RMS

Puerto Rico

PRNC - 91-A
Revised June 1970

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 15 - July 3
1970

OPERATED BY UNIVERSITY OF PUERTO RICO UNDER CONTRACT
NO. AT (40-1)-1833 FOR U. S. ATOMIC ENERGY COMMISSION

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THIS COURSE IS GIVEN

IN COLLABORATION WITH

THE DEPARTMENT OF MICROBIOLOGY

OF THE UPR SCHOOL OF MEDICINE

TISSUE CULTURE COURSE

First Week: Introduction to Tissue Culture Techniques
(June 15 to 19, 1970)

Date	Time		
Monday 15	9:00 to 9:05 a.m.	Introductory Remarks	Dr. J. Chiriboga, Director Medical Sciences and Radiobiology Division
	9:05 to 10:00 a.m.	Lecture - History of Tissue Culture	Dr. R. Martínez-Silva
	10:00 a.m. to 1:00 p.m.	Laboratory Exercise #1 Preparation of Material, Sterilization and Sterility Tests, Staining of Cells.	Dr. R. Martínez-Silva
Tuesday 16	9:00 to 10:00 a.m.	Lecture Cells: Morphology and Functions	Dr. R. Martínez-Silva
	10:00 a.m. to 1:00 p.m.	Laboratory Exercise #2 Preparation of Tissue Culture Constituents	Dr. R. Martínez-Silva
Wednesday 17	9:00 to 10:00 a.m.	Lecture Dynamics of Cell Populations	Dr. R. Martínez-Silva
	10:00 a.m. to 1:00 p.m.	Laboratory Exercise #3 Monolayer Cultures of Chick Embryo Cells	Dr. R. Martínez-Silva
Thursday 18	9:00 to 10:00 a.m.	Lecture Diploid Cells and Cell Lines	Dr. R. Martínez-Silva
	10:00 a.m. to 1:00 p.m.	Laboratory Exercise #4 Techniques for Growing Cell Lines in Tissue Cultures	Dr. R. Martínez-Silva
Friday 19	9:00 to 10:00 a.m.	Lecture Conservation and Transport of Cell Cultures	Dr. R. Martínez-Silva
	10:00 a.m. to 1:00 p.m.	Laboratory Exercise #5 Conservation and Transport of Cell Cultures	Dr. R. Martínez-Silva

TISSUE CULTURE COURSE

June 15 to July 3, 1970

Second Week: Use of Isotopes at Cellular and Molecular Level (June 22 to 26, 1970)

Date	Time			
Monday 22	9:00 to 10:00 am.	Lecture	Labelling of Polysomes	Dr. Raymond A. Brown
	10:00 am. to 1:00 pm.	Laboratory	" " "	Dr. Raymond A. Brown
Tuesday 23	9:00 to 10:00 am.	Lecture	Nucleic Acid and Protein Synthesis I	Dr. Jorge Chiriboga
	10:00 am. to 1:00 pm.	Laboratory	Labelling of Polysomes (cont.)	Dr. Raymond A. Brown
Wednesday 24	9:00 to 10:00 am.	Lecture	Metabollic pathways in cells Use of Isotopes	Dr. Roger Ramos-Aliaga
	10:00 am. to 1:00 pm.	Laboratory	Metabollic pathways in cells Use of Isotopes	Dr. Roger Ramos-Aliaga
Thursday 25	9:00 to 10:00 am.	Lecture	Nucleic Acid and Protein Synthesis II	Dr. Jorge Chiriboga
	10:00 am. to 1:00 pm.	Laboratory	Metabollic pathways in cells Use of isotopes	Dr. Roger Ramos-Aliaga
Friday 26	9:00 to 10:00 pm.	Lecture	Analytical Methods in Cell and Molecular Biology	Dr. Raymond A. Brown
	10:00 to 1:00 pm.	Laboratory	Continuation	Drs. Raymond A. Brown and Roger Ramos-Aliaga

TISSUE CULTURE COURSE

Third Week: Application of Tissue Culture Techniques to Virology.
(June 29 to July 2, 1970)

