3. MATERIALS AND METHODOLOGY
SAMPLE SELECTION

The subjects for this study were randomly selected 520 non-pregnant, non-lactating women from different areas of Bombay city. They were all from the lower middle income group. The criteria for selection was their ability and willingness to participate and co-operate in the study. All the subjects were apparently healthy and did not suffer from any pathological or physical illness during the experimental period.

Detailed information about each subject was collected for the study by using the following measurements.

A. ANTHROPOMETRY

1. Height: Measurements on height were recorded by using anthropometer. The subjects was made to stand without the shoes with heels together and back straight. The heels, buttocks, shoulders and head touched the vertical surface and the head was held straight, with the line of sight horizontal. A level was slid down until the bottom surface touched the subjects head and a reading was taken to the nearest 0.05 cm (Marcus, 1968).

2. Weight: The equipment used to record the weight was of a single beam type scale. This was checked for accuracy to the nearest 0.05 kg. After calibrating the scale to zero the subject was made to stand on the centre of the platform. The weight was noted to the nearest 0.05 kg. The
subjects wore ordinary light clothing but removed their shoes (Jellife, 1966).

Using the height and weight measurement, percentage over/under weight was calculated by comparing the values obtained with the standard values for weight and for height (Marcus, 1968).

\[
\text{Percentage Standard} = \left( \frac{\text{Actual Weight (kg)}}{\text{Standard Weight}} \right) \times 100
\]

- >120% Standard = Excess
- 80 - 90% Standard = Marginal deficiency
- 60 - 80% Standard = Moderate deficiency
- <60% Standard = Severe

**BODY MASS INDEX (BMI)** also called as Quetlet Index, was calculated by using the formula:

\[
\text{BMI} = \frac{\text{WEIGHT (kg)}}{\text{HEIGHT (m)}^2}
\]

This value indicates whether the person is over or under weight.

3. **Body Frame Size:** This was assessed using height and wrist circumference ratio and comparing the values from the standard table given below. The wrist was measured where it bends (distal to the styloid Process) on the right arm (Marcus, 1968).

**Table 3.1**

**ESTIMATING FRAME SIZE FROM HEIGHT/WRIST CIRCUMFERENCE RATIOS**

<table>
<thead>
<tr>
<th>FRAME SIZE</th>
<th>WOMEN</th>
<th>MEN</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMALL</td>
<td>&gt;11.0</td>
<td>&gt;10.4</td>
</tr>
<tr>
<td>MEDIUM</td>
<td>10.1 -11.0</td>
<td>9.6 - 10.4</td>
</tr>
<tr>
<td>LARGE</td>
<td>&lt;10.1</td>
<td>&lt;9.6</td>
</tr>
</tbody>
</table>
B. DIETARY INTAKE

The dietary data were collected as follows:

1. Assessment of Dietary Intake: Detailed information about each subject was collected by using pre-planned and pretested questionnaire. The dietary data was obtained by the 24 hour food recall method. Intake of various food items was recorded in terms of household measures: Puri, chapati, and parathas were recorded in numbers, rice, dhal, vegetable bhaji, curds were recorded as number of katoris, (small or big). To measure oil, sugar, butter etc., teaspoon and tablespoon were used. Description of the food items was also recorded like plain rice, boiled, pulao, bhaji, etc.

2. Calculation of the Nutritive value foods: The 24 hour food consumption data which was collected was then converted in terms of raw weights. The Nutritive value was calculated by using Nutritive value of Indian foods (Gopalan et al, 1982) and nutritive value of various food preparation (Basic Food Preparation, Dept. of Foods and Nutrition, New Delhi). Only calories, proteins, vitamin C and iron intake were calculated.

