

CELL PHYSIOLOGY

BIOLOGY 580

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Euglena EXPERIMENTS: EFFECTS OF PHYSICAL AND CHEMICAL FACTORS ON MOTILITY

EFFECT OF TEMPERATURE ON MOVEMENT:

The effect of light on movement

Euglena has a light spot at the anterior end at the base of the flagellum which has been implicated in phototactic responses. The eyespot is associated with the flagellum. The action spectrum of phototaxis corresponds to the absorbance of the eyespot with a peak at 480-500 between blue and aqua. Each group should apply different colored plastic to holes made as a vertical series of windows, (each with a different color) down the side of the test tube, where the fluid of the culture is in a black paper used to cover the sides of a test tube. Make sure no light can get into the tube except through the holes. Place lights within 20 inches of the tube and holes (don't get the tubes too close or hot, and look at the distribution of the organisms after 1-2 hrs. When making conclusions, remember that the color of something like the plastic shows what color is reflected, not sent through it. Do this by carefully decanting with the windows held up.

Effect of ions on movement

Euglena can switch to an amoeboid or more appropriately, a euglenoid movement under certain conditions since it has a rather soft pellicle. With the following experiments, try to determine what those conditions are. In these experiments use bottled water, not tap or distilled water. Place 1 ml of culture for the whole class into 1 ml of the following 1/16 M solutions;

NaCl, KCl, MgCl₂, CaCl₂, Na₂SO₄, NaI. Then each group should observe the rate of movement in one drop on a slide. Then different groups can each try 2 mixtures of the above with one drop in each of two different ions. Give the euglena a chance to equilibrate in the new mixture, or observe as they do, noting the difference when a divalent cation is mixed with a monovalent and another divalent cation. Look up the effect of gibbs donnan equilibrium changes on ions bound to microtubules and microfilaments due to such mixtures.

bound monovalent cation = free monovalent cation ----- bound
divalent cation SQUARE ROOT OF free divalent cation

THIS SAYS THE RATIO OF BOUND MONO TO DI-VALENT CATIONS EQUALS THE RATIO OF FREE MONO TO SQUARE ROOT OF FREE DIVALENT CATIONS

Can you get recovery of stopped ones by adding a drop of water? Observe for at least 5 minutes after dilution. Looking at the equation, you can see that dilution with water does not simply change this value in the equation, since the square root of the divalent ion concentration does not change at the same rate as the monovalent ions, by dilution.

Effects of pH on motility observe motility using a drop of culture and a drop of buffers ranging from 5.5-9. Each group works alone on this.

Effects of alcohol on motility Each group should do this alone. To .5 ml of culture, add drops one at a time of 2% alcohol solutions, wait 2 minutes and observe movement after each drop until it stops. If it stops with one drop, dilute the alcohol 1/10 and start again. Use all of these alcohols which differ in chain length, and therefore rate of penetration of the membrane: methyl, ethyl, propyl, butyl, amyl. See if they recover after dilution with water. Keep in mind when writing up your results that the cell membrane regulates intracellular ion concentrations important in motility, and that motility occurs due to microtubules or microfilaments and other associated proteins.

ACTIN POLYMERIZATION AS DETECTED BY DNAase INHIBITION

References: Cell 15:935-943. 1978; J. Biochem. 87:785-802. 1980.

Stock solutions: 1. DNAase I- .1 mg/ml in .5 mM CaCl₂, 50 mM TrisHCL pH 7.5 prepare at the time of the lab by adding solvent. 2. DNA (calf thymus) cut DNA fibers and dissolve by stirring overnight after homogenizing in a glass homogenizer in .1 M tris pH7.5, 4 mM MgSO₄, 1.8 mM CaCl₂. Measure absorbance at 260 nm, should be .5-.65 OD. This should be 1 mg/ml, dilute 1/25. 3. actin extracted from acetone muscle powder 4. guanidineHCL- 1.5 M GuHCL in 1 M sodium acetate, 1 mM CaCl₂, 20 mM trisHCL pH 7.5

Equipment: quartz cuvettes, micropipettes-10,15,30 ul; UV spectrophotometer Quartz cuvettes should only be cleaned with Q-tip cotton swabs, soap, distilled water. Handle with care- they cost \$30 each.

Purpose: to make a standard curve of inhibition of DNAase by G-actin, then to use that to detect how much G- or F-actin is present in an unknown (later we will use sea urchin eggs before, during and after cleavage)

Step 1. detection of DNAase without inhibition. This is done by mixing 10ul of DNAase with 3 ml DNA (solutions above) and immediately observing OD at 260 nm over a period of 5 min. We will have to use the spectrophotometer in the adjacent room. It holds four cuvettes, so several can be done at once from the different groups by reading in series. Plot the results and get a slope.

Step 2. detection of g-actin inhibition using known amounts of G-actin. Run a Biorad on your actin sample. Then prepare the following: (.5 ml each)

1. 20 ug/ml
2. 100 ug/ml

3. 300 ug/ml

4. 400 ug/ml

5. 600 ug/ml (if your actin is not this concentrated, make a similar but more dilute series.)

Now place 15-30 ul of the actin into a cuvette,(always use the same volume in your tests from then on) with 10 ul DNAase, stir immediately to mix the actin and enzyme and quickly add 3 ml DNA (have all these things ready) and observe in spectrophotometer and plot, get slope, and determine the amount of inhibition by the actin. Do this for each concentration of actin. Plot amount of inhibition (%) vs. actin concentration. Complete inhibition of 1ug DNAase by 1.35 ug actin according to the literature. The lower limit of detection of actin should be 10 ug/ml actin, upper limit 1.8 mg/ml.

Step 3. Now prepare some F-actin (someone else can be doing this ahead of time) by adding .5 ml 2M KCl to 4.5 ml G-actin. (This can then be used for viscometry). Save .5 ml for use in this test, using it about an hour after adding the salt. Dilute the F-actin, using a concentration of actin which gave at least 50% inhibition of DNAase, use the volume used previously with G-actin, doing an inhibition test (this will not inhibit as much because much of the actin will be in the F-form). Now take a volume of the F-actin (like .1 ml) and add the same volume of guanidineHCl and incubate at 0" for 5 min. Then take 2x the volume (of this treated actin) usually used in the inhibition test (since you diluted with GuHCl) and do the DNAase inhibition. This will give you the value of the total actin present, since the guanidine causes depolymerization. Subtract the amount of G-actin in the test without guanidine from the value in the test with guanidine, and that is the amount of F-actin present. Now we can use this test on extracts of cells where there is not enough actin to extract and do viscometry. Hand in the inhibition standard curve and the values for G-actin and F-actin and total actin in your polymerized actin.

VISCOSITY MEASUREMENTS OF ACTIN POLYMERIZATION TO F ACTIN

From the protein readings you took on the actin, figure out the mg/ml. If you have 5 mg/ml, for the experiments below, you would take 0.5 ml and dilute with 4.5 ml tris-ATP-CaCl₂ to give Actin at a final concentration of .5 mg/ml in .01 M TRIS pH 8, 10-4M ATP, 10-4 M CaCl₂. Actin can be induced to polymerize by addition of 2.5 M KCl to give a final concentration of 50mM. **DO NOT ADD THE KCl UNTIL YOU HAVE TAKEN A VISCOSITY MEASUREMENT OF THE DILUTED G-ACTIN WITH EVERYTHING ELSE, BUT WITHOUT THE KCl. PLACE 5 ML IN THE VISCOMETER AFTER HEATING IT TO ROOM TEMPERATURE BY IMMERSING IT'S CONTAINER IN TAP WATER AT 25 DEGREES. USE A STOP WATCH TO SEE HOW MUCH TIME ELASPED BETWEEN WHEN THE ACTIN PASSES THE TOP CROSS MARK TO WHEN IT PASSES THE BOTTOM MARK. REPEAT, AND AVERAGE THE RESULT.** Then add the amount of KCl to polymerize the 5 cc of actin (.05/2.5= 1/50 dilution or 0.1/5, so add 0.1 ml 2.5 M KCl to the actin in the bulb of the viscometer, **ROTATE** to mix, **RECORD THE TIME**, and do a viscosity measurement by reading the number of seconds for the actin to traverse the distance between the two cross marks and record it. Do that every 2 minutes or however long it takes to run the volume through in case that is more than two minutes. Stir the actin in the bulb between readings by rotating the viscometer. Keep the readings going for 15 minutes. Now try to see if actin will polymerize in the presence of an oxidizing agent. Prepare 5 ml of G-actin as above but with diamide in it. That means you will add to 0.5 ml actin

(or whatever volume in your case gives 2.5 mg actin)+ whatever volume of tris-ATP-CaCl₂ to make up to 5 ml, plus diamide at a concentration given by your instructor. Measure viscosity and add KCl as before and follow viscosity change. **MAKE SURE ALL SOLUTIONS ARE AT ROOM TEMPERATURE WHEN YOU START TO MEASURE THEIR VISCOSITY. ACTOMYOSIN VISCOSITY** Prepare the actomyosin by adding some unfrozen myosin (1/5 the concentration of the actin) to a diluted 5 ml preparation. Try to have a concentration which will give an initial flow time at room temperature of 2-4 minutes, using 5 ml in the Ostwald viscometer. figure out the protein concentration in mg/ml from your protein tests and the dilution. After determining this flow time, add 0.05 ml 0.1 M ATP and 0.05 ml of 0.01M MgCl₂. Keep doing readings until it reaches a steady state (this may take 40 minutes.) There should be an initial decrease and then a rise back to what it was when you started. Plot a graph of flow time versus time after addition of reagents. Try this at 20,25, and 30 degrees and calculate the Q10. What does this show about the reaction? Is the Q10 the same for the increase as for the decrease? (Remember in trying to explain the results that there is 10-4M ATP in the actin.)