Monday 29	9:00 to 10:00 a.m.	Lecture The Science of Virology	Dr. Julio I. Colón
	10:00 a.m. to 1:00 p.m.	Laboratory Exercise #8 Methods of Detecting and Measuring Virus Multiplication by Cytopathic Effect	Dr. Julio I. Colón
Tuesday 30	9:00 to 10:00 a.m.	Lecture Methods for Detecting Virus. Multiplication in Tissue Culture	Dr. Julio I. Colón
	10:00 a.m. to 1:00 p.m.	Laboratory Exercise #9 Plaque Method for Confluent Layers of Cells	Dr. Julio I. Colón
Wednesday 1	9:00 to 10:00 a.m.	Lecture Methods for Detecting Virus Multiplication in Tissue Culture	Dr. Julio I. Colón
	10:00 a.m. to 1:00 p.m.	Laboratory Exercise #10 Neutralizing Antibody Assays in Cell Cultures	Dr. Julio I. Colón
Thursday 2	9:00 to 10:00 a.m.	Lecture Application of Tissue Culture in Virus Isolation and Vaccine Production	Dr. Julio I. Colón
	10:00 a.m. to 1:00 p.m.	Laboratory Observation and Interpretation of Previous Experiments	Dr. Julio I. Colón

FINAL EXAM - FRIDAY, July 3, 1970

LABORATORY EXERCISE #1

PREPARATION OF MATERIAL. STERILIZATION AND STERILITY TESTS. STAINING OF CELLS

Objective:

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 L cells

Two tubes of HeLa cells

Two tubes of DC 2 cells

Six tubes of thyoglicolate medium

Six tubes of Sabouraud agar

Six tubes of PPLO broth

Twelve tubes of PPLO agar

Dienes' stain

Procedure:

1. Observe under the microscope and describe the different cell types.
2. Tests for sterility

   2:1 Inoculate 0.1 ml of each cell culture tube into 10 ml of thyoglicolate medium. Incubate at 37°C. Read and record results every 24 hours. If negative, discard after 5 days.

   2:2 Streak a loopfull of each cell culture on Sabouraud Agar. Incubate at room temperature. Read and record results during a week, at 24 hour intervals.

2:3 With a pipette deposit some drops of each cell culture fluid on PPLO broth and agar (Difco). 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.

2:4 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 a 10X objective and 10, 12.5 or 15 X 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 tissue 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 "feathery". PPLO colonies vary from 10 to 500 microns in diameter (0.01 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, they are usually marked out on the petri dish with a glass marking pencil.

Confirmation of PPLO colonies depends, in addition to morphological characters, on:

1. Inability to remove the imbedded 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 forms.
3. A requirement for native protein.
4. Reaction with the Dienes stain.

The Dienes stain is prepared by dissolving 2.5 gms methylene blue, 1.25 gms. azur II, 10.0 gms maltose, and 0.25 gm. 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 PPLO 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 PPLO colonies never decolorize the stain.

Staining of cells by Giemsa method.

1. Remove the growth medium of the tubes provided for sterility testing.

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.
4. 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 during 30 minutes, wash with tap water.
6. Remove the slide; let dry; mount following the instructions.

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:

4 Chick embryos

Scissors

Syringe 50 ml.

Centrifuge tubes

Graduated cylinder

Antibiotics (Penicillin and Streptomycin)

1. HANKS BALANCED SALT SOLUTION (BSS)

A. 10 X Solution

Unit #1 $NaHCO_3$ 3.5 gm.

Dissolve in 250 ml. distilled water. Dispense in a convenient bottle (50 ml. screw-cap prescription bottle) and autoclave at 120°C for 15 minutes.

Unit #2

NaCl	80.0 gm.
KCl	4.0 gm.
$MgSO_4 \cdot 7H_2O$	2.0 gm.
$Na_2HPO_4 \cdot 2H_2O$	0.6 gm.
Glucose	10.0 gm.
KH_2PO_4	0.6 gm.

Dissolve in 800 ml. distilled water.

Unit #3

$CaCl_2$	1.4 gm.

Dissolve in 100 ml. distilled water.

Unit #4

Phenol Red	0.4 gm.

Mix Phenol Red in a small amount of water until a paste,dilute to 150 ml. with distilled water, titrate to pH 7 with N/20 NaOH. Make up to final volume of 200 ml. Preserve with 1-2 ml. Chloroform.

Add 100 ml. of unit #4 to unit #2 and then add unit #3 to make 1,000 ml. Pour solution into glass stoppered bottle and add 3-4 ml. chloroform as a preservative. This solution may be kept at room temperature for 6 months-1 year.

NOTE: Minimize transfer of chloroform in preparation of the working solution. Be certain that bottle caps are loosened during autoclaving to insure that all chloroform is driven off.

B. Working solution

The working BSS 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 CO_2. The balanced salt solution is now ready for use. Do not tighten caps until pH of BSS is 7.4.

