Your countdown timer will allow you to determine the timing for your mitochondrial prep.

Step 6: SPECTROPHOTOMETRIC READINGS OF SDH ACTIVITY. SDH activity will be monitored through a decrease in A_{600} for your reactions, as DCIP is converted from its blue oxidized form to its colorless reduced form. The change in A_{600} reading (ΔA_{600}) will be determined by subtracting the reading for a tube at the end of the reaction time from the reading for the $t=0$ tube. This ΔA_{600} is then divided by the elapsed time for the reaction to calculate enzyme velocity. These velocities will then be plotted against the succinate concentration in the reaction to construct a plot for determining K_M and V_{max} for the SDH enzyme in the presence and absence of its inhibitor.

Re-blanking the Spectrophotometer. Re-blank the instrument by placing a glass test tube fill with $\sim 3\text{ml}$ H_2O in the cuvette holder of the spectrophotometer. **Push the 0 ABS button.** The instrument should now read 0.000.

Spectrophotometric Readings of Reaction Tubes. When the w/o Malonate reactions are complete, record the reaction time elapsed, and then dilute each reaction by adding 3 ml H_2O to each tube of this series and to the $t=0$ tube. Flick the bottom of each tube to mix and then immediately place it in the cuvette holder of the blanked spectrophotometer. Allow the A_{600} reading to settle for a few seconds before recording it in the Raw Data Chart on the next page. By the time you have completed this task, the w/ Malonate reactions will probably be complete. If so, record the elapsed reaction time, dilute the reactions, and take their A_{600} readings. It is probably best to divide the labor in this task. One lab partner should make the dilutions, while the other takes and records the A_{600} readings.

PLOTTING THE DATA. Use the data from the **RAW DATA CHART** to calculate the velocities to be plotted in the Michaelis-Menten and Lineweaver-Burke plots on pages 7 and 8. ΔA_{600} is calculated for each tube by subtracting its final A_{600} reading from the approximate beginning A_{600} reading (in $t=0$ tube). The velocity ($\Delta A_{600}/\text{min}$) of each reaction is then determined by dividing this number by the length of the reaction time in minutes as measured with your timer. (Use a time of 10 minutes for the w/o tubes and 20 minutes for w/ tubes, if you forgot to record this.) Enter these values in both the **RAW DATA** and **PLOTTED DATA** chart. Then calculate the succinate concentration in each reaction by multiplying the concentration of the succinate stock (0.3M) by the factor of its final dilution (taking into account its dilution both in the dilution series and in the final reaction). The velocity of each reaction is then plotted against the concentration of substrate (succinate) on the **Michaelis-Menten plot** (pg 7). The reciprocal of the velocity is plotted against the reciprocal of the substrate concentration on the **Lineweaver-Burke plot** (pg 8). Calculate these reciprocal values and enter them in the **PLOTTED DATA** chart. Draw a best fit line between the points on each plot (solid line for the uninhibited reactions and dotted line for the inhibited ones). The lines on the Michaelis-Menten plot should intersect 0 and will likely be sigmoidal in shape. Those on the Lineweaver-Burke plot will be straight lines with a Y intercept > 0 and X intercept < 0 . Some previously obtained data are supplied on page 10 for you to practice with before working with your own data. After plotting the practice data and your own data, use the instructions on page 13 to determine the K_M and V_{max} for the enzyme in your mitochondrial fraction.

Practice Data (each blank worth 1 pt) (#22-47)

RAW DATA CHART

	Reaction Tube Identity	A_{600} $t \sim 10$ m	ΔA_{600} $(t_0 - t_{-10})$	A_{600} $t \sim 20$ m	ΔA_{600} $(t_0 - t_{-20})$	Velocity $\Delta A_{600}/\Delta t$ $(\Delta t \sim 10\text{m})$	Velocity $\Delta A_{600}/\Delta t$ $(\Delta t \sim 20\text{m})$
Dilute & Read at $t=10$ min	t=0	2.449		NA	NA	NA	NA
	w/o-4	1.209		NA	NA		NA
	w/o-3	0.951		NA	NA		NA
	w/o-2	0.845		NA	NA		NA
	w/o-1	0.386		NA	NA		NA
Dilute & Read at $t=20$ min	w/-4	NA	NA	1.305		NA	
	w/-3	NA	NA	0.930		NA	
	w/-2	NA	NA	0.693		NA	
	w/-1	NA	NA	0.180		NA	

