Rhos PRNC - 91-A Rarioed done 1870 fuente PUERTO RICO NUCLEAR CENTER MEDICAL SCIENCES AND RADIOBIOLOGY DIVISION COURSE IN TISSUE CULTURE AND RADIOISOTOPE TECHNIQUES AT CELLULAR AND SUBCELLULAR LEVEL PROGRAM AND LABORATORY EXERCISE MANUAL June 18, daily 3 1970 ‘OPERATED BY UNIVERSITY OF PUERTO RICO UNDER CONTRACT No, AT (W8-1I-IEBD FOR U. & ATOMIC ENERGY COMMISSION ---Page Break--- PRNC - 91-0 COURSE IN TISSUE CULTURE AND RADIOISOTOPE TECHNIQUES AT CELLULAR AND SUBCELLULAR LEVEL PROGRAM AND LAB ---Page Break--- -_— ee OS Oe oho ee oe oe oe: oe: ee ee ee ee ee oe ee oe i ---Page Break--- -ae fur CURE eceRER, Introduction to Tissue Culture Techniques (June 15 to 29, 1970) Introductory Hem ~ History of Cell Culture Laboratory Preparation of Media, Sterilization and Sterility Tests, Staining of Cells, and 10:00 am. Preparation of Constituents Wednesday 17 to Thursday 28 Heereine is Frahepors of Cell Cultures _ Dr. Jorge Chiriboga, Director Medical Sciences and Radiobiology Division Dr. Re Hortfns Dr. Re Martfer-silva Dr. Re Martfnensitve Dr. Re Hinrtfaectivn Dr. Ry Kertines Dr. Re Martinen-Stva, Dr. Re itevtfnes- have Dr. R. Mertinex-Siva Dr. Re Martfaes Aiden ---Page Break--- a ae lS lS al ee. eee ae ae ee ee ae eee Tuesday 23 Wednesday 25 Thursday 26 1s373 cuysuRs cou June 15 to July 3, 1970 Use of Techniques at Cell Culture and Lecture labeling of Polysomes. Laboratory "OH Lecture Muscle Cell and Protein Synthesis 1 |. Laboratory Involving of Polyscose (cont) Lecture Metabolite Analysis in Cells Use of Techniques 10:00 am to 1:00 pm, Laboratory Metabolic pathways in Cells Use of Techniques 9:00 am to Lecture — Analytical Methods 10:00 am and Molecular Biology 10:00 am to Laboratory — Continuation 1:00 pm. Dr. Dr. Dr. Dr. Dr. Dr. Dr. Dr. Dr. Raymond Ay Brow Jorge Chiriboga Raymond

A, Tesvon-Aae Roge Roger Romo#-ALin Foxge Chirivoe Roper Raomh Lice Raymond As Bs Taymod A. Broun Roger Hanoe-A4 ---Page Break--- SS a eae eee rr eS Oe iim lee eee ee ee ee 3 ‘Tissue CULTURE Cour (June 29 to July 2, 1970) Vol. 29 9:00 to Lecture 39:00 am, the Balance of Virology 46 10:00 am to 1:00 pm, Measuring Virus Multiplication by Cytopathic Effect Laboratory Be 9:00 to Lecture 10:00 am. Methods for Detecting Virus Multiplication in Tissue culture 10:00 a.m. Laboratory exercise £9 Fleqie method for Taylors of Cell Lecture Methods for Detecting Multiplication in culture 10:00 a.m. Laboratory Bueroine 3.19 to Neutralizing 4 2:00 pm, An Cold Cultures ‘maveasy to Lecture 10:00 a.m. ‘Application of Tissue Culture in Virus Isolation and Vaccine Production Laboratory: validation and interpretation of Previous Experiments Application of Tissue Culture Techniques to Virology. Dr, Julio I. be. Julio Ts be, dviso I. Dr, dulso Te De. Julio Te be. Julio 1 Dr. Julio T. Dr. Julio I. coin coro conéa coro corn corén corn coréo ---Page Break--- LABORATORY REPORT #1 PREPARATION OF MATERIAL. STERILIZATION AND STERILITY TESTS. STANDARDS OF CELLS orsoctive ‘ALL components in a cell or organ culture system must be free of contaminant microorganisms. Routine testing Procedures should be carried out in order to rule out the Presence of bacteria, fungi, and mycoplasma. Materials: ‘Two tubes of 1 cell to these of tela cells SMe these of ne 2 cots Bie tubes of thioglycolate medium ‘Six tubes of Sabourand agar. Five tubes of PPL0 bot Twelve tests of PO agar tenes" stain Procedure: Observe under the microscope and describe the different cell types. 2 Tests for sterility 2:1 Inoculate 0.2 ml of each cell culture tube into 10 x1 of thioglycolate medium. Incubate at 37°C. Read and record results every 24 hours. If negative, discard after 5 days. 2:2 Streak a loopful of each cell culture on Sabourand Agar. Incubate at room temperature, Read and record results during a week, at 24 hour intervals, ---Page Break--- ach Yahoratony Benetse #1 With @

pipette deposit some drops of each cell culture “media” on Pio broth and agar (Bife0). streak the agar with a bacteriological loop, invert the plate and incubate at 37° for seven days. Incubate the

