CELL PHYSIOLOGY BIOLOGY 580

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580 LAB INTRODUCTION

1. We can assemble some systems to try working with artificial membranes and single cell microelectrodes.
2. We can purify some proteins. We can use centrifugation, columns, and salt extraction. (myosin, tubulin, actin, Na+K+ATPase)
3. We can do electrophoresis and Western blot with antibody detection of proteins

4. We can observe cell motility, organelle motility in cells and effects of drugs and salts.
5. We can do membrane lipid purification and analysis
6. We can study cell cycle and drugs which modify it in sea urchin eggs. We can do immunohistochemical detection with antibodies.
7. We can study protein kinases and phosphatases.
8. We can do protein and phosphate tests and standard curves.
THE WAY THINGS ARE CHANGED IN CELL PHYSIOLOGY LAB

We used to do experiments where we did not know how they were supposed to come out, then we tried to explain our results. We were given the hypothesis to test, we were given the chemicals, and we were given directions of how to do an experiment.

Now we are given a topic, let's say "How does cytoplasm move? " We are given an idea of available equipment, reagents, animals or plants. Then we are told- observe something about cell movement or at least something to do with cytoskeleton.
Decide how a certain system can be used to test an hypothesis: for example,
1. **A sea urchin sperm can only enter an egg if both of them can polymerize an available supply of G-actin.** Then you can test various ways to prevent actin polymerization to see if it prevents fertilization. You would have to do a library search using let's say: su:actin polymerization fertilization prevention or acrosome pH Calcium osmotic pressure. So instead of figuring out what 1M glycerol does, you would figure out what it might do before you use it- why do you use that instead of salt?
2. pollen cannot germinate without the assembly of actin (test in the presence of cytochalasin or colchicine or metabolic inhibitors.)
3. pollen cannot germinate without first altering the pollen coat, and the continual softening of the advancing cell wall tip (in the presence of a protease inhibitor or a cellulase inhibitor or a protein kinase inhibitor or EDTA)
4. Elodea chloroplasts can move around on an actin sheet in the presence of ATP and Ca++. Then you have to figure out how to cover a slide with actin filaments.
5.

WHAT TO USE IF YOU WANT TO TEST THE EFFECT OF SALT
Substitute equal osmotic material that does not ionize- glycerol, sucrose, urea, ficol.
Change the concentration, or substitute divalent for monvalent, or K or Li for Na.
Add EDTA or EGTD to tie up divalent cations.
Use sephadex to change ionic medium.
Use ion exchange chromatography and elute with different salts to see which has an effect.
Use ionophores.
Change ratio of monovalent and divalent ions.
Precipitate with ammonium sulfate- salting out.
Precipitate by dilution- myosin.

HOW TO TEST FOR INVOLVEMENT OF CA++
Use A23187.
Add EDTA or EGTA or citrate to chelate it.
Inject it.

Add Ca++ pump poison.
Poison Ca++ channels

WHAT TO DO TO TEST THE EFFECT OF METABOLISM
Add inhibitors of glycolysis or CAC or ETS.
Cut off O2 supply.
Cut off CO2 supply for plants
Remove light for plants.
Remove food.
Uncouple oxidative phosphorylation

HOW TO TEST THE EFFECT OF WATER- use heavy water or carbowax to cut down amount of water in cell.
Use glycerol to cut down water concentration.
HOW TO TEST EFFECT OF HYDROGEN BONDS
Break bonds with urea or heat. With actin and tubulin, depolymerize with cold.

FIND A SPECIFIC DRUG THAT PREVENTS AN ACTION Then try different concentrations or time of application.

To TEST FOR EFFECT OF PH. Carefully prepare buffers of different pH, or apply materials which alter internal pH like ammonium chloride.
Use amiloride to prevent H/Na exchange
HOW TO TEST FOR ACTION OF ATP OR G PROTEIN OR SECOND MESSENGERS:
Look up specific inhibitors of these in reactions. There is a handbook on inhibitors in the library. Vanadate effects ATP-utilizing proteins , inhibits phosphatase.
HOW TO TEST FOR EFFECTS OF PHOSPHORYLATION
Test for increases or decreases of P-amino acids.
Test effects of cAMP, cGMP, ATP, GTP in presence of permeator molecule such as digitonin.
Lead can trap phosphate broken off molecules, then be made black to observe.
TO TEST FOR MEMBRANE INVOLVEMENT
Use detergents, Triton-X, digitonin, ionophores.
Extract with chloroform-methanol.
Use centrifugation to pellet membranes to see if material soluble at certain times, attached at others.
Make membrane vesicles, isolate, turn inside out, see effect.
Teat for endocytosis of material, exocytosis prevention or initiation.
Get temperature effects due to lipid phase change.
HOW TO TEST FOR ACTION OF MEMBRANE POTENTIAL
Change salt gradients. Add channel poisons, change charge across membrane with voltage clamp.
HOW TO TEST FOR NUCLEAR INVOLVEMENT
Add actinomycinD to prevent RNA synthesis, puromycin to prevent protein synthesis
TEST FOR ENZYME INVOLVEMENT
Use many poisons, one at a time.
Precipitate out protein with TCA,
Test Q10 of reaction to see if just diffusion or if enzyme.\

HOW TO TEST FOR -SH INVOLVEMENT OR REDOX POTENTIAL
Use DTT, mercaptoethanol, diamide,

TECHNIQUES USED TO TEST CHANGE

centrifugation
thin layer chromatography
gas-liquid chromatography
electrophoresis
observation in microscope
antibody reaction for immunocytochemistry or gel detection
immunodiffusion plates
enzyme assay
spectrophotometric detection by wavelength
column chromatography
gel filtration
microelectrodes, voltage clamp
cell injection
image analysis
fixation, sectioning and staining
dialysis
salting out
alcohol precipitation
lipid extraction
fluorescence detection
drug treatment
milipore filtration
detergent extraction
high salt extraction
microdissection
isoelectric focusing
recombinant dna
reporter genes
make models or cell-free systems such as tubulin or actin on a slide with motors moving over them
affinity chromatography
hydrophobic chromatography
cell ph or ion concetration detection by dyes or microelectrodes specific to one ion
fluorescent or other analogue chemistry

TO PURIFY A PROTEIN, need a way to follow it through the procedure and to detect presence of contaminants:
enzyme assay, electrophoresis, antibody, radioactivity

INDIVIDUAL PROJECTS CAN BE SUBSTITUTED FOR STANDARD LAB WORK.

INTRODUCTION

This cell physiology lab is designed to do several things:

1. Acquaint you with biological experimentation as a non-exact science

2. reinforce your knowledge of the experimental method, force you to start out each experiment with an hypothesis to test, make you aware of the importance of control experiments for comparison;

3. Allow you to think of cell activities as products of control of the cytoskeleton, secretory and endocytotic mechanisms, and metabolic machinery which can result from nuclear or environmental stimuli which produce alterations in membrane structure or other changes which can result in ionic concentration differences or enzymatic alterations. Our most common experimental variables will be temperature, calcium and magnesium ion concentration, cytoskeletal disruptors, metabolic inhibitors and substrate concentration, and anesthetics that alter the membrane.

4. Allow you to learn some of the methods used in biological research in cell physiology. This will be a great advantage to those going into graduate work, or teaching, or professional school. Not very many universities have courses in cell physiology. That is why I had to write this manual, so you are fortunate to be able to have this opportunity as an undergraduate.

Occasionally we have labs which do not produce the expected results of the hypothesis. The common error of students is to think that these labs failed. It is just as important to your education in biological research to figure out why some experiments do not "work out" the way they were expected, as it is to get beautiful results. Most of research is spent getting the experimental conditions to the point where data can be collected and meaningful results collected. Research is not cookbook chemistry, and I have not attempted to write a cookbook. I have taken some experiments from the literature and tried to adapt them to classroom application. There are often time constraints, which make this difficult. Sometimes, we try a different species due to availability. Sometimes experimental animals die right before the class, sometimes mistakes in solution making can occur, since graduate students often are also doing this for the first time, and sometimes the lab temperature is very warm or very cold which can change rates of egg development, or cell motility.

No lab should be considered wasted, since you will learn to handle volumetric measurement, microscopic proficiency, quantitation, methods for handling proteins, enzymes, live cells. If you are consistently getting nonsensical results when everyone else is not, then you must ask for help. You may have bad pipetting methods, solution labeling methods, glassware washing methods, or data recording technique.

You can help insure good lab technique by the following:

1. Purchase a glass marking pen, some graph paper, a cheap stopwatch, and keep them in your backpack.

2. Take good notes during the experiment about any variation of technique and record your results accurately. You can save yourself enormous amounts of lab time by setting up tables ahead of time for filling in the results.

3. Read about the experiment in your text and any references in the lab manual before you come to the lab. You will get much more out of the experiment. You should write some of the possible conclusions to such experiments before you do them. For example, if you are going to vary the ion

concentration in a certain enzyme test, find out what that ion does in cells, in relation to that enzyme so that you will have a possible explanation of your results. You will not be able to figure out what your experiments mean unless you do this. You may want to bring your texts to class as resource books, or your biochemistry text may also help. If the experiment involves cytoskeletal disruptors, find out what each one does, and write it in your lab book, since that can be referred to many times. Since you always have to turn in your lab results at the end of the week, you must have some of the work done before the lab.

4. Don't rely on the instructor to tell you what this experiment is supposed to mean. I will be happy to answer questions, but I expect you to do some work before you come. It is not always possible to coordinate the lecture with the lab, so sometimes you will do something in lab we have not yet discussed in lecture. Therefore, you will have to read in your text to find out where it fits in the overall cell physiology. That reading will just make it easier when we come to that part in lecture.

5. Always use clean glassware. Most of the time you can see dirt in glassware. You will not have a problem if you always leave it clean at the end of the lab, to dry for the next time. Always take clean pipettes from stock for the day, and then put them back at sink to be washed at the end of the day. Glassware should be immersed in hot soapy water and brushed individually, then rinsed eight times in running tap water and three times in distilled water. Invert to dry. Disposable pipette tips and cover glasses need not be washed.

6. Keep your lab exercises after they have been graded. Your lab book will be handed in at the end of the semester for review and grade. The lab assistant will grade some experiments, but the instructor will make the final analysis. Do work with your lab partners, but write up your experiments by yourself, since they will be compared at the end. That is where the prelab work will show up. Don't copy material from books or articles without referencing them. Looking topics up on the web of on your text CD is also a great idea.