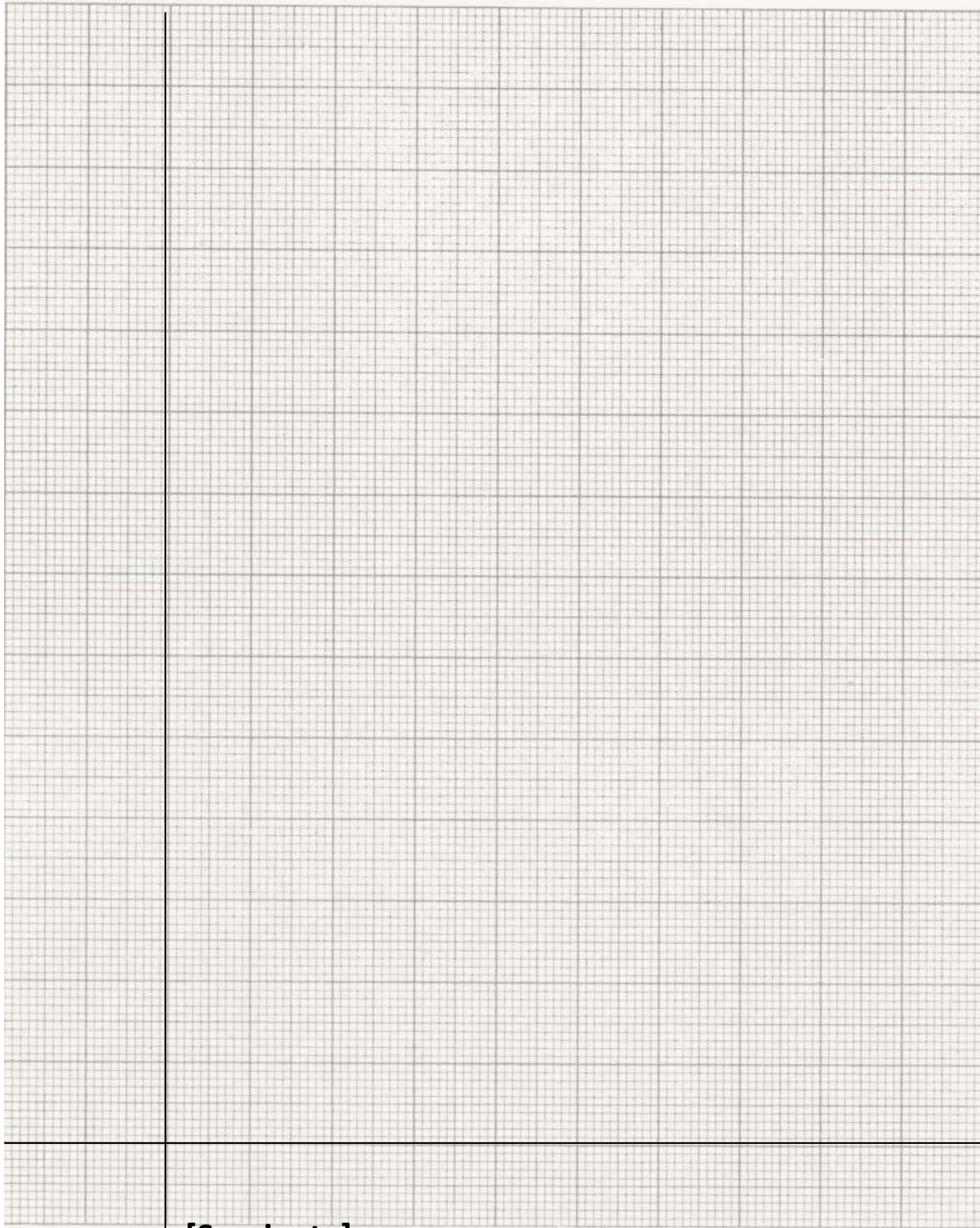
PLOTTED DATA ([Succinate] series different in this experiment) (#48-79)

