CHAPTER-II

MATERIALS AND METHODS
The following methods were used in the present study:

Dietary history
Anthropometry
Clinical assessment
Evaluation of psychomotor and physical performance
Biochemical measurements

DIETARY HISTORY

24 hour dietary recall method or the questionnaire method, whose validity has already been established (1) was used to assess the dietary intake of the children.

The raw amounts used, the total cooked amount and the cooked amount consumed by the child was assessed by using standardized cups. The cooked amount of each preparation served to the index child in terms of these standardized cups was assessed from the mother. The amount of each preparation consumed by the child was converted into raw food in the following way:

\[
\text{Total raw quantity of the foodstuff used in that preparation} \times \frac{\text{Intake of the cooked amount of that preparation}}{\text{Raw amount of a particular foodstuff consumed by the individual from a given population}} = \text{Total cooked quantity of the food preparation}
\]

The raw quantities of various foodstuffs consumed in different food preparations were calculated and summed up to obtain the total consumption of a particular food item during the past 24 hour period. These quantities were further converted into nutrients using food composition tables (2).

The proforma used for the 24 hr dietary recall method is given in the appendix I. The proforma was also useful to get the details of socio-economic status of the families.
ANTHROPOMETRY

Body mass as judged by weight and linear dimensions as judged by height were the two anthropometric measurements used to divide the children into supplemented and control groups.

Weights were taken using a lever type balance with a platform, and the measurements were noted to the nearest of a tenth kilogramme. Heights were measured with a vertical anthropometric rod, and the measurements were noted for the nearest 0.1 cm.

Height, weight and age were taken into consideration to determine the extent of undernutrition based on Waterlow's Classification (3).

(a) Those who had height for age equal to 90% of median or 50th percentile height for age, and weight for height more than or equal to 80% of NCHS standards (4) were considered normal (no stunting and no wasting).

(b) Boys with adequate height for age (greater than 90%) but subnormal weight for height (less than 80%) were considered as suffering from acute short duration malnutrition (wasted but not stunted).

(c) Those with adequate weight for height (more than 80%) but subnormal height for age (less than 90%) were considered as nutritional dwarfs (stunted but not wasted).

(d) Those with lower values for both parameters were taken as suffering from current long duration malnutrition (stunted and wasted).
CLINICAL ASSESSMENT

Clinical examination was done by seeing for the clinical signs of vitamin A deficiency - xerophthalmia, conjunctival xerosis, bitot's spots; riboflavin deficiency - cheilosis, angular stomatitis, and glossitis; phrynoderma (Appendix II).

PSYCHOMOTOR PERFORMANCE

(a) **Hand-steadiness test**: Hand-steadiness was measured by the accuracy with which a metal stylus can be placed into holes of small diameters cut in metal and electrically connected. The gadget contains 9 holes of different sizes arranged in a descending order. The diameter of the biggest hole was 1.8 cm and that of the seventh hole 0.3 cm. The subject has to keep the metal stylus in each hole for one minute without touching the rims of the holes. Contacts of stylus with rims of holes are recorded as errors (5) (Fig. 4).

(b) **Grip strength**: One of the oldest instruments for the measurement of individual differences in grip strength is the hand dynamometer. The instrument consists of an inner and an outer handle, a dial and a pointer. The subject grips these handles so that the second phalanges of the fingers press against the inner handle, while the outer handle presses against the heel of the hand. The subject then squeezes as hard as possible and the pointer indicates the grip strength in kg (6) (Fig. 5).

PHYSICAL PERFORMANCE

One mile or 1.6 km running test has a significant correlation with maximal oxygen consumption \( \text{VO}_{2\ max} \) and this test can
serve as a suitable field test for the assessment of physical fitness (7).

**Method:** Based on earlier reports, a mile or 1.6 km running test was chosen as the best possible field level simple test to measure physical fitness of school children. This test does not require any sophisticated equipment and trained personnel. The subjects were requested to cover the distance as fast as they could but cautioned to control their initial speed so that they would not collapse in the end due to exhaustion.

With a view to motivate through competition, all the subjects were made to start at the same time, and a prize to the winner of the race was also announced.

After taking the initial heart rates, the children were asked to run a distance of 400 meters four times (1600 metres total) in a football ground. The time taken to cover the distance was noted using a stop watch with a "second hand", exercise heart-rate and post-exercise heart rates were noted by measuring the pulse rates immediately after completing the run, and 3 mts after rest. Finger-prick blood samples were collected for haemoglobin analysis (8).

