MATERIALS AND METHODS
To fulfill the objectives mentioned in the earlier section, various studies were planned and divided into three parts. These broadly consisted of water and food analysis, animal experimentation and human studies.

PART-I

1. Determination of iodine content from water samples as well as foodstuff and salt samples which are commonly available in Anand and Vallabh Vidyanagar.

2. Effect of storage on the iodine content of salt.

3. Effect of cooking on the iodine content of salt.

PART-II

Three animal experiments were planned to study the effect of dietary iodine on the growth and metabolic aspects of weanling animals. In the first experiment the effects of dietary iodine on the growth of weanling animals was studied. Further the effect of the goitrogen thiocyanate was also studied on these animals. On a long term basis the reproductive performance of these animals as well as the first generation of these animals was studied in the second experiment. Further the protective effect of various vitamins as antioxidants was studied against methimazole (an antithyroid drug used to produce hypothyroidism), the vitamins studied being vitamins C, E, A and β-Carotene in the third experiment.

PART-III

1. Iodine status in school children (6-12 years old, N=1596)

2. Iodine status in adult subjects (normal subjects and subjects suffering from hypothyroidism).

PART-I

The studies were carried out in two cities namely Anand and Vallabh Vidyanagar.
Collection of water samples

A total of twenty three water samples from different bore wells of Anand Municipality and Municipality Borough, Vallabh Vidyanagar were collected in previously washed, cleaned and dried polyethylene bottles. About 13 samples were collected from Anand and 10 samples from Vallabh Vidyanagar. These were from the different tube wells run by the Municipality / Municipal Borough and thus covered the total geographical area under each. Plastic bottles were used for sample collection. These were washed with diluted nitric acid, followed by tap water and then rinsed in distilled water and inverted to dry before use. Sample water was collected at source before which the bottles were rinsed thoroughly with the water to be collected as sample. Samples were collected from the running tube wells. The bottles were completely filled with water, capped tightly and labeled. The details of the site of collection and tube well were recorded. The containers after labeling were brought to the laboratory for laboratory investigations. Each water sample was analyzed in triplicate for iodine content using the iodide electrode method described later in this chapter.

Collection of food samples

Samples of foodstuff were collected from the local market of Vallabh Vidyanagar in plastic bags and duly labeled. Food samples purchased for analysis are listed in Table-7. The samples were then brought to the laboratory for laboratory analysis. Samples were analyzed for iodine content.

Preparation and storage of samples

Cereals and pulses were powdered individually in a Waring blender. Fruits and vegetables were cleaned, peeled and deseeded, if necessary and then crushed in a Waring blender. Three samples were weighed for each foodstuff for ashing purpose. Samples were analysed freshly as far as possible. Whenever crushed sample had to be stored, all dry powders were stored in clean, dry polyethylene bags while crushed fruits or vegetables were stored in bottles which were previously washed with concentrated nitric acid and rinsed thoroughly with
### Table-7: List of food samples analysed for iodine content.