EGG CORTEX ISOLATION

Unfertilized or fertilized eggs prepared according to the fertilization exercise are washed after settling in 5 volumes of 0.1 M MgCl₂, 1 mM Tris pH 8 to stabilize the cell membranes and cortex. They are centrifuged at 5000 rpm for 5 min, and homogenized in 10 mM MgCl₂- 1 mMTris pH 8. The cortices are sedimented again at the same rate and resuspended in the same medium and rehomogenized, and this is again repeated. The final pellet contains the ghosts or cortical hulls. These hulls can then be used for the extraction of actin, tubulin or the assay for enzymes such as G6PD, Na+K+ATPase.

NA+K+ATPASE FROM MEMBRANES OF SEA URCHIN EGGS

OBTAIN EGGS, FERTILIZE THEM, AND PREPARE CORTICAL HULLS AS IN OTHER EXPERIMENTS.

ASSAY THE ATPASE WITH THE FOLLOWING REACTION MIXTURE:

MOLARITY VOL FINAL CONC

MgCl ₂	0.6M	.05 mL	
NaCl	1.5M	.9	150mM
KCl	1.5	.2	33 mM
ATP	.02	1.3	2.88 mM
TRIS	1 M	1.8mL	200 mM
PROTEIN	*	1.2 mL	
WATER		3.55 mL	
TOTAL		9.0 mL	

* TO BE DETERMINED BY BIORAD USING MATERIAL LEFTOVER

THIS GIVES YOU ENOUGH FOR FOUR 2 ML SAMPLES, A ZERO AND THREE OTHERS.

TAKE OUT SAMPLES AS FOR MYOSIN ATPASE AND DETERMINE THE P_i CONTENT AS FOR MYOSIN ATPASE. THIS ENZYME IS LESS ACTIVE, SO USE LONG TIME PERIODS LIKE 5,10,15 MINUTES AT 35 DEGREES.

SUBSTITUTE NITROPHENYLPHOSPHATE IN THE REACTION AND READ ON THE SPECTROPHOTOMETER DIRECTLY, WITHOUT THE ADDITION OF THE P_i DETECTION REAGENTS.

VARY THE ASSAY BY LEAVING OUT BOTH Na^+ AND K^+ TO SEE THE LEVEL OF Mg^{++} ATPASE. TO MAKE SURE IT IS NOT A Ca^{++} ATPASE, SUBSTITUTE Ca^{++} FOR Mg^{++} IN ONE EXPERIMENT, OR ADD EGTA TO THE SAME MOLARITY AS THE Mg^{++} .

MITOSIS REGULATION

Prepare fertilized eggs as in the fertilization exercise. Resuspend .1 ml eggs into ten ml of the following drugs in seawater and pour into a petri dish; all at 10 μ G/ML

1. D-ACTINOMYCIN
2. PUROMYCIN
3. COLCHICINE
4. VINBLASTINE
5. CYTOCHALASIN
6. DEXAMETHASONE
7. DNP
8. ARSENIC
9. CYANIDE
10. Place .1 ml eggs into normal seawater as a control.
11. Place a similar control on ice.
12. Place a control at 37 degrees.

Observe to see when cleavage occurs, or if it occurs. Determine whether each drug or treatment should work at the nuclear level or spindle level, or cleavage furrow level, or metabolic level.

ISOLATION OF MITOTIC APPARATUS

Remove the vitelline membrane from unfertilized eggs by treatment in 3 mM dithiothreitol (DTT), pH 9 for ten minutes. Decant off the supernatant from the settled eggs (they will settle during the ten minutes.) Wash 2x with sea water and then fertilize as in fertilization exercise. You will not be able to see a membrane elevate, since it is gone, so it would be a good idea to reserve a few eggs that are not treated with dtt to fertilize at the same time, to be sure the sperm is good. After fertilized eggs settle, resuspend them in Ca-Mg-free sea water (CMFSW) which contains 10 mM EDTA, pH 7.5. Settle, repeat. When nuclear membrane breaks down in 1 1/2 to 2 hours remove the supernate and replace with 5 volumes of 90% 1M dextrose, 10% cmfsw. When they reach metaphase, suspend the cells in 10 volumes of isolation medium, stand 2 mins, then shake moderately to break cells, or pass through 54 um nitex cloth, place in an ice bath, and observe to see that cells are broken. Spin at 200 g to sediment mitotic apparatus (at 0 degrees for 30 min.) Add CaCl₂ to a concentration of .1 mM to halve the mitotic apparatus. Observe and freeze

ISOLATION MEDIUM 1 M SUCROSE, .15 M DITHIODIGLYCOL, 1 mM EDTA pH6.4. An alternative is 20 mM MES, 10 mM EGTA, 1 mM MgCl₂ pH 6.4.

REFERENCES: Mazia et al. The direct isolation of the mitotic apparatus. J. Biophys biochem cytol. 10: 467-74. 1961. Silver et al. Isolation of mitotic apparatus containing vesicles with calcium sequestration activity. Cell 19:505-16, 1980.

ISOLATION OF TUBULIN

One half the class will use chick brains and one half will use sea urchin eggs as a source of tubulin. Dissection kits will be needed for chick brain preparation. From chick brains ten chicks 15-17 days old (ours are 19 days) in incubation (hatching is 21 days) should be sacrificed by decapitation and the brains cut out and weighed. (Save the heart,liver, eyes of the chick for later lipid experiments, freeze in separate containers, well labelled.) After mincing with scissors, brains will be homogenized in 1.5 Volumes (1.5 ml x g weight) buffer

20 mM NaPO₄, pH 6.75, 10mM Na glutamate, .02% sodium amide, 0.1mM ATP or 0.1M PIPES, containing 1 mM EGTA and 0.1 mM GTP pH 6.94. Centrifuge at 20,000 rpm in Sorval for 60 min. Keep the supernate, measure the volume, and adjust to 1 mM GTP. Dialyze overnight or 2 days against PIPES buffer. This is a crude prep of tubulin. Raise the temperature to 37 'C. Run a turbidometric assay. Polymerize for 30 min at 37 degrees, then run another set of assays. Now add 2 mM taxol and

polymerize for another 30 min, run assays. Spin at 18,000 rpm for 30 min. Save the pellet. Resuspend in .14 the volume that was present in the original homogenate. Store in refrigerator or freeze by placing tube in dry ice and acetone and store in freezer. To use, thaw. Run proteins to determine concentration: do 1/5, 1/10, 1/50 dilution. Then to calculate concentrations for all these dilutions, figure ug/ml protein for the OD you got for each dilution, then multiply by the dilution (5,10,or 50) and divide by 1000, that equals mg protein/ ml. Make a table of these values. Reference; Borisy et al, NY Acad Sci Proc. 1974 p 107. Purification of tubulin, etc.

Store in freezer, well labelled. For polymerization, other optimal conditions are: 5mM BES ph 6.5 ; .5 mM MgSO₄ ; 1 mM GTP ; 1 mM EGTA ; 50 mM KCl ; 1.6 mg/ml tubulin (final concentration, not before adding the other reagents). **Stop read the next paragraphs before adding anything to reach these concentrations, add more concentrated reagents, and bring protein concentration from what it was before dilution.** You need 5 ml for viscometer and you may only have enough protein for one run so be careful of the order of addition of reagents. Addition of Ca⁺⁺ and colchicine can inhibit polymer formation use final concentrations of: 1.6 mM CaCl₂, 10⁻⁵ M colchicine addition of deuterated water can promote polymerization. Dilute the tubulin prep with 30% the volume of deuterated water(D₂O). So when designing an experiment: make your reaction mix in the cold, then run viscosity, in the warm water bath, then add Ca and run again, then try the same whole series again, but add colchicine instead of Ca⁺⁺.Run viscosity measurements for at least 20 min after adding reagent. Note change in viscosity with time (hopefully). Turbidometric assay of polymerization follow optical density at 350 and 450 nm. You should get an increase as the temperature goes up. Cool to 10 degrees to get a decrease, which takes only 5 min at that temperature. You could also try the effect of redox agents on this change in OD or see if they reverse it.

ANOTHER WAY TO ISOLATE FROM BRAINS: 0.1M MES, 1mM EGTA, 1mM GTP, 0.5M MgCl₂ pH 6.4. homog in ice. centrifuge 100,00 g 1 hr 4 degrees, discard pellet, mix with equal vol assembly buffer below.

ANOTHER WAY TO GET POLYMERS Reference: Y Song, S Heins, E Mandelkow, E Mandelkow. Aluminum fluoride, microtubule stability and kinesin rigor. In MotorProteins Ed. RA Cross and J Kendrick-Jones. J. of Cell Science 1991 Supplement 14. The assembly buffer from this paper is as follows:

0.1M PIPES pH 6.9, 1mM each MgSO₄, EGTA, DTT, and GTP. Add NaF and AlCl₃ ratio of 4:1- try 1mM AlCl₃, 4mM NaF. Add final conc 8M glycerol. These MT should have attached kinesin.

TUBULIN PREPARATION FROM SEA URCHIN EGGS This is the method of Kuriyama (J. Biochem 81:1115-1125. 1977. You can use unfertilized eggs or eggs at first metaphase for the isolation. Eggs are washed 2x with 10 mM Na phosphate buffer, 10mM MgCl₂, .24 M sucrose, pH 6.8. Pellet eggs, resuspend in 2-5 vol of 0.1 M Na phosphate, 1 mM EGTA, .5 mM MgSO₄, 1 mM ATP, pH 6.7 and homogenize and spin 20,000g 30 min., in the cold. Do a batch adsorption onto DEAE-sephadex A50 ion exchanger by adding to 30 ml of supernate 10 ml of resin (activated in PMA), stir occasionally for 30 min. Pellet the resin with attached tubulin at 2000g for 1 min. Save this supernatant for electrophoresis, label it egg homog minus tubulin. Wash the resin in 30 ml 0.4 M NaCl-PMA by allowing it to stand for 10 min in the solution with occasional stirring. Spin

(save the first wash for electrophoresis) and wash 2x more. The tubulin is still on the resin (hopefully). Now suspend the resin in 10 ml 0.6 M NaCl-PMA to elute the tubulin. Add glycerol to stabilize during dialysis overnight against polymerization solution, in the cold. Concentrate by pressure dialysis. Freeze for electrophoresis after assay.

