MATERIAL & METHOD
MATERIAL AND METHODS.

The present study was carried out in the Department of Paediatrics in collaboration with the Department of Biochemistry, M.L.B. Medical College and Hospital, Jhansi (U.P.) from May 1982 to March 1983. 48 Infants (more than 3 months of age) and pre-school children (1 to 5 years of age) attending the Well Baby Clinic and those admitted in the Paediatric Ward were selected for the study.

Cases were grouped as follows:

A. Control group.
B. Study group.

Selection for Control group -

10 Infants and pre-school children weighing more than 80% of the 50th percentile of Harvard Standard for their age, who were apparently healthy or convalescing after a mild attack of Polio, were selected for the Control group.

Selection for Study group -

38 Infants and pre-school children suffering
from Protein Energy Malnutrition (P E M), weighing less than 80% of the 50th percentile of Harvard Standard for their age, comprised the study group.

Children suffering from Primary liver disorders or diseases like Diabetes mellitus, Primary hypertension, Myxoedema, Renal disorders and Malaria etc. affecting the total lipids or its fractions were excluded from both the control as well as the study group.

Besides name, age, sex, address, socio-economic status, occupation of parents, educational qualifications of parents, birth order of child in the family and per-capita income, following facts were recorded in each case on specially designed proforma.

**Dietary history -**

A detailed dietary history since birth till the completion of this study was recorded with special emphasis on the following points.

1) The age upto which breast milk was given.

2) The age when artificial milk was started, the type of artificial milk, its dilution and the average quantity consumed daily.

3) The age when semi solids and solids were started.
iv) The present dietetic history in terms of quantity and quality of food used for feeding the child.

An average of total calories and protein intake per day were recorded in each case to know the quantum of deficiency for that age.

Birth History -

A detailed birth history was recorded with special emphasis on low birth weight (including pre-matures and small for dates).

Milestones -

The developmental history of each child was recorded in all the four fields i.e. Gross motor, Manipulative or fine motor, Adaptive or social and speech. The age at which the child attained different milestones was ascertained by subjective and objective assessment.

Present and Past Illnesses -

From the parents or other family members, detailed history was obtained regarding present and past illnesses. Efforts were made to find out the occurrence of any acute or chronic illness like Tuberculosis, worm infestation, pertussis, measles and Malaria
etc. that might have affected the nutritional status of the child and also the lipid and its fractions in the blood.

**Family history** -

An enquiry was made about the history of diabetes, tuberculosis, hypertension etc. in the family members.

All patients of study group were put on nutritional therapy to raise the daily intake of food to 200 Calories per kg. with 3-4 gms. proteins per kg. of the present body weight. (Walia and Rupmini, 1962), with vitamins and minerals supplementation. Drugs for infections and infestations, and intravenous fluids were used as and when required.

An attempt was made to follow up the cases upto 20 days.

**Clinical examination** -

A thorough clinical examination was done including general appearance, psychomotor changes, hair changes, facial look like moon facies or wizened look. Eyes were examined for the presence of conjunctival xerosis, bitot’s spot, pallor and any other abnormality. Lips, gums and tongue were examined for the presence of
angular stomatitis, cheilosis, bleeding and spongy gums, glossitis etc. Total number of teeth present in the oral cavity and their health status was noted. Skin was examined for the presence of hyperkeratosis, depigmentation, xerosis and various dermatoses. Skeletal system was examined for the presence of any deformity and signs of vitamin D deficiency such as cranio-tubes, bossing of skull, Harrison sulcus, knock knee or bow legs.

Clinical assessment for the loss of subcutaneous fat and muscle wasting was done in each child. Thyroid gland was examined to find out any abnormality.

A thorough examination was done to detect any systemic abnormality.

**Anthropometric measurements** -

Measurements were conducted on the day of admission, on 10th day and on 20th day.

**Weight** - Infant weighing scale capable of measuring weight to the nearest of 0.050 kg. was used for children who could not stand, and adult type spring weighing machine was used for recording weight nearest to 0.250 kg in older children. Weight was recorded
with minimum clothes and before each measurement zero error was corrected. Same machines were used for subsequent followup.

**Length** - By using an infantometer, recumbent length was measured to the nearest of 0.1 cm., by placing the child supine on the infantometer. Head was held firmly against the fixed upright head board and legs straightened, keeping feet at right angles to legs with toe upwards. The free foot board was brought into firm contact with the child's heel.

**Head circumference** - With the help of a narrow flexible steel tape, head circumference was measured by applying it firmly over the glabella and supra-orbital ridges anteriorly and the occipital prominence posteriorly, giving the maximal circumference.