<table>
<thead>
<tr>
<th>Sr.No</th>
<th>Foods</th>
<th>Scientific name</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td><strong>Cereals</strong></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Bajra (whole)</td>
<td>Pennisetum typhoideum</td>
</tr>
<tr>
<td>2</td>
<td>Wheat (whole)</td>
<td>Triticum aestivum</td>
</tr>
<tr>
<td>3</td>
<td>Rice (whole)</td>
<td>Oryza sativa</td>
</tr>
<tr>
<td>4</td>
<td>Jowar (sorghum) (whole)</td>
<td>Sorghum vulgare</td>
</tr>
<tr>
<td>5</td>
<td>Maize (whole)</td>
<td>Zea mays</td>
</tr>
<tr>
<td>II</td>
<td><strong>Pulses</strong></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Green gram (whole)</td>
<td>Phaseolus aureus Roxb.</td>
</tr>
<tr>
<td>2</td>
<td>Cow pea (whole)</td>
<td>Vigna catjang</td>
</tr>
<tr>
<td>3</td>
<td>Moth bean (whole)</td>
<td>Phaseolus aconitifolius Jacq.</td>
</tr>
<tr>
<td>4</td>
<td>Red gram (tender)</td>
<td>Cajanus cajan</td>
</tr>
<tr>
<td>5</td>
<td>Bengal gram (whole)</td>
<td>Cicer arietinum</td>
</tr>
<tr>
<td>6</td>
<td>Bengal gram (Kabuli)</td>
<td>Cicer arietinum</td>
</tr>
<tr>
<td>7</td>
<td>Peas (whole)</td>
<td>Pisum sativum</td>
</tr>
<tr>
<td>8</td>
<td>Field bean (whole)</td>
<td>Dolichos lablab</td>
</tr>
<tr>
<td>9</td>
<td>Raj mah (whole)</td>
<td>Phaseolus vulgaris</td>
</tr>
<tr>
<td>10</td>
<td>Soya bean (whole)</td>
<td>Glycine max Merr.</td>
</tr>
<tr>
<td>III</td>
<td><strong>Milk powder</strong></td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td><strong>Vegetables</strong></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Brinjal</td>
<td>Solanum melongena</td>
</tr>
<tr>
<td>2</td>
<td>Bottle gourd*</td>
<td>Lagenaria vulgaris</td>
</tr>
<tr>
<td>3</td>
<td>Tomato (Ripe)</td>
<td>Lycopersicon esculentum</td>
</tr>
<tr>
<td>4</td>
<td>Cabbage</td>
<td>Brassica oleracea var. capitata</td>
</tr>
<tr>
<td>5</td>
<td>Cauliflower</td>
<td>Brassica oleracea var. botrytis</td>
</tr>
<tr>
<td>6</td>
<td>Potato</td>
<td>Solanum tuberosum</td>
</tr>
<tr>
<td>7</td>
<td>Onion</td>
<td>Allium cepa</td>
</tr>
<tr>
<td>8</td>
<td>Cow pea pods</td>
<td>Vigna catjang</td>
</tr>
<tr>
<td>9</td>
<td>Cluster beans</td>
<td>Cyamopsis tetragonoloba</td>
</tr>
<tr>
<td>10</td>
<td>Tinda</td>
<td>Citrullus vulgaris</td>
</tr>
<tr>
<td>11</td>
<td>Broad bean</td>
<td>Vicia faba</td>
</tr>
<tr>
<td>12</td>
<td>Ladies finger</td>
<td>Abelmoschus esculentus</td>
</tr>
<tr>
<td>13</td>
<td>Bitter gourd**</td>
<td>Momordica charantia</td>
</tr>
<tr>
<td>V</td>
<td><strong>Fruits</strong></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Pomegranate*</td>
<td>Punica granatum</td>
</tr>
<tr>
<td>2</td>
<td>Sapota **</td>
<td>Achras sapota</td>
</tr>
<tr>
<td>3</td>
<td>Apple</td>
<td>Malus sylvestris</td>
</tr>
<tr>
<td>4</td>
<td>Plum</td>
<td>Prunus domestica</td>
</tr>
<tr>
<td>5</td>
<td>Guava</td>
<td>Psidium guajava</td>
</tr>
<tr>
<td>6</td>
<td>Orange *</td>
<td>Citrus aurantium</td>
</tr>
<tr>
<td>7</td>
<td>Mango **</td>
<td>Magnifera indica</td>
</tr>
<tr>
<td>8</td>
<td>Papaya **</td>
<td>Carica papaya</td>
</tr>
<tr>
<td>9</td>
<td>Banana *</td>
<td>Musa paradisiaca</td>
</tr>
<tr>
<td>10</td>
<td>Pine apple**</td>
<td>Ananas comosus</td>
</tr>
</tbody>
</table>

Samples were purchased once from the local market.

* Peeled before blending
** Peeled and deseeded before crushing.
distilled water before use. Iodine content was estimated by the colorimetric method (Moxon and Dixon, 1980) and is described later in this chapter.

Collection of salt samples

Ten (10) varieties of salt samples were collected from the local market (shopkeepers) for iodine estimation. Brands included were Tata salt, Nature salt, Dandi salt, Saffola salt, Shudh salt, Annapurna salt, Captain Cook salt, Dharati salt, Amrut salt and Crystal salt which was an unbranded, crystalline salt. Salt samples were stored in a labeled glass jar. The details of the samples were recorded and iodine estimation was carried out from fresh samples and from stored samples using the titrametric method (DeMaeyer et al, 1979).

(The various market brands [9 in no.] and the natural crystalline salt [1 in no.] purchased from the market were coded as follows: A. Tata salt, B. Nature salt, C. Dandi salt, D. Saffola salt, E. Shudh salt, F. Annapurna salt, G. Captain Cook salt, H. Dharati salt, I. Amrut salt and J. Crystalline salt.)

