MATERIAL AND METHODS
The present study consists of 115 cases admitted or attended M.L.B. Medical College, Jhansi in the department of Gynaecology and Obstetrics during the period of July 1961 to March 1962. The cases were divided into following groups:

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Type of cases</th>
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</thead>
<tbody>
<tr>
<td>I</td>
<td>Cases of Normal pregnancy</td>
</tr>
<tr>
<td>II</td>
<td>Cases of abnormal pregnancy</td>
</tr>
</tbody>
</table>

Group I - Cases were further classified into two subgroups:

<table>
<thead>
<tr>
<th>Sub Group</th>
<th>Type of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a)</td>
<td>Cases followed upto delivery</td>
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<tr>
<td>(b)</td>
<td>O.P.D. cases and cases which could not be followed upto delivery.</td>
</tr>
</tbody>
</table>

Group II - Cases of complicated pregnancy were selected including:

1. Prematurity
2.ental distress
3. Low maturity
4. Twins
5. Hydrocephalus
6. Toxaemia of pregnancy
7. Ante-partum haemorrhage
8. Hydramnios
9. Abort disease
Diabetes Mellitus

Detailed history was taken including present, past, family, obstetric and personal histories.

Proper general, systemic and antenatal examination was done specially to judge the foetal maturity clinically.

A thorough study was made to see the stages of labour and complete examination of new born was done to assess the actual maturity after birth.

**AMNIOCENTESIS**

Liquor amnii collected by either of the following methods avoiding contamination:

1. Abdominal amniocentesis - during antenatal period
2. Vaginal amniocentesis - during labour
3. Collection during cesarean section.

**Procedures**

**Abdominal route**

Preliminary procedure: Amniocentesis may be safely undertaken as an out patient procedure without pre-medication of the patient. The patient was told about the procedure and the reason for it.

**Equipment and the materials required**

1. The 20/21 gauge needle forceful spinal needles
   (length 2.5" - 6 or 6.5 - 165 mm)
2. one pair syringe holding syringes.
Picture No. 1. Amniocentesis (Per abdominal route)
(iii) Sterile sanae and sponges.
(iv) Small abdominal towel with a slit
(v) Antiseptic solution and container
(vi) Sterile 5 ml. and 10 ml syringes.
(vii) Appropriate clean bottles to receive the samples.

Preparation of the patient:— The patient was asked to void urine and was made comfortable in the dorsal position on an examination table, with the head and shoulders slightly elevated to promote relaxation of abdominal muscles.

Selection of the site for amniocentesis:— The abdomen was gently palpated to determine the size of the uterus, height of fundus, fetal limbs etc. Fetal heart rate counted at this time.

The area between fetal arms and legs and of the largest of necks were the most suitable sites for insertion of the needle. Scar areas were avoided if any.

Procedure:— Having selected the puncture site, part was painted and draped. With full aseptic precautions the needle with stylet was passed with a quick thrust through the abdominal wall and uterine wall into the amniotic cavity at the selected site. Usually a sensation of 'give' was obtained as the needle point entered the amniotic cavity. The stylet was removed from the needle. The amniotic fluid flowed through the needle. 10 ml. amniotic fluid was withdrawn.
After aspiration of the fluid, the needle was quickly withdrawn and puncture site was sealed with tincture-benzoin. The patient should remain on the table for 10 minutes. Postal heart was auscultated again. She was told to report any fever, pain, chills, bleeding or leakage of fluid.

Following aspiration, amniotic fluid was aseptically transferred from syringe to the properly labelled sample container.

Vaginal Amnioentesis :- The transvaginal approach was applied when patient was in labour with membranes present and cervix adequately dilated.

Patient was put on table in dorsolithotomy position. Vagina and Vulva properly cleaned. Fine speculum was placed in posterior vagina and if needed cervix was held by sponge holding forceps. A 20 number L-D needle with stilet was inserted directly into the bag of waters. After removing the stilet amniotic fluid was aspirated into the syringe.

Collection during Cesarean section :- After opening the abdominal cavity, needle was inserted under vision at suitable site in uterus and amniotic fluid was aspirated with the help of sterile syringe.

Amniotic fluid was used immediately or kept at -20°C for storage if delay was expected.
Standard - Leathin and sphingomyelin were obtained from V.P. Chauk Institute, New Delhi and kept at -20°C.

Chemicals - All the reagents were analytical grade (A.R) or guaranteed reagents (G.R)

1. Silica Gel - G
2. Chloroform
3. Methanol
4. Normal saline (0.9%)
5. Acetic acid
6. Perchloric acid (60%)

Reagents -

1. Standard Phosphorus -- Concentration 0.0125 mg/0.5 ml. 0.2197 grams of potassium dihydrogen phosphate \((\text{KH}_2\text{PO}_4)\) was dissolved in water and made upto one litre. A few drops of chloroform were added.

2. Metal (N-methylacetoacetic acid sulphate), 1 gm in 100 ml of 2% solution of sodium bisulphite.

3. Ammonium - Molybdate solution -- 7.5 gm was dissolved in 200 ml of water, 100 ml of 0.1N sulphuric acid was added and made upto 400 ml with water.

EXTRACTION OF PHOSPHOLIPIDS FROM AMNIOTIC FLUID

Modified method of Gluck et al (1971) was used and L/S ratio measured by molar - method.

Fresh amniotic fluid or that stored at -30°C was centrifuged to remove cells and sediments 2000 at r.p.m. for 5-10 minutes.
5 ml. of supernatant amniotic fluid was extracted with equal volume of methanol (5 ml.) and 2 volumes (10 ml.) of chloroform.