broth at the same temperature for four days and after this period, place some drops on a PPLO agar plate, spreading with a bacteriological loop. Without removing the cover of the plates, inoculated the 1st and 4th days, look for colonies of PPLO under the microscope stage focusing through the agar. Use 40x objective and 10, 12.5 or 15x ocular. Most PPLO colonies are round, with a dense center and a less dense periphery, giving the appearance of a fried egg. PPLO colonies have been isolated from diverse cultures, however, they do not conform strictly to this appearance on primary isolation. They may appear to lack a distinct periphery and appear to be totally embedded in the agar. These colonies are usually very small and look "granular" or "leathery". PPLO colonies vary from 10 to 500 microns in diameter (0.02 to 0.5 mm) and characteristically the center only, or all of the colony, is embedded in the agar. Individual organisms cannot be resolved since they are the size of an average virus particle. Occasionally, at the periphery of PPLO colonies, "large bodies" characteristic of this group of organisms are found. After locating the colonies, ---Page Break--- mBReeEe ee eS eS es as a2 eeCUlcer EY OE eeOUDT,llOllU Inboratory Frereise #1 they are usually marked out on the petri dish with a glass “parking pen”. Confirmation of PPLO colonies depends, in addition to morphological characters, on: 1. Inability to remove the embedded portion of the colony from the agar surface by stroking the colony with a loop. This demonstrates the fact that the colony is embedded. Bacterial colonies will rub off. 2. The non-reversion to bacteria which subsequent passages of the colonies will reveal. Reversion to bacterial form would be typical of L form. 3. A requirement for native protein. 4. Reaction with the Dienes stain. The Dienes

Stain is prepared by dissolving 2.5 g of methylene blue, 1.25 g of azur IT, 10.0 g of maltose, and 0.25 g of sodium carbonate in 100 ml of distilled water. With a cotton swab moistened in the stain, stroke the area of an agar plate just adjacent to the suspected colony. The stain will diffuse to the colony which is then examined under the microscope as described above. The FPLO colonies stand out distinctly with densely blue staining centers and light blue peripheries. Bacterial colonies are also stained but these are decolorized in about 30 minutes. The FPLO colonies never decolorize the stain. Staining of colonies by Giemsa method. Remove the growth medium of the tubes provided for sterility testing. ---Page Break--- Laboratory Exercise #1 2. Wash twice with Hank's solution. 3. Add methyl alcohol covering the surface of the slide. After 5 minutes, the cellular sheet will be fixed. Remove the alcohol and add 1 ml of Giemsa stain (make a fresh stain by diluting 1 drop into 1 ml of distilled water). 5. After staining for 30 minutes, wash with tap water. 6. Remove the slide; let dry; mount following the instructions. ---Page Break--- 8. LABORATORY EXERCISE #2 PREPARATION OF TISSUE CULTURE CONSTITUENTS Objective: To obtain the components which will provide the cell "in vitro" with the organic and inorganic substances necessary for its nutrition, and will protect it against changes in physical state, temperature, pH, etc. Materials: 7 4 cm thick embryo scissors syringe 50 ml. Centrifuge tubes graduated cylinder antibiotics (penicillin and streptomycin) 11, HANKS BALANCED SALT SOLUTION (RBBS) A. 10 X Solution: Wait A. NaCl; 3.5 g. Dissolve in 250 ml of distilled water. Dispense in a convenient bottle (50 ml screw-cap prescription bottle) and autoclave at 121°C for 15 minutes. Unit #2: 60.0 g. NaCl, 1.0 g. KCl, 2.0 g. $NaHCO_3$, 28.0 g. glucose, 0.6 g. sodium phosphate, 30.0 g. sodium sulfate, 0.6 g. potassium phosphate. Dissolve in 800 ml of distilled water. ---Page Break--- Laboratory Exercise #2 Unit #3: 0.0 g. Dissolve in 100 ml of distilled water. Unit # Phenol Red O.M.

ame Nix Phenol Red in a small amount of water until a paste, dilute to 150 ml. with distilled water, titrate to pH 7 with 1/20 acetic acid. Make up to final volume of 200 ml. Preserve with 1-2 ml. Chloroform. Add 100 ml. of unit #1 to unit #2 and then add unit #3 to make 1,000 ml. Pour solution into a glass stoppered bottle and add 3-4 ml. chloroform as a preservative. The solution may be

kept at room temperature for 6 months to 1 year. NOTE: Minimize transfer of chloroform in the preparation of the working solution. Be certain that bottle caps are loosened during autoclaving to ensure that all chloroform is driven off. The working solution is prepared by diluting 10X Stock 1:10 with distilled water. Dispense in convenient size screw cap bottles and autoclave at 120°C for 15 minutes. Aseptically add 2.5 ml. of sterile sodium bicarbonate solution (Unit #1) to each 100 ml. of BSS. The pH may be adjusted with CO2. The balanced salt solution is now ready for use. Do not tighten caps until pH of BSS is 7.4.

NUTRIENT MEDIA
Eagle's minimum essential medium contains higher concentrations of amino acids than the basal medium first described by Eagle. The medium may be prepared with Hank's BSS. The medium is prepared. At the time of use, redistilled water (20°C) and NaHCO3 are added to the 1X solution.