(i) Storage loss of iodine from salt

To observe the loss of iodine content from the different varieties of salt, monthly loss of iodine content from salt samples during storage were studied. The experiment was carried out for a period of 12 months at one-month intervals. All the ten samples were purchased from the retail market within one month of manufacture and stored at room temperature in glass bottles. Samples were weighed in triplicate on every occasion and used for the estimation of iodine content.

(ii) Cooking loss of iodine from salt

Salt samples were also tested for iodine loss during closed and open cooking. Three varieties of salt namely, Tata salt, Shudh salt, and Annapurna salt (commonly used by the local population) samples were used for the cooking procedure for different time periods i.e. 2, 5, 7, 10, 15, 20, 25 and 30 minutes, at boiling temperature. Triplicate samples (10 g) were cooked with 50 ml double
distilled water, in stainless steel vessels with and without lids. After this iodine content of samples were estimated by using the titrimetric method (DeMaeyer et al, 1979).

PART- II

The present series of investigations (3 in number) were attempted to explore the effects of different dietary levels of iodine on the developmental, metabolic, and teratogenic aspects of albino rats. Two of the animal experiments were planned and conducted with deficient, normal and excess iodine in the diets with and without potassium thiocyanate on weanling and adult animals. Reproductive performance was also assessed. A third experiment was also planned to observe the effect of antioxidants on hypothyroidism-induced rats. For all the three experiments the basal diet was a cornstarch based 10 % casein protein diet supplemented with salt mixture, vitamin mixture and vitaminised oil. This is described below.

Preparation of basal diet

Basal diet was prepared by mixing cornstarch and casein obtained from the local market (Darshan Chemicals, Vallabh Vidyanagar). Casein was found to contain 86 g % protein; therefore 11.627 g of casein was mixed with 71.322 g of starch to obtain 10 g % protein in the mixture. The diet was supplemented with salt mixture, vitamin mixture and vitaminised oil at the time of feeding. The composition of basal diet is given in Table-8. Salt mixture (Table-9) was added at 3.5 % level and vitamin mixture (Table-10) was added at 1% level, prepared according to AIN-93G-Growth Diet for Rodents prescribed by the American Institute of Nutrition. Vitaminised groundnut oil (Table-11) was prepared according to AIN-93G and added at 7% level.

Animals were housed individually in previously cleaned, galvanized iron cages. Food and water for drinking were given ad libitum throughout the experimental period. Animals were weighed initially and thereafter once in a week until the feeding period, using a triple beam balance (Ohaus, USA).
### Table-8: Composition of basal diet

<table>
<thead>
<tr>
<th>Sr.No</th>
<th>Ingredients (g)</th>
<th>(g/100 g diet)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Corn starch</td>
<td>71.322</td>
</tr>
<tr>
<td>2.</td>
<td>Casein (86 % protein)</td>
<td>11.627</td>
</tr>
<tr>
<td>3.</td>
<td>Oil</td>
<td>7.000</td>
</tr>
<tr>
<td>4.</td>
<td>Fiber (cellulose)</td>
<td>5.000</td>
</tr>
<tr>
<td>5.</td>
<td>Mineral mix (AIN-93G-MX)</td>
<td>3.500</td>
</tr>
<tr>
<td>6.</td>
<td>Vitamin mix (AIN-93G-MX)</td>
<td>1.000</td>
</tr>
<tr>
<td>7.</td>
<td>L-cystine</td>
<td>0.300</td>
</tr>
<tr>
<td>8.</td>
<td>Choline Bitartarate (41.1 % choline)</td>
<td>0.250</td>
</tr>
<tr>
<td>9.</td>
<td>Tert-butylhydroquinone</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td><strong>Total (g diet)</strong></td>
<td><strong>100.0</strong></td>
</tr>
</tbody>
</table>

Source: AIN-93 G. The Growth Diet for Rodents recommended by the American Institute of Nutrition. It is modified from a previous version of AIN-76 A.
Table-9: Composition of mineral mixture