SOME LAB TIPS

PIPETTING. For good control of the pipette use your index finger on the tip of the pipette, not your thumb if using the blue bulbs. Pull fluid up into the pipette until slightly above the place where you want the volume to be. Release your BULB at the same time as you place you finger on the tip. If the tip of the pipette is wet, you will not be able to easily release a part of the volume to get it to the volume you wish to deliver, so don't have wet fingers. Probably the most accurate way to pipette is by the blowout method, where you only take up the amount you wish to release, each time you place a volume in a tube, then blow out the fluid using the bulb after it flows to a stop. With the black bulbs, always make sure you remove the cotton from the pipette, and for small volumes don't have a high vacuum on the bulb or you will suck fluid into the bulb. Always use the size pipette near the volume you want (use a 1 ml for .1-1, use a 5ml for 1.1-5, a 10 ml for 5.1-10.0. Each time you pipette, you magnify the error. Don't use large pipettes for small amounts to deliver, because you cannot read the large diameter as accurately as the smaller diameter pipettes. For amounts less than .1 ml use the automatic pipettes with disposable tips. During the same lab, you can use the same tips, if you label them by marking them with a marker to prevent contamination of the reagents.

For all materials use rubber bulbs. Most lab chemicals are not toxic, but by law we cannot mouth pipette.

DILUTIONS. To make dilutions, divide the desired concentration by the present concentration, and then use that value to determine the desired volume of concentrated reagent to dilute with water to the final volume.

Example: reagent prepared is 1 M. Reagent concentration desired in your reaction mixture is 6 mM or .006M. Divide .006 by 1= .006. You need 25 ml of reaction mixture, so multiply 25 by .006= .15 ml you need to add before bringing the mixture to a final volume of 25 ml. Often in making reaction mixtures you have to add several reagents, so add up all the reagent volumes added, subtract from the 25 ml, then make up the difference by adding water. **Bringing a calculator to lab is essential**. IT IS IMPORTANT NOT TO DILUTE REAGENTS TO THE FINAL CONCENTRATION DESIRED BEFORE ADDING THEM TO THE REACTION MIXTURE BECAUSE THAT WOULD DILUTE THEM FURTHER.

pH. The pH of reactions is very important and must be controlled during experiments. Make sure that all reagents are the proper pH, and then add buffer at the controlled pH desired. Usually, use 0.01M or less final concentration.

SPECTROPHOTOMETERS. Always check to be sure the filter system is correct for the wavelength you wish to use. Then turn it on 20 min before you need to use it, so it can stabilize. Use only special tubes, and insert them so the markings are at the front. Make sure you know how to zero the instrument, and have a control tube for doing that.

WATER BATHS. Fill the water bath with water the approximate temperature you want, and then adjust it with ice and a thermometer. For heat, use warm water, then turn on the heating element and adjust it so the light just comes on at the desired temperature, and goes off when you turn it a little bit lower. Then watch it for a few minutes, to be sure it is right.

REAGENTS. Since the whole class has to use the reagents, it is important that dirty pipettes not contaminate them. Take the amount of reagent you have figured out that you need for the whole day, placing it in an Erlenmeyer flask, well marked. Since there are 16-20 students and 8-10 pairs of partners, never take more than 1/10 of the total volume. At the end of the day, you can put back reagent that you feel is uncontaminated, particularly expensive ones like ATP, cytoskeletal disruptors, protein standard reagents, phosphate standard reagents, GTP, buffer, sucrose. **Always keep high-energy compounds like ATP, GTP, G6P, acetylcholine, or proteins isolated from cells on ice** during the day since they break down at room temperature. Never add them to your reaction mix until the last minute.

ABSENCES. There will be two absences allowed, with library work to makeup labs. Habitual tardiness, or long coffee breaks, or allowing your lab partner to do the work, is always noted by the instructor and will be reflected in your final grade.

GRADING will be subjective. An A grade will be for those who do the work, write the reports, including drawing correct conclusions about the experiments. This will include getting results on unknown samples given by the instructor. To be able to do all of this in 3 hours takes organization, done largely before coming to lab. The instructor will do spot checks to see if you have done your prelab work. It is possible for everyone in lab to get an A, if everyone does the work well.

REFERENCES FOR LABORATORY EXPERIMENTS RESERVE ROOM SOUTH LIBRARY UNDER SPARLING, BIO580L

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GET NEW LIST FROM INSTRUCTOR

ERROR ANALYSIS FOR BIOLOGY 580 LAB I.

How can one evaluate the expected error for an experiment? Break up this answer into parts:
1. figure out the expected error for each step in the test See Analytical Chemistry by Christian.

A. **Purity of chemicals**: analytical reagents are 99.95% pure B. **Weighing**: the last digit of the scale gives the sensitivity analytical balance to .1 mg,
larger scale to .1 g or .01g (see scale)
so the accuracy depends upon the amount weighed since .1 g is a lot less of the total weight when you have 30g than when you have .3 g total.
C. **Pipettes**: blowout two rings around top
10 ml error .02
5 ml .01
1 ml .006
automatic pipettes to 1-2% or .01-.02
D. **Volumetric glassware**:
100-1000 ml volumetric flask .0003
graduated cylinders .01
To calculate the expected error in your experiments you use these values X the total you are using (g,ml) and that squared gives you the variance for what you really measure out- expected error.
E. calculate the total error- all the errors combined, some of which may cancel out others by adding up all the variance (error squared.)
See Taylor, or Christian, or Bevington, or Freund
We will do this on a spreadsheet where you will enter
1) the proper expected error from the list above, and the amount measured (ml,g) and it will automatically give you the expected error which will then be squared to give you variance for that sample or solution in the columns so marked. Then in the end, you will get the total for each sample in the standard curve or enzyme analysis.
2) When you are doing a standard curve you have more than one sample, so you have to multiply the total variance by the number of samples to get the total variance for the test.
3) to get the expected error for the test, which includes getting the regression line and equation, you take the square root of the total variance.
What is the difference between accuracy and precision? The accuracy of a test is just what we were just talking about, but the precision is a matter of how repeatable your results are.
What is the difference between standard deviation and variance? How can variance be used to determine the total error in additive tests?

		EQUIP	experr	amt-gorml	ERR	VARIANCE	NUM STEPS	NUM SAMP		
							#####	#####		
phosphate	TCA	balance			#####	#####	##	#####		
test		vol			#####	#####				
		pipette			#####	#####				
	po4	balance			#####	#####				
		vol			#####	#####				
		pipette			#####	#####				
		water			#####	#####				
	sulf-mo	vol			#####	#####				
		gradcyl			#####	#####				
		gradcyl			#####	#####				
		bal			#####	#####				
		vol			#####	#####				
	sncl	bal			#####	#####				
		PIP			#####	#####				
		pip			#####	#####				
							#DIV/0!	#DIV/0!	TOTAL VAR	PHOSPHATE
		vol			#####	#####				
									TOTAL	
protein	soln3	bal			#####	#####	X		VAR	PROTEIN
							#####	#####		
		bal			#####	#####	##	#####		
		vol			#####	#####				
	tart	bal			#####	#####				
		vol			#####	#####				
	cop	bal			#####	#####				
		vol			#####	#####				
	solnA	grad			#####	#####				
		pip			#####	#####				
		pip			#####	#####				
	BSA	BAL			#####	#####				
		GRAD			#####	#####				
	FOLIN	GRAD			#####	#####				
		GRAD			#####	#####				
	BSATEST	PIP			#####	#####				
	H2O	PIP			#####	#####				
	DUP	PIP			#####	#####				
	SOLNA	PIP			#####	#####				
							#DIV/0!	TOTVAR		
	SOLNB	PIP			#####	#####				
							#DIV/0!	EXP SD		
						#####				

c. <u>Regression analysis of linear expressions</u> We will do a regression analysis of our standard curves. Today we will enter data and do regression. same references

These would be the errors expected from volumetric and weight errors. There are left instrumental errors (usually .01) and operational errors. How can one control for that?

COMPUTER ANALYSIS OF DATA FOR 580L

We will use the apple computers to do regression analysis and statistics on our protein curves, phosphate curves, and enzyme activity curves.

First we will enter our data into a spread sheet, using Cricket.
The IBM can be used for Excel spreadsheets.

LIST OF CHEMICALS AND MOLECULAR WEIGHTS

chemical	VOL	MW	desired conc
standard sodium phosphate			
NaH2PO4:H2O		138	
PO4	1 L	31	10ugPi/ml
trichloracetic acid TCA	500 ml		5%

Phosphate detection reagents:

1) sulfuric acid 5N 141ml conc/l titrate to check
sodium molybdate 500 ml- 18.76g in 2.5N H2SO4
2) stannous chloride stock solution 40% 25ml in conc HCl
use diluted .25 ml/50 ml H2O

protein detection reagents:

3) sodium carbonate 1 L 2% in 0.1 M NaOH
sodium hydroxide MW=40

4) sodiumpotassium tartrate 100 ml 1%
5) copper sulfate 100 ml .5%
6) solution A- 48 ml soln 3 +1ml soln 4+ 1ml soln 5

7) std bovine serum albumin 150 ml 1mg/ml
8) Folin phenol reagent dilute 1/2

Buffers:

tris buffer pH 8.0 50 ml 121.14 1M

CALCULATIONS:

Figure out the weights or measures for all the solutions needed.
Then using the spreadsheet figure out the expected error in such solutions and in the protein test and phosphate test by entering the error for each part of making the solution or test:

protein test. Look at the reagents for protein detection test above. Figure out whether you use the balance, pipettes, what kind of glassware to see the possible error. If you have a good calculator, you can use that instead of the computer.

For reagent 3 you first make the three solutions, then you combine them. Then you use 5 ml (pipette) for each sample.

so you will use a 1 liter volumetric, weigh out the sodium carbonate and sodium hydroxide and bring the volume to 1 l. Then the solutions 4,5 are made in 100 ml volumetric flasks, and you pipette out 1 ml of each. Now add up all the possible errors for all these processes by getting the variance and then

summing them. Now try the whole thing using graduated cylinders instead of volumtric flasks, and see the difference in the expected error. Record your results in your lab book.

Find out how much difference it makes to use a 5 ml pipette for pipetting 1 ml, compared of using a 1 ml one.

So what you need is a worksheet which has kind of measure in the first column, the known error for the step in the second column, the amount measured in the 3rd, column 2 x 3 to give the measurement error at this step in col 4; col 5 has this value squared and this is the variance.