**BIOCHEMICAL MEASUREMENTS**

1. **Blood sampling:**

   Finger-prick blood samples from children were collected in heparinized capillary tubes. They were centrifuged in a microhaematore-crit and stored at -20°C till analysed. Simultaneously 20 ul of blood
was collected in haemoglobin pipette for haemoglobin estimation. Mouse blood was drawn from the ocular plexus in heparinized tubes.

2. **Haemoglobin estimation:**

**Principle:** Haemoglobin is converted into cyanmethaemoglobin by the addition of potassium cyanide and ferricyanide. The colour of cyanmethaemoglobin is read in a colorimeter at 540 nm against reagent blank (8).

**Reagents:**

Drabkin's solution was prepared by dissolving 0.5 g potassium cyanide, 0.2 g potassium ferricyanide and 1.0 g of sodium carbonate in 1 litre of distilled water.

**Procedure:** Twenty microliters of blood from the finger prick collected in a calibrated haemoglobin pipette was added to 5 ml of Drabkin's solution, mixed well and read in Klett Summerson Colorimeter, using 54 number filter against reagent blank (Drabkin's solution).

**Calculation:** A standard curve was constructed with the cyanmethaemoglobin solution obtained from BDH. The concentration of the sample was calculated from the standard graph.

3. **Protein estimation:**

Protein was estimated by the method of Lowry et al (9).

**Principle:** (i) Peptide bonds in the protein form a purple coloured complex with copper salts in alkaline solution. (ii) Tyrosine and tryptophan present in the protein reduce the phosphomolybdic-phosphotungstic reagent. The final colour obtained is a result of the above two reactions.
Reagents:

(a) 2 per cent sodium carbonate in 0.1 N NaOH
(b) 1 per cent copper sulphate
(c) 2 per cent sodium potassium tartrate. 0.5 ml of 'b' and 0.5 ml of 'c' are added to 50 ml of 'a'. This is solution 'd'.

Folin's reagent: Folin Ciocalteu reagent was diluted with an equal volume of 0.1N NaOH.

Standard protein solution: Bovine serum albumin (Sigma) 100 μg/ml was prepared.

Procedure: The test sample containing about 50-100 μg protein/ml and standard (20 to 100 μg) were taken in 10 ml test tube. The necessary amount of 0.1N NaOH was added to bring the volume to 1.5 ml. To this, 1.5 ml of the solution 'c' was added. Each tube was shaken well and allowed to stand for 10 minutes, after which 0.15 ml of diluted folin's reagent was added with continuous shaking. The tubes were allowed to stand for half an hour and the OD was read at 520 nm. The protein concentration of the sample was calculated from the standard graph.

4. Urinary creatinine:

Principle: Creatinine forms an orange coloured complex with picric acid in the presence of strong alkali. This creatinine picrate has an absorption maxima at 540 μm (10).
Reagents:
Picric acid (BDH) - 1%
Sodium hydroxide - 10%

Standard creatinine solution: 1 g pure creatinine (E. Merck) was dissolved in 0.1N HCl and the volume was made up to 1 litre.

Working standard solution: 1 ml of stock solution was diluted to 100 ml with distilled water to yield a solution containing 10 μg/ml.

Procedure: One ml of urine was diluted with distilled water to 100 ml, and mixed well. An aliquot of 5 ml was pipetted out into a test tube. To this 2.5 ml of picric acid and 0.5 ml of 10% NaOH were added. The contents of the tube were mixed well and allowed to stand for 20 minutes. The volume was made up to 10 ml by adding 2 ml of water and then the optical density was read at 540 μm. A reagent blank substituting distilled water for test sample was included with each batch of determination.

Different levels (10-60 μg) of a standard creatinine solution were similarly treated and the creatinine content of the sample was calculated from the standard curve.

5. Riboflavin estimation in blood, liver and urine:

Principle: Riboflavin has a strong absorption band in the region of 440 to 470 μm wave length and fluorescent band around 530 μm. The intensity of fluorescence is proportional to the concentration of riboflavin in dilute solutions. Riboflavin is measured in terms of the differences between the fluorescence before and after chemical reduction by sodium dithionate.
(i) **Red cell riboflavin**: Red cell riboflavin was estimated by a fluorometric method of Bessey et al (1949) as modified by Ramji et al (1973) (11).