ELECTROPHORESIS PRE-LAB

COMPONENTS:

What is the function of the

- a) acrylamide
- b) bis-acrylamide
- c) ammonium persulfate
- d) tris buffer
- e) SDS
- f) TEMED

What is the difference between and the purpose of the stacking gel and the separating gel?

Look through your textbook to find 2 different protein electrophoretic gels. What were they trying to demonstrate with the gel?

What is the purpose of running standards with your unknowns?

What kinds of stains can be used on gels to distinguish between sugar groups, phosphate groups, lipid groups on proteins?

How can you fix proteins in the gel after they are run?

When you look at a gel, how would you know which were small and which were large in MW?

Which end of the gel was at the anode?

How would that differ if you wanted to run basic proteins like histones?

How would you decide which %gel to use for your particular protein?

What is the difference between isoelectric focusing and SDS gel preparations?

What is a Western blot? How can that be more useful than just the gel?

When would you use a gradient gel?

When you want to use an enzyme assay to detect which band is your protein, what kind of electrophoresis would you use?

What is the advantage to 2-D gel electrophoresis?

SAFETY:

What safety measures are needed?

1. Use plastic gloves with acrylamide when pouring gels. Wear safety glasses when doing it in case it splashes while removing bubbles from under the template.
2. Measure TEMED in running hood with autopipette.
3. Use plastic gloves with CuCl_2 stain.
4. Wash hands after all these manouvers.

ACRYLAMIDE SLAB GEL ELECTROPHORESIS

STOCK SOLUTIONS

ACRYLAMIDE 30% use plastic gloves to make up acrylamide solutions.

Acrylamide 30 g

bis .8 g

water up to 100ml

After stirring for 1 hr on magnetic stirrer (not so hard that you splash) filter through #1 whatman filter paper, store in refrigerator with your name and the date made.

LOWER TRIS (1.5 M TRIS-CL, PH 8.8) 18.17 G TRIS in about 80 ml water. Adjust to pH 8.8 with conc. HCl. Use pH meter with magnetic stirrer. Bring up to 100 ml in a volumetric. Store in a reagent bottle in refrigerator, mark name and date.

UPPER TRIS 0.5 M PH 6.8 6.06 g tris in about 80 ml water. Adjust as lower tris with pH meter, magnetic stirrer, conc. HCl to pH 6.8, bring to 100 ml with a volumetric, store as above.

BATH BUFFER pH 8.4 4X 12.0 g Tris 57.6 g glycine these do not have to be reagent grade. adjust pH when dissolved in about 500ml water, then bring to 1 L.

USE 160 ML PER ELECTROPHORESIS CHAMBER, DILUTE TO 600 ML. For SDS runs, add ml SDS to give a final concentration of 0.1%.

GEL OVERLAY 10 UL 10% AP, 4 ml water

10% AP .1G AMMONIUM PERSULFATE+1ML WATER, KEEP ONLY FOR TWO DAYS.

DESTAIN SOLUTION for 1 liter

INGREDIENT	FINAL CONCENTRATION	VOLUME
METHANOL	10%	100ml
ACETIC ACID	10%	100 ml
WATER	80%	800 ml
STAINING SOLUTION FOR 1 L		
ISOPROPYL ALCOHOL	25%	250 ML
ACETIC ACID	10%	100 ml
0.1% COOMASSIE BLUE		1 g
WATER	65%	650 ml

GEL PREPARATION for isozyme gels

10% SEPARATING (LOWER) GEL

	FOR THICK PLATES		FOR THIN PLATES
	1	2	1
LOWER TRIS	5.5ML	11.0	4.5
ACRYLAMIDE	7.3	14.6	6
WATER	9.2	18.3	7.5
10%AP	100UL	200UL	50 UL
TEMED	10 UL	20 ul	10 ul

total	22.11	44.12	18.06
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do not pipette acrylamide by mouth, IT IS A NEUROTOXIN, leave ingredients at room temperature until they warm up, before using. Add TEMED in fume hood, IT IS CARCINOGENIC. If you get any acrylamide on you, wash your skin, as it can be absorbed through the skin.

STACKING GEL 4.5% gel		
	thin	thick
UPPER TRIS	1.5	2.5
ACRYLAMIDE	.9	1.5
10%AP	30ul	50ul
WATER	3.6ml	6 ml
TEMED	10ul	20ul

allow gels to polymerize undisturbed. Leave some in the pipette to see when it polymerizes. Do not disturb the pipette for at least 15 min, as pipetting prevents polymer formation.

SAMPLE PREPARATION FOR NATIVE ISOZYME GELS Homogenize eggs or chick embryo parts in 0.3M NaCl, tris pH 8; or in .3M sucrose, tris pH8. Weigh the tissue and use 4x dilution.

Add 10 ul tracking dye, 5 crystals of sucrose per .2 ml sample. Use 60 ug of a mixture of proteins, use 4 ug of a pure one.

Run gels for isozyme study at 10 ma/gel overnight until approximately 3 or 4 hours after the tracking dye goes off the end.

G6PD ENZYME ASSAY ON GELS AFTER THEY ARE RUN.

REACTION MIXTURE

A NADP 1MG/.4 ML 10X TEA- MAKE FRESH EVERY DAY

B KCN 13.2 MG/2ML WATER (MULTIPLY AMOUNT BY NUMBERS OF GELS RUN

C NITRO-BLUE TETRAZOLIUM 4.9 MG/2 ML WATER

D PHENAZINE METHOSULFATE 2.14 MG/10ML WATER

KEEP B,C,D IN DARK BOTTLES make up ABCD EVERY DAY FRESH

E G6P (ADJUST TO pH 7.4 (10x kept in freezer)

F

TEA BUFFER 10X KEPT IN FREEZER
.05M TRIETHANOLAMINE 7.46G
.01M MgCl ₂ .952 g
.005 M EDTA 1.46G
ADJUST TO pH 7.4 and then bring to 100 ml and freeze in small aliquots

After stopping current, remove gels by separating plates with spatula. BE SURE TO MARK YOUR GEL AT SLOT NUMBER 1 WITH CUTTING OFF THE CORNER DIAGONALLY. Cut off the stacking gel just above the top of the separating gel. Drop into 20 ml 1 x TEA and place on shaker for 10 min.

Prepare reaction mixture as follows:

NADP	0.16 ML
KCN	2 ML
NITROBLUE	2 ML
PHENAZINE	2 ML
G6P	2 ML
BUFFER	2 ML
WATER	10 ML
FINAL VOLUME	20 ML

Pour off TEA, holding gel in plate with rubber gloves (finger marks will appear on gel otherwise. Place gel on slowly moving shaker (in a covered box, must be in the dark) for about 2 hr. Rinse 5-6x with distilled water. Shake gently with enough 10% methanol, 1% glycerol to cover for at least 4 hr, then mount for drying under vacuum.

MEASUREMENT OF MOLECULAR WEIGHT USING PAGE ISOZYME SEPARATIONS

REFERENCE: J.L. Hedrick, A.J. Smith. 1968. Arch. Biochem.

Biophys. 126:155-64. Size and charge isomer separation and estimation of molecular weights of proteins by disc gel electrophoresis.

Purpose: to distinguish between polymer isozymes differing mainly in size and distinct isozyme proteins differing in size and charge.

1. Calculate the relative mobility of the isozyme band or standard protein stained band relative to the tracking dye for gels of different % acrylamide concentration.
2. Plot $\log R_m$ (relative mobility) versus % gel on graph paper or plot relative mobility vs % gel on semilog paper. Calculate the slope and compare for each protein standard and isozyme. Lines which are non-parallel and converge on one y intercept are polymers of one subunit. Proteins with similar size, but different charge have parallel lines with the same slope. Proteins differing in both molecular size and charge will have non-parallel lines which do not intersect the y axis together.
3. Calculate the molecular weight of the unknowns by reading it off of a plot of standard molecular weight proteins vs slope.

ESTIMATION OF SUBSTRATE SPECIFICITY

Record which isozymes are present in various substrates and cofactors, and their relative intensities . Draw some conclusions about specificity from your data.

LACTIC DEHYDROGENASE ISOZYMES

Comparison of isozymes can be made from different tissues, especially heart and skeletal muscle, or from different stages of embryonic development. Different tissues have different tolerances for high levels of lactic acid buildup and their enzymes are different.

Homogenize the tissue with sucrose homogenizing medium. Run the electrophoresis as for G6PD. Make sure that the electrophoresis was allowed to run for about 4 hours after the tracking dye went off the bottom of the 10% gel. (6 ma overnight from 6pm -9Am then add 4 more hours.) Cut identifying corners off of sections of the gel (for example if you had 4 repeats of three tissues, cut the gel so that each piece has the three tissues represented, that means you will have 4 pieces. Cut a square corner, triangle corner, etc at lower right hand corner of each.) Now you can treat each piece differently, varying the substrate or some other factor- temperature, time of incubation, or stain for protein with Coomassie blue. First Place all pieces into a bath with .014 M tris buffer, pH 8.3 for five minutes on the shaker in a large dish (this is to remove the electrophoresis buffer system.)

Next place the gel into the reaction mixture containing the following reagents: (make sure this is kept in the dark box once it is mixed together as it is light sensitive.)