- Then after listing every step, you add up all the variances, take the square root of the total to get expected error per sample.
- For the error of the whole regression line, you take the total variance, multiply by the number of samples and take the square root.
- Expected STD DEV will be square root of (total variance x Number samples)/n-1).

LOWRY PROTEIN STANDARD CURVE

read the introduction and lab clues.

Start out by thinking to yourself: "What is the purpose of doing a standard curve for protein?"

How does one prepare a standard curve from data? What is the purpose of having duplicate samples?

Answer in brief: When we have a preparation of protein with unknown protein content and we want to know how much is there, we can use this test if we have a standard curve from which we can calculate how much protein is there from the amount of blue color detected. When we have duplicates, there is leeway for an occasional mistake such as a dirty tube or a dropped tube or pipetting error since we can average values or throw out tubes that are obviously wrong.

MATERIALS TO HAVE CLEAN AND READY: 30 testubes, testube rack, pipettes, 100 ml graduated cylinder. Marking pencil (bring your own felttip marker to lab from now on.) You may work with one lab partner, but make sure that you each do part of the pipetting and dilution. We will use the black rubber bulbs for these experiments for the pipetting. Let the air out the top by squeezing the two top buttons together. Let the fluid in to the level you want by squeezing the two bottom buttons right above the pipette that say S (Suction). To let the fluid out place the tip of the pipette on the side of the vessel, above the fluid level and press the two buttons together marked E (empty). To get out the last little bit cover the tip of the small rubber bulb with a finger and squeeze the small bulb while holding the S buttons open. We get better curves with regular pipettes than we get with automatic pipettes, since there is difficulty getting used to automatic pipettes so that consistent samples are taken.

Preparation of solutions

REAGENTS: Solutions may contain more than one chemical. The Reagent A contains four things which are not stable when mixed, so they are mixed each day from solutions which are stable.

reagent A 1%NaK TARTRATE 2cc
0.5%CuSO4 2cc

mix these two, in a 100 ml graduated cylinder,then add

2% Na2CO3 in 0.1N NaOH TO MAKE A TOTAL OF 100ML

the solution must be used the same day. 100ml will be enough for 19 samples.

reagent B Folin phenol reagent (0.5cc per sample x number of samples)

add an equal quantity of distilled water

Example: 5cc Folin reagent + 5 cc water will be enough for 19 samples- you never can recover all of a solution.

Bovine serum albumin: If the BSA stock solution you need is supposed to be 1 mg/ml but the one provided is 0.1 g/ml (100 mg/ml) dilute it 1/100 (since you don't need 100 ml, use .5/50ml then we will have 1000 ug/ml (1 mg/ml) stock solution .

PRELAB PREPARATION Standard curves are graphs of optical density (absorbance) versus concentration of some compound. We are not particularly interested in how the chemical part of these tests work, we are only interested in learning how to determine quantitatively how much of various unknown things we have in different cell fractions or preparations.

For standard curves to work right we have to have a constant volume of material to be tested. For the Lowry protein test we used 0.5 ml samples with known amounts of protein; for the Pi test, we will use 2 cc samples diluted in 1.6 ml TCA. We must have the approximate proper range of concentrations of material in our known samples or they will be too dark or too light to make a good curve. For proteins we should have between 50 and 600 ug/ml for good readings, so we usually have to dilute a cell fraction preparation 1/5 or 1/10 before testing them for the Lowry test.

important: you will use this standard curve for the rest of the semester to figure out how much protein you have in solutions. So be very careful; do a good job, or you will have to repeat it.

PREPARATION OF PROTEIN SOLUTION DILUTIONS

Now you are going to set up two sets of tubes: if possible use different racks for 1 and 2.

- one set of dilutions of BSA each with a volume of 2 ml, these will be used to make up the second set;

All measurements should be as accurate as possible, with graduated pipettes for amounts under 10 ml and graduated cylinders for larger quantities.

- label 7 tubes with the concentrations in the left column below and make these combinations. This will be your first set of dilutions in rack one.

BSA DESIRED CONC STOCK	AMOUNT 1000uG/ml BSA	AMOUNT distilled water
600 uG/ml	1.2 ml	0.8 ml
500	1.0	1.0
400	0.8	1.2
300	0.6	1.4
200	0.4	1.6
100	0.2	1.8
50	0.1	1.9

- set up sets of duplicate tubes, one set behind the other in the second rack, marked for those same dilutions: you will take duplicate 0.5 ml samples of each dilution from rack one to be pipetted to each duplicate two tubes in rack 2. Set up a single water blank tube.

PREPARE 7 PAIRS OF TUBES LABELLED WITH THE DILUTION AND ONE TUBE FOR THE WATER BLANK. Take 0.5 ml of the firdt set of dilutions with their known concentration of bovine serum albumin (BSA) and place in each of the paired tubes (do this in duplicate, two samples per concentration using labeled tubes) as listed above,) This will be rack 2.

- plus one water blank 0.5 ml) in rack 2. Don't make the common mistake of using plain water as the final water blank! The water blank is .5 ml water plus all the reagents used in the test.
- Keep the first rack dilution set separate now so you don't get mixed up and use them for the color test. You can set these away, now, but save them for the Biorad test.

STUDENTS OFTEN USE THE WRONG SET OF TUBES TO DO THE FINAL TEST. MARK THEM WELL. KEEP THOSE DILUTIONS in rack 1 FOR THE BIORAD TEST.

- So now you have a rack with duplicate sets of tubes with 0.5 ml in each and a water-blank marked -blank-. <u>This tube will always have everything added first</u> since it will be used first to zero the spectrophotometer.
- You will also be given 2 unknowns with numbers on them which will tell us which are the correct answers to the quantity in the unknown. Prepare 0.5 ml duplicates of unknowns, mark tubes.
- You must **warm up the spectrophotometer now for 30 min** as you do the rest of the experiment. Turn on with left knob. Make sure you have one with a **red filter and red tube**.

- *WARNING!!!!!FOR THE NEXT STEP* ***Mix each tube swiftly****, energetically* *right after adding each reagent or the proper color will not develop. Do not wait and mix all after adding all.* I you have a lab partner, one person should pipette, one stir. Get a demonstration of how to stir tubes from the instructor.
- **ALWAYS START WITH THE WATER BLANK Add 5 ml of reagent A** to all of the tubes in rack 2 containing the .5 ml aliquots, SINCE YOU WILL USE THAT FIRST TO ZERO THE SPECTROPHOTOMETER.
- **Mark down the time**. Wait ten minutes after all additions, mixing. At this step you will have 14 BSA tubes and one water blank and 4 unknowns.
- **Add 0.5ml reagent B** (already diluted with water) mix very thoroughly, immediately after adding to each tube, rapidly,
- Note the time you finish, **wait 30 minutes. Read at 650 nm** on spectrophotometer. Zero the instrument without the cuvette using the left knob, for the left side of the scale with the blank tube out (0.5 ml water plus all the reagents). Then set at 0 absorbance with the right knob for the right side of the scale, using that water blank in the cuvette. Read the unknowns and knowns starting from the lowest concentration, pouring each into the same cuvette, shaking out excess from the cuvette after pouring samples back. Read duplicates one right after the other without changing the settings of the spectrophotometer. Make sure the densitometer is set at the proper wavelength and has a red filter in place. Wipe the tubes with tissue before inserting them in the well. There is a mark on the front of the tube so they are inserted correctly. Make sure you use the proper cuvettes for reading the optical density.
- Record your data on the lab sheet that will be turned in at the end of the lab. Data includes all values, not just the averages of the duplicates.
- Make a table of the original data using Cricket or other computer program. Average the two values for each known concentration. 4. Plot the optical density versus the protein concentration in ug/ml using the computer. Do not divide by two even though you only used 0.5 ml in your test, and later when you use this on an unknown you will not have to worry about multiplying by 2 to get the concentration in ug/ml. However, when you use less than .5 ml for your unknown tests in later experiments, and then add water for dilution to make up the rest, you will have to take that into account in your unknown, multiplying by the dilution, in that .5 ml.

You should get a fairly straight line with a little dropping off at the top of the curve. Compare your results with others in the lab.

- Prepare a permanent lab book for the semester and place these results into it after they are graded. You should always bring your lab book to lab and put your results directly into the book from now on. Also bring a disc for the MAC to record your data. You will use some of these results in later experiments. Do not loan your results to anyone else, unless given permission by the instructor. That will be considered cheating by both people.
- Everyone is allowed to miss two labs or throw out the lowest score. Other missed labs will be counted as zeros. To get credit for a lab you must attend the whole lab period, unless you finish early. You should always read the experiment before you come to class.

- Clean your glassware with dilute soap (too much soap is hard to get off), 8 rinses with tap water and 3 rinses in distilled water. Set upside down to drain before next lab period.

In cases where we use many tubes, such as today, we will use the dishwasher. Place tubes upside down in dishwasher racks.

BIORAD PROTEIN ASSAY

This assay is for samples which are not very dilute. Use the Lowry test for dilute solutions

1. Prepare dye reagent. **IT TAKES AT LEAST HALF AN HOUR TO FILTER THE REAGENT SO DO IT EARLY.** It is supplied as a 5x concentrate. Dilute 1/5 (1+4), stir and filter through #1 filter paper. This lasts for 2 weeks in a dark bottle in refrig.

- Prepare standards for a standard curve with Labeled tubes. You can use the dilutions from the Lowry test you already made. Make dilutions of unknowns so that they may be in this range (it is usually safe to do a 1/10,1/50 dilution of cell homogenates), this means there will be 200 to 1400 ug/ml. YOU CAN USE YOUR STANDARD DILUTIONS FROM THE LOWRY TEST, except for the dilutions below 200 ug/ml
- Place .1 ml of each into marked duplicate pairs of tubes. Prepare a water blank of the same volume.
- Add 5 ml diluted dye reagent to each tube. Vortex or mix, but avoid foaming.
- Measure OD at 595 nm (with water blank (plus biorad reagent) adjusted to 0,) after 5 minutes or up to an hour.
- Plot OD versus concentration for the standards, plot in Cricket to get the equation and then read your unknown concentration and multiply by the dilution for the final concentration.

THE SPECTROPHOTOMETER IN THIS CASE DOES NOT USE A RED FILTER OR TUBE. CHECK TO MAKE SURE THEY ARE NOT PRESENT. THIS STANDARD CURVE WILL BE USED FOR THE REST OF THE SEMESTER. BE CAREFUL.