**Reagents:**

(a) Trichloroacetic acid (Merck Analar) - 20%
(b) Potassium phosphate (Dibasic) (BDH Analar) - 4M
(c) Standard stock riboflavin (Sigma) - 1 mg/10 ml in 0.1N HCl
(d) Anhydrous sodium dithionate crystals (LOBA) - 20 mg

**Red cells preparation**: Using microcrit heparinised capillary tubes, blood was drawn from the ocular plexus of mice by the retino-orbital puncture and collected in normal saline. This sample was spun at 3000 rpm for 15 minutes to separate RBC from the plasma. The RBC fraction was washed twice with normal saline.

**Procedure**: One tenth ml of haemolysate (1:1 diluted) was diluted to 1 ml with water and deproteinized by the addition of 1 ml of 20 per cent TCA. This was extracted for half an hour by repeatedly mixing in a Vortex mixer. The solution was then filtered and incubated at 37°C overnight for complete hydrolysis of FAD and FMN to free riboflavin.

The hydrolyzed sample was centrifuged for 10 minutes and 1 ml of the clear supernatant was transferred into a second test tube. The hydrolyzed sample was neutralized with half its volume of 4M potassium phosphate. The reducible fluorescence was measured using a Hitachi Spectrofluorometer set at 470 nm excitation and 530 nm.
fluorescence. Reduction of riboflavin was carried out with solid sodium dithionate crystals. After the crystals get dissolved, the second reading was taken with in half a minute after the addition of sodium dithionate.

Different levels of (10 to 40 ng) of a standard riboflavin solution was similarly treated and the riboflavin concentration of the sample was obtained by referring to the standard graph. The entire procedure was carried out in dim light. The values were expressed as μg riboflavin per 100 ml RBC.

(ii) Liver riboflavin: The fluorometric procedure described above was adopted for the estimation of total flavin in liver by taking suitable aliquots.

Preparation of homogenate: 0.1 ml of 25% liver homogenate was made up to 2.5 ml. Then from this diluted sample, 0.2 ml was taken and made up to 1 ml. Extraction of riboflavin was done as described earlier for red cell riboflavin.

Urinary riboflavin: Analysis of riboflavin in urine was carried out by the method described by Morell and Slater (12).

Procedure: Urine sample was filtered, and diluted 10 times. From the diluted sample, an aliquot of 0.5 ml was taken into a test tube, to which an equal volume of 4M potassium phosphate (dibasic) solution was added, and mixed well. The rest of the procedure followed was same as described above.
6. Enzyme assays:

(i) **Erythrocyte glutathione reductase activity**: The activity was measured by the method of Bayomy and Roeslki (1976) which is a modification of the method introduced by Beutler (13).

**Principle**: Glutathione reductase catalyses the reduction of oxidized glutathione to reduced glutathione which can be measured by monitoring the oxidation of NADPH at 340 nm.

\[
\text{Oxidized glutathione} + \text{NADPH} + \text{H}^+ \xrightarrow{\text{GR}} \text{Reduced glutathione} + \text{NADP}^+ + \text{FAD}
\]

The activity was measured in the presence and absence of added FAD, and was expressed as per cent activity coefficient.

**Reagents**

Phosphate buffer pH 7.4: (a) 2.722 g of KH$_2$PO$_4$ was dissolved in distilled water and made upto 200 ml, and stored at 4°C.

(b) 22.8 g of K$_2$HPO$_4$·3H$_2$O was dissolved in water and made upto 1000 ml, and stored at 4°C.

The two components were allowed to reach room temperature. 100 ml of component (b) was taken in a beaker and titrated with component (a) to a pH 7.4, and stored in refrigerator at 4°C.

Sodium bicarbonate solution, 0.1M: 8.4 g of NaHCO$_3$ was dissolved in distilled water and made upto 1 litre. It was prepared fresh every 14 days and stored at 4°C.
GSSG solution (80 μmol/l): 243 mg of GSSG (Sigma) was weighed in a small beaker. 5 ml of distilled water and 50 ul of 0.8M NaOH (32 g/l) were added. They were mixed well and placed in an ice bath. It was prepared fresh daily.

Flavin adenine dinucleotide solution (250 μmol/l): 1.0 mg of FAD (Sigma) was dissolved in 5 ml of distilled water and was protected from light. It was prepared fresh daily.

NADPH + H⁺ solution (4 μmol/l): 33.2 mg of NADPH (Sigma) was dissolved in 10 ml of 0.1 M NaHCO₃ solution, mixed well and was kept in an ice bath. It was prepared fresh daily.

