BIOCHEMICAL FRACTIONATION OF CHLOROPLASTS

During the past thirty years, a series of protocols has been developed that permits the homogenization of tissues and the subsequent fractionation and purification of cellular constituents. The thrust of the current exercise is centered around the isolation and subsequent fractionation of chloroplasts from pea leaves. Featured prominently in virtually all homogenization procedures are devices, such as the commercial blendors, the French Press, and the Probe Sonicator, all of which generate intense shear forces across the length of a cell, literally ripping the cell apart. However, excessive shear force will destroy the organelle we seek in any isolation protocol. In the current exercise, we will use a commercial blendor to homogenize plant cells. Cell disruption will release a variety of proteases, nucleases, lipases, and other molecules that may denature or destroy the cellular constituents that we wish to study. A variety of precautions (*e.g.*, keeping everything cold, including proteolytic enzyme inhibitors, etc.) have been incorporated into all isolation protocols to avoid these problems. The current lab exercise utilizes these same precautions. The purification of subcellular components on the basis of density takes advantage of differential sedimentation in high-intensity gravitational fields, induced by centrifugation and ultracentrifugation. In the current exercise, we will utilize high-speed differential velocity centrifugation to fractionate mixtures of cellular organelles. Purification of fractionated organelles can be performed on the basis of size, charge, or composition, using chromatographic or electrophoretic approaches. We will use gel electrophoresis to separate proteins present in the organelle fractions. In sum, this exercise incorporates all of the essential facets of cell homogenization, and all of the essential facets of organelle purification, fractionation and characterization.

Isolation of chloroplasts and fractionation into stromal and thylakoid protein fractions. The chloroplast from higher plants can be a large and abundant organelle that is green in color and is the site of photosynthesis in the organism. Photosynthetic reactions occur in and on the internal membranes of the chloroplast and involve two light absorption steps (*i.e.*, the light reactions). The absorption of light energy is essential for the generation of reducing power (*i.e.*, the dark reactions), that is used for the production of reduced compounds such as $NADPH_2$. These reduced compounds provide the chemical energy necessary for the synthesis of carbohydrates from CO_2 and H_20 in a process known as carbon fixation. In previous courses, you may have isolated chloroplasts and determined photosynthetic activity *in vitro* in various light regimes through the measurement O_2 production or the oxidation/reduction of the blue dye DCPIP.

Within the chloroplast, several compartments are delimited by internal membranes and membrane systems. The reactions that occur at these different sites within the chloroplast are mediated by the enzymes and light harvesting complexes found on the membranes; *the purpose of this exercise is to produce several fractions that are enriched in each of the different internal chloroplast membrane systems, with the intention of characterizing the proteins that reside at each site*.

Because chloroplasts are green, they are easily visualized during complex isolation and fractionation protocols. Because chloroplasts are found in plants, we can perform complex isolation protocols that do not start out with the unnecessary slaughter of any animals. **This laboratory exercise will demonstrate to you the basic underlying principles for both organelle isolation from intact cells in living tissues, and the subsequent fractionation of those isolated organelles. The same principles and similar protocols are used in the isolation and fractionation of organelles from all kinds of eukaryotic cells.** It should be intuitively obvious that different parts of the chloroplast contain different polypeptides. Using

current technology, it is possible for you to isolate and then fractionate these complex organelles. You will analyze the fractionated chloroplasts after the electrophoretic separation of their constituent proteins. An ancillary part of organelle isolation protocols is the on-line analysis of protein content or chlorophyll content (*i.e.*, yield) from the various fractions as you proceed from step to step. Assessments of yield at multiple intermediate steps of the protocol are often the primary indicators of whether or not your final isolates will contain what you expect them to contain. For this reason, the protocol calls for protein analysis. Since chlorophyll content is also a quantitative indicator of yield in the fractions, its analysis is also included.

An essential facet of this protocol is that you arrive organized and then keep close track of each fraction that you save for electrophoretic separation. With the current isolation scheme, you will accumulate 6 separate samples during the course of this fractionation protocol (see the FLOW CHART below). It is unreasonable to expect that your memory will good enough to remember which fraction was 'lower band' and which fraction was 'upper band' in the absence of the clear labelling of tubes and good record keeping.