STANDARD PHOSPHATE CURVE

For the Pi test, we want between .2 and 7 ug/ml of standard or unknown phosphate. We will use NaH2PO4:H2O as our standard solution. To get a stock solution of 10 ug/ml or 10 mg/l of P, we know the MW of the whole molecule is 138, and of the P is 31, therefore 31/138=.01/x= .045g/l of NaH2PO4 will contain 0.01 g/l P. That will be our stock solution.

MAKE TWO RACK AS WE DID LAST TIME- ONE FOR DILUTIONS, DUPLICATES IN SECOND.

TO MAKE THE DILUTIONS OF THE STOCK SOLUTIONS:

TUBE MARKING	**ML STOCK**	**ML H2O**
P CONC ug/ml	10 ug/ml	
0 BLANK	0	5
0.2	.1	4.9
0.4	.2	4.8
0.6	.3	4.7
0.8	.4	4.6
1.0	.5	4.5
2.0	1	4
3.0	1.5	3.5
4.0	2	3
5.0	2.5	2.5
6.	3	2

- YOU HAVE PREPARED 11 TUBES WITH DIFFERENT DILUTIONS.
- We will now prepare PAIRS, two SEPARATE duplicates, EXCEPT FOR THE WATER BLANK. 1 ml samples from each of these dilutions, a total of 21 tubes, to be used directly to test for color development.
- To each of the 1 ml samples, add 1 ml of 5% TCA to each tube for a total of 2 ml.
- Then to each tube add 1.6 ml sodium molybdate
- Shake immediately, if not sooner.
- Dilute fresh each day .25 ml Stannous Chloride in concentrated HCl reagent (just let reagent run into pipette and then let it run out until it is at the .75 on a 1 ml pipette, then blow it out into a 50 ml graduate cylinder, fill to 50 ml with H2O. Be careful not to contaminate this reagent with a pipette containing P (detergent has lots of P).
- Add 0.4 ml of this diluted reagent to each tube, but only do a few tubes at a time, because they must be read 2 minutes later

Plot curves using Cricket, store data on disc.

FERTILIZATION, SEA URCHIN

PREPARATION AND MATERIALS PER GROUP OF FOUR STUDENTS:two sea urchins, syringe, 2 small beakers (50 ml), filtered sea water, ph 5,7,8 sea water, CA++Mg++free sea water, hand centrifuge, container of ice.

THIS INVESTIGATION REQUIRES: SLIDES and coverslips ANIMALS:sea urchins (Echinodermata) UTENSILS:50 ml beakers, syringe and needle for injection of sea urchins, centrifuge tubes, pipettes, petri plates.

PRELAB; LOOK UP FERTILIZIN, ANTIFERTILIZIN, BINDIN, VITELLINE MEMBRANE, SPERM MOTILITY, ACROSOME REACTION, CORTICAL REACTION.

OBJECTIVES: to see sperm movement, attachment to egg, lifting away of fertilization membrane from surface of egg, first cleavage and to investigate mechanisms involved.

REQUIRED TASKS: fertilize eggs, observe fertilization membrane lifting off the surface of egg.

OBSERVE EFFECTS OF PH, TEMPERATURE, CA++ CONTENT ON FERTILIZATION. TEST THE EFFECTS OF -SH AGENTS ON FERTILIZATION MEMBRANE HARDENING. Students to work in pairs and turn in completed lab sheet at the end of the lab

PROCEDURES: In this section you will determine the relationship between eggs and sperm and correct environmental conditions required for normal fertilization, fertilization membrane formation, and first cleavage LABEL ALL DISHES OF EGG FERTILIZED WITH THE TIME OF SPERM ADDITION, AND ANY VARIABLE CONDITION. MAKE SURE YOU HAVE AN UNTREATED CONTROL WHICH HAS NO SPERM ADDED, IF THAT IS REQUIRED IN YOUR EXPERIMENT.

EACH GROUP OF 2 STUDENTS;

1) 1.inject two sea urchins with 1 cc of .55 M KCl and then give them a good shake and wait to see what sex they are: the gametes come out five gonopores at the aboral surface (opposite the oral surface where the mouth is) if the gametes are white, it is sperm and should be shed by turning the male upside down in a petri dish placed on ice; if the gametes are yellow, it is a female and the eggs should be shed into filtered sea water (pH 8) by inverting them over a beaker full of sea water. The beaker should be of smaller diameter than the sea urchin so that it won't fall in. Keep injecting until you get both sexes, after appropriate waits to see what the next one is. You can trade with other groups, also.

ALWAYS KEEP THE EGGS AT ROOM TEMPERATURE AND THE SPERM ON ICE This means use room temperature sea water in all egg treatments and at fertilization. If it is cold, warm it by setting it in a pan of warm water. Give another 1 cc injection when egg spawning slows down, then leave the female to complete the spawning for about ten more minutes. In the meantime, design your experiments and label your dishes.

2) After spawning is complete pour off the sea water into another beaker, so you don't lose your eggs by mistake. Pour the remainder into a centrifuge tube and spin 15 rotations on the hand centrifuge (make sure you have a balance tube opposite.) REMOVAL OF EGGS FROM THE CENTRIFUGE TUBES CAN DAMAGE THEM UNLESS IT IS DONE CORRECTLY! Always pour some fluid over the eggs, then using a pasteur pipette with a rubber bulb, fill the

pipette with fluid from the top of the tube, then gently force the fluid out into the bottom of the tube to stir the eggs. Do not let air bubbles do the stirring, and don't creat air bubbles or suck the eggs up into the pipette and then back out- it homogenizes them.

Removal of jelly: Add 25 ml ph5 sea water to empty the eggs from the tube into the beaker, and allow it to stand for three minutes before adding .5 ml 1 M tris pH 8 to raise the pH rapidly. (the pH 5 treatment removes the egg jelly, but prolonged treatment leads to damage to the eggs.) Now we must remove the dissolved egg jelly by washing the eggs. So do centrifugations as above, SAVING THE FIRST SUPERNATE, LABEL IT JELLY, and wash the eggs with a tube full of sea water pH 8, 2 times, suspending the eggs each time as above.

3) Now we have our eggs prepared for the experiment. Sperm are much more delicate than the eggs, so they must be kept undiluted in ice (the seminal fluid has substances in it to keep the sperm inactive and the ice also helps to do that.) They must be diluted prior to their addition to the eggs, or we get polyspermy and abnormal cleavage. Add one drop of concentrated sperm to 10 cc of sea water in a graduated cylinder, stir to disperse evenly. This diluted sperm suspension will only last for 20 minutes, so do it right before you need it, and keep it in the ice bath. Prepare a slide with a drop of eggs, have it set up on a microscope prior to seprm addition adjusting the light the eggs on the slide easily visible. Add a drop of the sperm, slam the cover slip on the slide and place under the low power objective and observe fertilization. If you don't get fertilization try it again with another batch of sperm from someone who found good fertilization. Then proceed to fertilize your aliquots of eggs.
4) Fertilization: ADD 0.1 ML DILUTED SPERM TO 10 ML OF SEA WATER CONTAINING .1 ML EGGS. Observe, using dissection microscope and dark field (obtained by putting mirror on its side until field appears black and eggs appear white) or compound microscope. The fertilization membrane is formed by a lifting away of the vitelline membrane, and an addition of material to it from the ruptured and released contents of the cortical granules. Look at the number of sperm around one egg, record for a few eggs. Observe and record the sperm shape and size compared to the egg, movement of the flagellum. For polyspermy, get one dish of eggs with about 100 sperm per egg. Check the effect on cleavage, as compared to the control.
5) Test the effect of pH on fertilization. Fertilize a DISH of eggs in the same manner, but use pH 5 sea water in one dish, pH 7 in another. Record the % fertilization membranes formed after 1 min, 5 min. What is your control?

IF YOU ARE GOING TO CULTURE THE EMBRYOS TO LATER STAGES, DECANT OFF THE SPERM LADEN SEA WATER ATER THEY SETTLE, AND POUR A SHALLOW LAYER INTO PYREX BAKING DISHES AND INCUBATE THEM AT 15 DEGREES C FOR ANYTHING PAST THE 2 CELL STAGE OR THE CLEAVAGES WILL NOT BE SYNCHRONOUS. IT TAKES ABOUT 1 1/2 HRS TO GET TO THE FIRST CLEAVAGE. KEEP CHECKING TO SEE IF YOU CAN SEE THE MITOTIC APPARATUS DEVELOPING. The fertilization membrane is formed by a lifting away of the vitelline membrane, and an addition of material to it from the ruptured and released contents of the cortical granules. Look at the number of sperm around one egg, record for a few eggs. Observe and record the sperm shape and size compared to the egg, movement of the flagellum. What is your control? Why is the lifting of the fertilization membrane a good indication that a sperm has penetrated the egg?

If you are going to culture the embryos to later stages, decant off the sperm laden sea water after they settle, and pour a shallow layer into pyrex baking dishes and incubate them at 15 degrees c for anything

past the 2 cell stage or the cleavages will not be synchronous. It takes about 1 1/2 hrs to get to the first cleavage. Keep checking to see if you can see the mitotic apparatus developing.

Keep in mind that we have two sets of factors working in fertilization: 1) the activation of the sperm acrosome reaction, polymerization of actin filament 2) activation of egg and cortical reaction. Try to figure out which require Ca++ release and which require a pH change. Calcium ionophore and NH4 can be used to answer these questions.

reference-Nucciteli: How do sperm activate eggs? Current topics in developmental biology vol 25. Chapter 1. 1991.

1) EFFECT OF TEMPERATURE ON FERTILIZATION. Fertilize a dish of eggs that has been sitting on ice for ten minutes. Compare the percent fertilization after and 5 min. with the control. .UL EFFECT OF CA++ ON FERTILIZATION. Fertilize a dish of eggs using Ca++Mg++free sea water instead of regular sea water. Compare the results with the control.

2) EFFECT OF -SH REAGENTS ON VITELLINE MEMBRANE AND FERTILIZATION MEMBRANE. treat the eggs with DTT or glutathione prior to fertilization, and check them after addition of sperm.
3) METHODS FOR REMOVING VITELLINE MEMBRANE:

1. Epel, D., AM Weaver, D. Mazia. 1970. Exp cell res 61:64-68. Methods for removal of the vitelline membrane of sea urchin eggs. I. Use of Dithiothreitol (Cleland Reagent.)