Solution Per Liter
10X solution:
L-Arginine, 1.05 gm
L-Cystine, 0.33 gm
L-ysine, 0.58 gm
Tryptophan, 0.20 gm
L-Phenylalanine, 0.32 gm
L-Threonine, 0.48 gm
L-Serine, 0.52 gm
L-Valine, 0.06 gm
L-Isoleucine, 0.52 gm
L-Methionine, 0.15 gm

Solution B:
Tyrosine, 0.32 gm
Acetylcysteine, 0.24 gm
These amino acids are dissolved in 200 ml. of 0.075 M HCl with gentle heating (60°C).

Solution C:
Myo-inositol, 200 mg
Pyridoxal, 200 mg
Thiamine, 200 mg
Pantothenic Acid, 200 mg
Choline, 200 mg
Sodium, 400 mg
Riboflavin, 20 mg

Laboratory Exercise
Components are dissolved in approximately 175 ml. of double distilled water and then brought to a final volume of 200 ml. with double distilled water.

The solution is dispensed in 10 mL amounts and stored at -20°C. 10 mL of Solution C are added to each liter of 10X media. Solution B: 200 mg of Biotin are dissolved in 150 mL of double-distilled water. To increase stability during storage, 1.0 mL of 1.0 HCl is added. The total volume is brought to 200 mL with double-distilled water and the solution is dispensed in 10 mL amounts and stored at

-20°C. 10 mL of Solution D are added to each liter of 20X media. Solution: 200 mg of folic acid (crystalline) are dissolved in 200 mL of 1X Hanks' BES pH 7.8. The solution is dispensed in 20 mL amounts and stored at -20°C. 10 mL of Solution Y are added to each liter of 10X media. Glutamine Solution 36 - (To be added at the time of use): 12 g of L-Glutamine are dissolved in 400 mL of double-distilled water and sterilized by filtration through a filter-type pad. The solution is stored at -20°C and 1.0 mL is added to each 100 mL of 1X Bagle's medium. Preparation of the final mixture is BSS. The following are dissolved in solution B: Wac 80.0 g, KCl 4.0 g, NaCl 2.0 g. ---Page Break--- a2 Laboratory Exercise #2 B. The following are dissolved in 50 mL double-distilled water and added to the pool: aHPO4 12.0 g, WLP 0.60 g. 20 grams of Glucose are dissolved in 50 mL of double-distilled water with 20 mL of 141 Phenol Red solution and added to the pool. 4. The volume of the pool is brought to 600 mL with double-distilled water and the following solutions are added: Per 1.0 Liter 10X medium: Solution C 10.0 mL, Solution D 10.0 mL, Solution F 20.0 mL. In 8 separate flasks containing 160 mL double-distilled water, 2.0 g anhydrous CaCl2 are dissolved and added to the pool slowly with vigorous shaking. 6. The amino acids of Solution A are added to the pool and the volume is brought to approximately 950 mL with double-distilled water. 7. A solution containing 20,000 units of Penicillin and 20,000 micrograms of Streptomycin per mL is added in a volume of 5.0 mL per liter and the mixture is held in the refrigerator.

overnight. The total volume is brought to exactly 2,000 ml with double distilled water and the solution is sterilized through a Seitz type pad. For use, the solution is diluted to 1X with sterile double distilled water and 24 of the 3% Glutamine Solution and 1.25 to 2.5% of a 2.04 NaCl are added. ---Page Break--- Laboratory Exercise #2 D. The following are dissolved in 50 ml double distilled water and added to the pool: Na2HPO4 12.90 g (101%), 0.60 g. 20 grams of Glucose are dissolved in 50 ml of double distilled water with 20 µl of 1% Phenol Red solution and added to the pool. 4. The volume of the pool is brought to 600 ml with double distilled water and the following solutions are added: Per 1.0 liter 10X medium Solution A 10.0 ml solution D 10.0 ml solution E 10.0 ml. 6. In a separate flask containing 160 ml double distilled water, 2.0 gms anhydrous CaCl2 are dissolved and added to the pool slowly with vigorous shaking. 5. The amino acids of Solution A are added to the pool and the volume is brought to approximately 950 ml with double distilled water. 6. A solution containing 20,000 units of Penicillin and 20,000 micrograms of Streptomycin per ml is added in a volume of 5.0 ml or liter and the mixture is held in the refrigerator overnight. Then, the total volume is brought to exactly 2,000 ml with double distilled water and the solution is sterilized through a Seitz type pad. 4. For use, the solution is diluted to 1X with sterile double distilled water and 1% of the 3% Glutamine Solution and 1.25 to 2.5% of a 2.84 NaCl are added. ---Page Break--- Laboratory Exercise #2 For laboratories occasionally using small amounts of Eagle's medium, it is recommended that the 1X medium be prepared by diluting a 10X Stock Solution of the amino acids and the 20K stock solution of the vitamins (stored at -20°C) appropriately in Eagle or Hanks’ 299 adding Glutamine, antibiotics, and NaHCO3 as indicated above. This prevents deterioration of the vitamins during long-term storage at icebox temperature. 3.