These experiments are based on exercises and procedures developed by Drs. Robert Infantino and Steven Wolniak (University of Maryland, College Park).

References

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BFC LAB 1: Isolation of Chloroplasts and Fractionation

Preparation of intact chloroplasts from pea seedlings (modified from Schuler & Zielinski, 1989):

1. Pipet 35 ml of Percoll gradient solution into four 50 ml centrifuge tubes. Spin the solution at 40,000x*g* (*e.g.,* 17,000 rpm in a Sorvall SS-34 rotor in the RC-2B centrifuge) at 4°C for 40 min. Make sure that tubes are balanced with an identical tube on the opposite side of the rotor. While the Percoll is spinning, plan the isolation protocol carefully.

Percoll gradient solution: 50% PBF-Percoll (17.5 ml/tube)
50% grinding buffer (17.5 ml/tube)

2. Begin the dissection of the pea seedlings-(45-50g) immediately after you begin the initial Percoll spin. Chill the pea seedlings in ice water for 15 min. Place the chilled seedlings in a large crystallizing dish containing 50 ml of Grinding Buffer and chop the seedlings into small segments. Transfer the segments plus the grinding buffer into a chilled plastic box. Add ~450 ml of additional (slushy) Grinding Buffer. Distribute the segments plus the grinding buffer uniformly in the box and homogenize the segments for 5s at a setting of "6" using the Polytron Tissue Homogenizer.

Grinding buffer: (600 ml)
0.35 M sorbitol
50 mM HEPES/KOH, pH 7.5
2 mM EDTA
1 mM $MgCl_2$
1 mM MnCl2
add 1 g sodium ascorbate immediately before use

3. Filter the slurry through two layers of moistened miracloth plus one layer of 20 : nylon bolting cloth into a 1000 ml beaker that is sitting on ice. Transfer the filtrate to two 250-ml centrifuge bottles and centrifuge the filtrate at 2500 x *g* for four min at 4°C.

4. Decant and discard the supernatant. Gently resuspend the crude pellets with a moistened brush in a total of 6 ml of fresh, pre-chilled grinding medium. When the resuspension is complete, place duplicate 100 : l samples into two 1.5 ml microfuge tubes labeled "**1**". Store them on ice. *For each sample in the protocol, you must obtain two aliquots in separate microfuge tubes. One aliquot will be used either for a BCA protein assay or a chlorophyll assay* (Lab 2)*, and the other will be used for SDS-polyacrylamide gel electrophoresis (*Lab 3).

5. Carefully place the remainder of your crude suspension on top of each Percoll gradient. It is essential that the suspension not disturb the gradient; if the solution is added gently to the top of gradient by pipetting along the wall of the tube, you will obtain a suitable loading onto the gradient.

6. Centrifuge the samples at 9000 x *g* at 4°C for 15 min in a swinging bucket rotor (*e.g.*, Sorvall HB-4 at 9000 RPM) without the brake on.

7. When the centrifuge stops, carefully remove the gradients from the rotor. You should observe two green bands. The **upper band** (**U**) contains thylakoids(broken chloroplast remnants) and

the **lower band** (**L**) contains intact chloroplasts. If the upper band is far larger than the lower band, your homogenization was excessive. Remove each of the bands from the gradient with a Pasteur pipette, being careful not to mix (or mix up) fractions.

8. Place each fraction into a fresh 50 ml centrifuge tube. Add 30 ml of grinding medium to each tube. (This step is designed to remove the Percoll from the fractions.) Cap each tube with a square of Parafilm and gently invert each of the tubes several times to mix the fraction with the buffer. Remove the Parafilm and centrifuge the tubes at 2500 x *g* (3700 rpm) for 4 min. at 4°C. Carefully decant the supernatant and resuspend the pellets in ~3 ml of pre-chilled grinding buffer. Keep the samples on ice. Place duplicate 100 µl samples in microfuge tubes labeled "**2U**" and "**2L**".