14 cc .014 M tris pH 8.3

6 cc 0.1 M lactic acid, sodium salt, pH 8.3 (2cc for invertebrate material)

8 mg nitrobluetetrazolium

3 mg NAD

1.4 mg phenazine methosulfate

A good control is to run one with everything except the lactic acid.

Incubate in the light-tight box overnight for invertebrate material at room temperature. For 9 day chick embryo parts it only takes 10 min at room temperature for lines to develop. After lines develop to your satisfaction, using rubber gloves, hold gels in dish and pour off incubation mixture, wash with distilled water several times. Then store in distilled water. Treat them as protein stained gels before drying them (methanol-glycerol solution).

Compare the protein stained and isozymes for the different tissues.

If you get good bands, you could cut them out and freeze them to run on SDS gels as compared to whole homogenates to see whether the different isozymes have different molecular weights.

To make up lactic acid solution: MW=90.08, 88.3% pure. $.255\text{g}/25\text{ml} = 1\text{M}$, this is made by weighing out the liquid lactic acid in a beaker, bringing to 25 ml.

To make .1 M dilute 1cc/5cc, adjust pH with 1 M NaOH to 8.3 and dilute to final volume of 10 ml.

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FOR SDS GRADIENT GELS FOR SEPARATION FOR PROTEINS FOR MOLECULAR WEIGHT DETERMINATION: these gels are different from the ones above because they contain SDS in the buffers.

FOR 5-12.5% RESOLVING GEL GRADIENT

for thin plates 5%

12.5%

LOWER TRIS CONTAINING SDS	2 ML		2 ML
ACRYLAMIDE STOCK	1.7		4.2
WATER	6.2		3.7
TEMED (USE HOOD)	3 ul		3 ul
10% AP	25UL		25UL

ADD 7 ML OF EACH TO GRADIENT MAKER AND PUMP IMMEDIATELY INTO PREPARED PLATES. OVERLAY GEL OVERLAY SOLUTION (0.1% SDS, AP, H₂O). CAN BE PREPARED A DAY AHEAD. POUR OFF WATER AND DRY WITH FILTER PAPER. MOVE SPACERS IN TIGHT.

FOR STACKING GEL: 3%

2.5 ML UPPER TRIS WITH SDS

1.5 ML ACRYLAMIDE

6 ML WATER

30 uL 10% AP

10 uL TEMED

pour into plate until it covers comb template and is even with the top of the gel. If you get any on you, wash it off immediately as it is a neurotoxin. Pull out comb when it hardens.

Put 4 ml SDS in 600 ml diluted tris-glycine reservoir buffer.

Prepare protein samples in 2 % SDS, 10% GLYCEROL, WITH OR WITHOUT 5% Mercaptoethanol, plus a drop of tracking dye. Boil 5 min in water bath. Use prepared standards. Place samples into wells, recording wells for each different sample. Always place a std in the middle and another different std near it.

SAMPLE PREPARATION FOR SDS GELS

The point of this experiment is to check the purity of those protein preparations we have made, and to look for proteins we thought we had present in various homogenates we made for enzyme assays. Use your myosin, F and G-actin, tubulin, actomyosin, acetylcholine esterase homogenate, egg cortices, spindles, brain and muscle pellets, sperm.

Run molecular weight determination gels only until tracking dye reaches within 1 cm of the bottom of the plate—for two setups use 60 volts until sample in stacking gel, then 120 V about 5 more hrs. Someone from each group must come back to remove gels and place in destain.

Stain at least 2 hrs or overnight with coomassie blue (after washing in destain for 1 hr), then place in destain with foam overnight on a shaker. Dry under vacuum when bands clearly seen and after soaking in drying solution (10% methanol, 1% glycerol) for a few hours.

CALCULATE MW Measure the distance traveled into the gel by each band. Plot the knowns in the standard using semilog paper with distance in cm along the bottom, MW on semilog side (or regular paper plot the log of MW) . Use this graph to determine MW of unknowns.

Make a list of unknown MWs and possible proteins from each well.

Known molecular weights of some proteins

MYOSIN HEAVYCHAIN 205,000-220,000

MYOSIN 470,000

H-MEROMYOSIN 350,000

DYNEIN 300,000

MAPS 200-300,000

SHEATH PROTEIN 220,000

NEXIN 165,000

IgG 150,000

SPOKES 118,000

TUBULIN DIMER 100,000

PHOSPHORYLASE A 100,000

TROPOMYOSIN 70,000
BOVINE SERUM ALBUMIN 66,000
SMALL MAPS 60,000-70,000
CATALASE (LIVER) 57,500
PYRUVATE KINASE 57,000
GLUTAMATE DEHYDROGENASE 53,000
OVALBUMIN 43,000-45,000
G-ACTIN 42,000
ENOLASE 41,000
ALDOLASE 38,994
GLYCERALDEHYDE-3PD 36,000
LACTATE DEHYDROGENASE 36,180
CARBONIC ANHYDRASE 29,000
CHYMOTRYPSINOGEN 25,666
TRYPSINOGEN 24,000
LIGHT CHAIN MYOSIN 20,000,16,000
TRYPSIN INHIBITOR 20,100
CALMODULIN 19,000
TROPONIN-C 18,000
MYOGLOBIN 17,200
PROFILIN 16,000
AVIDIN 16,000
HEMOGLOBIN 15,500

RIBONUCLEASE B 14,700

LYSOZYME 14,310

LACTALBUMIN 14,200

When you look at the pattern in the gel, how do you know which are small and which are large? Where was the anode in the experiment, top or bottom? How do gradient gels results differ from non-gradient gels? What is a Western blot and how can it be used to learn more about a protein?

MEMBRANE LIPID ANALYSIS BY GLC

Folsch extraction membranes, whole eggs, isolated mitotic apparatus, cortical hulls, sperm, can all be extracted with chloroform-methanol to yield soluble lipids. Weigh out a known quantity of these. Place this into a glass conical centrifuge tube. Add an equal vol of chloroform, 2 vol of methanol, and sonify in an ice bath for 1 min. Add 1 vol chloroform and resonify for 30 sec. Add 1 vol water and resonify for 30 sec. Spin full speed in a clinical centrifuge for 10 min. Remove and keep the bottom chloroform layer. Dry it down under a stream of nitrogen in a warm bath. Redissolve in a very small quantity of 2:1 chlor:meth (.1 MI).

FATTY ACID METHYLATION for gas chromatography

Have a source of the fatty acid, either on TLC gel scraped off plate, or evaporated or powder.

1. add 3 ml 5% methanolic HCl.

2. heat in teflon capped (tightly closed) tube for 2-4 hrs at 80 degrees C.

3. add 3 ml pentane, shake on vortex stirrer for at least 30 sec, spin for 5 min, remove the pentane from the bottom to a new tube, add 2 ml pentane to original tube and repeat the extraction, combine pentane with the first pentane.

4. Add 1 ml water to the pentane, shake and remove water from the bottom (this should remove any non-methylated FA.) Add a small amount of MgSO₄ powder to the tube and shake, spin 5 min, pour off pentane to storage vial. (The magnesium takes up water and dries the pentane). Evaporate the pentane to the desired volume so that the sample size used in GLC (1 ul for the glass capillary column) will contain the proper amount of lipid. if trying this for inositol methylation, use 5 mg powder as your starting material.

TLC. place a 10 ul sample on two marked tlc plates. Place other samples on the same plate. Be sure to place a standard mixture on each plate, one of neutral and one of polar lipids. Develop the set with neutral lipids (set the plate into a container with 80 ml petroleum ether, 20 ml ether, 1 ml conc acetic acid) and it will approach the scribe at the top in about 30 minutes. Develop one for polar lipids (container has 64 ml chloroform, 25 ml methanol, 10.5 ml acetic acid.) This will take up to an hour to develop. Keep the top tightly closed to make it go evenly. Remove from the container and place in a hood to dry. Spray with detectors or pour over the gel 3% Cu acetate in 8.5% Phosphoric acid (concentrated is 85%). Heat in a preheated oven at 140 degrees for 30 min. Remove and store in the dark covered by foil until you can look at the spots. Otherwise, decide the relative darkness of the various spots in each sample and between samples.

GLC the fatty acids from the lipids must be cleaved off the lipids and methylated overnight with acidic methanol, in an oven. See the instructor. Then the sample can be injected into the GLC to determine the chain lengths and degree of unsaturation.

Alternates lipids can be treated with various phospholipases before being analyzed.

GLUCOSE-6-PHOSPHATE DEHYDROGENASE ASSAY AND EFFECTS OF REDOX AGENTS

G6PD is the first enzyme in the hexose monophosphate shunt which is important in control of the redox potential of the cell, and in the maintenance of the -SH state on proteins, as well as in synthesis of ribose and deoxyribose for nucleic acid synthesis and the NADPH needed for synthesis of large molecules such as lipids.

MATERIALS AND METHODS Unfertilized eggs (U) and fertilized zygotes (F) of Lytechinus pictus or Strongylocentrotus purpuratus are obtained, and washed in 0.1 M MgCl₂, 0.01 M Tris pH 8. Homogenates prepared (1:4 V/V) in 0.01 M MgCl₂ 0.01 M Tris will provide our enzyme.

RUN A BIORAD PROTEIN TEST ON THE HOMOGENATE.

Electrophoretic separations and detection of G6PD isozymes can also be done on this homogenate.

SPECTROPHOTOMETRIC ASSAY Spectrophotometric G6PD assays measuring the amount of NADPH produced can be used. The reaction mixture contains:

0.2 ML HOMOGENATE

4.5 ML TEA-MgCl₂ BUFFER (0.05 M TRIETHANOLAMINE, pH 7.4,

0.1 M MgCl₂, 5 mM EDTA)

0.2 ML NADP 2 mM, PH 7.4

To start the reaction add:

0.2 ML G6P 25 mM run this at room temperature. Read the optical density at 340 nm, (be sure there is no red filter) setting the initial reading on zero and recording the od increase with time. Plot on answer sheet. To calculate enzyme activity, divide the od change/min by the protein concentration of the homogenate, with its final dilution of .2/5 ml (1/25).