CELL DISPERSING AGENTS A, Hanks' Solution 1.0% 2 g of powdered trypsin is dissolved in 100 ml of phosphate buffer saline and the solution is passed through ash-free filter paper (Schleicher and Schull #589). The solution is then sterilized by filtration through a diatomaceous earth pad and stored at -20°C. B, Versene Solution (Ethylenediamine tetraacetic acid) 8.0 ml = HPO, 0.2 ml KCl, 0.2 ml TiangPO, 0.35 ml Versene 0.20 g. Dissolve in 1,000 ml of distilled water. Dispense in convenient amounts and sterilize by autoclaving at 120°C for 15 minutes. 4, ANTIBIOTICS SOLUTION Penicillin (20,000 units per ml) and Streptomycin (20,000 micrograms per ml) 1. Add 10 ml of Hanks' solution to 1,000,000 units of Penicillin, 2. Add 210 ml of Hanks' solution to 1 vial with

a gram of Streptomycin. ---Page Break--- Laboratory Exercise #2 3. Mix the contents of both vials and add up to 50 ml of Hanks' solution. 1. Dispense into vials and keep at -20°C. 5. CHICK EMBRYO EXTRACT 50% Each student will be provided with 5 embryos 9-10 days of age. A. Harvest the embryos and place them in a sterile Petri dish where the eyes, beak, legs and wings are removed. B. The remaining tissues are washed in a beaker containing Hanks' BSS, then minced with uterine scissors. C. The minced tissue is passed through a 50 ml syringe into a graduated cylinder or centrifuge tube. D. An equal volume of Hanks' BSS is added to the tissue culture and the mixture is stirred and allowed to stand for 30 minutes. E. The suspension is centrifuged at 1,500 rpm for 20 minutes and the supernatant fluid (constituting the 50% extract) is removed and stored at -20°C. F. After thawing for use, the extract is clarified by centrifugation at 2,000 rpm for 10 minutes. ---Page Break--- Laboratory Exercise #3 MONOLAYER CULTURES OF CHICK EMBRYO CELLS consist of standard culture media that have been optimized for the propagation and development of chicken embryo fibroblasts from fertilized eggs. Material, i 4

enick embryos Petes dimes Beaker S| scissors Forceps syringe Erlenmeyer flask magnetic stirrer Centrifuge tubes Procedure 1. Chick embryos 9 days old are harvested and placed in a sterile Petri dish where eyes, beaks, legs, and wings are removed and discarded. 2. The embryos are transferred to a beaker containing Hanks’ BSS and washed in 3 changes of the solution. 3. The embryos are minced into pieces approximately 3 mm in diameter, with uterine scissors, and the minced tissue is washed with 3 changes of Hanks’ BSS. 4. The minced tissue is passed through a 50 ml. syringe (without needle) into a 500 ml. Erlenmeyer flask, where it is washed twice with 90-100 ml. of Hanks' BSS. ---Page Break--- Laboratory Exercise #3 5. After the fluid from the second washing has been removed, an appropriate volume of 0.25% solution of trypsin in Hanks' BSS is added. The volume of trypsin solution employed is determined by the number of embryos being processed (5-20 embryos, 200 ml., 21-30 embryos, 300 ml.) 6. A magnetic stirring bar is added to the flask which is placed on the magnetic stirrer and the suspension agitated for 1 hour at room temperature. After one hour of trypsinization, the flask is slanted to sediment large tissue particles and the supernatant fluid (cell suspension) is decanted through a stainless steel wire cloth (72 mesh wire diameter 0.0037 inches) into centrifuge tubes. 8. The cell suspension is centrifuged horizontally at 600 rpm for 20 minutes and the supernatant fluid containing trypsin is aspirated immediately. The cells are washed once by resuspending in Hanks' BSS, centrifuging at 600 x g for 10 minutes, and aspirating the supernatant fluid. 9. The cells are then resuspended in 15 to 30 ml. of Hanks’ BSS, transferred to 15 ml. or 30 ml. graduated, conical centrifuge tubes and centrifuged horizontally at 600 x g for 10 minutes. 10. The volume of packed cells is noted and after removal of the supernatant fluid, the cells are diluted in the following medium: Porcine serum 2.0 ml. Lactalbumin hydrolysate in

physiological saline 5.0 ml, ---Page Break--- an. ae Laboratory Exercise #3 50% Chick Embryo Extract 4.0 ml. Hank's BSS 0.5 ml. 2.84 WalGO, 2.5 ml Penicillin-streptomycin solution 1.0 ml. For tube culture the cells were diluted 1 to 200 and dispensed in ml volumes. For plating in stoppered bottles, the cells were also diluted 1 to 200 and dispensed in ml volumes into three ounces prescription bottles. For plating in 60 mm Petri dishes 5 ml of 1:200 dilution of the cells were added and incubation is conducted in a 95% atmosphere. After incubation at 37°C for 1 to 2 days, complete monolayers of cells are formed and the cultures are ready for inoculation with viruses or clinical material. ---Page Break--- LABORATORY EXERCISE 3-1 TECHNIQUES FOR GROWING CELL LINES IN TISSUE CULTURE The experiment is designed to maintain a cell line (e.g., 2 or 1) for the duration of the course. Each student will be provided with 1 bottle of cells. Materials: 1 bottle with a confluent cell monolayer Hank's Solution trypan blue 14 tubes Procedure: 1. Observe the