Potassium - EDTA solution (60 mmol/l): 3.2 g of potassium salt of EDTA was dissolved in distilled water and made up to 100 ml. It was prepared fresh weekly and stored at 4°C.

Preparation of haemolysate: The capillary tubes containing erythrocytes were cut at the phase of red cells and plasma. The portion containing the red cells was crushed with a glass rod and to it 0.5 ml of glass distilled water was added, and centrifuged for 15 mts to separate out the crushed glass tube. From this haemolysate, 50 ul was taken for the enzyme assay.
The tubes were set up as follows:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Tube - I (ml)</th>
<th>Tube - II (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate buffer</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.025</td>
<td>0.025</td>
</tr>
<tr>
<td>Distilled water</td>
<td>0.05</td>
<td>--</td>
</tr>
<tr>
<td>FAD</td>
<td>--</td>
<td>0.05</td>
</tr>
<tr>
<td>GSSG</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>Haemolysate</td>
<td>0.05</td>
<td>0.05</td>
</tr>
</tbody>
</table>

The tubes were incubated at 37°C for 15 minutes following which 0.05 ml of NADPH + H⁺ solution was added to each tube. The reaction rate was continuously monitored at 340 nm for 5 minutes and the linear absorbance change was measured. Haemoglobin was estimated by the cyanmethaemoglobin in 50 μl of haemolysate.

**Calculation:**

The enzyme activity was expressed as micromoles of NADPH oxidized per hour per gram of haemoglobin.

\[
\text{EGR Activity} = \frac{A \times 1.225 \times 60}{6.22 \times 0.05} \times \frac{100}{\text{Hb g%}}
\]

\(A\) = decrease in absorbance in one minute at 340 nm. Molar extinction of NADPH = 6.22 x 10³. The activity coefficient is the ratio of stimulated to basal activity.
Activity coefficient (AC) =

\[
\frac{\text{Reduction of absorbance with added FAD}}{\text{Reduction of absorbance without FAD}}
\]

(ii) **Liver D-amino acid oxidase activity:** A colorimetric procedure described by Seifter et al (14) was used for the estimation of liver D-amino acid oxidase activity (EC 1.4.3.3)

**Principle:** D-amino acid oxidase catalyses the following reaction:

\[
\text{FAD} \quad \begin{array}{c}
\text{D-amino acid} + \text{H}_2\text{O} + \text{O}_2 \\
\rightarrow \\
\text{Keto acid} + \text{NH}_3 + \text{H}_2\text{O}_2
\end{array}
\]

When D-alanine is the substrate, the keto acid formed will be pyruvate. The pyruvate forms a reddish coloured hydrazone with 2,4-dinitrophenylhydrazine which can be measured at 520 nm.

**Reagents:**

(a) DL-alanine (Sigma) - 0.04 M (equivalent to 0.02 M D-alanine)

(b) Sodium arsenite - 0.04 M in 0.11 M NaCl

(c) Sodium pyrophosphate - 0.033 M

(d) 2:4-dinitrophenylhydrazine (DNP) (BDH) - 20 mg of 2:4 dinitrophenylhydrazine was dissolved in 20 ml of hydrochloric acid by warming and the solution was made upto 100 ml.

(e) 2 N sodium hydroxide (BDH)

(f) Trichloroacetic acid (Merck Analar) - 20%

**Preparation of sample:** Fresh liver sample was washed free of blood with 0.9% saline. One gram of the liver tissue was homogenized in 0.02 M sodium phosphate buffer of pH 8.3. The homogenate was made upto 10 ml. Aliquots of this 10% homogenate were taken for the enzyme assay.
Procedure: The assay was run in duplicates in 25 ml tubes which were marked 'blank' and 'test'. The additions were done according to the following protocol:

<table>
<thead>
<tr>
<th>Additions</th>
<th>Blank (ml)</th>
<th>Test (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Arsenite</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>Sodium pyrophosphate</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Liver homogenate</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td>TCA</td>
<td>0.50</td>
<td>--</td>
</tr>
<tr>
<td>DL-alanine</td>
<td>0.25</td>
<td>0.25</td>
</tr>
</tbody>
</table>

The tubes were flushed with oxygen for 3 minutes, capped and incubated at 37°C in a shaking waterbath for 5 hours.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Blank (ml)</th>
<th>Test (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCA</td>
<td>--</td>
<td>0.50</td>
</tr>
<tr>
<td>Water</td>
<td>2.50</td>
<td>2.50</td>
</tr>
</tbody>
</table>