Redox detection of enzyme activity Redox potential can be measured using Lazar Research Laboratories model ORP146 microelectrode and DJM146 reference electrode and reference solutions (0.1 M potassium ferrocyanide and 0.05 M potassium ferricyanide-set to 192mv and 0.01 M potassium ferrocyanide, 0.05 M potassium fluoride about 66 mv higher.) Solution potentials can be measured in a test tube using the same reaction mixtures as for the spectrophotometer readings. Read using a Beckman Zeromatic II pH meter, using the change in mV.

EFFECT OF REDOX AGENTS ON G6PD Treatment of samples with redox agents: 20ul of 10x redox agent was added to 0.2 ml of homogenate. Final concentrations are as follows: diamide-0.4 mM; GSH or GSSG-0.1 mM; ascorbate 6 uM, 1/2 concentrated ascorbate 3 uM; DTT- 4 mM and all are in 0.1 M Tris-HCl, pH 8. Controls should be mixed with 0.2 ml Tris buffer. Samples should be incubated for 1/2 hr at room temperature before the assay EITHER with the electrode or spectrophotometer assay.

CHICK CELL DISSOCIATION AND REAGGREGATION

PURPOSE: To compare various early embryonic tissues in terms of how easily they can be dissociated, and what kinds of mechanisms are involved.

Materials: Chick Ringers solution, Hank's Balanced salt solution (a phosphate buffered saline with glucose and phenol red (a detector of dead cells)); scalpel, petri dishes, microscope, at least two early chick embryos.

Method: Embryos can be candled if they are over 2 days old. On the earlier ones, you just have to open them to see if they are alive. Either break them into chick ringers or break the shell back to expose embryo. When you are ready to remove the embryo, firmly clasp the edge of the embryo blastodisc with forceps in your left hand. Do not let go at any time until this operation is complete or you will lose the embryo into the yolk. Cut with scissors around the blastodisc using your right hand, including around the forceps so they are holding the embryo you are cutting off. After cutting completely around, remove the blastodisc by picking up the forceps and place it in a petri dish with ringers and gently flatten it out. You must keep the embryo warm with your light for the rest of the experiment, or place in the warm incubator between periods of observation. Now we will do the same so that we have two embryos of approximately the same stage.

The embryo will be placed in Ca-Mg-free Hank's solution and cut into pieces such as brain, heart, somites, yolk sac, and if any amnion is present, use that. Now cut each piece into 2. That means you will have a total of four pieces of tissue of each type from the two embryos. We will place one of each set of four in a control Hank's solution, and the other three in different experimental solutions used to dissociate cells.

The kinds of solutions used will be 1) Ca-Mg-free Hank's plus 0.05% trypsin, pH 7.2, 2) Ca-Mg-free Hank's plus 0.05% trypsin and 0.005M EDTA and 3) Ca-Mg-free Hank's plus 0.005 M EDTA. Now use vaseline to separate the petri dishes into four compartments; mark the dish and each well, after placing 1 ml of the different solutions on different pieces of the same tissue in the different quadrants of the petri dishes, record the time, cover, and place in the incubator at 37 degrees.

Decide how you are going to score your results, for example by the size of clumps under the microscope, number of cells per clump, effect of mechanical stirring with needle, effect of gentle pipetting with a pasteur pipette.

After 10 min observe each piece and record any change in appearance. Using a needle test the tissue for firmness, see if it is starting to fall apart. Replace in incubator and reobserve at 10 min intervals for 1 hour recording the exact time of observation. If nothing happens after 30 min, use some mechanical method to aid, for example stir with needle or gently pipette. Record the methods used. If a particular tissue falls apart much faster than the others, take note of it. Record what kinds of adhesion mechanisms you think are being interfered with in each case, using your text book as a resource.

Experiment with dissociated cells: pipette some dissociated cells into a centrifuge tube, add ten times the volume of Ca-Mg-free Hanks and spin down gently, remove and toss fluid. Now add 1 ml Hank's with Calcium back and see if they adhere after 30 min in the incubator in the centrifuge tube or in a petri dish

on the shaker at 37 degrees. If you have several kinds of tissue dissociated, mix some of them and see if they reassociate with Ca^{++} .

Questions to answer: At what time did each tissue start to dissociate? Which tissue was the fastest to dissociate? Which solution did it the fastest? Which did it the most completely? Check the size of the clumps and number of cells per clump unde

r the microscope to answer that question. How would doing the experiment at room temperature effect the experiment? Did the heart continue to beat in the solutions used? When did it stop?

ACETYLCHOLINESTERASE

Materials required per group: Acetylthiocholine chloride (ACh)- 2 ml (2.167 mg/ml), butyrylthiocholine chloride (BCh)- 2 ml (2.167 mg/ml), DTNB- 5 ml (39.6 mg/10ml); buffer 0.1 M phosphate buffer pH 7.8, 30 ml. Test tubes, marker, rack, spectronic tubes. Homogenizing medium: 0.1 M MgCl₂, .01 M Tris pH 8.

This enzyme is present in nerve and muscle cells and is released into the extracellular space at the postsynaptic cell of the synapse or neuromuscular junction. It is important in breaking down the neurotransmitter after an impulse. We will use muscle as our source of material. Weigh out 2 g of frog or other muscle. Add 5 ml of homogenizing medium and grind in a glass homogenizer by pulling the

plunger up and down (IN ICE BATH) 20x. Centrifuge the homogenate in a refrigerated centrifuge (about 10,000 rpm for 10 min).

Prepare the following tubes for the assay, using the amounts indicated: DTNB detects the -SH released from ACH or BCH by enzyme action, giving a yellow color. Prepare the following as a control: Homogenate boiled for 2 min. That control will reveal and color development just due to intrinsic -SH in the tissue. The BCH is used to test for the presence of non-specific cholinesterase, so the value for that control must be subtracted from all the ACH values to get the true value.

Prepare only one tube at a time because these reagents are not stable at room temperature. Make sure all tubes are room temperature at the time you add the enzyme and DTNB to start the reaction.

assay #	homogenate	VOL	final	ACH	BCH	DTNB	Buffer
1	homogenate supernate	.4ml	4 ml	.4	-	.5	2.7
2	" "	.4	4	-	.4	.5	2.7
3	" "	.4	4	-	-	.5	3.1
4	boiled " "	.4	4	.4	-	.5	2.7
5	" " "		.4	4	-	.4	5
6		0	4	.4	-	.5	3.1
7		0	4	-	.4	.5	3.1
8	same as # 1 but add 20 ul eserine (this is poison do not mouth pipette).						

Zero the tubes as soon as possible after adding the homogenate, at 410nm (it takes the spectronic 30 min to warm up). Take reading at every 20 sec for the first min., leaving the tube in the spectronic, then every 30 sec until it stops changing (3 min is long enough.) In order to calculate the specific activity of the enzyme, you will need to run a Biorad protein on your homogenate supernate.

Next figure out which control values must be subtracted from the ACH value, and calculate change in OD/mg protein/min.

EXERCISE ON NERNST EQUATION AND GIBBS DONNAN EQUATION

AN ATTEMPT TO EXPLAIN IONIC EFFECTS ON CELL MOTILITY

Let's look first at the effect of changing ion concentrations on the membrane potential. This is done by the Nernst equation:

Rates of ion movement have to do with :

1. how steep a gradient we have from inside to outside concentration
2. permeability
3. membrane potential

When we have a negative inside to outside, as we normally do in cells, it favors the movement of + ions in, if there is a channel through which they can pass. It also causes a movement of - ions out.

When we have a concentration gradient that causes net diffusion down the gradient, or from higher to lower concentration. This will cause K^+ to move out and Na^+ to move in since the concentrations in and out are:

Inside Outside

K^+ 400 mM 20 mM

Na^+ 45 440

Ca^{++} .001 10

Cl^- 50 560

The Nernst equation for Na^+ would be

V (voltage) for Na^+ = $2.303 \frac{RT}{zF} \log_{10} \frac{(\text{conc out})}{(\text{conc in})} = 58\text{mv} \log_{10} \frac{440}{45} = 60\text{mv}$

zF (conc in) 45

for K^+ = $2.303 \frac{RT}{zF} \log_{10} \frac{(\text{conc out})}{(\text{conc in})} = 58\text{mv} \log_{10} \frac{20}{400} = -75\text{mv}$

zF (conc in) 400

so if the cell is completely permeable to Na⁺ and K⁺ it has to be inbetween, but if the permeability changes for each, membrane potential can go between -75 and +60mv. However, changing the gradient can also change the potential.

R=gas constant, T is absolute temp, z is valence or charge, F is 96,500 coulombs/gm equivalent. RT/zF is 25 at 18 degrees

The Goldman equation takes into account all ions permeabilities and concentrations inside and out:

$$V_m = 2.303 \frac{RT}{F} \log \frac{P_K [K^+]_{out} + P_{Na} [Na^+]_{out} + P_{Cl} [Cl^-]_{in}}{P_K [K^+]_{in} + P_{Na} [Na^+]_{in} + P_{Cl} [Cl^-]_{out}}$$

$$V_m = 58 \log \frac{100 + 0.04(440) + 0.45(50)}{400 + 0.04(50) + 0.45(560)} = -60.1 \text{ mV}$$

$$400 + 0.04(50) + 0.45(560) = -60.1 \text{ mV}$$

permeability- relative

K⁺ 1

Na⁺ 0.04

Ca⁺⁺

Cl⁻ 0.45

Using the formula for frog and mammal Ringer's solution, artificial sea water, plasma, figure out the membrane potential for frog ciliary cell, sea urchin egg, mammal heart cell.