3. Comparison with RDA: The calculated values were compared with recommended dietary allowances (Gopalan et al, 1982) for the specific nutrients in order to assess their adequacy. The results were expressed as percentage of RDA.
C. PHYSICAL WORK CAPACITY

The physical work capacity was assessed by using a "nine inch Harvard step test" (Skubie and Hodgins, 1964). The various steps of performing this test were as follows:

1. Determination of resting pulse by counting the pulse for a minimum of one minute.
2. Counting the number of steps taken in a period of three minutes. This was done by making the subject climb up and down a pair of nine inch staircase. The subject had to put both the feet on one step each at a time and only then proceed to the next.
3. Post exercise pulse rate was recorded within the first ten seconds for a period of at least thirty seconds. Subsequent readings were made every one minute, each time for thirty seconds till the basal pulse rate returned.
4. The time for recovery of the pulse rate to the basal rate was noted as recovery time.
5. Time monitoring was done with the help of a stop watch.

This test was done both before and after the supplementation. Figure - 3.1.

D. BIOCHEMICAL PARAMETERS

To assess the stages of iron deficiency the following tests were carried out (1) Haemoglobin (2) Serum Iron (3) Total Iron-Binding Capacity (4) Serum Ferritin.
Blood Collection

A 10 ml. sample of blood was collected from each subject by venipuncture by a trained technician. One ml of blood were placed in a glass bulb containing EDTA (Ethylene Diamide Tetra Acetate). This was used for analysis of haemoglobin (g/dl). Remaining 9 ml of blood were placed in an iron free glass tubes and centrifuged at 2500 rpm for 5 minutes. The separated serum was then decanted into iron free glass tubes and frozen. This was used for analysis of serum iron, T.I.B.C. and serum ferritin.

1. Haemoglobin

The Cyanmethemoglobin method was used for the analysis of haemoglobin (Bharucha et al, 1979).
Principle

In the presence of potassium ferricyanide of alkaline PH, haemoglobin and its derivatives (Except sulfhemoglobin) are oxidised to methemoglobin. Methemoglobin so produced reacts with potassium cyanide to form cyanmethemoglobin, a red coloured complex which is measured colorimetrically at 540 nm or green filter. The colour intensity is proportional to the haemoglobin concentration of the blood sample.

Reagents: i. Drabkin’s Solution.
ii. Standard Haemoglobin solution

Procedure

i. Measured 5 ml of Drabkin’s solution in cuvet. Transferred 20 mcl (0.02 ml) well mixed blood (collected with anticoagulant) by a Sahli type haemoglobin pipette or its equivalent. After filling to the mark, the outside was wiped clean with absorbent tissue or cotton and the contents rinsed into the diluent three times and Mixed.

ii. Allowed the cuvet to stand for at least 5 minutes at room temperature. Measured absorbance against haemoglobin reagent blank at 540 nm or using a green filter.

Calculations

\[ \text{Haemoglobin (g%) } = \frac{\text{O.D. Test-O.D. Blank}}{\text{O.D. Standard-O.D. Blank}} \times \text{Concentration of standard} \]

Normal Value - Haemoglobin : 12-16 g/100 ml

2. Serum Iron

The serum iron was analysed using the SPAN diagnostic kit by the ferrozine method (Bharucha et al, 1979).
Principle

Serum proteins were precipitated with a reagent containing hydrochloric acid (to dissociate iron), thioglycolic acid (to reduce iron to ferrous state) and trichloroacetic acid (to precipitate proteins). The iron in the ferrous state reacts with chromogen, to give pink coloured complex which is measured colorimetrically.

Sample

2.0 ml of serum was taken

Reagents:

i. Protein Precipitant
ii. Chromogen
iii. Stock iron standard (10mg%)

Preparation of working solution - working iron standard:

Diluted the stock iron standard (10 mg%) 1 to 50 with distilled water. Prepared it fresh, before use.

Precautions:

i. Used acid washed (with two-fold diluted HCl) and thoroughly rinsed (with deionized water) glassware.
ii. Used good quality deionized or distilled water.
iii. Used fresh unhemolyzed samples.
iv. During deproteinization of test sample, used centrifuge for separating precipitate and supernatant. Did not use filter paper, since it was likely that the filter paper may have added iron contamination leading to erroneous results.
v. Since this method was very sensitive and normal
values were in micrograms, accurate pipetting and cleanliness of glassware was very essential.