Reaction Tube Identity	[Succinate]	Velocity $(\Delta A_{600}/\Delta t)$	1/[Succinate]	1/Velocity
w/o-4	0.006 mol/l			
w/o-3	0.012 mol/l			
w/o-2	0.018 mol/l			
w/o-1	0.024 mol/l			
w/-4	0.006 mol/l			
w/-3	0.012 mol/l			
w/-2	0.018 mol/l			
w/-1	0.024 mol/l			

Michaelis-Menten Plot (Velocity vs. [Succinate])

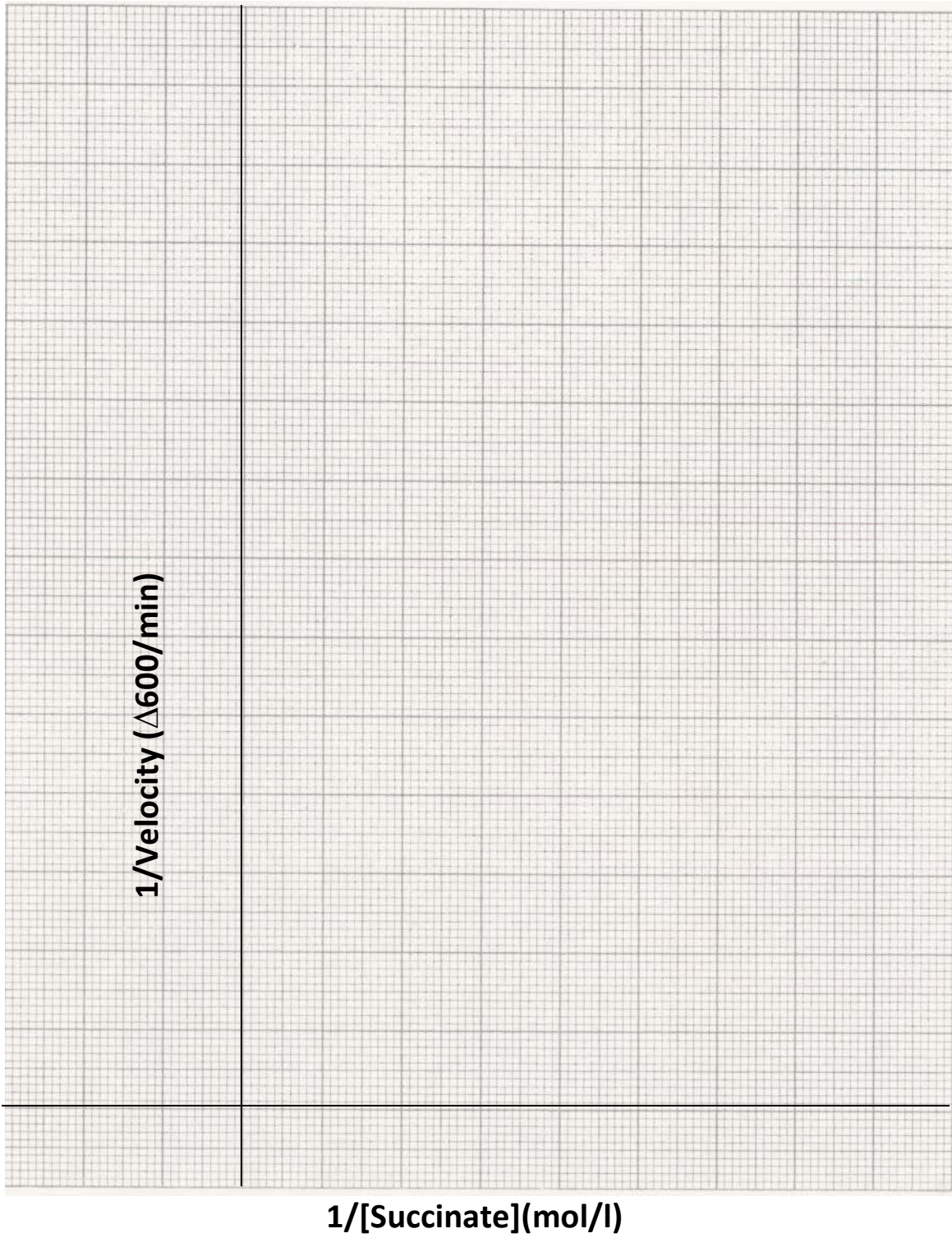
(#80, 81)