<table>
<thead>
<tr>
<th>MINERAL</th>
<th>g / kg.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium carbonate</td>
<td>357.00</td>
</tr>
<tr>
<td>Potassium phosphate</td>
<td>196.00</td>
</tr>
<tr>
<td>Potassium citrate</td>
<td>70.78</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>74.00</td>
</tr>
<tr>
<td>Potassium sulphate</td>
<td>46.60</td>
</tr>
<tr>
<td>Magnesium oxide</td>
<td>24.00</td>
</tr>
<tr>
<td>Ferric citrate</td>
<td>6.06</td>
</tr>
<tr>
<td>Zinc carbonate</td>
<td>1.65</td>
</tr>
<tr>
<td>Magnesium carbonate</td>
<td>0.63</td>
</tr>
<tr>
<td>Cupric carbonate</td>
<td>0.30</td>
</tr>
<tr>
<td>Potassium iodate</td>
<td>0.01</td>
</tr>
<tr>
<td>Sodium selenate, anhydrous</td>
<td>0.01025</td>
</tr>
<tr>
<td>Ammonium paramolybdate</td>
<td>0.00795</td>
</tr>
</tbody>
</table>

POTENTIALLY BENEFICIAL MINERAL ELEMENTS

<table>
<thead>
<tr>
<th>MINERAL</th>
<th>g / kg.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium metasilicate</td>
<td>1.4500</td>
</tr>
<tr>
<td>Chromium potassium sulphate</td>
<td>0.2750</td>
</tr>
<tr>
<td>Lithium chloride</td>
<td>0.0174</td>
</tr>
<tr>
<td>Boric acid</td>
<td>0.0815</td>
</tr>
<tr>
<td>Sodium fluoride</td>
<td>0.0635</td>
</tr>
<tr>
<td>Nickel carbonate</td>
<td>0.0318</td>
</tr>
<tr>
<td>Ammonium vanadate</td>
<td>0.0066</td>
</tr>
<tr>
<td>Powdered sucrose</td>
<td>221.026</td>
</tr>
<tr>
<td>Total</td>
<td>1000.00</td>
</tr>
</tbody>
</table>

Source: AIN 93 G-MX Mineral Mix.

- 3.5 g of salt mixture was added per 100 g diet.
- Choline Chloride: 100 g of choline chloride was dissolved in 1000 ml of distilled water out of which 10 ml per 100 g diet was given daily.
### Table-10: Composition of vitamin mixture

<table>
<thead>
<tr>
<th>VITAMINS*</th>
<th>(g / kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotinic acid</td>
<td>3.00</td>
</tr>
<tr>
<td>Calcium pantothenate</td>
<td>1.60</td>
</tr>
<tr>
<td>Pyridoxine HCl</td>
<td>0.70</td>
</tr>
<tr>
<td>Thiamine HCl</td>
<td>0.60</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>0.60</td>
</tr>
<tr>
<td>Powdered sucrose</td>
<td>974.655</td>
</tr>
</tbody>
</table>

**VITAMINS IN WATER**

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>(g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Folic acid</td>
<td>0.20</td>
</tr>
<tr>
<td>D. Biotin</td>
<td>0.02</td>
</tr>
<tr>
<td>Vitamin B₁₂ (0.1% in mannitol)</td>
<td>2.50</td>
</tr>
</tbody>
</table>

Source: AIN 93 G.

* 1 g of vitamin mixture was added per 100 g diet.

** Vitamins in water were prepared and used as follows:

200 mg Folic acid, 20 mg D-Biotin and 2.5 mg Vitamin-B₁₂ were dissolved in 1000 ml of distilled water out of which 1 ml solution was directly mixed per 100 g diet.
**Table-11: Composition of vitaminised oil**

<table>
<thead>
<tr>
<th>Vitamins in Oil*(stock)</th>
<th>g / liter oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin E (all-rac-alpha-tocopheryl-tocopherol-acetate) 500 I.U / g.</td>
<td>15.00</td>
</tr>
<tr>
<td>Vitamin A (all-trans retinly-palmitate) 500000 I.U / g.</td>
<td>0.800</td>
</tr>
<tr>
<td>Vitamin D₃ (400000 I.U / g.)</td>
<td>0.250</td>
</tr>
<tr>
<td>Vitamin K (menadione)</td>
<td>0.075</td>
</tr>
<tr>
<td>Groundnut oil (to make up to)</td>
<td>1.000</td>
</tr>
</tbody>
</table>

Source: AIN- 93 –Vx Vitamin Mix

* Vitaminised oil was prepared as follows:

14 mg of TBHQ (Tert Butyl Hydroxyquinone) was added as an antioxidant per one litre vitaminised oil. From this 1 ml of stock vitaminised oil was mixed with 6 ml of groundnut oil and used as working.
EXPERIMENTAL PLANS

EXPERIMENT-1

This experiment was divided into two phases.