Incubate unfertilized S. purp or L. Pictus or Dendraster excentricus in 5 mM DTT at pH 8.0. Releases jelly, and vitelline m and accessory cells of starfish. 5 min to remove jelly and VM. pH very important. higher pH (9.2) requires 1/2 the time.

2. BM Shapiro. 1981. Awakening of the invertebrate egg at fertilization. In Fertilization and embryonic development in vitro. Eds. L Mastroianni, Jr. and JD Biggers. Plenum Press, NY. pp233-255.

used 3 amino-1,2,4-triazole to inhibit ovoperoxidase activity, FM remains soft and can be removed anytime (Showman and Foerder, 1979:Exp Cell Res. 120:253-255. Removal of the fertilization membrane of sea urchin embryos employing aminotriazole.)

3. SG Ernst, BR Hough-Evans, RJ Britten, and EH Davidson. 1980. Limited complexity of the RNA in micromeres of sixteen-cell sea urchin embryos. Devel. Biol. 79:119-127.

fert at 5x10 E5 eggs/ml add equal vol of following after membrane elevates (about 90 sec):freshly prepared .08% papain, .40%glutathione, pH 7.8, swirl gently and look until membranes begin to disappear 90-120 sec later. After 7-9 min dilute to 1-3x10 E4.

4. RO Hynes and PR Gross. 1970. A method for separating cells from early sea urchin embryos. Devel. Biol. 21:383-402.

fert eggs in 0.04% papain, .2% cysteine, sea water, pH 7.8. Make fresh daily. Keep eggs suspended 4-5 min, spin down gently, remove supernate, wash 2x with filtered SW

What do these methods tell us about how the vitelline membrane is held on the egg and what happens at fertilization to lift it off as the fertilization membrane?

ARTIFICIAL ACTIVATION BY A23187 AND PAF

PLATELET-ACTIVATING FACTOR CAN ACTIVATE THE CORTICAL REACTION IN SEA URCHIN UNFERTILIZED EGGS AND MEIOSIS IN STARFISH EGGS

Mary Lee Sparling, Biology Dept Jan, 2001

Platelet-activating factor (PAF) is a choline phospholipid similar to phosphatidyl-choline, a membrane lipid, but having an acetyl group on its second carbon instead of a long acyl hydrocarbon chain. It causes secretion of materials stored in secretory granules in many kinds of cells (blood platelets, neurons, immune cells, and follicle cells) and causes the acrosome reaction of sperm. The effect of PAF is due to a PAF-receptor which when occupied can cause activation of many enzymes producing lipid second messengers or lipid substrates. PAF is a very ancient and important lipid signal in reproduction and may aid gamete union as well as prevent apoptosis of egg and sperm once they are united since PAF Receptor can activate PLA, PLC, PLD, PI3K, GTPase, Ca++ channel, and PKC. Production of PIP2 by PI3K or activation of PKC, to phosphorylate many cell proteins, prevents apoptosis. PAF is produced after fertilization by sea urchin egg homogenates and after stimulation of meiosis in starfish egg homogenates. It is considered an autocoid, or substance that when secreted can cause changes to the cell that produced it. Fluorescent PAF and other related lipids (LPAF, PC, PE) were applied to eggs to see where they go. The effect of PAF does not seem to require entry into the cell. Application of $5X10^{-4}$ M PAF caused secretion of the cortical granules which usually only occurs in eggs which have been fertilized. A similar activation of secretion can be caused by calcium ionophore A23187 which also activates the enzyme pathway which synthesizes PAF. Normal PAF production at the time of fertilization may be the cause of the secretion of the acrosome by sperm and the cortical reaction in normal eggs, and a part of the program for activation of cell division and differentiation.

take .2 ml eggs, add following add all to tube before eggs except sperm

	20ul		1ul	1ul	1ul	20ul		5ul	
	sperm		alc	PAF 10-3M	PC 10-3M	HEPES		BSA .1%	
E1									
row 1	x								do first to check

row 2	x		x			x		x	control for second
row 3			x			x		x	
row 4			x		x	x		x	
row 5			x	x		x		x	
row 6	x		x		x	x		x	do second to check

Database: MEDLINE

Author(s): Brandriff B ; Hinegardner RI ; Steinhardt R

Title: Development and life cycle of the parthenogenetically activated sea urchin embryo.

Source: J Exp Zool (JOURNAL OF EXPERIMENTAL ZOOLOGY) 1975 Apr; 192 (1): 13-24 Journal Code: I47

Abstract: A method is reported for inducing parthenogenetic development in eggs of the sea urchin Lytechinus pictus, a species which previously could not be artificially activated. NH4OH or the calcium ionophore A23187 are used as activating agents followed by hypertonic treatment. The ionophore is superior in activating large numbers of unfertilized eggs, whereas NH4OH produces a larger percent of embryos able to undergo gastrulation. Both feeding larvae and urchins arising from these artificially activated eggs are diploid. All individuals in which sex has been identified have been female. The viability of these completely homozygous organisms is low compared to their fertilized counterparts.

- Author(s): Kamata Y ; Mita M ; Fujiwara A ; Tojo T ; Takano J ; Ide A ; Yasumasu I
  Address: Department of Biology, School of Education, Waseda University, Tokyo, Japan.

Title: Probable participation of phospholipase A2 reaction in the process of fertilization-induced activation of sea urchin eggs.

Source: Dev Growth Differ (DEVELOPMENT GROWTH AND DIFFERENTIATION) 1997 Aug; 39 (4): 419-28 Journal

Abstract: In sea urchin eggs activated by sperm, A23187 or melittin, BPB (4-bromophenacyl bromide, a phospholipase A2 inhibitor) blocked fertilization envelope formation and transient CN(-)-insensitive respiration in a concentration-dependent manner. BPB had virtually no effect on the increase in [Ca2+]i (cytosolic Ca2+ level), the activity of phosphorylase a and the rate of protein synthesis, as well as acid production and augmentation of CN(-)-sensitive respiration. BPB also inhibited fertilization envelope formation and augmentation of CN(-)-insensitive respiration induced by melittin. Melittin, known to be an activator of phospholipase A2, induced the envelope formation, acid production, augmentation of CN(-)-insensitive and sensitive respiration, but did not cause any increase in [Ca2+]i, the phosphorylase a activity and the rate of protein synthesis. An activation of phospholipase A2 induced by Ca2+ or melittin seems to result in cortical vesicle discharge and production of fatty acids, which are to be utilized in CN(-)-insensitive lipid peroxidase reactions.

Activation of other examined cell functions in eggs activated by sperm or A23187, probably results from Ca(2+)-triggered sequential reactions other than Ca(2+)-caused activation of phospholipase A2.

Author(s): Elhai J ; Scandella CJ

Title: Arachidonic acid and other fatty acids inhibit secretion from sea urchin eggs.

Source: Exp Cell Res (EXPERIMENTAL CELL RESEARCH) 1983 Oct; 148 (1): 63-71 Journal Code: EPB

Abstract: Massive secretion at the egg surface follows fertilization of sea urchin eggs or parthenogenetic activation by the calcium ionophore A23187. The secretory products are used to construct the fertilization envelope around the egg. Arachidonic acid prevents the raising of the fertilization envelope induced by either sperm or A23187. We developed a secretion assay based on the ability of A23187 to raise fertilization envelopes from the surface of unfertilized eggs. Arachidonate delays the onset of this reaction in a dose-dependent fashion. 5 microM arachidonate produces a two-fold delay in the standard assay. In contrast, the propagation of secretion over the surface of the egg is unaffected at all concentrations that have been tested. Some closely related fatty acids (e.g. 11, 14, 17 C20:3 and linoleate, 9, 12 C18:2) share with arachidonate the ability to inhibit secretion, whereas others (e.g., 8, 11, 14 C20:3 and linolenate, 9, 12, 15 C18:3) do not. The results are not easily reconciled with a cyclooxygenase- or a lipoxygenase-mediated action. Despite the sensitivity of this phenomenon to small changes in fatty acid structure, it is suggested that the fatty acids exert their effect by altering the structure or dynamics of the membrane lipid bilayer.

Database: MEDLINE

- Author(s): Chambers EL ; Hinkley RE

Title: Non-propagated cortical reactions induced by the divalent ionophore A23187 in eggs of the sea urchin, Lytechinus variegatus.

Source: Exp Cell Res (EXPERIMENTAL CELL RESEARCH) 1979 Dec; 124 (2): 441-6 Journal Code: EPB

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

PHOSPHOLIPASE D ASSAY

This assay will take advantage of the transphosphatidylation by the enzyme, taking choline off PC and putting the ethanol on it as a head group. The Ptd Eth can be detected on TLC. REFERENCES: D. Zoukhri, DA Dartt. 1995. Cholinergic activation of PLD in lacrimal gland acini is independent of PKC and calcium. Am. J Physiol 269 (3 pt1);c713-20.

Ella, KM, Dolan, JW, Meier, KE. 1995. Characterization of a regulated form of PLD in the yeast S. Cerevisiae. Biochem. J. 307;799-805.

SPRECIFIC EXPERIMENT

Change in phospholipases at fertilization of sea urchin eggs.

NEED: SEA URCHIN EGGS AND SPERM, If continuing from the previous fertilization lab start at the ***#4 below

SEA WATER, pH 8,

HOMOGENIZING MEDIUM:(100 mM KCl, 5 mM MgCl2, 1 mM ATP, 10 mM benzamidine, 25 mM tris Cl pH 9.6), (Need 50 ml)

ETHANOL,

DETERGENT- octylglucoside

PLD reaction mix: (one with and and one without 2% ethanol)

150 mM NaCl
25 mM HEPES pH 7
5 mM EDTA
1 MM EGTA
1 mM DTT
10 mM substrate such as PC sonified in 200uM octylglucoside (make 10x, 2 ml) (Triton-x inhibits enzyme, as does freezing)
ethanol or not

Each group needs 4 ml. (20 ml total of solution.)

1. shed sea urchin gametes into sea water by .6M KCl (in class had 15 ml eggs. treat with pH 5 3 min, adjust to pH 8 with tris, settle, then add new sea water so each group has 15 ml diluted eggs.) Take out 30 ml for unfert with and without Ca++.