2. NUTRIENT MEDIA

Eagle's minimum essential medium contains higher concentrations of amino acids than the basal medium first described by Eagle, which permits cultures to be kept for longer periods of time without feeding. The medium may be prepared with Hanks BSS base. The medium is prepared, concentrated 10X and stored in the refrigerator. At the time of use, glutamine and antibiotics (stored at -20°C) and $NaHCO_3$ are added to the 1X solution.

Solution A: Per liter 10X medium

l-Arginine. HCl	1.05 gm.
l-Hystidine. HCl	0.31 gm.
l-Lysine. HCl	0.58 gm.
l-Tryptophane	0.10 gm.
l-Phenylalanine	0.32 gm.
l-Threonine	0.48 gm.
l-Leucine	0.52 gm.
l-Valine	0.46 gm.
l-Isoleucine	0.52 gm.
l-Methionine	0.15 gm.

Solution B:

l-Tyrosine	0.32 gm.
l-Cystine	0.24 gm.

These amino acids are dissolved in 200 ml. of 0.075 HCl with gentle heating (80°C).

Solution C:

Nicotinamide	200 mg.
Pyridoxal	200 mg.
Thiamine	200 mg.
Pantothenic Acid	200 mg.
Choline	200 mg.
i-Inositol	400 mg.
Riboflavin	20 mg.

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 medium.

Solution D:

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

Solution E:

200 mg. folic acid (crystalline) are dissolved in 200 ml. 1X Hanks' BSS pH 7.8. The solution is dispensed in 10 ml. amounts and stored at -20°C; 10 ml. of Solution E are added to each liter of 10X medium.

Glutamine Solution 3% - (To be added at the time of use) 12 gms. of l-Glutamine are dissolved in 400 ml. of double distilled water and sterilized by filtration through a Seitz-type pad. The solution is stored at -20°C and 1.0 ml. is added to each 100 ml. of 1X Eagle's medium.

Preparation of the final mixture of 10X Eagle's medium in Hanks' BSS

a. The following are dissolved in solution B:

NaCl	80.0 gm.
KCl	4.0 gm.
$MgSO_4 . 7H_2O$	2.0 gm.

b. The following are dissolved in 50 ml. double distilled water and added to the pool.

$Na_2HPO_4 \cdot 12H_2O$	1.52 gm.
KH_2PO_4	0.60 gm.

c. 10 grams of Glucose are dissolved in 50 ml. of double distilled water with 20 ml. of 1% Phenol Red solution and added to the pool.

d. 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 E	10.0 ml.

e. In a separate flask containing 160 ml. double distilled water 2.0 gms. anhydrous $CaCl_2$ are dissolved and added to the pool slowly with vigorous shaking.

f. The amino acids of Solution A are added to the pool and the volume is brought to approximately 950 ml. with double distilled water.

g. 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.

h. The total volume is brought to exactly 1,000 ml. with double distilled water and the solution is sterilized through a Seitz type pad.

i. 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 2.8% $NaHCO_3$ are added.

b. The following are dissolved in 50 ml. double distilled water and added to the pool.

$Na_2HPO_4 \cdot 12H_2O$	1.52 gm.
KH_2PO_4	0.60 gm.

c. 10 grams of Glucose are dissolved in 50 ml. of double distilled water with 20 ml. of 1% Phenol Red solution and added to the pool.

d. 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 E	10.0 ml.

e. In a separate flask containing 160 ml. double distilled water 2.0 gms. anhydrous $CaCl_2$ are dissolved and added to the pool slowly with vigorous shaking.

f. The amino acids of Solution A are added to the pool and the volume is brought to approximately 950 ml. with double distilled water.

g. 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.

h. The total volume is brought to exactly 1,000 ml. with double distilled water and the solution is sterilized through a Seitz type pad.

i. 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 2.8% $NaHCO_3$ are added.

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 10X stock solution of the Vitamins (stored at -20°C) appropriately in Earle or Hanks' BSS adding Glutamine, antibiotics and $NaHCO_3$ as indicated above. This prevents deterioration of the vitamins during long term storage at ice box temperature.

3. CELL DISPERSING AGENTS

A. Trypsin Solution 1.0%

1 gm. 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 Seitz-type pad and stored at -20°C.

B. Versene Solution (Ethyelenediamine tetraacetic acid)

NaCl	8.0 gm.
KH_2PO_4	0.2 gm.
KCl	0.2 gm.
Na_2HPO_4	0.15 gm.
Versene	0.20 gm.

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 microgammas per ml.)