PHASE-1  Growth study

The present study was planned to determine the effect of different dietary levels of iodine with or without goitrogen on the growth of weanling albino rats for six weeks.

Thirty-two albino rats weighing about 48-72 g (mean wt. 60g) were grouped into four groups, consisting of eight animals in each group (6 females and 2 males). Group-I was fed a low iodine diet (LID), Group-II was fed a normal iodine diet (NID), Group-III was fed a normal iodine diet (NID) along with potassium thiocyanate (goitrogen) and Group-IV was fed a high iodine diet (HID). Composition of diets fed is given in Table-12. Animals were fed the respective diets for a period of six weeks. Figure-8 shows the division of groups. Weekly diet and iodine intake as well as change in body weight were recorded.

PHASE-2  Generation study

After six weeks of the growth period (as mentioned above), animals were continued on the same respective diets for another 31 weeks (total period 37 weeks). During the period of thirty-seven weeks, the animals were bred three times to observe their reproductive performance, after which they were sacrificed. Parameters assessed include organ weights, blood haemoglobin, serum glucose, enzymes, lipid profile, T₃, T₄ and TSH. Figure-9 shows the details more clearly.

EXPERIMENT-2

The progeny obtained from experiment-1 (first generation) were again taken for the next experiment conducted to assess the growth, reproductive performance and tissue hormonal levels. Twenty-eight albino rats weighing about 49
Figure-8: Experimental design for rat experiment –1.

Phase I

Growth study
32 Animals
(mean weight 60 g)

- Group-I
  LID
  (6F+2M)
- Group-II
  NID
  (6F+2M)
- Group-III
  NID+Golitrogen
  (6F+2M)
- Group-IV
  HIG
  (6F+2M)

Feeding period six weeks

Phase II

Generation study
(32 Animals)

1st mating after
12 weeks of

- 4 females from G-II conceived and delivered
- 2 nd mating after 22 weeks of feeding
- 3 females from G-II conceived and delivered
- 3 rd mating after 31 weeks of feeding

Sacrificed after 37 weeks of feeding

Taken for Experiment-II

Parameters
- Body weight g
- Diet intake g
- Protein intake g
- Iodine intake ug

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Pups</th>
<th>No. of pups</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-I</td>
<td>12</td>
<td>26</td>
</tr>
<tr>
<td>G-II</td>
<td>24</td>
<td>31</td>
</tr>
</tbody>
</table>

Parameters

Physical
- Diet intake
- Body weight
- Liver weight
- Kidney weight
- Thyroid weight

Biochemical
- Blood Hemoglobin
- Serum Protein, ALP, LDH
- T3, T4, TSH
- Cholesterol

Triglyceride
HDL
Liver Protein
Liver MDA
Table-12: Composition of control and experimental diets containing different levels of iodine.

<table>
<thead>
<tr>
<th>Sr.No</th>
<th>Ingredients (g / 100 g diet)</th>
<th>Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>I (LID)</td>
</tr>
<tr>
<td>1.</td>
<td>Corn starch</td>
<td>71.322</td>
</tr>
<tr>
<td>2.</td>
<td>Casein (86 % protein)</td>
<td>11.627</td>
</tr>
<tr>
<td>3.</td>
<td>Oil (ground nut)</td>
<td>7.000</td>
</tr>
<tr>
<td>4.</td>
<td>Fiber (Cellulose)</td>
<td>5.000</td>
</tr>
<tr>
<td>6.</td>
<td>Vitamin mix (AIN-93G-MX)</td>
<td>1.000</td>
</tr>
<tr>
<td>7.</td>
<td>L-cystine</td>
<td>0.300</td>
</tr>
<tr>
<td>8.</td>
<td>Choline Bitartarate (41.1 % choline)</td>
<td>0.250</td>
</tr>
<tr>
<td>9.</td>
<td>Tert-butylhydroquinone*</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Total (g diet)</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>Iodine (µg/100 g diet)</td>
<td>4.00</td>
</tr>
<tr>
<td></td>
<td>Potassium thiocyanate (mg/100g diet)</td>
<td>0.00</td>
</tr>
</tbody>
</table>

(n=6 females+2 males)
* Added to the stock vitaminised oil.
- Group – I Low iodine diet (4 µg/100 g diet).
- Group – II Normal iodine diet (20 µg/100 g diet).
- Group – III Normal iodine diet (20 µg/100 g diet) + goitrogen potassium thiocyanate (250mg/100 g diet)
- Group – IV High iodine diet (100 µg/100 g diet).