2. fertilize the rest, (add .1 ml diluted sperm per 10 ml) check to see if membrane lift away, place on ice at a timed interval, like 5 min to stop development after

3. spin 15 ml in hand centrifuge to pellet eggs, measure volume (about 1.5 ml each),

****4.homogenize both unfert and fert batches using 10 ml homog medium, keep cold. spin 20,000 rpm 10 min to get the membrane pellet.

5. place each pellet (fert and unfert) in 2 ml PLD **reaction mixture** and homogenize. KEEP COLD.

REACTION:prepare in a small eppendorf tube:

40 ul resuspended pellet with phospholipases
2ul fluorescent PAF in detergent
2 ul ethanol (add last)
add additional things like 1 ul Ca++

This will make a fluorescent assay possible.

So have a control with only Plipase, PAF, place in tube,label well CONTROL. You should have one for the unfert, one for the fert.; place a control with Plipase, PAF, ethanol in a different tube and mark

ETHANOL, one for fert, one for unfert, and an experimental with Ca++, Plipase, PAF, ethanol in a different tube for both fert and unfert, mark Ca++. Now warm them up!

7. Incub in white incubator for 20-30 min at 35 degrees.

place 5 ul of sample on G60 silica gel plates (no Fluor marker)
Develop plate: chlor:meth:water/aCETIC ACID (45;45:10;1
PHOTO UNDER LONG WAVE UV WITH plate reader

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

scolpoletin 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 aliqouts 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, NH4. Ionophore chemilum is delayed. Inhibited by triazole.

Procedure for Labeling DNA using DAPI

Reference: Spector, D., Goldman, R. Leinwand, L. (1998). Cells-A Laboratory Manual: Subcellular Localization of Genes and Their Products. volume 3. p. 101.10

Labeling Fixed Cells

1. Prepare a stock solution at 10 mg/ml of **DAPI** (m.w. 350) in distilled H20, protect from light, and store at 4^0C. Prepare a 5000-fold dilution in PBS to be used for labeling.

2. Prepare a *fresh* 3.7% **formaldehyde** solution for fixation. Also prepare a 0.2% Triton x-100 solution of permeabilization.

3. Aspirate the cell medium.

4. Rinse cells three times with PBS (+).

5. Fix the cells for 10 minutes in 3.7% formaldehyde solution.

6. Aspirate and rinse the cells three times for five minutes each in TBS

7. Permeabilize the cells by immersion in 0.2% Triton X-100 for five minutes.

8. Aspirate and rinse three times for five minutes each in TBS.

9. Incubate the cells at room temperature for 1-5 minutes in the DAPI labeling solution.
   -Preparation for DAPI solution: 1ul in 5ml of distilled water
   -add 1ml to each tube and let sit for 1-5 minutes.

10. Aspirate off the labeling medium, rinse at least once in TBS and mount.
    -Observe under microscope using UV lighting.

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 aphipathic, 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 concnetration 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-glucopyranocide (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 blendor approach is not so good.

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

MEMBRANE LIPID ANALYSIS

- **Folsch extraction membranes**, whole eggs, isolated mitotic apparatus, cortical hulls, sperm, can all be extracted with chloroform:methanol
- **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.
- 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
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

???

- **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.

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

PROTOPLASMIC STREAMING IN PLANTS

Read about protoplasmic streaming before coming to class
http://www.cells.de/cellseng/medienarchiv/index/3_funk.htm
http://www.cells.de/cellseng/medienarchiv/archiv/bp1c1562d/1562_b11.htm
http://www.cells.de/cellseng/medienarchiv/archiv/bp1c1562d/1562_b19.htm

Work in groups of TWO. Organisms to be used are plants: TRADESCANTIA AND ELODEA which have been exposed to light for an hour before use place a **stamen of Tradescantia** (the male part of the flower) or a **leaf of Elodea** torn to expose some thinner layers on a slide and observe. See which has the

best streaming and then prepare 10 duplicates. Keep them wet with water. Observe all slides to make sure they are alive before doing any experiment. Keep them under a light, but don't get them hot or dry them out by having them too close.

Effect of vital stains:

immerse the Tradescantia stamen or Elodea leaves in 0.05% Vital stains by dropping some stain on the slide. Observe whether there is a difference in the staining by :

1. Methylene blue
2. Neutral red
3. Janus green b.

Are the particles stained so that you can see them moving? In the unstained control can you see any particles moving? Which ever one does the best, use it for the other experiments so that it is the easiest to see the particles moving.

Effect of amino acids on movement

effect of histidine 10 mg/ml in 0.05M tris buffer. Observe the movement after placing one drop on a drained preparation (use a piece of filter paper to drain off any soluions when it says replace fluid.) If there is an effect, add water until it goes back to normal.

Effect of temperature on movement

place one slide on ice and leave for 15 minutes before observing movement, then observe immediately and as it warms up. Effect of light on movement:

place one slide in your cabinet with foil around it (make sure you had water on the prep) and observe after 1 hr.

Effect of pH drain a prep and then add water buffered to pH 8.3 And observe movement. See if you can quantitate the rate. Drain the preparation and replace fluid with pH 5.8, Doing that twice to wash away the pH 8. Observe and quantitate, repeat at pH 7.

Effect of anesthetics add a drop of 2% methanol, 2% ethanol, other alcohols and observe movement.

Effect of cytoskeletal disruptors these agents are expensive, so take only what you need, or bring back what you don't use. Look up the effects of these drugs on cells before coming to lab.

Using

1. Cytochalasin in 4% DMSO. A control here must be 4%DMSO alone.

2. Colchicine 1uM

3. VINBLASTINE 1 mG/mL
4. EGTA .1mM
5. CaCl2 10uM add 3 drops
6. ATP 2 x 10 -3 M
7. caffeine

References: annual review plant physiology 25:309

How do you explain the effects of vital dyes, histidine, and cytoskeletal disruptors?
How are microfilaments or microtubules involved in the movement? What kinds of motors are present in plants to move cell particles?

Pollen Germination in Tradescantia , impatiens, jasmine, lily, pomegranate, California poppy, and protoplasmic streaming in hair cells of Tradescantia and leaves of Elodea.

These two experiments are done together with the idea of relating them in terms of mechanisms of protoplasmic movement.
Ref: Rapid germination of pollen in vitro. D.J. Schimpf. The American Biology Teacher 54:168-9. 1992.

Germination solution: 10 g sucrose, .01 g boric acid, .03g calcium nitrate in 100 ml distilled water. This is GS.

Dust the pollen powder over the bottom of a tiny disposable plastic petri dish (best results occur when there is oxygen, not under a coverslip.) Replace cover, and label. Add a few drops of one of the solutions and observe under 10x, and when germination starts, take out a sample and place on a slide with a coverslip so you can look at it with 40x. It is important to get Impatiens flowers with colored anthers at the center of the flower, not a green solid structure. If you want to study the difference between immature and mature pollen you can use the anthers from an unopened bud, and try to see what will make it germinate.

Some pollen germinates in 5-10 minutes, continues to lengthen for an hour. Others germinate after an hour. Be sure to write down the time you placed the pollen in solution. After seeing the germination in your control pollen, pick the flowers which worked the best, and continue the rest of the experiment, but also do Tradescantia since that will be used in the second half of the experiment.
CAN YOU MAKE AN HYPOTHESIS ABOUT WHAT CAUSES POLLEN GERMINATION?

Pollen lands many places, but the only place where it will do any good is on the stigma of the ovary. There a sugary secretion is sticky and holds it on the surface, and then after germination, the pollen grains digest their way through the tissues of the style, perhaps using the digestion products as an energy source,

until reaching the egg where the sperm nuclei move from the pollen tube to fertilize it in preparation for seed formation. NEXT: TEST YOUR HYPOTHESIS.

Prepare the control as above, in the germination solution. Also try some experimental dishes to get at the signaling mechanism and the outgrowth mechanism. The following solutions can be used:

1. GS without sucrose
2. GS without Calcium
3. GS without boric acid
4. Chelating solution: .35M sucrose, .001 M EDTA, HEPES pH 7.2 (EDTA BINDS CALCIUM SO IT IS NOT AVAILABLE)
5. calcium ionophore A23187 makes channels in the membrane so calcium can enter or leave the cell. 1mg/4ml GS.
6. GS with colchicine 25 uM (.o1 mg/ml)
7. GS with cytochalasin 2.4 mg/ 2ml GS + 4 drops DMSO
8. .3 M KCL (cells have high K+ inside and low outside, so the gradient across the membrane which usually determines membrane potential will be changed.)
9. 1M glycerol (osmotically like sea water)
10. distilled water
11. apple peel in water (ripe apple peel has ethylene)
12. ammonium chloride solution (alters cell pH) 10 mM (8mg/15 ml GS)
13. peroxide solution, a powerful oxidizing agent 500 uM
14. chlorox solution (dissolves some complex carbohydrates such as chitin, removing fly egg coats) use 1:1 with water, leave on only briefly, suck off or dilute with GS.

The following physical factors can also be tested: cold or hot temperatures; light of different colors by wrapping in different colors of cellophane; dark; lack of oxygen; bubble oxygen through solution; bubble carbon dioxide through.

Protoplasmic streaming.

The same samples of pollen tubes can be used for examination of vesicle transport in white Tradescantia, pigment granule motion in purple Tradescantia. The purple ones are easier to see, but I could see movement in all tubes with GS solution, and in hair cells of Tradescantia.

This takes 20x or better yet 40x magnification.

Cell movements usually have an actin filament-myosin motor combo as in amoeboid movement or tubulin microtubule-dynein or tubulin-kinesin motor combo as in pigment granule motion or vesicles in neurons. They have rather opposite optimal conditions. Actin-myosin interaction requires calcium release to start it and actin can be depolymerized by cytochalasin or cold. Tubulin-dynein works best at low calcium. Tubulin is depolymerized by colchicine and cold. Particles are moved toward the center of the cell by dynein, outward by kinesin. Both kinds of motion require ATP. If the outgrowth is due to osmotic factors, such as uptake of water, using different concentrations of sugar, glycerol, salt can test for those effects. Using the solutions listed on the previous page, do experiments to test which mechanism might be used

here. So all you need to do is take samples from the petri dishes and make slides to look for streaming in all the experimentals. Always look at your control first, so you know what to look for in the experimentals.

Discuss the results with your partners. The object of this exercise is not to have fun and see pollen grow, though we do allow it. The object is to study protoplasmic motion mechanisms. Gather careful data. Figure out how best to display it. Can you draw any conclusions? Write them in your summary report.