1. Add 10 ml. of Hanks' solution to 1,000,000 units of Penicillin.
2. Add 10 ml. of Hanks' solution to 1 vial with a gram of Streptomycin.

3. Mix the contents of both vials and add up to 50 ml. of Hanks' solution.

4. Dispense in 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, beaks, 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.

LABORATORY EXERCISE #3
MONOLAYER CULTURES OF CHICK EMBRYO CELLS

Objective:

Maitland type of cell cultures from chick embryo were among the first to be used for viral propagation. Development of new techniques has increased the use of chick embryo fibroblast for virus isolation and antigen production.

Materials:

4 chick embryos

Petri dishes

Beaker

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 50-100 ml. of Hanks' BSS.

5. After the fluid from the second washing has been removed, an appropriate volume of .25% solution of trypsin in Hanks' BSS is added. The volume of trypsin solution employed is determined by the number of embryos being processed (5-10 embryos, 200 ml., 11-20 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.

7. 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 .0037 inches) into centrifuge tubes.

8. The cell suspension is centrifuged horizontally at 600 rpm for 10 minutes and the supernatant fluid containing trypsin is aspirated immediately. The cells are washed once by resuspending in Hanks' BSS, centrifuging at 600 rpm 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 rpm 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:

Bovine serum	2.0 ml.
5% Lactalbumin hydrolysate in physiological saline	5.0 ml.

50% Chick Embryo Extract	4.0 ml.
Hanks' BSS	85.5 ml.
2.8% $NaHCO_3$	2.5 ml.
Penicillin-Streptomycin solution	1.0 ml.

11. For tube culture the cells are diluted 1 to 200 and dispensed in 1 ml. volumes. For plaquing in stoppered bottles, the cells are also diluted 1 to 200 and dispensed in 8 ml. volumes into three ounces prescription bottles. For plaquing in 60 mm. Petri dishes 5 ml. of 1:200 dilution of the cells are added and incubation is conducted in 5% atmosphere.

12. 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.

LABORATORY EXERCISE #4

TECHNIQUES FOR GROWING CELL LINES IN TISSUE CULTURE

Objective:

This experiment is designed to maintain a cell line (HeLa, DC 2 or L) for the duration of the course. Each student will be provided with 1 bottle of cells.

Materials:

1 bottle with a confluent cell monolayer

Haemocytometer

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 per centtrypsin 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 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.
3. Count the cells in the haemocytometer

   3-1. 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 haemocytometer coverglass, avoiding any overflow into the moat.

3-2. Determine the average number of viable cells (dead cells stain 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.

3-3. Tranfer 50,000 cells into each of 14 test tubes. Stopper tubes with rubber stoppers. Incubate at 37°C in an horizontal plane.

4. Keep record and observe cells every day.

LABORATORY EXERCISE #5
CONSERVATION AND TRANSPORT 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

Ampoules

A. Storage

1. Obtain a bottle with a culture of 5 day old cells from which the out-growth medium has been removed and replaced with 10 ml. of fresh medium, consisting of 10 per cent horse serum and 90 per cent lactalbumin 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 10^6 cells, otherwise the cells are not suitable for storage.
2. The pH of the medium is adjusted to 7.4 by means of an 8.8% $NaHCO_3$ solution. Add 1.0 ml. of sterile glycerol to the 0 ml. of medium in each culture.
3. Transfer the cell suspension to ampoules (which can be flame-sealed) or to tubes, tightening the stoppers and sealing with adhesive tape.
4. Bring to 4°C during 1 hour and then place the tubes (or ampoules) in the Revco (-70°C) where the temperature will drop 1°C per minute. Once the temperature inside the ampoule has reached -20°C, the cells

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.

B. Transport

1. Trypsine 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 4°C for 24 hours.
3. Centrifuge at 200 rpm for 30 minutes and discard the supernatant.
4. Add medium to obtain a cell suspension of 2.4 x 10^6 per ml.
5. 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 filling 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.

LABORATORY EXERCISE #6
LABELING OF POLYSOMES

Objective:

To study the incorporation of RNA precursors into cellular polysomes.

Material:

1 liter RSB buffer

0.01 M tris-H Cl pH 7.4

0.01 M K Cl

0.0015 M mg Cl_2

100 ml 10% sucrose w/w in RSB

100 ml 10% sucrose w/w in RSR

10 ml RSB containing 5 x 10^{-7} gm/m/ hydrocortisone

500 ml liquid scintillator

2 Blake bottles with confluent

monolayer of L-cells

Trypsin for removal of cells

Procedure:

Monday:

1. Make up solutions.
2. Add 30 μ c H^3 uridine to each Blake bottle.
3. Make 6 10-20% sucrose gradients.
4. Set up pump and flow cell for monitoring gradients.
5. Set up paper discs for fractionation.