cells under the microscope and describe them. 2. Remove medium with a pipette. Wash 2 times with Hank's BSS. Add 10 ml of 0.25 percent trypsin solution, allowing the trypsin to cover the cells for exactly one minute at room temperature. Remove all of the trypsin and place the tubes in a 37°C incubator for 10 minutes. At the end of this time the cells should be almost completely detached from the wall of the bottle. Add 10 ml of fresh medium and suspend the cells homogeneously with a pipette. Count the cells in the hemocytometer. With a Pasteur pipette carefully express a drop of the cell suspension made up of 0.5 ml cells plus 1.0 ml of trypan blue under the hemocytometer coverslip, avoiding any overflow into the moat. ---Page Break--- Laboratory Exercise 3-2. Determine the average number of viable cells (dead cells stained blue) in the 4 large corner squares used for counting white blood cells, multiply by 10,000 the number of viable cells to

obtain the number of cells per ml. Adjust to 50,000 cells/ml. using Eagle's medium, transfer 50,000 cells into each of 1 test tube. Stopper tubes with rubber stoppers. Incubate at 37°C in a horizontal plane. Keep record and observe cells every day. ---Page Break--- LABORATORY EXERCISE 45 CONSERVATION AND TRANSFER OF CELL CULTURES Objectives: To maintain in the laboratory with a minimum of handling viable cell lines not in continuous use. Materials: Cell culture Haemacytometer, Trypan blue solution. A. Storage 1. Obtain a bottle with a culture of 5-day-old cells from which the outgrowth medium has been removed and replaced with 10 ml. of fresh medium, consisting of 10 percent horse serum and 90 percent casein hydrolysate yeast extract medium. After a 2-day incubation period, the medium is removed, the cells trypsinized and counted. A bottle should yield between 10 and 20 x 10^8 cells, otherwise the cells are not suitable for storage. 2. The pH of the medium is adjusted to 7.4 by means of an 8.0% HEPES solution. Add 21.0 ml. of sterile glycerol to the 0 ml. of medium from each culture. 3. Transfer the cell suspension to ampoules (which can be frozen) or to tubes, tightening the stoppers and sealing with adhesive tape. 4. Bring to -80°C during 1 hour and then place the tubes (or ampoules) in the freezer (-70°C) where the temperature will drop 1°C per minute. Once the temperature inside the ampoule has reached -20°C, the cells ---Page Break--- Laboratory Exercise #5 can be stored at -197°C in the Liquid nitrogen refrigerator. Under these conditions cells can be stored for periods up to 5 years. 5. To revive the frozen cells, the ampoule is removed from the Liquid nitrogen refrigerator and thawed rapidly in a 37°C water bath. A volume of the cell suspension containing 1.5 to 2.0 x 10^6 is added to 10 ml. of outgrowth medium and cultures initiated in a 200 ml. bottle. 1B. Transport 1. Trypsinize a bottle of Hela cells and dilute in growth medium to obtain a suspension of no more than 0.6 x 10^6 cells/ml. 2.

Refrigerate at °C for 24 hours, centrifuge at 200 rpm for 30 minutes and discard the supernatant. Add medium to obtain a cell suspension of 2.4 x 10^6 per ml. In this state, the cells can be shipped in an iced container and upon receipt, sedimented by centrifugation at 200 rpm for 30 minutes and resuspended in fresh growth medium at a concentration of 0.6 x 10^6 cells/ml. A satisfactory method to ship cell cultures is obtained by placing the vessel with nutrient medium, preventing the trauma to the cells by the movement of the medium. Upon receipt, the medium should be removed and the cells fed with a new one. ---Page Break--- 22. LABORATORY EXERCISE #6 INCORPORATION OF POLYSOMES Objective: To study the incorporation of RNA precursors into cellular polysomes. Material: 1 Liter RSB buffer 0.02 M tris-HCl pH 7. 0.01 M KCl 0.0015 M MgCl2 100 ml 10% sucrose w/w in RSB 1100 ml 10% sucrose w/w in RSB 20 ml RSB containing 5 x 10^-7 g/ml hydrocortisone 500 ml Liquid scintillator. 2 flake bottles with confluent monolayer of L-cells. Trypsin for removal of cells. Procedure Monday: 1. Take up solution tone. 2. Add 30 µg uridine to each flake bottle. 3. Take 6 x 10^-2 sucrose gradients. 4. Set up minipump and flow cell for

monitoring gradients. 5. Set up paper discs for fractionation. Tuesday: 1. Add 10 µg cM uridine to flake bottles and incubate 10 min. 2. Trypsinize cells, ---Page Break--- -23 Laboratory Exercise #6 3. Centrifuge cells in conical centrifuge bottle. 4. Take up in 1.0 ml RSB containing 5 x 10^-7 g/ml hydrocortisone. 5. Homogenize in glass homogenizer. 6. Add 0.33 ml 20% sucrose to homogenate and centrifuge at 8000 RPM for 5 min. 7. Add liquid scintillator to supernatant and place 0.4 ml on each of 3 sucrose gradients. 8. Centrifuge in SW 39 rotor at 35,000 RPM for 30 min. 9. Pump gradients through flow cell and on to appropriate analysis. 10. Wash slices 10 minutes in 10% TCA and 5 min. in 95% ethanol. 11. Incubate and add to vial. 12. Count 1 min/vial. ---Page Break--- This is a laboratory exercise, whose purpose is to