DESIGN OF ENDOCYTOSIS EXPERIMENT

EFFECTS OF DRUGS ON MEMBRANES, CYTOSKELETONS AND THEIR ACTIVITIES

ENDOCYTOSIS

Monensin: inhibits pinocytosis in rat embryo fibroblasts. Tested by horseradish peroxidase uptake, and anti-5'nuc-ase antibody uptake inhibition detected after 3 hr, .3 uM for ½ max inhib. Exchange of 5'nucleotidase between cyto organelles and membrane inhibit 90%. The density where enzymes found different after monensin. Galatosyl transferase and 5'nuc increased density from 1.128 to 1.148, and peroxidase taken up in vesicles changed from 1.194 to 1.160. Alters density of lysosomes and Golgi.

Effect probably due to ionophore effect on Golgi. Inhibits secretion as well. Wilcox, DK, Kitson, RP and CC Widnell. 1982 Inhibition of pinocytosis in rat embryo fibroblasts treated with monensin.Cell Biol 92:859-64

Schroeder,F. Altered phospholipid composition affects endocytosis in cultured LM fibroblasts. Biochim. Biophys. Acta 649:162-174.1981. the lipid content of membrane regulates amount of pinocytosis. Phagosome lipid content same as PM, as was ATPase and 5'nuc-ase. However, the quantity of PC is elevated in phagosomes when cells given choline.

ENDOCYTOSIS

PURPOSE: to see if endocytosis is tied to loss of ATPase, and if prevention of it prevents ATPase loss. We already have seen in our slides that ammonia treated eggs had dark color, never lightened, and ammonia or high pH is supposed to inhibit endocytosis. Also we can try to trigger all secretion granule exocytosis to see if that makes it go away, A23187 did. ATPase can be inhibited by ouabain 10 mM. For activation, A23187 50 ug/ml; ammonium chloride (NH_4) 10 mM in sea water, pH 9.0 (Epel, D., Steinhardt, RA, Humphreys, T, and D. Mazia. (1974) Dev. Biol. 40:245-55).

We can also use cytoskeleton disruptors to prevent endocytosis. Microtubules involved, so vinblastine 10 uM colchicine should prevent endocytosis since it disassembles MT. Cold should also prevent. Apply ATPase antibodies to outside of cell, fertilize, detect antibodies. First we need to see if any stick to outside, also could try a lawn of cortical granules. In my eggs when I lowered pH for jelly removal, the stain was dark inside egg, and butyric acid is one way to artificially activate eggs.

PROBLEMS

1. REMOVAL OF VM, HYALINE, FM

If endocytosis is to be done on UF, as well, must be removed before fert.

How to tell if really fertilized? Must keep sample to see if cleaved.

2. HOW LONG TO EXPOSE

Swanson et al, 1985 **lucifer yellow used 1mg/ml**, after 5 min punctate stain in cortex, after 1 hr, large vesicle or in live cells long strands. Detected in microscope, Tri-x film after 3.7% formaldehyde, rinsed, mount in glycerol. Used fluorescein filter set. Cell viability measured by trypan blue 0.1%. Peroxidase using method with H2O2 and diansidine.

Used sephadex G25 at pH 5 to measure bound LY, after triton-x100 8% of it bound to high molecular wt material. **PMA-phorbol ester inc pinocytosis 1.8x.**

LY not taken up at 0 degrees

3. DOES FIXATION CHANGE THE INTERNAL CONTENT THAT WAS TAKEN UP?

in Swanson et al fixed cells (see above) and did fluorescence

4. How to measure

Microscpoe- correct filters for **lucifer yellow**

Dot blot for peroxidase

dextran blue-microscope Exposure of FM free embryos to **Blue dextran at 2 mg/ml in sea water for 20 min to 1 hr**

sediment membranes, how quantitate- absorbance in microcuvette

Fluorescence spectrophotometer- use .35 ml lysed cells, bring to 1.5 ml Lee and Epel (1983) with **acridine orange.**

5. How use NH4 inhibition of endocytosis

add NH4Cl after fert Lee and Epel (1983) after 2 min, dye turned green, so pH changed- caused 40% efflux of dye

acetic acid (10 mM potassium acetate buffer) to pH 6.0 caused loss of dye in 0Na SW

Cells treated with **NH4Cl 30-40 mM for 30 mi**n then washed away, lowers pH to 6.5, inhibits coated pit pathway but not lucifer yellow. (Sandvig et al 1989)

6. Try ruthenium red, heparin to see effect . **Ruthenium red (0.2 mg/ml in sea water for 1 hr in the refrigerator)** used to label cell surfaces in embryos, after FM removal.

7. Victoria blu can be used to see cortical granules to see if egg fertilized, stain after fixed, so could do fluorescence of endocytosis, then add **victoria blue** to same cell to see if truly fertilized (Schroeder, TE, 1979.) **0.3% alcoholic VB**
8. ionophore A23187 used with 0 Na sea water by Lee and epel (1983) **Used 460mM choline Cl and 2.5 mM KHCO3** for artificial SW to substitute for Na.
9. Sardet,C. 1984 isolated unfert cortex has CG, acidic ves and elaborate ER, assoc with mito, yolk, acidicves. Plaques on PM where clathrin coats can assemble for endocytosis after fert. After fert, has coated pits, actin mesh with acid ves in it

REFERENCES:

Lee, HC and Epel,D. 1983 Changes in intracellular acidic compartments in sea urchin eggs after activation. Devel. Biol. 98:446-54.

Sandvig, K, Olsnes, S, Peterson, OW and Van Deurs, B. 1989 Control of coated pit function by cytoplasmic pH. Methods Cell Biol 32: 365-82.

Sardet,C. 1984 The ultrastructure of the sea urchin egg cortex isolated before and after fertilization. Dev Biol 105:196-210

Schroeder, TE 1979 Surface area change at fertilization: resorption of the mosaic membrane. Devel. Biol. 70:306-26.

Swanson, JA, Yirinec, BD and Silverstein, SC. 1985 Phorbol esters and horseradish peroxidase stimulate pinocytosis and redirect the flow of pinocytosed fluid in macrophages. J. Cell Biol. 100:851-9.

CILIARY MOVEMENT

We will observe the ciliary movement under various conditions, using frog mouth cilia, planaria and sea urchin blastula stage embryos. Sea urchin blastula can be prepared by the fertilization exercise and then culture overnight. Gastrula stage can also be used, and they require 2 days of culture.

Frog mouth cilia

pith and decapitate a cold frog. Prepare the roof of the mouth for observation by separating it from the lower jaw. Keep it moist with frog ringers solution. Cut a very tiny piece of aluminum foil to be carried by the ciliary beat (about 1mm.)

To test effect of temperature:placing it on ice, recording the surface temperature and the change in rate. Calculate the Q10. (REMEMBER TO USE RATE, NOT JUST DISTANCE OR TIME.)

PLANARIA place a planarian in a petri dish and set a lamp next to it. See which way the planarian moves. Set the lamp in a new position and see how it moves again. Do this until you develop a hypothesis about how it moves in relation to light. Now observe its movements in the same solutions used on the frog mouth. Have an equal amount of test drug and water that the planarian is in. Make sure that is enough for it to move. Is cordination of the ciliary movement affected?

Sea urchin embryos

place some embryos in 2% ficol

PREPARATION OF FISH MYOSIN, ACTIN POWDER, Ref.

Martone, CB, Busconi, L, Folco, BJ, Trucco, RE, Sanchez, JJ 1986 J. Food Sci. 51:1554-5.

- Materials needed: fish, cold distilled water, applicator sticks, thread, centrifuge bottles and tubes, cheesecloth,filter paper for buchner funnels, 50% glycerol,ice, solutions, meat grinder. Each group of students will need a pan of ice, some cold distilled water,

and 60 g of ground muscle. **soln A**: 0.01M Kcl, 20 mM tris buffer pH 7.4. **soln b**: 0.45M Kcl, 5mM mercapto-ethanol, 0.2M Mg acetate, 1mM EGTA, 20 mM tris pH6.8. **1 mM NaHCO3** for diluting myosin. **0.1 M ATP. Soln. C** 0.5 M KCl, 5 Mm mercaptoethanol , 20 mM tris pH 7.5. **soln D**: 0.8 M KCL, 5 mM mercaptoethanol, 0.2 M Mg acetate

SOLUTIONS FOR MUSCLE EXPERIMENT

SOLN A).01M KCl, .02 M tris pH 7.4
use 1M tris, 1.5 M KCl for one liter 20 ml tris, 6.7 ml Kcl, adjust pH

	MW	VOL	G/L	G/VOL
SOLN B:		500ML		
.45M KCl,	74.55		33.55	16.77
5 mM mercaptoethanol,	78.13		.39G	.2ML
.2M Mg acetate	214.46		42.9	21.45
.02m TRIS	121.1		2OML 1m	10ML
.001 M EGTA	380.4		.38G	.19G

WEIGH OUT ATP TO make a 50x sln to ADD RIGHT PRIOR TO USE:
MW 605.2 .1M= 60.5MG/ml OR 605 MG/10ML. Dissolve in tris .001M pH7. adjust

0.1 mM NaHCO3-	MW 84	8.4g/l	4.2 g/500ml

SOLN C: 100ml

0.5 M Kcl		37.28	mg/ml 3.7 g
5mM mercap			40ul/100
.o2M tris pH 7.5			2ml

SOLN D:	100ml		
0.8M Kcl		59.6g/l	5.96 g /100
5 mM mercap			40 ul
0.2 M Mg acetate			4.29 g/100

0.2M Nahco3 100ml 1.68g/100

-

- The fish will be numbed in ice, killed and skinned and the muscle removed from the skeleton and placed on ice except for the long muscle in the back which will be used for glycerinated fibers.
- 1.Each group of 3 students should weigh out 30-50 g of muscle ground in a meat grinder into a beaker.
- 2.Extract this muscle on ice in 10 vol of soln A: 0.01M Kcl, 20 mM tris buffer pH 7.4. Place in a beaker and keep on ice for 1/2 hr, This will wash away contaminant proteins, etc. Pour through cheesecloth and throw away the super (unless the muscle disintegrates so not caught on cheescloth in which case you must centrifuge), keep muscle in cheesecloth.
- 3. Place in a beaker (cheesecloth and all) with 5 vol soln b: 0.45M Kcl, 5mM mercapto-ethanol, 0.2M Mg acetate, 1mM EGTA, 20 mM tris pH6.8. Add ATP to 10mM. Incubate on ice for 1 hr. Stir

periodically and get muscle off the cheesecloth. (Ask yourself why we are using ATP and this pH, and EGTA.)