Tuesday:

1. Add 10 μ c C^{14} uridine to Blake bottles and incubate 10 min.
2. Trypsinize cells.

3. Centrifuge cells in conical centrifuge bottle.
4. Take up in 1.0 ml RSB containing 5 x 10^{-7} gm/ml hydrocortisone.
5. Homogenize in glass homogenizer.
6. Add 0.33 ml 20% sucrose to homogenate and 8000 RPM for 5 min.
7. Add 4 mg DOC 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 proper discs.
10. Wash discs 10 minutes in 10% TCA and 5 min. in 95% ethanol.
11. Dry discs and add to vials.
12. Count 1 min/vial.

LABELING OF POLYSOMES

This is a laboratory exercise, whose purpose is to familiarize the student with some 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 and, consequently, of the peptide to be synthesized.

It would appear that the ribosomes are stable and can be reused by the cell, whereas most messenger RNA is unstable. Hence the ribosomes are best labeled by a long exposure to an RNA precursor (in this case H^3 uridine) whereas the messenger RNA can be labeled by a short exposure to precursor (C^{14} 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 nuclei acid can be measured by allowing the displaced fluid to flow through a flow cell which is monitored at 260 mμ 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 β -emitters and the most frequently used technique for separation of double label. By an appropriate setting of the discriminators in two separate channels, both H^3 and C^{14} can be counted accurately and efficiently.

LABORATORY EXERCISE #7
METABOLIC STUDIES USING ISOTOPES IN TISSUE CULTURE

Objective:

Use of L-methionine-$C^{14}H_3$ in the biosynthesis of phosphatidil—choline. Incorporation of C^{14} for transmetilation.

Materials:

Human liver cell cultures (Chang's cell line)

L-methionine-$C^{14}H_3$ (New England Nuclear Corp., Mass.), sterile solution (0.1 ml = 2.5 μc).

Trypsin solution 0.25% in pH 7.4 phosphate buffer.

n-butanol.

Isotonic sodium chloride solution (0.9%).

Developing solvent for chromatography: chloroform, methanol, water (65:25:4).

Glass plates with 250 μ layer of silica-gel G.
Preparation: Silica-gel G 15 gms., 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 (PPO 0.4%, POPOP 0.01% in toluene).

Radioactivity counting vials.

Beckman liquid scintillator.

Stirring rods and Pasteur pipettes.

Procedure:

1. Add 0.1 ml (2.5 μc) of L-methionine-$C^{14}H_3$ dissolved in 10 ml of culture medium to a Chang's cell culture. Incubate for 24 hours at 37°C.

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.
5. Repeat the above procedure twice.
6. Resuspend the cells in 1.0 ml saline and transfer to a Potter Elvejehm 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 15 minutes.
10. Transfer 20 ℓ of the butanol phase to a counting vial adding 5.0 ml of the scintillating liquid.
11. Prepare a silica-gel plate.
12. Dry in a 100°C oven for 1 hour.
13. Allow the plate to cool and place an aliquot of the butanol phase 2 cm from the border, the volume depending on the radioactivity count. Next to this sample spot a standard of 20 ℓ of L-α -lecithine.
14. Place 50 ml of the developing solvent in the chamber and then the silica-gel plate.
15. Allow to develop until the solvent level has reached the top border.

16. Allow to dry at room temperature. Develop the sample components in an iodine vapor chamber.

17. Once the spots are identified the gel is collected from each one and placed in individual vials. Add 5 ml scintillating liquid and count in the Beckman liquid scintillator using the carbon 14 tracer.

18. Discussion of the results obtained.

USE OF ISOTOPES IN THE STUDY OF METABOLISM IN LIVER CELLS (CHANG CELLS).

INCORPORATION OF C^{14} OF THE L-METHIONINE-METHYL-C^{14} IN THE MOLECULE OF PHOSPHATIDILCHOLINE

Previous work: a) Use of isotopes - The use of isotopes in the study of cellular functions is based on the property of these elements to serve as molecular markers, whose fate is followed through the metabolic pathways in which these compounds are utilized. The property of differentiating a stable or radioactive isotope from the ordinary elements, due to their different mass values or by particle or radiation emission, makes it possible to easily identify these elements within the cellular structure or when an intermediate or final compound of the metabolism is isolated. Also, by this same property it is possible to know the interchange values of a marked compound within the cell or other level of higher organization.