9. Re-pellet the intact chloroplast sample by centrifugation at 2500 x *g* for 5 min at 4°C. Discard the supernatant.

10. Resuspend the pellet in 2 ml of Chloroplast Lysis Buffer. Incubate the sample for 15 min. on ice with occasional vortexing. At the end of the incubation period, place two 100 µl samples in microfuge tubes labeled "**3L**".

Chloroplast Lysis Buffer: 62.5 mM Tris-Cl, pH 7.5
2 mM $MgCl_2$

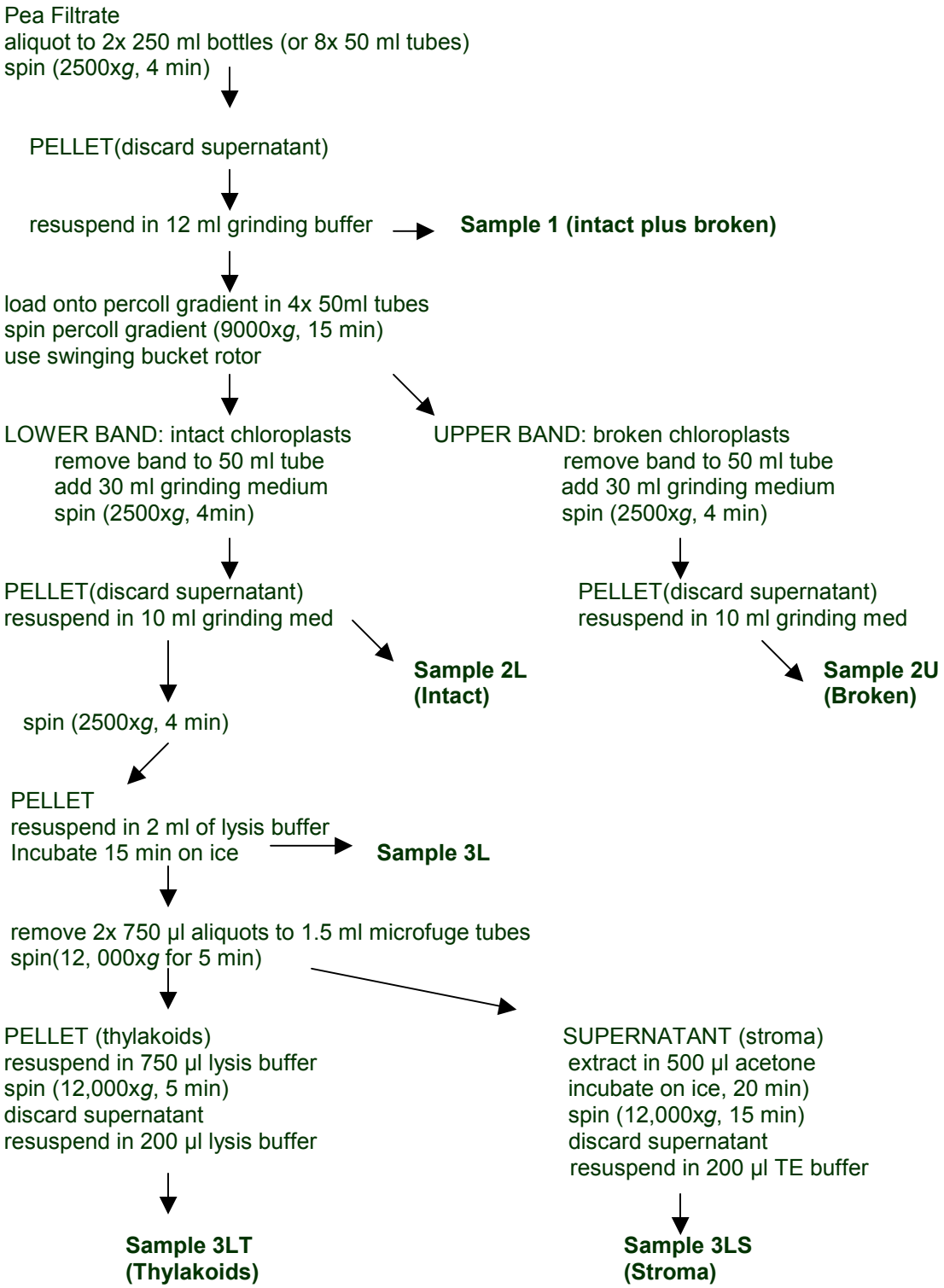
11. Place duplicate 750 µl samples of the lysed, intact chloroplasts into fresh 1.5 ml microfuge tubes. Collect the thylakoid membranes by centrifuging in the microfuge at 12,000 x *g* for 5 min at 4°C. Transfer the supernatant into a fresh microfuge tube. The supernatant contains the proteins from the stromal portion of the chloroplast (**do not discard).** Resuspend the thylakoid pellet in 750 µl of chloroplast lysis buffer. Re-centrifuge at 12,000 x *g* for 5 min. Discard the supernatant and resuspend the pellet in 200 µl of lysis buffer and then divide the resuspended sample equally into two microfuge tubes labeled "**3LT**".