Velocity ($\Delta 600/\text{min}$)



[Succinate]

Lineweaver-Burke Plot (1/Velocity vs. 1/[Succinate] (#82, 83)



Your Data

RAW DATA CHART Each open blank should be filled and is worth 1 point each (#84-#109).

	Reaction Tube Identity	A_{600} $t \sim 10$ m	ΔA_{600} $(t_0 - t_{-10})$	A_{600} $t \sim 20$ m	ΔA_{600} $(t_0 - t_{-20})$	Velocity $\Delta A_{600}/\Delta t$ $(\Delta t \sim 10\text{m})$	Velocity $\Delta A_{600}/\Delta t$ $(\Delta t \sim 20\text{m})$
Dilute & Read at $t=10$ min	t=0		NA		NA	NA	NA
	w/o-4			NA	NA		NA
	w/o-3			NA	NA		NA
	w/o-2			NA	NA		NA
	w/o-1			NA	NA		NA
Dilute & Read at $t=20$ min	w/-4	NA	NA			NA	
	w/-3	NA	NA			NA	
	w/-2	NA	NA			NA	
	w/-1	NA	NA			NA	

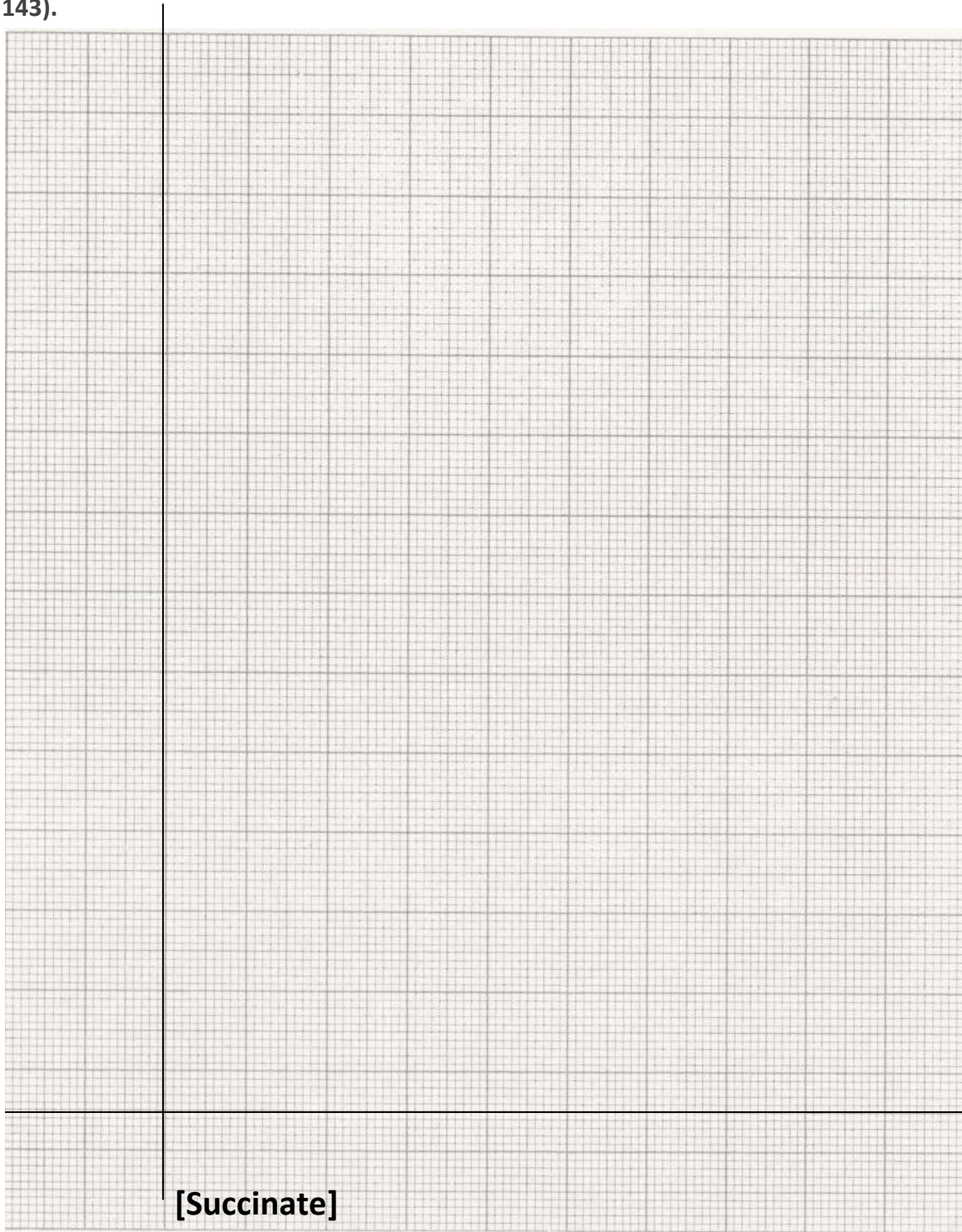
PLOTTED DATA Each open blank should be filled and is worth 1 point each (#110-#141).