**Chest circumference** - In the mid respiration the chest circumference was measured with the help of a steel tape at the level of xiphoid cartilage in a plane at right angles to the vertebral column. The measurement was recorded in recumbent position to the nearest of 0.1 cm.
**Mid arm circumference** - Circumference of left upper arm at the point midway between the tip of acromion process of scapula and olecranon process of ulna, was measured to the nearest of 0.1 cm. while the arm was hanging freely by the side.

**Mid calf circumference** - Maximal calf circumference was measured nearest to 0.1 cm. on the left lower limb when the child was bearing weight on it.

**INVESTIGATIONS.**

Each child was subjected to the following laboratory investigations.

**Blood** - Haemoglobin, Total and Differential leucocyte counts.

  Total Serum Proteins.

  Serum Albumin.

  Serum Globulin.

**Sugar.**

**Urine** - Routine examination.

**Stool** - Routine examination for ova and Cyst.

Mantoux Test and Radiological investigations were performed when necessary.

Besides above investigations lipid status was assessed by estimating.
Total Serum Cholesterol level

Serum Total Lipids and
Serum Free Fatty Acids.

Collection of Blood samples -

Blood samples were collected from the peripheral vein or femoral vein of each child by venipuncture on the day of admission, 10th day and 20th day under all aseptic precautions and after taking consent from the parents.

Blood specimen was collected in plain vial, and was allowed to clot. The serum was then transferred to a test tube and centrifuged. The upper clear layer of serum was transferred to another test tube which was stored in deep freezer for analysis.

ESTIMATION OF TOTAL SERUM CHOLESTEROL

On the principle of Zlatkis, Zak and Boyle (1953), serum total cholesterol was estimated by Henley's method (1957).

Principle - Cholesterol in acetic acid solution gives red colour when treated with ferric chloride and sulphuric acid.

Reagents -

1. Acetic Acid - Glacial acetic acid (AR) aldehyde free.
2. Ferric chloride reagent - 0.05% solution.

This reagent was prepared by dissolving 50 mg. of FeCl₃·6H₂O salt in 100 ml. of glacial acetic acid (A.R.). The prepared solution was stored at room temperature.


4. Stock cholesterol standard solution -

100 mg. of cholesterol powder was dissolved in 100 ml. of glacial acetic acid (A.R.) to prepare stock cholesterol standard solution. The solution thus prepared was kept in cool and dark place.

5. Cholesterol standard for use -

The above cholesterol standard solution was diluted 1 to 25 with ferric chloride reagent. This solution was also kept in cool and dark place.

**Technique** -

0.1 ml. of serum was added to 10 ml. of ferric chloride reagent in a test tube. The mixture was mixed well and was kept for 10-15 minutes for the proteins to flocculate. The whole mixture was centrifuged at 2,500 rpm. for 10-15 minutes and then from the clear supernatant fluid 5 ml. was transferred to another
test tube. 3 ml. of sulphuric acid ( A R ) was added by the side of the test tube and the mixture was mixed thoroughly by shaking, and then allowed to stand for 20-30 minutes.

For the Standard-0.1 ml. of physiological saline was mixed with 10 ml. of cholesterol standard solution for use and then out of this mixture 5 ml. was transferred to another test tube.

For the Blank - 5 ml. of ferric chloride reagent was taken in a third test tube.

The samples were simultaneously treated for Blank and Standard.

Values of unknown ( Test Sera ) and Standard were read on colorimeter against Blank to set zero using a yellow filter.

Calculations -

Total cholesterol in mg / 100 ml. serum

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times \frac{100}{0.05} \times 2$$

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 400.$$

In general total cholesterol was performed on each specimen, however to test reproducibility duplicate determinations were performed at intervals throughout the study.
ESTIMATION OF SERUM TOTAL LIPIDS

Phosphovanillin method (Frings and Fendley, 1972 and Annino, 1976) was employed to estimate serum total lipids by using Span Diagnostic Kit (Art. No. 926).

Principle - Serum lipids on heating with concentrated sulphuric acid and phosphovanilline reagent produce pink colour which can be measured colorimetrically.

Reagents - The kit contained the following reagents -

1. Lipid Standard (700 mg/dl.)
2. Sulphuric acid (36 N)
3. Phosphovanilline Reagent.

Vanilline Reagent - 6.0 gms of Vanilline was dissolved in water in 1 litre volumetric flask and diluted to give a total volume of 1 litre. The solution was stored in brown bottle.