Procedure

A. **Deproteinization of Test Sample** - prepared the test sample, standard and an experimental blank as follows:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Exp. Blank (EB)</th>
<th>Standard (S)</th>
<th>Test sample (T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>-</td>
<td>-</td>
<td>2.0 ml.</td>
</tr>
<tr>
<td>Working iron standard</td>
<td>-</td>
<td>2.0 ml.</td>
<td>-</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>2.0 ml.</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Protein precipitant</td>
<td>2.0 ml.</td>
<td>2.0 ml.</td>
<td>2.0 ml.</td>
</tr>
</tbody>
</table>

mixed well using vortex mixer, allowed to stand at room temperature for 10 minutes and centrifuged the test sample (T), till a clear supernatant was obtained (Step A).

B. **Colour Development** : Prepared a blank for the colorimeter and proceeded as follows for the blank, experimental blank, standard and test sample:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Blank (B)</th>
<th>Exp. Blank (EB)</th>
<th>Standard (S)</th>
<th>Test sample (T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant (from Step A)</td>
<td>-</td>
<td>2.0 ml.</td>
<td>2.0 ml.</td>
<td>2.0 ml.</td>
</tr>
<tr>
<td>Distilled water</td>
<td>3.0 ml.</td>
<td>1.0 ml.</td>
<td>1.0 ml.</td>
<td>1.0 ml.</td>
</tr>
<tr>
<td>Chromogen</td>
<td>2.0 ml.</td>
<td>2.0 ml.</td>
<td>2.0 ml.</td>
<td>2.0 ml.</td>
</tr>
</tbody>
</table>

mixed well using vortex mixer and incubated at 37°C for 30 minutes.
Measured the O.D. of experimental blank (EB), Standard (S), Test sample (T) on a colorimeter with a yellow filter against Blank (B).

For spectrophotometer - All the volumes mentioned under colorimetric procedure in all steps could be halved. Rest of the procedure would remain unchanged. Measure the O.D. at 570 nm.

Calculations

Serum iron in mcg/100 ml. = \( \frac{\text{O.D. Test} - \text{O.D. Exp. Blank}}{\text{O.D. Standard} - \text{O.D. Exp. Blank}} \times 200 \)

Normal Values of serum iron = 60 - 150 mcg/100 ml.

3. Total Iron Binding Capacity (T.I.B.C.)

Total Iron Binding Capacity was estimated by the ferrozine method (Tietz, 1976) using SPAN diagnostic kit.

Principle

Serum is treated with iron standard and excess of iron is removed by absorption on magnesium carbonate. The iron content of this serum is a measure of the total iron binding capacity.

Reagents : 1. Protein precipitant
2. Chromogen
3. Magnesium carbonate (light)
4. Working iron standard (10 mg %)
5. Working iron standard (prepared as a mentioned in serum iron estimation procedure).

Precautions Taken : Same as for serum iron estimation.
Procedure

Step A. Saturation of Sample with Iron

1. 2.0 ml of accurately measured serum was mixed with 4.0 ml of working iron standard and allowed to stand for 10 minutes at room temperature.

2. Then magnesium carbonate, 200mg, (approx) was added to the mixture and mixed well with vortex mixer and allowed to stand for 30 minutes at room temperature. Intermittent vigorous shakings were done at every 5 minutes interval.

Step B. Deproteinization of the Test Sample

1. The test sample, standard and experimental blank were proceeded accordingly.

<table>
<thead>
<tr>
<th></th>
<th>Exp. Blank (EB)</th>
<th>Standard (S)</th>
<th>Test Sample (T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Working Iron Standard</td>
<td>-</td>
<td>2.0 ml</td>
<td>-</td>
</tr>
<tr>
<td>Supernatant (from step A)</td>
<td>-</td>
<td>-</td>
<td>3.0 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>2.0 ml</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Protein precipitant</td>
<td>2.0 ml</td>
<td>2.0 ml</td>
<td>3.0 ml</td>
</tr>
</tbody>
</table>

2. The tubes were mixed well on vortex mixer and allowed to stand at room temperature for 10 minutes and centrifuged to obtain clear supernatant (only test sample).