fantasize the student with cone techniques widely used in cellular and molecular biology. In the cell, proteins are synthesized on the polysomes. These consist of individual ribosomes held together by the messenger RNA which specifies the amino acid composition of the protein. The size of the polysome is proportional to the size of messenger RNA, consequently, of the peptide to be synthesized. It would appear that the ribosomes are stable and can be reused by the cells whereas most messenger RNA is unstable. Hence the ribosomes are very labeled by a long exposure to an RNA precursor (in this case 13 wridine). Whereas the messenger RNA can be labeled by a short exposure to precursor (cl uridine). After breakage of the cell and removal of nuclei, mitochondria, and cell wall by low speed centrifugation, the principal particulate matter left in the supernatant is the polysomes. These are best analyzed by sucrose density gradient centrifugation. In this technique the sample is layered on top of a linear sucrose gradient in a centrifuge tube. There is a linear increase of sucrose with depth of the tube in order to stabilize the process of centrifugation. After centrifugation in a swinging bucket rotor, it is necessary to analyze the contents of the tube. This can be done in various ways. However, we shall displace the contents of the tube by injecting a more dense liquid into the bottom of the tube. Total nucleic acid can be measured by allowing the displaced fluid to flow through a flow cell which is monitored at 260 nm in a spectrophotometer. Fractions are collected on paper discs for analysis of radioactivity. It is convenient to collect samples on discs since in this form they can be easily processed to yield defined biochemical fractions with uniform physical properties for counting (no necessity for quenching correction). Liquid scintillation counting currently provides the simplest method for detection of beta emitters and the most frequently used technique for separation of double label. By an

appropriate setting of the discriminators to separate channels, both 13 and clean can be counted accurately and efficiently. ---Page Break--- 26. ‘LABORATORY EXERCISE #7 METABOLIC STUDIES USING ISOTOPES IN TISSUE CULTURE Subjects Use of Methionine-cM, in the biosynthesis of phosphatidylcholine. Incorporation of Cle for transdehydrogenations. Materials: Human Liver cell cultures (Chang's cell Line) Methionine-cUn, (New England Nuclear Corp., Meds.), sterile Glutamine (0-1 mM, 2.5 ye). Trypsin solution 0.25% in pH 7.4 phosphate buffer. n-butanol. Tetranitro sodium chloride solution (0.94). Developing solvent for chromatography: chloroform, methanol, water (65:25: Gass plates with 250 µm layer of silica gel G. Preparation: Silica gel 6-15 g, 30 ml distilled water; emulsified and layered in each plate. Chromatography chambers. Iodine vapor chambers. 15 ml centrifuge tubes. Sterile pipettes (0.5, 5.0 and 10.0 ml) Scintillating Liquid (FPO 0.4f, FOROP 0.00f in toluene). Radioactivity counting vials. Packman Liquid scintillator. stirring rod and Pasteur pipettes. Procedure: Add 0.1 mM (2.5 ye) of L-methionine-cM dissolved in 10 µl of culture medium to a Chang's cell culture. Incubate for 2 hours at 37°C. ---Page Break--- Laboratory Exercise #7 2. Trypsinize the cell culture with 5.0 ml of trypsin solution. Resuspend the cells in 5.0 ml of culture medium. 3. Centrifuge at 1,000 RPM for 10 minutes. 4. Resuspend the cell pack in 2 ml saline with gentle agitation. Centrifuge at 1,000 RPM

for 10 minutes. Repeat the above procedure twice. 6. Resuspend the cells in 1.0 ml saline and transfer to a Teflon homogenizer vial with a Pasteur pipette. Rinse the centrifuge tube with an additional 0.5 ml saline portion. 7. Homogenize 3-5 minutes. 8. Add 1.0 ml of n-butanol. Shake for 2 hours. 9. Centrifuge at 3,000 RPM for 25 minutes. 10. Transfer 20 µl of the butanol phase to a counting vial adding 5.0 ml of the scintillating medium. 11. Prepare a standard agar plate. 12. Dry in a 200°C oven for 2 hours. 13. Allow the plate.

to coo and place an aliquot of ‘the hand Phase 2 em from the Border, the volume depending on the Pedloactivity count. Next to this sample spot a standard of 208 of the -tecithine. Place 50 ml of the developing solvent in the chamber and then the stirring plate. Allow to develop until the solvent level has reached the top border. ---Page Break--- Laboratory Beeresce #7 1G. Now to dry at room temperature. Develop the sample components in uniodine vapor chamber. 1. Once the spots are identified, the gel is collected from each one and placed in individual vials. Add 5 ml of scintillating liquid and count in the Bechnen Liquid scintillator using the carbon 1 tracer. 28. Discuss the results obtained. ---Page Break--- retov vot a) Use of Santee the use of Suction in the study of certain functions is based on the property of these elements to serve as molecular markers, whose fate is followed through the metabolic pathways. In the case of the connie are visible, the property of differentiating a state or radioactive shape from the ordinary elements, due to inherent nist vast or by part of radiation, makes it saga to determine or find a compound of the nealinn 1 Setatd. false, any property is pointing to more the interrelationship of marked compounds within the cell or other levels of higher organization. Instruments of varying detection ability guide scintillation systems have a great advantage over others in the case of radioactive emissions (radiations of 13, oc). D) Transmethylation mechanism to form phosphatidylcholine - this transmethylation process has been studied at different levels of organization, of low energy in intact animals, in homogenates, in cell fractions, in the presence of isolated enzyme systems, etc. In intact animals (Du Vigneoul et. al.), the first studies made on rats fed on a diet deficient in methionine and cystine but in the presence of choline and homocystine, led to a demonstration of the existence of a metabolic process that forms choline from methionine. ---Page Break--- in evident proof of

