SEMIMICROMETHODS FOR MAJOR MACROMOLECULES ANALYSIS FROM PC12 CELLS IN CULTURE (10 CM PLATE, $4*10^6$ CELLS).

A. Lipid Extraction

1. Scrap off cells with rubber policeman in 1 ml Phosphate buffer saline (PBS)
2. Collect cells in ependorf microfuge tube (1.5 ml size)
3. Rinse culture dish with 0.4 ml PBS
4. Centrifuge (Beckman centrifuge) for 2 min. (50v, low speed)
5. Discard PBS
6. Add 0.6 ml methanol (M) and vortemix
7. Add 0.3 ml chloroform (C)
8. Seal coverlid and sonicate 10 min (water bath cleanser)
9. Centrifuge 2 min (220V high speed)
10. Transfer lipid extract to another eppendorf tube
11. Reextract pellet with 0.4 ml CM (1:2 by vol).
12. Sonicate, centrifuge (as in 8-10)
13. Combined lipid extract: evaporate to dryness under air (Lipid Extract)
14. Save delipidated pellet for protein, DNA determination or gel analysis.

B. Lipid Extract

1. Dissolve residue in 0.1 ml CM (2:1)
2. Mix well and centrifuge
3. Transfer aliquots with a Hamilton syringe for the following analyses:

(a) 2*25 µl for sialic acid determination (see IV)

(b) 2*5-10 µl for total lipid phosphorus (see V)

(c) Apply remaining for thin layer chromatography (see VI)

C. Delipidated Pellet

1. Add promptly, after the 2nd extraction (see A-14)
   0.05 ml of 5M NaOH (note paragraph 5 below)
2. Heat 10-15 min. at 60° C until pellet is dissolved
3. Add 0.45 ml H_2O and reheat (60°) if necessary
4. Transfer aliquots with a Hamilton syringe for:

(a) 5-10 µl for protein (Lowry) analysis (see I)

(b) 0.1 ml for modified DNA (Burton) analysis (see II)

5. For gel analysis of the delipidated pellet add SDS/mercaphthoethanol and boil 15 min. (promptly).

I. LOWRY DETERMINATION

Reagents:

(a) 3% $NaCO_3$ in 0.1M NaOH + 1% $CuSO_4$ + 2% Na^+K^+ Tartarate (98+1+1 volumes mixed before use)

(b) 2M Folin-Cicolteau

(c) Albumin 1mg/ml

To sample

(a) Add 0.5M NaOH and complete volume to 0.1 ml

Add 0.2 ml H_2O

Add 1.0 ml of Reagent A and incubate exactly 10 min at RT

Add 0.05 ml of 2M Folin reagent (undiluted) with continuous mixing

Read color after 30 min. at 650 nm.

II. DNA DETERMINATION (Std 23 u/μg)

(a) To 0.1 ml sample in 0.5M NaOH add 0.1 ml H_2O followed by 0.5 ml DPA (20 vol of diphenylamine and 0.1 vol 1% acetaldehyde)

24-48 hrs at room temperature

Read color at 588 nm

(b) ^3H Thymidine S.R.A. analysis (> 50,000 cells seeded per well)

1. Pulse label with ^3H Thymidine 1 μlCi/ml for 2 hrs.
2. Discard isotope and wash once in PBS
3. Add 1 ml 5% TCA (10 min at 4°C)
4. Wash twice with 5% TCA at 4°C (ensure removal of TCA)
5. Add 0.4 ml (the volumes can be reduced to 1/2) of 0.5% SDS in 0.1M NaOH
6. After 10 min take aliquots (5-10%) for H3-Thymidine counting in Dioxane scintillation fluid/
7. To the remaining solution in the well add 0.1 ml respectively of Diphenylamine reagent.
8. Cover 24 well plate (to prevent evaporation and allow for > 24 hrs for color to develop

9. Read at 588 nm against blanks and a standard curve of 5,10,15 µg DNA (by weight, Salmon sperm – calibrated by phosphorus analysis).

(c) Diphenylamine reagent

Dissolve 5g diphenylamine in 490 ml AcA glacial and 10ml H_2SO_4

Store (Brown bottle at R.T.).

(d) Befor use

Take 20 volumes of diphenylamine and 0.1 volume of 1% acetaldehyde. (Acetaldehyde is stored at 4° C).

IV. Procedure For Sialic Acid (free) Determination (Std 54 u/nmol)

Reagents:

(a) 0.1M H_2SO_4 hydrolysis solution

(b) 0.2M $NaIO_4$ (metaperiodate-M.W 213.9) in 9M H_3PO_4 (prepared fresh before assay).

(c) 0.77M NaAsO/O, 5M Na_2SO_4 in 0.05M H_2SO_4

(d) 44 mM TBA/0.5M $NaSO_4$

(e) Distilled cyclohexanone

Hydrolysis:

(a) Dry ganglioside samples in Eppendorf tubes

(b) Add 0.1 ml of 0.1M H_2SO_4

(c) Heat 2 hrs at 78° C

(d) Cool/centrifuge

(e) Run free sialic acid standartd under same conditions.

After Hydrolysis: To sample in 0.1 ml

(1) Add 0.2M $NaIO_4$ (10µl)

(2) Incubate 20 min at RT

(3) Add 0.77M NaAsO/0.5M Na_2SO_4 (50µl)

(4) Shake well (vortex) until color disappears

(5) Add 44 mM TBA (150µl)