Phosphovanilline Reagent - In 350 ml of vanilline reagent 50 ml of water and 600 ml of conc. phosphoric acid was added with constant stirring in a 2 litre flask. The solution was finally stored in brown bottle in refrigerator between 2 to 8°C.

Procedure -

Estimations were carried out in test serum
along with blank and lipid standard.

To 2 ml of 36W sulphuric acid 0.1 ml of serum was added and mixed thoroughly. The mixture was kept in a boiling water bath for 10 min and then cooled to room temperature. From the above mixture 0.2 ml fluid was transferred to another test tube and 6.0 ml of phospho-vanilline reagent was added. The mixture, thoroughly mixed was incubated at 37°C for 15 min time and then cooled to room temperature.

For standard - 0.1 ml of lipid standard and
For Blank - 0.1 ml distilled water was used.

Optical density of the test serum and standard lipid solution were measured at 540 nm by using green filter against blank to set zero.

Calculation -

Serum total lipids in mg/dl = \frac{\text{Reading of test}}{\text{Reading of standard}} \times 700

**ESTIMATION OF FREE FATTY ACIDS IN SERUM**

Millian Novaks technique (1965) was employed for the estimation of free fatty acids in serum.

**Principle** - Free fatty acids are extracted from the serum and then esterified with the help of cobalt reagent which are then estimated colorimetrically with the help of the indicator.
Reagents -

1. Cobalt reagent -
   Solution A - Cobalt nitrate-acetic acid-potassium sulphate, was prepared by adding 6 gms of cobalt nitrate \( \text{Co(NO}_3\text{)}_2 \cdot 6\text{H}_2\text{O} \) and 0.8 ml of glacial acetic acid to a saturated solution of potassium sulphate (saturated while boiling, with excessive crystals at storage, and filtered before use) to give a total volume of 100 ml at 37°C.

   Solution B - A saturated sodium sulphate \( (\text{Na}_2\text{SO}_4) \) solution was prepared by adding sodium sulphate powder to boiling water. It was stored at 37°C.

   Preparation of Cobalt reagent - Triethanolamine 1.35 volume was made upto 10 volumes with solution A. 7 volumes of solution B were added and then the mixture was shaken well. This reagent was prepared every time fresh for the analysis, as it was not stable.

2. Indicator -
   Stock solution - 0.4% alpha-nitroso beta-naphthol in 96% ethanol was prepared by dissolving 0.4 gm of it in 100 ml of ethanol.

   For use - 4 ml of stock indicator solution was diluted with 46 ml of 96% ethanol.
3. Dole's extraction mixture - This mixture was prepared by mixing redistilled isopropyl alcohol 40 parts, heptane 10 parts and N H₂SO₄ 1 part.

4. Chloroform-heptane solution - Redistilled chloroform and heptane were mixed in the ratio of 5:1 (v/v) to prepare chloroform-heptane solution.

5. Standard Palmitic Acid solution - 0.05M palmitic acid solution was prepared by dissolving 1.3 gms of palmitic acid in 100 ml of Dole's extraction mixture and was stored at 0°C.

Procedure -

Estimations were carried out in duplicate along with blank and palmitic acid as the standard.

To 2.5 ml of Dole's extraction mixture in test tube 1 ml of serum was added. The liquids were mixed by shaking. The test tube was cooled for 10 min in ice cooled water. To this 3 ml heptane was added followed by 4 ml of glass distilled water. The contents were thoroughly mixed and then allowed to stand for 10 min. After the phases had separated, 2 ml was drawn from the upper heptane phase and transferred to another test tube. 4 ml of chloroform-heptane mixture was added to it followed by 5 ml of freshly prepared cobalt reagent and the solution was thoroughly mixed
for 3 min. The mixture was centrifuged for 15 min at 2,500 rpm and then 4 ml of upper chloroform-heptane phase was transferred to a test tube containing a pinch of anhydrous sodium sulphate powder. From this 3 ml of the dehydrated chloroform-heptane mixture was transferred to a test tube containing 3.5 ml of the indicator solution.

For standard-1 ml palmitic acid solution and
For blank- 1 ml distilled water was used and these tubes were treated exactly as for serum as described above. Values were read 30 min later at 500 millimicron (nm) in a spectrophotometer.

Calculations -

Standard solution: 1.3 gms of palmitic acid/ml

Dole's extraction mixture (5.07 mEq/l).

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\text{FFA in mEq/l} = \frac{\text{Reading of unknown (test)}}{\text{Reading of Standard solution}} \times 5.07
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In each analysis, two standard solutions were treated simultaneously to reduce the error.

In general FFA estimation was performed on each specimen, however to test reproducibility triplicate determinations were performed at intervals throughout the study.