12. Place duplicate 500 µl samples of the stromal extract in fresh microfuge tubes. Precipitate each sample by adding 500 µl of acetone in the hood, mixing well and incubating on ice for 20 min. Collect the precipitated proteins by centrifuging in the microfuge at 12,000 x *g* for 5 min at 4°C. Decant the supernatants and resuspend the pellets in 200 µl of TE (Tris-EDTA, pH 7.5) buffer. Place duplicate 100 µl samples in microfuge tubes labeled "**3LS**".

You have generated a duplicate set of samples, numbered 1, 2U, 2L, 3L, 3LT, 3LS. One set of these samples should be stored at -20°C, without further treatment. They will be used in Lab 2 for the chlorophyll and BCA assays. To each tube in the other set, add 100 µl of 2X SDS sample buffer, boil for 5 min, cool, and store at -20°C. These samples will be used for electrophoretic analysis in Lab 3.

2X Sample Buffer: 2 ml 0.5 M Tris-HCl, pH 6.8
2 ml 20% SDS
200 µl 0.2 M EDTA
1 ml 1M dithiothreitol(DTT)
2 ml glycerol
2.8 ml H_2O
a few crystal of bromophenol blue (BPB)

FLOW SHEET FOR THE ISOLATION AND FRACTIONATION OF CHLOROPLASTS



BFC LAB 2: Analysis of Chlorophyll and Protein Content

Chlorophyll determination:

13. Label six 5-ml volumetric flasks tubes "1, 2U, 2L, 3L, 3LT". Transfer 50 µl of each sample into their respective flask and fill the flask with 80% acetone(v/v) up to the fill line. Cap and invert each flask 10-20 times. Filter the green acetone extract through Whatman #1 filter paper into a fresh labeled 14-ml test tube.

14. Determine the chlorophyll content of the filtrates by measuring A_{663} and A_{645} in the spectrophotometer. Absorbance of the samples should be read against the blank. If the extract is too concentrated for accurate absorbance readings, dilutions should be made with 80% acetone. Calculate the amount of chlorophyll by substituting the values you measured against the following equations.

$$\text{Chl } (\mu g/ml) = [20.2 \times (A_{645}) + 8.02 \times A_{663})] \times \text{dilution factor } (x100)$$

BCA protein determination:

The bicinchoninic acid (BCA) assay for protein (Pierce Chemical Co. BCA Kit) is a derivative of the biuret reaction (protein reducing Cu^{2+} in an alkaline medium to produce Cu^{+}). Peptide bonds and four amino acids (cysteine, cystine, tryptophan and tyrosine) are reportedly responsible for this reduction reaction. Two molecules of BCA interact with one cuprous ion (Cu^{+}) to form a water soluble product that exhibits a strong absorbance at 562 nm. This allows the spectrophotometric quantitation of protein concentration in aqueous solution.

15. Prepare a set of eleven microtubes containing the following BSA protein standard dilutions (made from a 1 mg/ml BSA solution) and dilutions of your sample 3LS.