Next figure out change in potential for added Ca⁺⁺ or Mg⁺⁺, or Na⁺, or K⁺ or Cl⁻ as we did them. How can the active transport pumps of the cell change these values?

Gated ion channels: For example, charged proteins could be gates or channels.

Na⁺ open at ligand binding

K⁺ open at 0 mV

Ca⁺⁺ open at -25 to 5 mV

Na⁺/H⁺ open at phosphorylation due to DAG, Ca⁺⁺ activation of protein

kinase-C

The 58 mV used for the constants in the equation was for standard room temperature try the equation with lowered temperature, increased pressure.

Solutions which are isosmotic with sea water include:

.52M NaCl

.55M KCl

.278 M CaCl₂

Na⁺ can be replaced by choline, a non-permeable molecule

Contents of sea water millimolar

Na⁺ 468

CaCl₂ 20

MgCl 45

References:

Geise- Cell Physiology

Hendry-Membrane physiology and cell excitation

Gibbs Donnan-

Ratio of bound monovalent to bound divalent cations is equal to the ratio of free monovalent to square root of free divalent.

Play with the numbers in sea water, Ca, Mg free sea water, cell cytoplasm; then see how changing the concentration by altering the membrane potential, or opening membrane channels allowing usually non-permeable ions in, can change the ratio of ions bound. Relate that to muscle contraction. Could there be waves of membrane potential change and then Ca⁺⁺ pore opening and closing to relate to ciliary motion?

Absolute temp = C + 273.15 = Kelvin

Gas Constant = R = 8.315 joules/degree

Ln natural log = 2.303 x log 10

F = 96,500 coulombs/gm equivalent

z = valence or charge of ion

PATCH CLAMP NECESSITIES

150 mV POTENTIAL for membrane

10 mV steps as electrode approaches membrane

firepolished glass pipettes with $1 \times 10^9 \Omega$ tip diameter 1 micron

suction 10×20 cm water

seal 10^9 to the $10^9 \Omega$ resistance

subpicoamperes for pore in membrane current

can tell pipette resistance from amplitude of current pulse, it increases when pipette approaches cell, zero when get seal

sensitivity depends on background noise, pipette capacitance,

holder noise, computer assoc noise

using a 50 G Ω feedback resistor for 1×300 Hz, get 0.1 pA root mean

square

action potential detected as capacitative current across patch

nystatin added to pipette, add conductive channels for monovalent

cations, then can voltage clamp the membrane and record the

current, when membrane left on cell has the advantage of allowing

second messenger control

membrane $1 \mu\text{F}/\text{cm}^2$ 1pF capacitance represents $100 \mu\text{m}^2$ membrane

Faraday cage to collect interference to ground

STAINING NUCLEI TO CHECK FOR ONE NUCLEUS PER CELL WITH POLYSPERMY OR ARTIFICIAL ACTIVATION

ORCEIN STAIN: ref: DL Poccia, GR Green 1986 Methods in Cell Biology 27:Chapter 9 Nuclei and chromosomal proteins, 157-175

Remove an aliquot of eggs, hand centrifuge

Resuspend in a few drops of sea water and several ml of ethanol: acetic acid (3:1)

After 10 min place a drop of suspension on slide and dry

Add orcein 2% in 75% acetic acid and place cover slip

After 10 min, remove excess fluid with paper, observe under microscope Nuclei are red

Fuelgen stain

Methylene blue drop of cells

Hematoxylin on drop of cells

PRECIPITIN TEST USING antibodies and TITER TUBES AND STAND

diluted antiserum 1/2 with saline, according to how much needed to keep antigen in solution at interface, then tube with normal serum diluted same way. Some diluted with 8% saline, others 16%, others .2M saline it still worked for myosin, actomyosin.

Then layer in bottom of tube to level above curved area. Can incorporate antibody in

20% sucrose and to diffuse the mixture into serial two-fold dilutions antigen overlaid on the sugar-antibody.

The dilutions are of the antigen, started with about 10 mg/ml before dilution, so use 20 mg/ml before add detergent., extract with detergent final 10x by adding equal amount of 20% detergent, then to get 1% detergent and 1/10 antigen, dilute with water, make other dilutions from this with TBS with 1% triton or octylgluc to 1/100, 250, 500, 750, 1000, 1250, 500, 2000. Layer carefully on top of the serum, use plain saline or detergent for control. Try using supernate as well as detergent extracted pellet. So do one rack

with normal serum, one rack per serum. For unknown titer antibody, use twofold dilution instead-
2,4,8,16,32,64,128,256,512,1024

Incubate 30 m 36 degrees, 30 min refrig record positives

IMMUNOHISTOCHEMICAL DETECTION AND LOCALIZATION OF ANTIGENS

We will attempt to localize actin and tubulin in dividing sea urchin eggs, glycerinated rabbit muscle, or chicken muscle. We will use the indirect, layered approach with unlabeled primary antibody which detects the antigen, then layer a fluorescein labeled secondary antibody or linker antibody:enzyme-antibody which gives a color.

Caution: SODIUM AZIDE. a preservative and poison is in these reagents. AEC is a preservative in the substrate and is a carcinogen, and can cause skin irritation, so if materials get on skin, flush with water. Drying at any of the stages of slide development leads to a poor final result. Don't take a break while incubating slides, to come back and find them dried out.

Peroxidase-antiperoxidase will be used to detect actin, to bind to the linker and then a red to brown color will develop when chromogen is supplied to the antigen-antibody- enzyme-antibody localized in the tissue. For some tissues, it is necessary to treat with .2% Triton x-100, to make holes in the membrane so the antibody can penetrate. This is common for cytoskeleton preparations, but would extract membrane enzymes. Stabilization buffer is used 5 min to keep microtubules intact, followed by 5 min the same medium containing the triton- x-100 when preparing for antitubulin stain. Then the tissue is fixed in paraformaldehyde for 5 min. For cross-linked fixed tissues, 0.1% trypsin treatment for 15 min may be necessary for antibody attachment to deparaffinized sections. a negative control must be run for each tissue, to check for non-specific staining by normal serum, instead of the primary antiserum. A positive control must be used which tests for the antigen in a tissue known to have the antigen (muscle for actin, chick brain or dividing eggs for tubulin.) We cannot use rabbit muscle if the primary antibody was developed in rabbits. We can use gently homogenized frog or chicken muscle, which we stick to protamine coated slides. An alternative is to use 1 ml test tubes with the cells (50 ul) and 50ul of each treatment in the numbered steps below, centrifuging in between, and suctioning off the supernatant.

1. attach unfertilized eggs to protamine sulfate treated slides. Transfer them from beaker to beaker with solutions. Fertilize the eggs, culture in sea water for one and a half hours so that spindles are visible. Fix eggs in paraformaldehyde for 5 min. Wash eggs in CMFSW. For positive controls use thin sections of muscle or brain. Expose slides to 0.3% hydrogen peroxide for five minutes to eliminate endogenous peroxidase room temperature. Rinse well with PBS 2x for 5 min.

2. Apply blocking reagent to anti-actin experimental slides. Blot the slides around the eggs (not on the eggs) to dry the slide. Apply 1 drop of reagent (vial 2), incubate 20 minutes at room temperature or 5 min at 37 degrees. Shake off liquid, no need to wash.

3. blot slide around tissue. Add 2 drops primary antibody (For rabbit anti-chicken actin, dilution is 1:75 in 1% BSA in PBS, FOR mouse anti-chick brain tubulin, the dilution is 1:250). Add 2 drops of normal serum (rabbit or mouse for the two controls) for your negative control. Apply two drops antisera to positive controls. 20 minutes at room temp or 5 min at 37.
4. rinse 2x with PBS or CMFSW according to tissue.
5. Blot around eggs. Add 2 drops linker antibody vial 4 to anti-actin and its control slides. Add 2 drops fluorescent goat anti-mouse to anti-tubulin slides and controls. Rinse 2x CMFSW OR PBS.
6. BLOT AROUND TISSUE. Add two drops labelling antibody (vial 5). Incubate 20 min room temp, 5 min 37. Rinse 2x with PBS OR CMFSW.
7. Blot around tissue, add substrate, incubate 10-40 min room temp, 5-10 min at 37. Monitor color development in the microscope. Rinse in CMFSW OR PBS. Return UNUSED SUBSTRATE to front desk at the end of the lab.
8. mount slides in 50% glycerol. Observe location of stain.

PROTOCOL FOR ANTIBODY STAIN

PROTOCOL FOR INDIRECT ANTIBODY STAIN

PREPARE EGGS:

Treat unfert eggs with:

pH 5 sea water, wash pH 8 SW

0.15% DTT (30 mg) and 0.1% (20mg) pronase (20 ml CMgfreeSW pH9) for

long enough to settle

wash 3x (1xCMFSW, 2x SW)

Treat with :

blocking agent-

1. anti-alpha ATPase-goat 1/100
2. anti-alpha ATPase rabbit 1/100
3. anti-alpha 2F sup-mouse
4. anti-alpha 5 ascites 1/100
5. anti-kinesin mouse
6. anti-beta 24 mouse
- , 7. anti-beta B4 mouse
8. suk-normal super
9. NRS

MEMBRANE PERMEABILIZATION

Use saponin or digitonin. Make digitonin 10% in DMSO, then dilute in cell suspension, 4 degrees 30 min. Use .008-.01% (it is 80% pure as powder)

Used cells fertilized and fixed 2/27/93

1. Wash 3 times with PBS, .5 ml. Use .4 ml of fixed cells to start. Hand centrifuge (HC) all samples for entire experiment.
2. Incubate 15 minutes with PBS containing .1% BSA and 1:25 normal goat serum. 38C, .5 ml. (made 2.6 ml of 1/25)
- 2a. Wash 3 times with PBS, .5ml, divide up into 50 ul tubes-6 for each 1 min and 10 min.
3. Incubate 1 hour with primary antibody at 37C, undiluted & 1:10 dilution, antibody is diluted with .5% BSA-PBS.