Step C. The tubes were then prepared for colour development in the following manner.
1. The tubes were mixed well again and incubated at 37° for 30 minutes.

2. The O.D. of the experimental blank (EB), Standard (S) and test sample (T) were measured on a colorimeter with a yellow filter against the Blank (B).

Note. For spectrophotometer all the volumes mentioned in this procedure can be haved, with no change in the procedure and O.D. measured at 570 nm.

Calculations

\[
\text{Serum T.I.B.C. mcg/100ml} = \frac{\text{O.D. Test} - \text{O.D. Exp. Blank}}{\text{O.D. Std.} - \text{O.D. Exp. Blank}} \times 400
\]

Normal Values: Serum T.I.B.C. = 270 - 380 mcg/100 ml.

Transferrin Saturation: was calculated as (Tietz, 1976)

\[
\text{percentage saturation} = \frac{\text{Serum Iron (mcg/dl)}}{\text{T.I.B.C.}} \times 100
\]

Normal Values = 20-25%

4. Serum Ferritin

Serum ferritin was quantitatively analysed by the enzyme Immuno assay procedure (Tietz, 1976).
Principle

The enzyme immuno assay for the quantitative determination of serum ferritin, is basically a two stage reaction.

Stage I  The binding of human serum ferritin to a solid phase anti-human ferritin, and the simultaneous binding of purified anti-human ferritin conjugated with alkaline phosphatase to the insoluble reaction immune complex.

Stage II  Reaction of alkaline phosphatase with a substrate solution consisting of para-nitrophenyl phosphate. The reaction is stopped with 0.1M sodium hydroxide. The optical density (405 nm) of yellow colour is directly proportional to the ferritin concentration in the sample.

Reagents

1. **Pre-diluted ferritin Calibrator Solutions** : Ferritin concentrations.... 6, 20, 60, 200, 600 and 2000 ng/ml. of human spleen ferritin in borate buffer containing bovine serum albumin, rabbit serum, sodium chloride, and inert colouring agents with sodium azide as a preservative.

2. **Solid Phase Anti-human Ferritin** : Plastic beads coated with rabbit anti-human spleen ferritin. Stored in borate buffer containing bovine serum albumin, rabbit serum, and sodium chloride, with sodium azide as a preservative.

3. **Sample Diluting Buffer** : Borate buffer containing bovine serum albumin, rabbit serum, sodium chloride, ethylene diamine-tetra acetate and inert colouring agents with sodium azide as a preservative.
4. **Conjugated Anti-human Ferritin**: 27 ml. of alkaline phosphatase conjugated rabbit anti-human spleen ferritin dissolved in 0.15 M phosphate buffered saline containing 1% normal rabbit serum, and sodium azide as a preservative.

5. **Substrate Buffer**: 10% diethanolamine, PH 10.0 with magnesium chloride, and sodium azide as a preservative.

**Serum Ferritin Assay Procedure**: Reaction trays were labelled according to the test schedule. Reagents, control sera, and patient samples were allowed to reach room temperature before use.

1. Wells A1 and A2 were skipped. Beginning with well A3, 10mcgL of calibrator of sample was pipetted into each reaction well. A1 and A2 were used to measure nonspecific binding only and contained conjugated solution and anti-human ferritin coated beads.

2. 200 mcgL of conjugated anti-human ferritin was pipetted into each well and the trays were placed on a clinical rotator. Trays were shaken for 2 minutes to assure adequate mixing.

3. Beads and grippers were added to each reaction well.

4. The trays were incubated on a clinical rotator for 2 hours at room temperature.

5. The 200/mcgL of substrate was pipetted into a second set of reaction wells.

6. The beads and grippers were removed and washed under running deionized water. They were shaken to remove excess of water.