Reaction Tube Identity	[Succinate]*	Velocity $(\Delta A_{600}/\Delta t)$	1/[Succinate]	1/Velocity
w/o-4				
w/o-3				
w/o-2				
w/o-1				
w/-4				
w/-3				
w/-2				
w/-1				

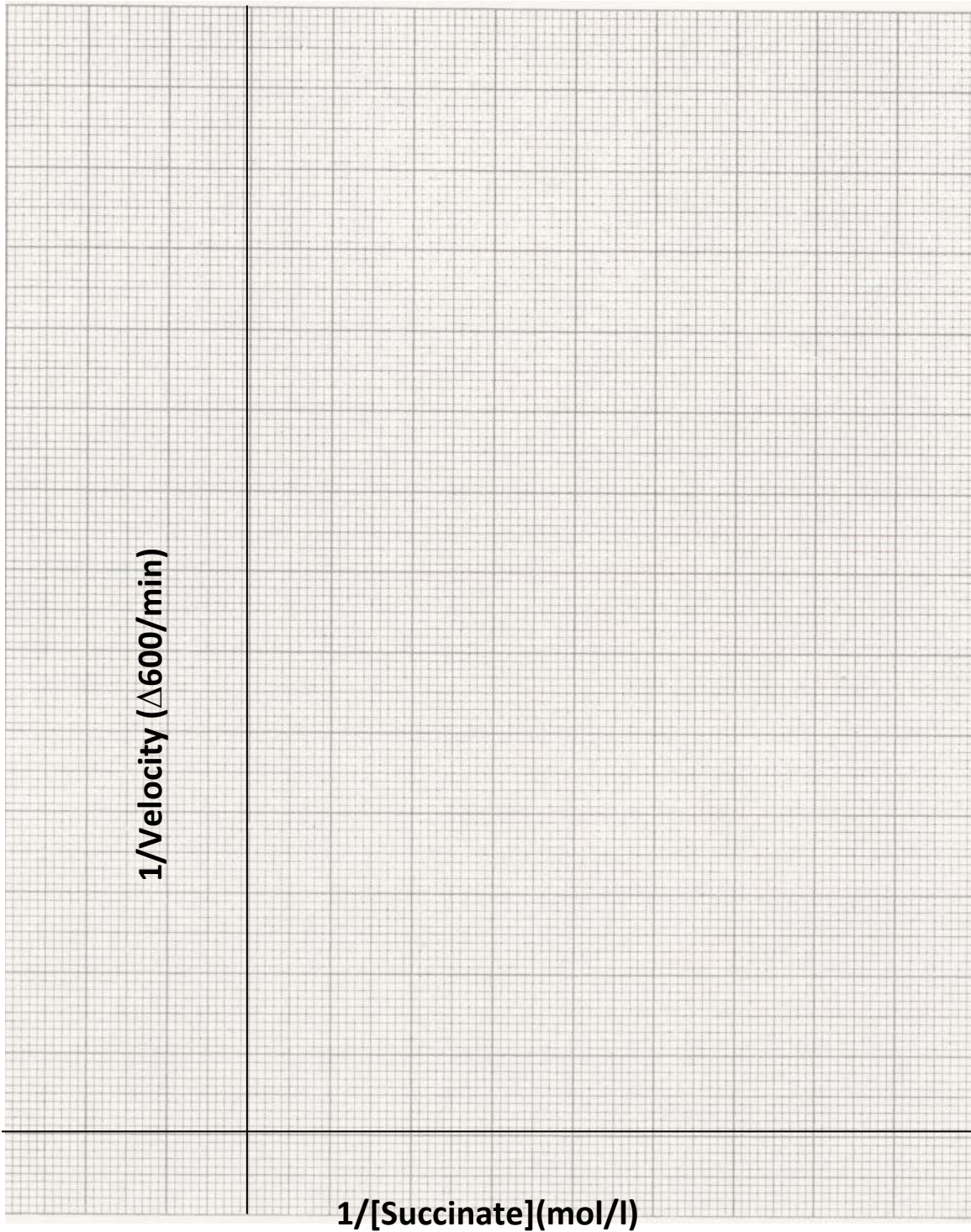
*[Succinate] = 0.3M (stock) x 1:3 dilution factor series x 0.2 dilution factor in reaction

Michaelis-Menten Plot (Velocity vs. [Succinate] 1 pt credit for each line (#142, 143).

Velocity ($\Delta 600/\text{min}$)



Lineweaver-Burke Plot (1/Velocity vs. 1/[Succinate]) 1 pt credit for each line (#144, 145).



Step 8. DETERMINING V_{max} and K_m FOR SDH FROM EACH PLOT:

Michaelis-Menton Plot: V_{max} can be determined from the Michaelis-Menton plot as the velocity at which the sigmoidal curve peaks (when an increase in [Succinate] no longer produces an increase in velocity). If your curve is not sigmoidal, use the highest velocity reached as V_{max} . To determine K_M from this plot, determine the [Succinate] giving $\frac{1}{2}$ that maximal velocity. Do this for the uninhibited reaction and the inhibited reaction and write these values in the chart below.

Lineweaver-Burke Plot: The x- and y- intercepts of the lines generated in the Lineweaver-Burke plots can be used to derive the K_M and V_{max} , respectively. ($K_M = -1/x$ -intercept and $V_{max} = 1/y$ - intercept).

PRACTICE DATA (#146-153)

	Michaelis-Menten Plot		Lineweaver-Burke Plot	
	K_M	V_{max}	K_M	V_{max}
uninhibited				
inhibited				

YOUR DATA Each open blank should be filled and is worth 1 point each (#154-161).

	Michaelis-Menten Plot		Lineweaver-Burke Plot	
	K_M	V_{max}	K_M	V_{max}
uninhibited				
inhibited				

Questions

1. The V_{max} and K_m values determined from the two types of plots of the practice data do not agree. Which do you feel more confident with and why? (#162)
2. Based on the K_m values determined from the Lineweaver-Burke plot, did any of your reactions contain a saturating concentration of succinate required to obtain the maximal velocity? (#163)