Sample	Addition	Amount of Protein	A_{562}
A.	50µl of H_2O	0 µg	
B.	5 µl BSA + 45 µl H_2O	5 µg	
C.	10 µl BSA + 40 µl H_2O	10 µg	
D.	15 µl BSA + 35 µl H_2O	15 µg	
E.	20 µl BSA + 30 µl H_2O	20 µg	
F.	25 µl BSA + 25 µl H_2O	25 µg	
G	50 µl BSA	50 µg	
H.	5 µl lysis buffer + 45 µl H_2O		
I.	1 µl 3LS + 4 µl lysis buffer + 45 µl H_2O		
J.	2.5 µl 3LS + 2.5 µl lysis buffer + 45 µl H_2O		
K.	5 :l 3LS + 45 :l H_2O		

16. Prepare Working Reagent by mixing 50 parts of Reagent A with 1 part Reagent B. The working reagent is stable for up to one day.

17. Pipet 1 ml of Working Reagent into each microtube, cap and mix.

18. Place the tubes at 37^{o}C in a constant temperature water bath for 30 min.

19. Allow the tubes to cool to room temperature.

20. Measure the absorbance at 562 nm for each sample (B-K) in the spectrophotometer using the "zero protein sample" (sample A) as the reference.

21. Prepare a standard curve by plotting absorbance *versus* protein concentration of the standards.

22. Use the standard curve to determine the protein content of sample 3LS (1.0, 2.5, 5.0 µl aliquots) .

BFC LAB 3: SDS Polyacrylamide Gel Electrophoresis

Electrophoresis is a process in which charged molecules migrate in an electric field. It is a powerful tool that permits separation of nucleic acids and proteins on the basis of their size, shape and charge. Under denaturing conditions, nucleic acids and proteins may be converted to essentially linear structures and they will be electrophoretically separated primarily according to their size and, perhaps, charge.

Sodium dodecyl sulfate (SDS) PAGE is used to separate protein mixtures according to the size of the constituent proteins. SDS is a detergent that acts as a powerful protein denaturant. The aliphatic dodecyl group tightly associates with the backbone of polypeptide chains, eliminating the secondary and tertiary structures of the protein and linearizing the polypeptide. Moreover, the sulfate moiety contributes a uniform negative charge to the complex, causing the polypeptides to migrate toward the positive electrode in an electric field. In PAGE, the sample is separated from the positive electrode by a porous, inert matrix of cross-linked polyacrylamide such that linearized polypeptides will migrate through the matrix with their individual mobilities determined by their size: small molecules will migrate more quickly, their movement will be permitted by a wide range of pore dimensions within the matrix; large molecules will move more slowly, their movement limited to those pores within matrix that can accommodate their size. Molecules of the same size will migrate the same distance, occupying identical positions at the end of the electrophoretic run. After staining the gel with a protein-binding dye, polypeptides appear as "bands" at various distances from their electrophoretic start point. By carrying out parallel electrophoresis of a group of marker polypeptides of known size, comparison of the mobilities of experimental and marker polypeptides permits estimation of the size of the experimental polypeptides (Weber & Osborn, 1969).

If a sample is directly loaded onto a polyacrylamide gel in a certain volume, polypeptides nearest to the gel surface when electrophoresis commences will enter the gel ahead of polypeptides more distant from the gel within the volume loaded; thus, bands obtained at the end can be a broad as the depth of the volume loaded. To obtain clear resolution of bands, Laemmli (1970) devised a 'discontinuous gel' system that is almost universally used today. Between the primary gel and the sample, one places a very large porosity "stacking" gel (offering no resistance to even large polypeptides) containing a low charge so that all polypeptides will move very slowly through it. This slow movement through the stacking gel forces the polypeptide mixture to occupy a minimal volume as it approaches the interface with the main, "separatory" gel. Thus, all polypeptides in the sample appear to enter the separatory gel simultaneously, yielding very distinctive bands after electrophoresis.

The electric field used in electrophoresis is provided by direct current generated from a power supply. As in any electrical circuit, Ohm's law applies:

$V = IR$, where V = voltage
I = current
R = resistance.

For most electrophoretic applications, either voltage or current is held constant. At constant voltage, the rate of polypeptide migration will be proportional to the current. However, the gel provides resistance to current and, like any resistor, it will heat up as current is increased. Power, a measure of current times voltage, is a measure of gel heating.