. Used B4B4, NS (normal culture medium), and 24. Both these antibodies are to beta subunit Na+K+ATPase.
4. Negative control incubate with culture medium.

(primary antibody can be done in the refrigerator overnight.)
5. Wash 3 times with PBS wait 5 min between.

6. Treat with secondary antibody, no dilution of link reagent supplied as for part of kit for dispensing as is, one drop or

50 ul, for 1 hour at 37C.

7. Wash 3 times with PBS wait 5 min between leave at room temp.

8. Incubate 15 minutes with streptavidin enzyme at 37C.

9. Wash 3 times with PBS.

10. Add substrate and incubate for 10 minutes.

do reaction in dark.

11. Wash 3 times with pbs

12. put cells in 50% glycerol.

1 min. after fertilization 10 min.

b4,24,normal serum: b4,24,normal serum:

1/1 and 1/10 dilution for each 1/1 and 1/10 dilution for each.

substrate was prepared by mixing ten drops of x-gal substrate with a bottle of 2.5 ml iron-buffer.

PBS solution - 100ml

0.806g NaCl

0.020g KCl

1ml 1M PO₄ pH 7.5 - make 1M solution of monobasic (0.140g NaH₂PO₄ & add d.w. to 10ml) and 1M solution of dibasic (0.140g Na₂HPO₄ and water to 10ml). Mix solutions to get to 7.5 pH.

DOT BLOT

PURPOSE: Which fraction is the enzyme in?

1 2 3 4 fert. unfert.

Which stage?

first 1 1/2

1. Purification of enzymes

Incubate the fractions with sodium dodecylsulphate for 30 minutes at 20 deg. at a protein concentration of 1.35-1.40mg/mL with .58mg SDS, 3mM ATP, 2mM EDTA and 50mM imidazole at a pH of 7.5.

*First mix the fractions with a solution of imidazole, EDTA, and ATP and then start incubation by the addition of SDS with continuous stirring. Then centrifuge at 19,000RPM for 40 minutes. (1)

2. Assembly of the apparatus (Bio-Dot):

A. Clean and dry apparatus and gasket.

B. Attach tubing to the outlet (runs from vacuum manifold through the 3-way flow valve to a trap for collection of the waste liquid).

C. Place the membrane support plate into position in the vacuum manifold.

D. Place sealing gasket (make sure holes are properly aligned.)

E. Place the membrane: (use forceps and gloves when handling the membrane).

But first:

a. pre-wet by sliding at a 45 deg. into TBS for protein binding.

-TBS (Tris Buffered Solution): 1* TBS, 2L

20mM Tris- HCl pH 7.5

500mM NaCl

Dissolve 4.84g Tris, 58.48g NaCl in 1.5L dd water.

Adjust to pH 7.5 with HCl. Adjust the volume to 2L with dd water. (2)

b. complete wetting is necessary to insure proper draining of solution.

c. remove and let excess liquid drain from membrane. (Blot membrane on a sheet of filter paper).

d. lay on gasket so it covers all of the holes. (make sure not to expose to the atmosphere when apparatus is assembled).

F. Next place sample template. Finger tighten 4 screws. Use a diagonal crossing pattern to insure even application of pressure on the membrane.

G. Turn on vacuum. Set 3-way valve to apply vacuum to apparatus.

While vacuum is applied tighten the apparatus using the diagonal crossing pattern to insure a tight seal.

H. Open to the atmospheric pressure.

I. Apply 100 micro-liter buffer to all 96 holes to rehydrate the membrane following the vacuum procedure in step G. Remove buffer gently by vacuum, watching over it carefully. You can regulate this by putting your finger over the part exposed to the atmosphere.

J. When drained, adjust the flow valve so that the unit is exposed to the atmospheric pressure and then disconnect the vacuum.

Ready for sample application!

3. PROTEIN BLOTTING

A. Adjust flow valve so that the vacuum chamber is open to the atmosphere.

B. Fill wells with antigen (protein) solution any volume up to 500 micro-liters per well. (most efficient is achieved by applying the required amount in a minimal sample volume. To each well add the same amount).

C. Allow entire sample to filter through the membrane by gravity flow. The flow valve should be positioned at a level below the sample wells to insure proper drainage during filter applications. Takes 30-40 minutes for 100 micro-liters to filter through the membrane. (If the antigen is very dilute look at optional wash on page 7 of the instruction manual of Bio-Dot Microfiltration Apparatus (2)).

D. Add 200-300 micro-liters of blocking solution. (also look at page 439, Volume 184 of Methods in Enzymology 3).

-Blocking solution, 100mL

1% BSA-TBS (BSA is bovine serum albumin)

add 1.0g BSA to 100mL TBS. Stir to dissolve. (2) Allow gravity filtration to occur until solution has completely drained. This should take about 60 minutes. No vacuum.

E. Adjust the flow valve so that the vacuum chamber is exposed to the atmosphere.

F. Add 200-400 micro-liters of the TTBS wash solution to each well.

-Tween 20 wash solution, 1* TTBS, 1L

20mM Tris , pH 7.5

500mM NaCl

.05% Tween 20

add .5mL Tween 20 to 1L of TBS. (2) Adjust flow valve to vacuum position and pull the wash solution through the membrane. Disconnect vacuum as soon as the wash solution has drained from all the sample wells. Repeat the wash step.

G. Open the valve to the atmosphere.

H. Add 100 micro-liter of the first antibody solution to each sample well. (If want to dilute it further look at page 16 of the instruction manual 2). Allow gravity filtration to occur until completely drained. It should take approximately 30-40 minutes. I. Apply vacuum to the apparatus to remove excess liquid from the sample wells.

J. Open flow valve to the atmosphere.

K. Add 200-400 micro-liter of TTBS to each well. Apply vacuum. Repeat for three wash cycles.

L. Turn the vacuum off and open the valve to the atmosphere.

M. Add 100 micro-liter of secondary biotinylated antibody solution.

N. Allow gravity filtration to occur until all is drained. This should take from 30 to 40 minutes.

O. Turn the vacuum on and drain the wells.

P. Add 200-400 microliter of TTBS to each well and drain completely. Repeat for two washes.

Q. Add one drop of label (enzyme) and then wash three times. Add the substrate.

R. Ready for color development.

S. Wash each well twice with 200 micro-liter TBS to eliminate excess Tween 20.

T. Add 100-200 micro-liter of the color developing solution to each well.

U. The reagent may be allowed to react while the solution is slowly drained by gravity filtration or the reaction time can be extended by closing the flow valve prior to adding the substrate.

R. When color development is completed, the excess substrate should be removed by vacuum and all the sample wells should be vacuum washed with 20 micro-liters of dd water to stop the reaction.

S. Remove the membrane from the apparatus, rinse it in dd water and allow it to air dry on filter paper.

*The basic instructions were obtained from the two instruction manuals **2 and 5**)

REFERENCES

1- Peter Leth Jorgensen

Purification and Characterization of (Na-K)-ATPase

Biochimica et Biophysica Acta, 356 (1974) P.37

2- Instruction Manual

Bio-Dot Microfiltration Apparatus

3- C.L.B., M.S.B., and K.G.

Streptavidin-Enzyme Complexes in Detection of Antigens on Western Blots Methods in Enzymology:
Avidin-Biotin Vol:184, P.439

Harcourt Brace Jovanovich, New York, 1990

4- Instruction Manual Ready-to-Use Link and Labels

Accu-Specs Sheet

5- Instruction Manual

Immun-Blot Assay Kit

Both of these experiments described in Foerder et al Proc Natl Acad Sci 75:3183-87.1978.

FLUORESCENCE EXPERIMENT

Measures peroxide production-eggs at fertilization or macrophages

Requires: scopoletin to be oxidized 10 uM added 10 s after HRP, dissolved in DMSO, so DMSO final conc is 7mM

3-amino-1,2,4-triazole to inhibit ovoperoxidase

horseradish peroxidase Type II 1.7 ng/ml added 10 s after fertilization

catalase

method of Root et al. J Clin Invest 55:945-955.1975

scopoletin loses its fluorescence when oxidized by HRP and H₂O₂. 5mM procaine which blocks granule release inhibits oxidation of scop. 24% without eggs in presence of H₂O₂, but completely with eggs

fert eggs 1-2% suspension, after 10 s add HRP, after another 10 s add scopoletin, swirl, take 2.7ml aliquots at intervals- like every min through 15 min. Add aliquots to .3ml 100 mM NaCN in SW pH8 to

stop oxidation. Spin 1000g 10 min 4degrees, decant super and read in fluorometer excit 350nm, monitor 460nm

Chemiluminescence: use scintillation counter

eggs 1-2% vol/vol settled eggs/SW, incubate with sperm in 1:1000 dilution in scintillation vials 15 degrees in shaking water bath. Remove vials at intervals to count chemiluminescence- see Rosen and Klabanoff J Clin Invest 58:50-60. 1976.

Can try with A23187, NH_4 . Ionophore chemilum is delayed. Inhibited by triazole.

INHIBITORS

Use of inhibitors or initiators of each second messenger chain reaction can help distinguish which are effective in causing the cycling at different times. Inhibitors are as follows: Calmodulin reactions- W-7, calmidazolium; CG reaction- procaine; ;G-protein- GDP beta-S; protein kinase-C- sphingosine, retinoic acid, K252a, H-7; phospholipase-A2 - quinacrine, bromophenacyl bromide; peroxidase- phenylhydrazine; IP3- heparin competes for receptor; protease inhibitor- Initiators are: phospholipase-c- stimulated by GTP-analog, best activity at pH 5; CG reaction- A23187, DAG; protein kinase-C - phorbol ester; pH change- NH₄ or procaine.