7. The beads were placed into the second set of reaction trays
containing the substrate. These were then incubated for 45 minutes at room temperature.

8. The reaction was stopped by adding 1.0 ml of 0.1 N NaOH to each well. The bead holder was raised and lowered several times to mix the contents well. The beads were discared after this step.

9. The instrument was zeroed with the blank prepared with 200/mcgL of the substrate solution and 1 ml. of sodium hydroxide solution.

10. The absorbance of all samples was read at 405 nm.

Calculations

A calibration curve was constructed by plotting the net absorbance value obtained for each ferritin calibrator on the vertical axis (y) and the corresponding ferritin concentration in ng/ml on the horizontal axis (x). The concentration of each unknown and control was determined directly from the graph of the calibrator response curve.

Normal Values of Serum Ferritin

Normal values of serum ferritin are age and sex dependent. Serum ferritin concentrations greater than 300 ng/ml may indicate increased iron stores as seen in idiopathic hemochromatosis. In iron deficiency anaemia serum ferritin is very low <20 ng/ml.

E. BACKGROUND INFORMATION

Other background information of each subjects such as marital status, menstrual and obstetric history were also recorded.
F. IRON SUPPLEMENTATION

The anaemic subjects were divided into 5 different groups in such a way that there were no significant differences in the mean biochemical parameters between different treatment groups. Each group was given a different types of oral iron supplements, (Table-3.2). The supplements used differed from each other either in their elemental iron content or form of iron. The subjects were asked to take one capsule daily immediately after their meal (lunch or dinner). The supplements were given for 10 weeks. The supplements were distributed weekly. Baseline symptomology was recorded and any side effects reported by the subjects were also recorded. After the supplementation was over, a 10 ml. sample of blood was again drawn and all the biochemical measurements done earlier were repeated. The physical work capacity was also assessed after supplementation.

**TABLE 3.2 : COMPOSITION OF SUPPLEMENTS USED**

<table>
<thead>
<tr>
<th>Form of Iron</th>
<th>Subjects</th>
<th>Ferrous Calcium Citrate N=48</th>
<th>Ferrous Succinate N=46</th>
<th>Ferrous Fumarate N=41</th>
<th>Ferrous Sulphate N=43</th>
<th>Ferrous Fumarate N=45</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elemental Iron</td>
<td>25 mg</td>
<td>35 mg</td>
<td>50 mg</td>
<td>55 mg</td>
<td>115 mg</td>
<td></td>
</tr>
<tr>
<td>Folic Acid</td>
<td>0.3 mg</td>
<td>0.5 mg</td>
<td>0.15 mg</td>
<td>0.5 mg</td>
<td>1.5 mg</td>
<td></td>
</tr>
<tr>
<td>Vitamin B₁₂</td>
<td>1.0 mg</td>
<td>2.5 mg</td>
<td>10. mcg</td>
<td>-</td>
<td>15 mcg</td>
<td></td>
</tr>
<tr>
<td>Vitamin C</td>
<td>35 mg</td>
<td>25 mg</td>
<td>75 mg</td>
<td>-</td>
<td>0.15 g</td>
<td></td>
</tr>
<tr>
<td>Vitamin B₁</td>
<td>-</td>
<td>-</td>
<td>5 mg</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Vitamin B₂</td>
<td>2 mg</td>
<td>-</td>
<td>5 mg</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Vitamin B₆</td>
<td>1.0 mg</td>
<td>-</td>
<td>1.5 mg</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>-</td>
<td>15 mg</td>
<td>45 mg</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Calcium</td>
<td>85 (ca)</td>
<td>-</td>
<td>Panthothenate 5 mg</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Dried Yeast</td>
<td>-</td>
<td>-</td>
<td>25 mg</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>
G. STATISTICAL ANALYSIS

The data was analysed using, student's "t" test, ANOVA and chi-square test. Correlation coefficient between selected variables were also computed.