In the detection for the presence of radioactive isotopes, counts are made with a series of instruments of varying efficiencies, whereby the liquid scintillation system has a great advantage over others in the case of low energy radioactive emissions (β radiations of H^3, C^{14}).

b) Transmethylation mechanism to form phosphatidilcholine - This transmethylation process has been studied at different levels of organization. In intact animals, in homogenates, in cell fractions, in the presence of isolated enzyme systems, etc.

In intact animals (Du Vigneaud et. al.), the first studies made on rats fed on a diet defficient 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 fróm methionine.

An evident proof of this operational mechanism was gotten "in vivo" using radioactive and stable isotopes. The use of CD_3-methionine, $C^{14}H_3$ methionine and CD_3-choline showed that there was an "in toto" incorporating mechanism of the methyl groups from the methionine into the structure choline and viceversa and that the radioactivity remained in the phospholypids of the liver.

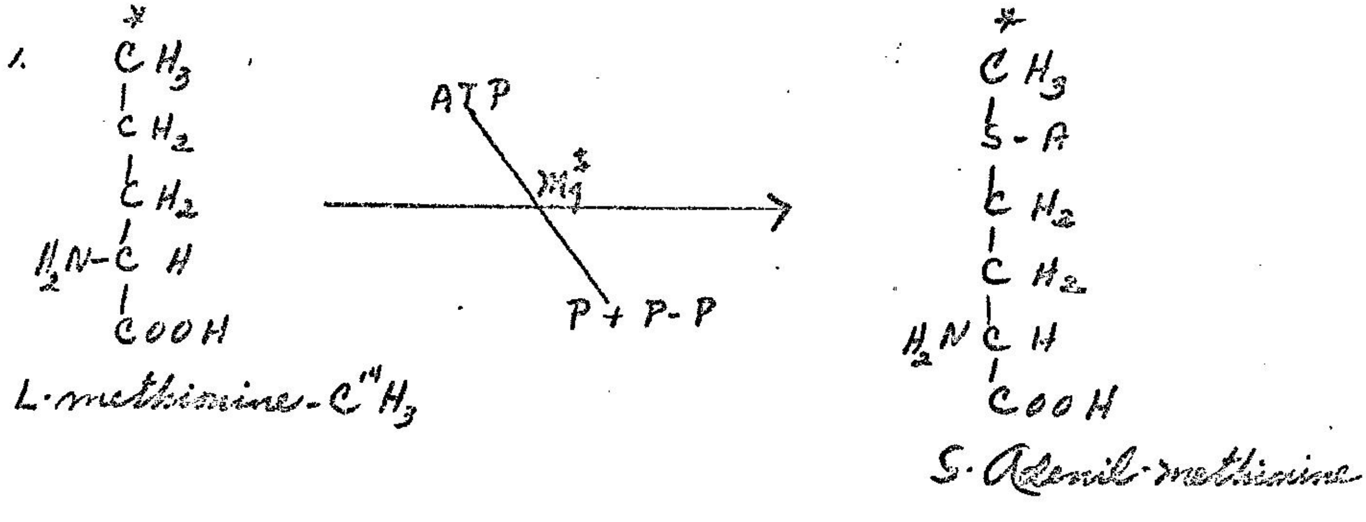
Other studies in liver homogenates and cell fractions led to the demonstration of the same type of molecules involved in this process (Bremer, J. and Greenberg, D.). In this way the radioactivity from $C^{14}H_3$-methionine was incorporated in the phospholypids of these fractions and $C^{14}H_3$-methionine in the presence of phosphatidilethanolamine, ATP, and Mg^{2+} as cofactors formed phosphatidilcholine, phosphatidilmonomethylethanolamine. Previously, work dealing with S-Adenosilmethionine (Cantoni et al.) allowed 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 phosphatidil choline by means of transmethylation using methionine and a phosphatidic derivative. One that catalyzes the formation of S-adenosil methionine using methionine, ATP, and Mg^{2+} (S-Adenosil methionine synthetase) and another that catalyzes the successive transmethylation steps (S-Adenosil methionine: phosphatidilethanolamine-methyl 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.

Reactions

1.