- 4. spin down pellet in sorval centrifuge for 15 min at 10,000 g. There are 8 places in the rotor, make sure you balance your tubes on the scale and put them opposite each other, and wait until the rotor is full before running. Save pellet for actin extraction (Step 6), keep pink supernate for myosin purification (sTEP 5.
- 5. Dilute all of the supernate from #4 with cold distilled water 1/25 (or 1 part plus 24 parts 1 mM NaHCO3 .) Stand on ice 1/4 hr. If you can see the myosin precipitating, suction off the top part which is just cell sol so that there will not be so much to centrifuge. Precipitation cannot occur unless this mixture is at pH 6.5, So measure the pH if you don't see any precipitate and adjust with 1/20 concentrated acetic acid, making sure you stir well while adding the acid for the adjustment.
- 5. Spin in large bottles in the centrifuge, save the pellet. Dissolve the pellet in 5 cc Soln C;0.5 M KCl, 5 Mm mercaptoethanol , 20 mM tris pH 7.5 by homogenizing in Potter-Elvehjem. Centrifuge at next lab start to remove insoluble material. Further precipitations by dilution and resuspension would further purify it.
- 6.<u>ACTIN PREPARATION</u> reextract the pellet from 4 with 5 volumes of soln D: 0.8 M KCL, 5 mM mercaptoethanol, 0.2 M Mg acetate; 5 min on ice. Centrif 10,000 g for 15 min. Discard super (has more myosin in it)
- reextract pellet with 5 vol of pellet wt of 2mM NaHCO3 in a beaker for 1 hr on ice, stir occasionally , centrifuge 48,000g 1 hr discard pellet.

SUPERPRECIPITATION OF ACTOMYOSIN

We will do light scattering at 540 nm to determine the presence of ppt. To a cuvette containing 0.5 cc of actomyosin add the following in two steps for each set, recording the light scattering change with each.

	H20	KCl`
1.	4.5 ML	0
2	2.5	2.5
3.	0	4.5

WHAT IS THE EFFECT OF SALT CONCENTRATION ON SUPERPRECIPITATION? TO LOOK AT THE EFFECT OF CA AND ATP AND MG, SET UP TUBES TO WHICH YOU ADD THE FOLLOWING:

	TRIS	CA (.01 M)	MG(.01 M)	MG (.1)	CA (.1)	H20	EDTA
1.	1 ML	.5 ML				3	
2.	1		.5 ML			3	
3.	1			.5		3	
4.	1				.5 ML	3	
5.	1	.5				2.5	.5

THEN ADD .5 ML ACTOMYOSIN TO ALL OF THEM AND MIX WITH A PASTEUR PIPETTE AND READ AT 540 NM. THEN ADD A DROP OF ATP TO EACH, MIX AND READ AFTER MIXING. THIS SHOULD BE DONE ON ICE THEN WIPE THE TUBE WITH A WIPETTE JUST PRIOR TO READING. IF YOU LET THE PELLET ALL FALL TO THE BOTTOM OF THE TUBE IT WON'T BE READ, SO THE MIXING IS IMPORTANT. INVERT IF ALL ELSE FAILS. FILL OUT THE ANSWER SHEET. Set these tubes in the refrigerator and look at them next time to measure the volume of the pellet.

ATPASE ACTIVITY DETERMINATION FOR MYOSIN OR ACTOMYOSIN:

1. dilute all myosin to 5 mg/ml by running a lowry protein test to determine the concentration, and then diluting it. If it is less concentrated than that, just note your concentration and use it undiluted.
2. Into a huge test tube prepare the reaction mixture (total vol 25 ml):

- 2.5 ml 1 M NaCl (final conc after dil=.1M)
- 2.5 ml 0.2 M TRIS PH 7.5 (final=.02M)
- 1.25 ml 0.1 M CaCl2 (Final=5 mM)
- ul mercaptoethanol (final 5 mM)
- 13.75 ml H2O
- 5ml myosin
- set this in 25 degree bath for 5 minutes to bring to temperature.
- In a separate test tube rack, prepare tubes marked 1-6., add 2 ml of 5% TCA to each tube. You will pipette 2 ml samples from the reaction mix into these tubes at different time intervals. These must be ready before the next step.
- Add 2.5 ml ATP to the reaction mixture (not the TCA tubes)and mix well and note time or press stop watch

4. Pipette out your first 2 ml sample as soon as you can to serve as a 0 time blank, but since it will not be zero time, record the time as you pipette it into the TCA. Take four other samples spaced over 30 minutes pipetting each into the TCA and recording the exact time. (It isn't important to get it done exactly at 5 min but it is important to know exactly the number of min and sec passed since adding the ATP to the myosin.)
5. Filter the TCA mixtures into clean tubes.
6. pipette 2 ml of the filtered TCA reaction mix into tubes, marked so you can tell which is which.
7. Add 1.6 ml sodium molybdate reagent, mix.
8. Add .4 ml stannous chloride diluted reagent, mix immediately, wait 2 min and read 1t 600 nm.
9. Go to your P std curve and look for the OD of each preparation, read off the concentration of P in ug/ml and then make a plot of P conc versus time elapsed in min. It should be a straight line, with the slope of the line being the enzyme activity in ug/ml/min. Usually enzyme activity is expressed as ug p/mg protein/min. Our activity is the slope of our line, divided by the final concentration in mg/ml after diluting it 1/10 by adding 2.5 ml to 25 ml reaction mix.

The effects of actin, calcium, ionic strength, pH, magnesium, and temperature can be studied on myosin ATPase. You can try assays without each of the above chemicals , or with half the amount, and the physical parameters can also be changed (room temp, pH 8 OR 5). Ionic strength can be changed by adding more or less NaCl. KCl causes precipitation of the Pi reagents, so don't use it. I want you to try the regular assay above and the effect of at least one of these bottom alterations. Do something different from the other groups around you so that you can all compare results. The slope of your plot for the assay under different conditions should be different. Put them all on the same graph but label them clearly. You should observe the reaction mix after you add your myosin (between the mad rush to pipette) to see if the myosin is soluble (clear solution) or precipitating (cloudy). See if it seems to work

best or worst when just starting to ppt. However, this means you must stir it before you pipette, so you don't draw off all the myosin in the first sample for the blank, or any subsequent sample.

Lowry protein determinations: try three samples: (remember always do duplicates and don't forget the water blank) full strength, 1/5, 1/10 on each preparation. That means you will use .5 cc prep full strength, .1 cc prep+.4cc water, and 0.05cc prep+ .45cc water. (Use a water blank). Record your results on the answer sheet using your standard curve from before to read the amount of protein present. Make sure you take into account the dilutions. How do your duplicates compare? How do your different dilutions compare in the answer they give you for the original protein content? If you don't get good results, do it over.

ACTIN POLYMERIZATION (see separate writeup in this manual) two of you can do the actin polymerization experiment, since it becomes more difficult to polymerize as it ages. Use the viscometry setup. Use your actin full strength. Do not dilute. When salt is added to give a concentration of 0.2 M KCl, F actin forms by polymerization.

DETECTION OF PHOSPHOPROTEINS

TRY TO SEPARATE PHOSPHORYLATED PROTEINS FROM NON

REF:DULCLOS, B., MARCANDIER, S., COZZONE, AJ. CHEMICAL PROPERTIES AND SEPARATION OF PHOSPHOAMININO ACIDS BY THIN-LAYER CHROMATOGRAPHY AND/OR ELECTROPHORESIS. Meth. Enzymol:201: 10-21.1991
Vacquier, V. and GW Moy Microchemical determination of phosphate in proteins isolated from polyacrylamide gels. same vol p 261-264.

O-phosphates on serine-threonine, tyrosine
N-phosphates arginine-histidine, lysine
acyl-phos aspartic and glutamic

Acid precipitation usually used and this destroys last two types, so only o-type can be done.
WHAT TO DO WITH THE SUPERNATE FROM CENTRIFUGATION to separate membranes.
From Vacquier- make homog 10% in TCA with powder w/v in cold, spin 10,000g 10min, wash in 90% v/v acetone, spin 3000g 10min. can dissolve in 10% w/v SDS for electrophoresis, but for ours
USE THE meth in 1st ref: hydrolysis of protein for 2 hr in 6N HCl at 110 degrees (shorter time get more P-Tyr, longer more PThr)

Get TLC plastic sheet cellulose plates 0.1 mm thickness, 20x20 cm Merck without fluorescent indicator. Spot

ascending chromatog in solvent A 10-12 hr or B 7-9 hr. Stain with ninhydrin prepared by mix 1 vol 0.33% ninhydrin in tert-butanol with 1 vol acetic acid-pyridine-water (1:5:5, v,v,v).

Do 2d- solvent a first, then b in second dimension

solvent a:5vol isobutyric acid-3vol 0.5M NH4OH
solvent B :7vol 2-propanol-1.5volHCL-1.5 vol H2O

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 uG/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 Cam 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 $CaCl_2$ 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 $MgCl_2$ pH 6.4.

REFERENCES: Mazia et al. The direct isolation of the mitotic apparatus. J. Biophys biochem cytol. 10: 467

ACTIN POLYMERIZATION AS DETECTED BY DNAase INHIBITION

References: Cell 15:935
Stock solutions: 1. DNAase I

Equipment: quartz cuvettes, micropipettes

Purpose: to make a standard curve of inhibition of DNAase by G

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

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

Step 3. Now prepare some Ft quanidine from the value in the test with guanidine, and that is the amount of F

VISCOSITY MEASUREMENTS OF ACTIN

POLYMERIZATION TO F ACTIN From the protein readings you took on the actin, figure out the mg/ml.
If it is cloudy or has particles in it, it must be centrifuged or it will clog the viscometer..
If you have 5 mg/ml, for the experiments below, you would take 5 ml and add tris KCl and ATP 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. CHECK 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 2e? (Remember in trying to explain the results that there is

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

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	VOLUME	FINAL CONC
MgCl2	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	amount TO BE DETERMINED BY BIORAD USING MATERIAL LEFTOVER
WATER		3.55 mL	
TOTAL		9.0 mL	

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

TAKE OUT SAMPLES AS FOR MYOSIN ATPASE AND DETERMINE THE Pi 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 Pi 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++.