DIRECTIONS FOR ULTRAVIOLET ANALYZER

1. Put in the correct filters and set proper range (.1-.25 For enzyme work)
2. Place a cuvette in each holder
3. Zero each a and b by using the top knobs on the channel alternator with baseline adjust knobs on zero
4. Place a baseline adjust knob carefully on .19 And set to .19 On chart for a, using the adj std knob for a when channel alternator is on a, then switch channel alternator to b and set baseline adjust on b to .18 Then using adj std knob b, set chart to .18 For b (if you offset b one line while zeroing , set it also at .19.
5. Set channel alternator on a&b
6. Set baseline adjust for a and b to zero again and see if they go back down to zero. If they don't, do the whole zeroing and adj std process over- this may take several times if you have changed wavelength filter. Once this is accomplished, never touch the adj std knobs the rest of the experiment. The zeroing knobs can be changed without altering anything on the calibration. This calibration is important and needs to be done each time the instrument is turned on so that the chart will show accurate quantitative results. Mark on your chart the date, wavelength, range, calibration set, and enzyme, at the beginning.

LIBRARY PROJECT

Each student should work alone to find independently an article published since 1990 (no older ones) about mechanisms of cell motility or membrane receptor effects. It could be about tubulin, myosin, actin, actinin, troponin, tropomyosin, maps, dynein, Na⁺K⁺ATPase, mitotic apparatus, neurotransmitters, hormones, cilia, flagella, poisons of these activities like colchicine, vinblastine, cytochalasin, calmodulin, phospholipase. Decide which topic you are interested in and look for an article (not the reverse, just finding any old article in a journal.) It must have a methods section to tell how the experiment was done, and you must copy the article to hand in along with your summary of it which must have these elements: **Purpose**, what is the paper trying to say?

Methods, you must figure out the actual weights and volumes of materials used to make each solution, as though you were going to do the experiment yourself. You must figure out what kind of equipment is needed, and if there are any animals or cells required, with a possible source, if given. You may have to look up some of the references in the paper to get the actual methods used, those should also be xeroxed and handed in. Tell why the method used was particularly appropriate (like electrophoresis, viscosity, enzyme assay, electron microscopy, etc.)

Results, tell what controls were used and the conditions used to test the dependent variables, and the effects of these. Mention which results you could not get using our lab equipment for biol 580.

Conclusions. Summarize and tell how that would be a good experiment to do for our class. Be sure that you write down the reference on the title page if it is not already there with journal, year, volume and pages.

CYTOCHEMICAL ASSAY FOR LOCALIZATION OF NA⁺K⁺ATPASE IN SEA URCHIN EGGS

Embryos are fertilized and cultured using the fertilization exercise to the stage desired. Aliquots of 1 ml are then fixed in 2% paraformaldehyde in cacodylate buffered sea water, pH 8.2 for 3 min on ice. The fixative is removed (after centrifugation for 1 min) using vacuum suction (don't suck out the eggs). Resuspend in 1 ml preincubation mixture (CMFSW , pH 7.6 with cacodylate, contains 10% DMSO, a toxic material) and stand on ice for 30 min. Include ouabain for the ones you want to test for inhibition, in this preincubation medium. Suction off. Now you are ready for the enzyme reaction:

1. add .5 ml reaction mixture which contains lead, ATP, Mg⁺⁺, Na, K, glycine, levamisole (an alkaline phosphatase inhibitor), DMSO. Make a control one by adding AMP reaction mix instead of ATP reaction mix.
2. place all tubes in 36 degree water bath for 20 min, stirring occasionally. Place in ice to stop reaction. suction off medium. Lead is toxic so it cannot be put down the drain, so we capture it in a vacuum flask.
3. Wash twice with 1 ml sea water, centrifuging or settling in between and suctioning off liquid. This fluid can be put down the drain.
4. In the hood, add room temperature 2% ammonium sulfide. Leave for 2 min, swirl once during that time to stir. Spin in the hood and decant fluid, wash 2x with sea water, settle on ice. Remove fluid, add 50% alcohol. Leave 30 min. Place a drop on slide, allow to dry. Add a drop of 50% glycerol, leave overnight, then observe under high (40x) power microscope.

FISH SCALE PIGMENT AGGREGATION AND DISPERSION

Ref. L.T. Haimo, MM Rozdzial. Lysed chromatophores: as model system for the study of bidirectional organelle transport. *Methods Cell Biol* 31:3-24.1989.

Work in pairs, but make sure both of you observe the scale.

We will anesthetize a marine fish and remove some scales and then place it back in its tank. The scales (a dozen or so) can be obtained by running a spatula from tail to head. The scales will be placed in Marine fish Ringer's solution with EDTA, the scales will be cut into strips still attached together at the base and then incubated for 1 hr with frequent agitation. Some students should try to add protease to digest away the extracellular material instead of just using EDTA. (It is best to use collagenase, but we don't have any, so we will use non-specific protease.) Then the epidermis will be loosened from the dermis and will be dissected off with the fine watchmaker's forceps (make sure you don't poke the dish with those forceps or the points will break. To keep the scale in position during dissection in a binocular microscope, use a drop of silicone vacuum grease to hold it stuck into it. Then after removal of epidermis, peeling down from the base or proximal end to the external end, there should be exposed the dermis with the chromatophores. See if you can find chromatophores. They are large compared to the rest of the cells. They will most probably be aggregated with the pigment in the center (the damsel fish are blue-black interchangeably). Now place the scale in the fish Ringer's to recover, keep watching it to see how it looks. Try adding some epinephrine or cAMP to cause aggregation or dispersion. To get an effect you have to have it do the opposite of what it is doing with nothing. Make a drawing of the cells. Then if nothing happens, try adding the scale to a detergent solution with Brij58 to break the membrane but leave the vesicular transport mechanism intact. Now if you add ATP it will aggregate. If you add ATP + CAMP then it will disperse. cAMP and ATP are expensive, so we would only suspend the scale in enough fluid of these to cover them.

Is there an effect with Ca^{++} ?

This lab will take patience, and watching more than one cell to see if any recover or move the vesicles. Sometimes they will recover vesicular transport but not pigment change.

Questions to answer. What is happening in the epinephrine and in the cAMP to cause reversal of pigment granule movement?

SEA URCHIN EMBRYO DISSOCIATION AND REASSOCIATION AND CULTURE. A way to study factors involved in cell adhesion.

1. Hand centrifuge embryos out of sea water (if later stages and still swimming, use clinical centrifuge 1/2 full speed 5 min.)
2. Resuspend in Ca^{++} free sea water, centrifuge.
Resuspend in .44 M sucrose, .001M EDTA, .05M citrate, pH 7.8 and dissociate embryos by [pipetting in and out of pasteur pipette 15 times.
3. centrifuge cells, keep pellet, dilute cells with 10 ml Ca^{++} free sea water and centrifuge again.
4. Resuspend in normal sea water with antibiotics or other media and observe cells under microscope. You can alter one factor in each dish to study effects on adhesion- put one dish on ice, one in warm, one at 15 degrees (their normal temperature), one on shaker, one with proteolytic enzymes, one with metabolic poison, one with fixed cells, one with no calcium, etc.
5. Leave in incubator at 15 degrees until next lab period . Figure out a way to measure cell aggregates to quantitate your results.

CELL INJECTION

We will have to assemble the microinjection system, but we have the components. A possible thing to do is to transfer maturation promoting factor (also mitosis promoting factor) from mature eggs to immature eggs of *Xenopus*.

References to read to see if you want to try this:

Partial purification and characterization of the maturation-promoting factor from eggs of *Xenopus laevis*. M Wu, JC Gerhart *Devel Biol* 79:465-477. 1980.

DETERGENT EXTRACTION OF MEMBRANES AND PARTICLES

We will use the extracted supernates and pellets to analyze how effective each detergent is in extraction. We will do the analysis of proteins by electrophoresis and lipids by TLC and GC. Read about the effect of detergent in your texts. Detergents form a hydrophilic coat on the outside of membrane proteins hydrophobic regions. Both molecules are amphipathic, so the hydrophilic part is facing outside to the water. Membranes are formed because the hydrophobic portions of the proteins and lipids intertwine as a bilayer, but the detergent can disperse them. The detergent concentration is very important because there is a critical micelle concentration which is the concentration when little bubbles of detergent with the hydrophobic portion is facing inward. The detergent replaces the lipid around the protein, dissolving them from the membrane. Ionic detergents bind to the proteins and lipids in such a way as to disrupt ionic and hydrogen bonds, often denaturing the protein. For example SDS binds to every side chain of proteins. Nonionic detergents such as Triton-X, lubrol, octylglucoside form mixed micelles at high concentrations (above critical micelle concentration) containing lipid, protein and detergent. At low concentration they bind to proteins hydrophobic regions allowing them to remain unaggregated during purification and dissolving them from membranes.

Find the critical micelle concentration (CMC) at room temperature for the detergents available:

Triton-X,
zwittergent,
octyl-glucopyranoside (contain alkyl group),
lubrol,
SDS,
tween-20
deoxycholate

We will want to try 2 concentrations of each, one 2X above, one 2x below CMC. The low concentration will probably extract peripheral proteins, the high the integral ones.

What kind of control do we need to run?

Calculate the amount of detergent to weigh out for these concentrations in 10 ml. Use the molecular weights in the Sigma chart.

We will homogenize tissue -1 gram- in 4 ml detergent (or any weight with 4x detergent volume/wt). Should there be salt or buffer present? The CMC varies with buffer. Temperature is also important since some detergents form a gel in the cold. We usually start the process on ice to prevent denaturation by proteolysis or lipolysis. Adding EDTA can prevent metalloproteases from breaking down the proteins once extracted.

Will the kind of homogenizer matter? Since detergents are foaming, the waring blender approach is not so good.

Design your experiment. Think about what equipment you need. For example tubes, centrifuge, what speed, pipettes, balance