$P = VI$, where P = power

Gel heating caused by excessive current causes smearing of the polypeptide bands and can cause equipment meltdown! Power supplies can provide constant voltage, constant current, and/or constant power. Constant current provides constant rate of polypeptide migration, at the expense of gel heating. Constant voltage reduces gel heating, at the cost of long separation times. With constant power, one gets constant heating but declining migration rates as the resistance of the gel increases.

Preparation of the electrophoresis apparatus and gel:

23. Prepare the following stock solutions. *Wear gloves. Acrylamide is a neurotoxin.*

Gel Solution A

30% acrylamide
0.8% bis-acrylamide

Gel Solution B

1.5 M Tris-HCl, pH 8.8
0.4% SDS

Gel Solution C

0.5 M Tris-HCl, pH 6.8
0.4% SDS

5% Ammonium persulfate (store dark)

0.5 g ammonium persulfate
10 ml H_2O

5X Electrode Buffer (~300 ml per gel apparatus)

94 g glycine
15.1 g Tris base
dissolve in ~800 ml H_2O
25 ml 20% SDS
40 ml 0.2 M EDTA
H_2O to 1 l

24. Wash the glass plates with soap and water. Clean inner surfaces with 70% ethanol and allow to dry.

25. Assembly of the apparatus will be demonstrated. Take special care to avoid leaks and to avoid uneven pressure on the glass plates that might crack them.

26. Insert the comb at the top of the small plate so that only the teeth enter the gap between the plates. Put a mark on the small plate 0.5 cm below the bottom of the teeth. This is the fill line for the separatory gel. Remove the comb.

27. *Wearing gloves*, measure the ingredients for the Separatory Gel into a 50 ml Erlenmeyer flask (~5 ml per gel):

12% Separatory gel:
4.0 ml Solution A
2.5 ml Solution B
100 µl 5% ammonium persulfate
3.6ml H_2O

Mix slowly. Ensure that your apparatus is completely assembled before proceeding with the next step.

28. Add 12 µl TEMED. Swirl a few seconds to mix. Pour the gel solution directly into the gap between the glass plates of your apparatus to the fill line mark. This should be done quickly, avoiding bubbles.

29. Quickly and gently pipette H_2O over the gel solution between the glass plates, minimizing mixing of the two solutions. This provides an air-free seal that will allow TEMED to catalyze polymerization of the acrylamide and bis.

30. Allow the gel to polymerize undisturbed for 30 min. Meanwhile, *wearing gloves*, measure ingredients for the Stacking Gel into a 50 ml Erlenmeyer flask (1.3 ml per gel):

Stacking gel: 0.6 ml Solution A
1.0 ml Solution C
50 µl 5% ammonium persulfate
2.35 ml H_2O

31. After the separatory gel has polymerized, pour off the water layer and rinse the gel surface with H_2O. Place paper towels under the glass plate sandwich to catch overflow from the next step.

32. Add 2.5 µl TEMED to the stacking gel solution. Swirl for a few seconds to mix. Pour the solution over the separatory gel, filling the gap between the two glass plates. Immediately insert the comb so that the teeth are submerged in the stacking gel solution. Avoid trapping bubbles. Allow the stacking gel to polymerize undisturbed for 20 min.

33. If you have not already done so, you must now use your BCA protein and chlorophyll assay data from Lab 2 to calculate protein concentrations for each of your samples. Remember that you added an equal volume of 2X Sample Buffer to each sample, so your actual sample protein concentration is one-half that determined in Lab 2.

Sample loading and electrophoresis:

34. Insert the glass plate sandwich into the electrophoresis apparatus. Dilute the 5X electrode buffer five-fold and add to the top (inner) chamber. Check for leaks.

35. Add 1X electrode buffer to the lower (outer) chamber. Gently, remove the comb and rinse the wells with electrode buffer.

36. Load samples through the upper electrode buffer into the gel wells with a micropipette (~10µg of protein equivalent samples #1, 2U, 2L, 3L, 3LT, 3LS; try to keep the volumes <20 µl)). Keep a record of which sample goes into which well. Because the sample contains glycerol it is more dense than the electrode buffer and should fall through the buffer in the well into the bottom of the well.