The contents of the tube were filtered through a Whatman No.1 filter paper. To 1.25 ml of the filtrate, or pyruvate standard containing (0 to 50 μg), 0.25 ml of 2:4-dinitrophenylhydrazine reagent was added and the mixture allowed to stand for 5 minutes. The colour was developed by the addition of 2.5 ml of 2N NaOH. All the additions were done, keeping the test tubes in ice. The samples were read in a Schimadzu Spectrophotometer at 510 mμ after removing from the ice for 2 minutes. The amount of pyruvate formed in the reaction was calculated from the standard graph. The enzyme activity was expressed as umoles of pyruvate formed per gram liver per 5 hours.
(iii) Liver pyridoxaminephosphate oxidase activity: Pyridoxaminephosphate or pyridoxinephosphate oxidase catalyzes the conversion of pyridoxaminephosphate or pyridoxinephosphate to pyridoxalphosphate (PLP). The enzyme activity was assayed by measuring the amount of product formed (15).

Both pyridoxal and PLP form yellow stable hydrazone (max=410 nm) when treated with phenyl hydrazine. To measure PLP in the presence of pyridoxal, colour development with pyridoxal was inhibited by decreasing the temperature to 0°C and increasing the acidity of the solution.

Reagents:

Tris buffer - 0.4 M, pH 9.0

Pyridoxaminephosphate (Sigma) - 10 mM

Liver homogenate - 25%

Phenyl hydrazine hydrochloride - 2% in 10 N H₂SO₄ (Baker Ltd., Dagenham, England)

18 N H₂SO₄

TCA (Merck Analar) - 100%

Procedure: The assay was carried out in 50 ml conical flasks. The complete reaction mixture contained:

Tris buffer 1.70 ml
Liver homogenate 0.40 ml
Distilled water 1.30 ml
Pyridoxaminephosphate 0.1 ml
Flasks containing the reaction mixture were incubated in a shaking water-bath at 37°C for 30 minutes. The reaction was stopped by adding 0.3 ml of 100% TCA. For the blank, TCA was added before adding the substrate. The precipitate was removed by centrifuging the samples for 10 minutes at 3000 rpm.

For the colour development, 3.3 ml of the supernatant or standard containing 0 to 10 µg PIP in 3.3 ml of water were taken in test tubes and tubes were placed in an ice-bath. To this, 0.5 ml of 18 N H₂SO₄ was added followed by 0.2 ml of phenylhydrazine reagent. The tubes were allowed to stand in ice for 30 minutes. The optical density of the colour was measured at 410 nm in Schimadzu Spectrophotometer. The enzyme activity was expressed as µg pyridoxal phosphate formed per g liver per 30 minutes.

(iv) Acyl-CoA Dehydrogenase Activity:

Mitochondria were prepared by the method of Mayer and Slater (15).

Fresh liver sample was washed free of blood with 250 mM ice cold sucrose solution. One gram of the liver tissue was first minced and then homogenised well with 10 ml of 0.25 M ice cold sucrose with a hand homogenizer. This was then spun at 600 x g in a cold centrifuge (Hitachi Ultra Centrifuge) for 10 minutes. The pellet consisting of the cell debris and the nuclei was discarded and the supernatant was spun again at 8000 x g for 12 minutes at 0-4°C. The supernatant, was decanted, and the pellet consisting the mitochondria was washed
twice with ice cold 0.25 M sucrose and spun again at the same speed for 12 minutes. The mitochondrial pellet was then suspended in 3 ml of 0.05 M phosphate buffer, pH 7.4 containing 1% cholic acid. Mitochondrial protein content was estimated by Lowry’s Method (1951) as described earlier.

**Acyl-CoA Dehydrogenase Activity:** The liver mitochondrial enzyme activity was assayed using palmitoyl-CoA as substrate (17).

The reaction medium contained 34 mM potassium phosphate buffer (pH 7.2), 0.15 mM cytochrome C, 1.5 mM potassium cyanide, and 3.75 µM rotenone, 50 µM palmitoyl CoA, 3 mM phenazine ethosulphate and 20 to 40 µg of mitochondrial protein in a total volume of 1.5 ml. The reaction was started by the addition of cytochrome C, and the cytochrome C reduction was monitored at 550 nm.