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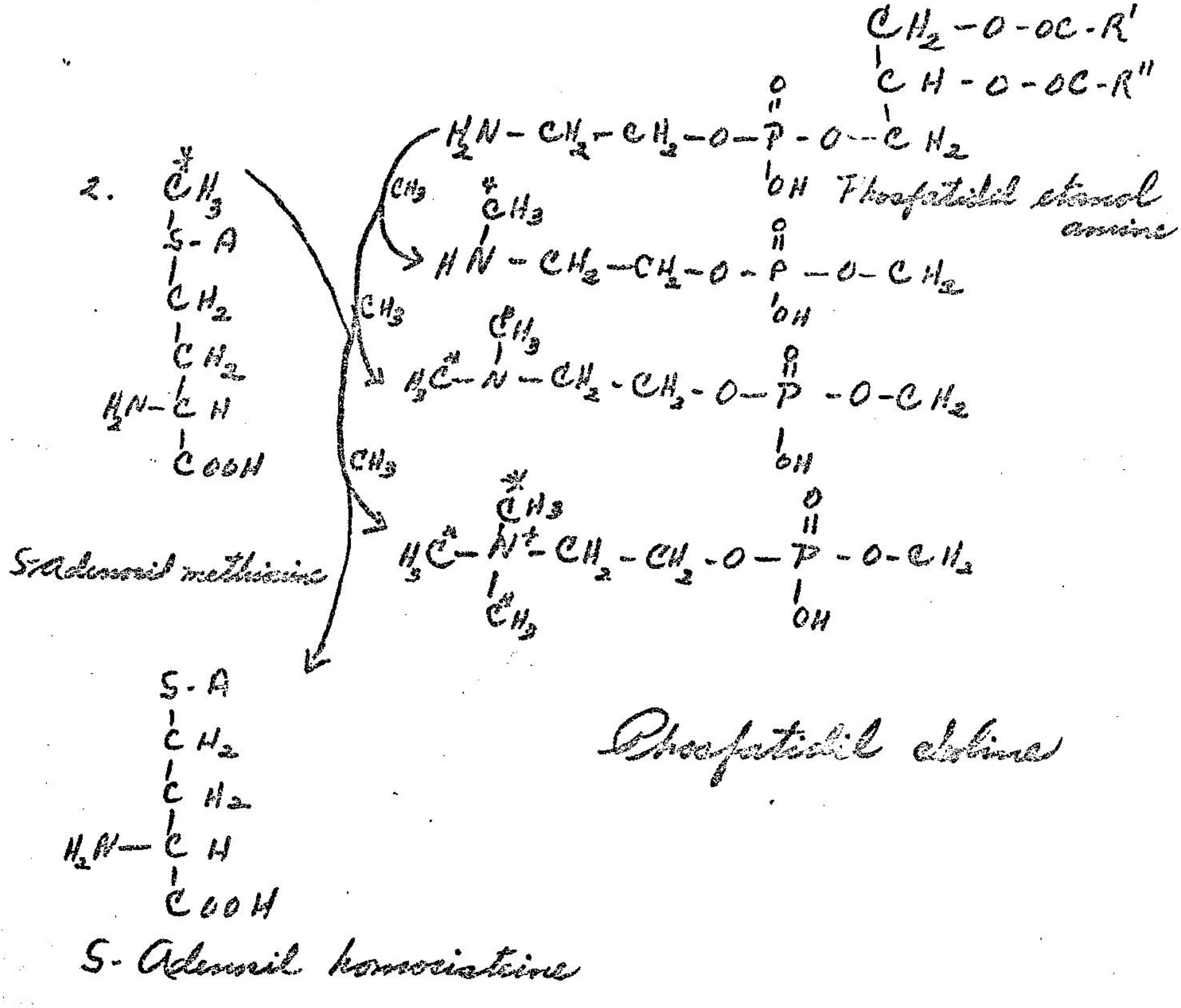

LABORATORY EXERCISE #8
METHODS OF DETECTING OR MEASURING VIRUS MULTIPLICATION IN TISSUE CULTURE
CYTOPATHIC EFFECT OF VIRUS MULTIPLICATION

Objective:

To determine the virus dilution that gives rise to cytopathic changes in 50 per cent of the inoculated cell cultures.

Materials:

Poliovirus suspension

Tubes

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 .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 4 Wasserman's tubes numbered 1 through 4 and dispense 1.8 ml. of media into each of them.
   b. Take 0.2 ml. of the poliovirus suspension and add to the first tube in the row. Mix thoroughly with a sterile pipette.
   c. Take 0.2 ml. from the dilution in the tube #1 and pass it to tube #2 mixing the contents.
   d. Repeat the operation with the remainder tubes.
4. Inoculate .1 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 glassware economy use one 0.2 ml. pipette for adding the virus dilution to the HeLa

cells beginning with the highest dilution and working back to the lower.