37. Load one lane with 5 µl of the BRL Protein Ladder mixture.

38. Place the cover on the gel apparatus. Attach power cords to the power supply. Turn on the power supply and adjust to 100V. Run until the dye front reaches within 0.5 cm of the bottom of the gel (1 hr). *Always turn off the power supply and disconnect the cords before opening the apparatus.*

Staining and visualizing polypeptides in the gel:

39. While the gel is running, prepare the stock solution:

***Coomassie Staining Solution:**	0.4 g Coomassie Blue R-250 (0.4%) 40 ml of methanol (40%) 10 ml of acetic acid (10%) Distilled water to 100 ml * Made up fresh

40. Stain gels for 30-60'. Rinse with distilled water.

Destaining Solution:	40 ml of methanol(40%) 10 ml of acetic acid(10%) Distilled water to 100 ml

41. Destain gel. Change destaining solution three times over a 1-3 hour period. Leave the gel in destain solution until the next lab.

BFC LAB 4: Analysis of Results

42. Photograph your gel next to a ruler, providing a linear scale with which to measure migration of various polypeptides.

43. You may "immortalize" your gel by drying it via the following procedure (derived from Matsudaira & Burgess, 1978):

a. Rinse the gel in H_2O then place in ~100 ml of 20% ethanol + 10% glycerol. Gently agitate at room temperature for 30 min.

b. Cut two pieces of porous cellophane slightly larger than the outer dimensions of the drying frame. Wet one of these with H_2O and smooth it over the solid member of the drying frame.

c. Center the gel on the cellophane-covered backing frame, avoiding trapping air bubbles.

d. Wet the second piece of cellophane and smooth it over the gel and cellophane-covered backing frame, again avoiding trapping air bubbles.

e. Clamp the open frame against the second piece of cellophane and place this sandwich in the hood to dry overnight. After drying you can trim the cellophane and the gel can be kept indefinitely under dry conditions.

44. The BRL Protein Ladder pattern should be similar to that shown on the handout. Plot the molecular weight of the standard proteins *versus* their individual migration distances.

Is the relationship linear or exponential? If the latter, a semi-log plot should produce a linear function.

45. Examine polypeptide bands within your experimental samples. Make a table listing sizes of detectable bands and the effect of the fractionation procedure upon the abundance of each polypeptide (*e.g.*, using a -, +, ++, *etc.* notation for band abundance).

Are there bands that show distinctive changes in abundance among your fractions? Describe those changes, referring to the polypeptides by molecular weight estimates that you calculate for each.

Note: Typical subunit MWs for some thylakoid and stromal proteins are shown in the table on the following page.

Thylakoid Membrane Proteins

Protein	Subunit Molecular Weights(kDa)*
LHC II	25
ATP Synthase	62(") 54($) 38(() 19(*) 13(,)

*Determined using our SDS-PAGE System

Calvin Cycle (stromal) Enzymes

Enzyme	Subunit Molecular Weights(kDa)
G-3-P Dehdyrogenase (NADP)	50-53
Transketolase	100
Epimerase	26
Phosphoribulokinase	44
RuBisCo	50-57(L) 11-16(S)

Outline for Lab Report C: Biochemical Fractionation of Chloroplasts

BIO 410

I. Introduction and Background (one-page limit)

Provide a brief description of our current understanding of the relationship between structure and function in intact chloroplasts.

II. Specific Aims

Concise list of the objectives of these experiments; can be a numbered list of single sentences.

III. Experimental Methods & Results

Describe what you did and present the results of those experiments. Experimental detail found in the manual need not be repeated. Include figures and tables presenting your results (*e.g.*, gel photos, graphs, tables).

IV. Conclusions & Discussion (one and one-half page limit)

Self-explanatory. How do the polypeptide patterns compare in the thylakoid and stromal fractions? Are there any polypeptides *(identified by estimated MW) that occur in both fractions? How might you approach the questions of whether the thylakoids and stroma contain exactly the "same" polypeptide?

V. Recommendations for Course Improvement

Give us three recommendations of means by which these experiments or the course in general can be improved in the future. Do not repeat recommendations that you gave before.