this operational mechanism was obtained "in vivo" using radioactive and stable isotopes. The use of cysteine, threonine, methionine, and choline showed that there was an incorporation of the methyl groups from the methionine into the structure of choline and vice versa, and that the radioactivity was retained in the phospholipid of the liver. Other studies in liver homogenates and tissue fractions led to the demonstration of the same type of molecules involved in this process (Armer, J. and Greenborg, D.). In this way, the radioactivity from CMH-methionine was incorporated into the phospholipid of these fractions, and Ubi-methionine in the presence of phosphatidylethanolamine, ATP, and Mg^{2}■ cofactors formed phosphatidylcholine, phosphatidylethanolamine. Previously, work dealing with S-adenosylmethionine (Cantoni et al.) showed methionine, ATP, and Mg^{2}■ to be replaced by said compound in cell fractions. These works established the participation of at least two enzymes that catalyze the formation of phosphatidylcholine by means of transmethylation using methionine and a phosphatidic derivative. One that catalyzes the formation (S-adenosylmethionine synthetase) and another that catalyzes the excessive formation of S-adenosylmethionine using methionine, ATP, and transmethylation steps (S-adenosylmethionine: phosphatidylethanolamine-ethyl transferase). There is no information available on the study of this metabolic process in animal or human cell cultures, but the studies that have been made and briefly published are valid for this level of organization, the cell level. ---Page Break--- CH, -0-0c-k' © bH-e-0e-a" e ¢ BN = Clee CI 0-P 0-8 Hy ey ou Phrafatects whsvecle aus Crene whe CH ~CH~9 ~ pH —O- CH, Cle on pie BOW-ON CN. OP ~0-0 Hy cu, by

Gus i rt 1 ~O- 4 HCW, - Cl oP O-eu, ey ow ---Page Break--- ae LABORATORY EXERCISE 48 Memions OF DETECTING OR MEASURING VIRUS MULTIPLICATION IN TISSUE CULTURE CYTOPATHIC EFFECT OF VIRUS MULTIPLICATION Objective: to determine the virus dilution that gives rise

to cytopathic changes in 50 percent of the inoculated eel cultures. Material: Poliovirus suspension, pipettes, procedure: 1. Observe the Hela cell cultures prepared during the third day of laboratory work. 2. Pipette off the growth media and replace it with 0.9 ml each of Eagle's medium containing 2% serum. 3. Prepare tenfold dilutions of poliovirus type 1 as follows: a. Set up a row of eight Wassermann tubes numbered 2 through 8. Add 1.8 ml of media into each of them. b. Take 0.2 ml of the poliovirus suspension and add it to the first tube in the row. Mix thoroughly with a sterile pipette. c. Take 0.2 ml from the dilution in tube #1 and pass it to tube #2, mixing the contents. d. Repeat the operation with the remainder of the tubes. 4. Inoculate 0.1 ml of each dilution of the virus and deliver to each of 4 tubes of Hela cells. A separate pipette should be used for each dilution; however, in the interest of laboratory resource economy, use one 0.2 ml pipette for adding the virus dilution to the Hela. ---Page Break--- Laboratory Exercise #8: Start with the highest dilution and work back to the lower. 5. Set up two Hela tubes for control without inoculation. 6. Bring to the incubator at 37°C and record the results every day. 7. Calculate the TCD50 by the Reed-Tench method. In the following table, an example is given of data derived from an ideal experiment for illustrating the procedure of accumulation:

ACCUMULATED VALUES
Dilution Ratio CPE No. CPE No. CPs Ratio Per Cent
0 1:2 6 32/2 100

Accumulated values for the total number of tubes that showed a CPE or were intact are obtained by adding in the direction indicated by the arrows. The accumulated CPE ratio represents the accumulated number of tubes with cytopathic changes over the accumulated total number inoculated. In this example, the cytopathic change in the 10^3 dilution is higher than 50%.