5. Set up to 2 HeLa tubes for control without inoculation.
6. Bring to the incubator at 37°C and read record the results every day.
7. Calculate the $TCID_{50}$ by the Reed-Muench method.

In the following table an example is given of data derived from an ideal experiment for illustrating the procedure of accumulation:

				ACCUMULATED VALUES			
Virus dilution	CPE Ratio	CPE	No. CPE	CPE	No. CPE	Ratio	Per Cent
10^{-1}	4/4	4	0	12	0	12/12	100
10^{-2}	4/4	4	0	8	0	8/8	100
10^{-3}	3/4	3	1	4	1	4/5	80
10^{-4}	1/4	1	3	1	4	1/5	20
10^{-5}	0/4	0	4	0	8	0/8	0

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%; in the next lower dilution, 10^{-4}, it is considerably lower. The necessary proportionate distance of the 50 per cent CPE end point lies between these two dilutions and is obtained as follows:

$$\frac{\text{(Per cent CPE at dilution next above 50\%)} - 50\%}{\text{(Per cent CPE at dilution next above 50\%)} - \text{(per cent CPE at dilution next below)}} = \text{Proportionate distance}$$

$$\text{or } \frac{80 - 50}{80 - 20} : \frac{30}{60} = 0.5$$

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 serial ten-fold dilution the factor is 1 (log 10 = 1) and thus is disregarded. In our example we have:

Negative logarithm of $TCID_{50}$ and point titer = negative logarithm of the dilution above the 50 per cent CPE plus the proportionate distance factor

that is:

Negative logarithm of the dilution above 50%	= -3
Proportionate distance (0.5 X dilution factor (log 10):	-0.5
$TCID_{50}$	-3.5

$$\text{Log } TCID_{50} = 10^{-3.5}$$

LABORATORY EXERCISE #9
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.

Materials:

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 .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^{-1}, 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.

Yeast Extract (Difco)	0.1 gm.
Lactalbumin hydrolysate	0.4 gm.
Horse serum	14.0 ml.
Hanks' BSS	85.0

Penicillin 50 u/ml. and 50 μ Streptomycin to complete medium.

5. Allow the agar to solidify and turn the plate upside down and incubate at 37°C for 3-4 days with the cell monolayer down.
6. Add 3 ml. of a 1/1000 1 + 7 neutral-red solution; incubate at room temperature for 2-4 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 correspondent dilution factor, the number of plaque forming units (PFU) per ml. of the virus suspension can be calculated.

LABORATORY EXERCISE #10
NEUTRALIZING ANTIBODIES ASSAYS IN CELL CULTURES

Objective:

To calculate the capacity of a serum to neutralize the cytopathic effect of a poliovirus in a HeLa cells systems.

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 non-specific virus 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 solutions or the maintenance media to be used in the cell cultures.
3. Poliovirus is diluted to contain 100 $TCID_{50}$ 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 dilutions.
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 normal serum) and incubated under the same conditions as the serum

virus mixture. For serum control, the 1/4 dilution is mixed with diluent. It is necessary to perform a concurrent titration of the virus to establish that a test dose actually contains approximately 100 $TCID_{50}$.

5. The conditions recommended for incubation of serum virus mixtures vary widely for certain agents; it has been demonstrated that some preliminary incubation does increase the neutralizing capacity of serum. The important consideration is to avoid incubation conditions under which the virus may be labelled for a sample for long periods of 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.
6. After incubation period, serum virus mixtures, virus controls and serum controls, are inoculated in volumes of .2 ml. into monolayer tube cultures. At least two cultures are employed for each serum mixture .
7. The inoculated cultures are incubated at 37°C and examined microscopically for ability of the serum to inhibit CPE of the virus.
8. 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

of the virus, as illustrated in the following example:

SERUM DILUTION	CPE Ratio	CPE	NO CEPE	CPE	NO CPE	MORTALITY	
						Ratio	Per Cent
1:4	0/2	0	2	0	6	0/6	0
1:16	0/2	0	2	0	4	0/4	0
1:64	1/2	1	1	1	2	1/3	33
1:256	1/2	1	1	2	1	2/3	67
1:1024	2/2	2	0	4	0	4/4	100

$$\frac{50\% \text{ - CPE at dilution next below}}{\text{CPE next above - CPE next below}} = \frac{50 - 33}{67 - 33} = \frac{17}{34} = 0.5$$

Logarithm 50 per cent neutralizing end point = -1.8 + (0.5 x (0.6)

= -1.8 + (-0.3)

= -2.1

antilogarithm -2.1 = 120