This mixture was mixed and kept for four hours with intermittent shaking.

The lower clear layer, containing phospholipids dissolved in chloroform, was separated in a beaker and supernatant again extracted with 3:1 chloroform : Methanol mixture. To extract most of the phospholipides this process was repeated three.

Now all these separated samples were mixed with an equal amount (volume) of 0.9% normal saline in a separating funnel for four hours, so as to separate proteins and other sediments which precipitate as a slummy layer in between the two solutions.

The lower chloroform layer containing phospholipides was drawn into a beaker very carefully without disturbing the intermediate slummy layer. This solution was evaporated almost to dryness on water bath. For separation of individuals phospholipides, the total extracted phospholipides were dissolved in known amount of chloroform (ml.).

**HIGH LEVEL CHROMATOGRAPHY**

Preparation of Silica for TLC: 10 g silica gel mixed with 100 ml of distilled water containing 0.05 M HCl (in a conical flask) and shaken rigorously for 20 seconds. This
Picture No. 2. Extraction of Membranlipids

(1) Extraction with Chloroform and Methanol
(2) Extraction with Normal saline
(3) Quantitative analysis

(a) Lecithin
(b) Sphingomyelin
Homogeneous slurry should be immediately poured for spreading as the binder hydrates and sets within 2-3 minutes.

**Preparation of the Plate**  
In order to prepare a satisfactory plate, the slurry must be spread evenly over the whole plate surface.

Standard size plate in T.L.C. was 20 x 20 cm, transparent glass plate of 5 mm thickness. Such plates were best spread with one of the commercially available T.L.C applicator containing spreader, sealer (gauge) and leveler.

After setting the plates, gate was fixed gauge at 0.25 mm thickness and slurry fed into the spreader which was then drawn along set of plates in a single smooth motion.

On completion of the spreading, the apparatus was left for 5 minutes for layer to 'set'. In this time layers containing binder usually change their appearance from shiny wet to a dry matte surface which indicated that gypsum had been formed.

**Layer thickness**  
In general layers of 0.25 - 0.30 mm thickness were used. This figure refers to the gap under the spreader (thickness of gel coating overglass plates).

**Drying the plate**  
The plate was dried by standing overnight at room temperature. Drying simply removes the water or other solvent used to form the slurry, and leaves the plate still containing a certain amount of water which is chemically bound.
Activation of plate: Activation involved drying the plate at an elevated temperature, usually 110-120°C for 1-2 hours.

Active plate usually pick up water rapidly from the atmosphere and even breathing on the plate was sufficient to change degree of activity greatly. So activated plate was usually reactivated immediately before use by a further heating for 30 minutes at 110°C temperature.

Cleaning plate for re-use: Soap or non-abrasive detergent with water was adequate for this purpose. A final rinse with distilled water followed by vertical draining and drying should ensure that plate was ready for re-use. Before coating the gel plates were cleaned with cotton soaked in acetone to remove any traces of lipoidal material over the plate.

Applying the sample: The sample applied by microsyringe used of applicator plate ensure evenly spaced spots 1 cm apart and 2.0 cm up from the edge. 0.03 ml. of extracted phospholipid from individual amniotic fluid sample applied with intermittent drying so that spot area was not more than a few mm in diameter.

Marking of plate: Standard lecithin, sphingomyelin and were marked by needle or pencil tip at the top of the plate with amount of quantity used.
Apparatus for I.L.G.  --  Blown glass tank with upward bowing was used so that the vertical plates will stand at an angle to the horizontal solvent surface. Plates should be erect and solvent solution calm and quiet.

**Solvent**  --  Chloroform : Methanol : Acetic acid : Distilled Water were taken in the ratio of 25 : 15 : 4 : 1

For the two plate tank as in the present study, 90 ml solvent was used. The solvent was made to run up to 17 cm height on plate from base, which used to take about 2-3 hours.

The plate was taken out and left to dry for 30-40 minutes at room temperature with a fast draught, till no solvent smell remained.

**Visualisation**  --  The dry chromatogram was placed in a dry tank containing crystals of Iodine which rapidly volatilise to purple vapours. A tank was kept permanently for this purpose.

Lipid compounds absorb iodine reversibly to produce brown spots on a faint yellow background. On removing the plate from the tank, the colour fades as the iodine evaporates and this may be hastened with a stream of air.
Recovery of compounds from plates: The sample spots, those corresponding in height to the spots of standard lecithin and sphingomyelin were encircled by needle. After complete evaporation of iodine, the spots were scraped off one by one help of spatula. The material was collected on butter paper, and then transferred into the test tubes separately for further determination.

**DETERMINATION OF PHOSPHOLIPIDS IN LECITHIN AND SPHINGOMYELIN FRACTIONS OF PHOSPHOLIPID**

This was done by modified method of Marinetti (1962). Silica gel scraped was directly digested with 1 ml of 60% per choleric acid (Mera 1960). Test tubes were kept on hot plate till it becomes clear. Few drops are distilled water were added and again the mixture boil for 2-3 minutes, to convert pyrophospholipids into inorganic phosphorus.

Then total amount was made to 10 ml by addition of distilled water, one ml of each ammonium molybdate and metal reagents were added and it was kept for 1/2 hours.

Known standard made by addition of 0.5 ml standard phospholipid solution to which 9.5 ml of distilled water, were added. 1 ml of ammonium molybdate was also added.

Control was prepared by taking 10 ml. distilled water including 1 ml of metal and 1 ml of ammonium molybdate reagents.

**Colorimetry** - The silica gel was allowed to settle down by centrifugation. Supernatant was used for measuring absorbance at 625 nm, using red filter in colorimeter.

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