lower @itution, 1074, it is considerably lower. The necessary proportionate distance of the 50 per cent CPE end ---Page Break--- -33+ Laboratory Brereise £8 8 obtained as follows: oint lies between these two dilutions and Per cent CPE at dilution next above 50% ~ Sof = Proportionate Per cent CPE at dilution next above 50% - (per cent CPR distance at dilution next below) or 80 - 50: 30 x 0.5 B20 bo Since logarithmically the distance between two dilutions is a function of the incremental steps used in preparing the series, it is necessary to correct the proportionate distance by the dilution factor. In the case of seriat ten-fold dilution the factor is 1 (log 10 = 2) and Yuin is disregarded. In our example we have: Negative logarithm of CTD, and point titer = negative logarithm of the D, 30 dilution above the 50 per cent CPE plus the proportionate distance factor that de: Negative logarithm of the dilution above 50% - Proportionate distance (0.5 X dilution factor (log 10): 05 TCTs 35 = 10-35 Log TOM yy = 10 ---Page Break--- THE LABORATORY EXERCISE 49 PLAQUE METHOD FOR CONFLUENT LAYERS OF CELLS Objective: To produce circumscribed infected areas by vaccinia virus in chick embryo fibroblasts which do not take the neutral red vital stain and appear as clear unstained areas against a background of viable stained cells, water. Chick embryo fibroblasts Vaccinia virus suspension Water bath “| Neutral red Petri dishes: Procedure 1. Set up three Wasserman's tubes numbered 1 through 3 and add 1.8 ml of Eagle's media to all tubes. From the pool of vaccinia virus supplied add 0.2 ml to tube #1 and mix thoroughly with a sterile pipette. Withdraw 0.2 ml and add to tube #2. Repeat the previous step with

new pipettes so that you will have virus dilutions 10^2, 10^-2, and 10^-3. 3. Remove the out-growth medium from the Petri dishes previously prepared with chick embryo fibroblasts and wash once with saline. Inoculate a plate with 0.5 ml of each virus dilution. 3. Incubate at 37°C for 2 hours for virus adsorption with

occasional rocking to distribute the virus particles: 4. After the adsorption period, remove the fluid and overlay the fibroblast sheets with 5 mL of the following agar medium: Agar 1.0 gm. ---Page Break--- 1 Yeast Extract (Difco) or animal albumin hydrolysate 10 gm. Horse serum 34.0 mL. Take 85.0 mL of Penicillin 50 u/mL and 50 Streptomycin to complete the medium. 5. Allow the agar to solidify and turn the plate upside down and incubate at 37°C for 3 days with the cell monolayer down. 6. Add 3 mL of a 1/1000 neutral-red solution; incubate at room temperature for 2 hours and overnight at 4°C. 7. The dilutions used should produce distinct and separated plaques. Observe against a white background. By counting the number of plaques at the dilution where they appear distinct, and by multiplying by the corresponding dilution factor, the number of plaque-forming units (PFU) per mL of the virus suspension can be calculated. ---Page Break--- LABORATORY EXERCISE 410 NEUTRALIZING ANTIBODIES ASSAYS IN CELL CULTURES Objective: to evaluate the capacity of a serum to neutralize the cytopathic effect of poliovirus in a HeLa cell system. Materials: Serum, Poliovirus suspension, Tubes with HeLa cells, Water bath at 56°C. Procedure: 1. The serum specimen is inactivated at 56°C for 30 minutes to destroy heat-sensitive viral inhibitory substances. 2. Set up a row of tubes to make serum dilutions of 1:4, 1:16, 1:64, 1:256, 1:1024 prepared in either balanced salt solution or the maintenance medium to be used in the cell cultures. 3. The virus is diluted to contain 100 PFU in a volume of 0.1 mL (as determined by a previous titration of the virus). The viral dilutions are made in the same medium employed for the preparation of the serum solutions. 4. Equal volumes of the serum dilutions (0.5 mL) and of the diluted test virus (0.5 mL) are mixed; the volume of serum-virus mixture prepared is dependent upon the number of cell cultures to be inoculated with the mixture. For virus control, the test virus dilution is mixed with an equal...

volume of diluent (or known serum) and incubated under the same conditions as the serum ---Page Break--- “a laboratory Breretse #20 virus mixture. For serum control, the 1/4 dilution is mixed with reagent. It is necessary to perform a concurrent titration of the virus to establish that a test dose actually contains approximately 100 TCID50. The conditions recommended for incubation of serum virus mixtures vary widely for certain agents. It has been demonstrated that some preliminary incubation doses increase the neutralizing capacity of serum. The important consideration is to avoid incubation conditions under which the virus may be labeled for a sample for long periods at 37°C. For most neutralization tests, incubation of the serum virus mixtures is conducted for 30 minutes to one hour at room temperature or at 4°C. After the incubation period, serum virus mixtures, virus controls, and serum controls are inoculated in volumes of 0.2 ml into monolayer tube cultures. At least two cultures are employed for each serum mixture. The inoculated cultures are incubated at 37°C and examined microscopically for the ability of the serum to inhibit CPE of the virus. The cytopathic effect of the inoculated tubes is recorded and the neutralizing end point is expressed as that dilution of serum which protects 50% of the tubes against the test dose ---Page Break--- ER EEE EE SE'SSE_— eo eel! lL 38 Laboratory Exercise #10 of the virus, as illustrated in the following example: sem DIRION * CRE‘ CRE "Ho cHPE + open inoculation | MORTALITY ae ‘sotto 1 1 MOS ratio for control Foe to 2 te 6 toe ° me ige fe la le la im los a:6h tye tas a ta te tap B wee tue fa fa ba fa tap lg 10h app eo tk te Am + 100 TCID50 gee oy os logarithm 50 percent neutralizing end point = -1.8 + (0.5 y (0. 6) «28+ 60.) = 2a antilogarithm -2.1 = 120 ---Page Break---