Iodine was added to the diet in the form of KI while thiocyanate, a goitrogen was added as potassium thiocyanate in double distilled water. Both were separately dissolved and stored in a plastic container at room temperature. They were added to the diet according to the experimental protocol at the time of feeding.
35-40 g (mean wt. 40 g) were grouped into four groups, consisting of seven animals (5 females and 2 males) in each group. All the four groups were fed the respective diets for a period of 31 weeks in the same manner as mentioned earlier in experiment-1. Animals were bred twice, at 12 weeks and at 22 weeks, to obtain progeny and to observe the reproductive performance, after which they were sacrificed. The composition of diets fed is the same as that fed in experiment-1 (Table-12). Figure-9 shows the experimental details more clearly. Parameters are also described at the end of experiment-3.

EXPERIMENT-3.

A total of thirty (30) male albino rats weighing about 150–185 g were fed the control and experimental diets for 6 weeks to observe the protective effect of vitamin C, vitamin A, vitamin E, and β-Carotene on methimazole (MMI) induced hypothyroidism. Group-I was fed the control diet (normal iodine), Group-II was fed the control diet along with MMI, Group-III was fed the control diet along with methimazole plus vitamin C, Group-IV was fed the control diet along with methimazole plus vitamin E, Group-V was fed the control diet along with methimazole plus vitamin A, and Group-VI was fed the control diet along with methimazole plus β-Carotene. Composition of diets fed is given in Table-13. Figure-10 shows the experimental details more clearly. Animals were fed the control and experiment diets for 6 weeks after which they were sacrificed.
Figure-9: Experimental design for rat experiment -2.

28 Animals
From 1st generation of Experiment-1
(7 animal in each group, 5 F+2 M, mean weight 40 g)

Group-I
LID
(5F+2M)

Group-II
NID
(5F+2M)

Group-III
NID+Goitrogen
(5F+2M)

Group-IV
HIG
(5F+2M)

Animals taken for Generation study

1st mating after
12 weeks of feeding

3 females from G-II
conceived and
delivered

2nd mating after
22 weeks of feeding

3 females from G-II
conceived and
delivered

Sacrificed
after 31 weeks
of feeding

Parameters

Physical
Body weight
Liver weight
Kidney weight
Thyroid weight

Biochemical
Blood Hemoglobin
Serum Protein
T3, T4, TSH,
Cholesterol
ALP, LDH

Triglyceride
HDL
Liver Protein
Liver MDA
Figure-10: Experimental design for rat experiment-3.

30 albino male rats
(mean Weight 167.00 g)

Group-I
Control diet (10% Casein protein diet)

Groups-II
Control diet + MMI (1 g/kg diet)

Group-III
Control diet + MMI (1 g/kg diet) + vitamin C (2.5 g/kg diet)

Group-IV
Control diet + MMI (1 g/kg diet) + vitamin E (2.5 g/kg diet)

Group-V
Control diet + MMI (1 g/kg diet) + vitamin A (80 mg/kg diet)

Group-VI
Control diet + MMI (1 g/kg diet) + β-carotene (24 mg/kg diet)

Experimental feeding period (six weeks)

Sacrificed all animals

<table>
<thead>
<tr>
<th>Physical</th>
<th>Biochemical</th>
<th>Liver MDA</th>
<th>Liver protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight</td>
<td>Blood Heamoglobin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver weight</td>
<td>Serum Protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidney weight</td>
<td>T3, T4, TSH,</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thyroid weight</td>
<td>Cholesterol</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Triglyceride</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HDL</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Serum vitamin C, E and A</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Liver vitamin C, E and A</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table-13: Composition of diets containing normal dietary iodine levels along with different antioxidants.

<table>
<thead>
<tr>
<th>Sr. no</th>
<th>Ingredients (g / 100 g diet)</th>
<th>Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>I</td>
</tr>
<tr>
<td>1</td>
<td>Corn starch</td>
<td>71.322</td>
</tr>
<tr>
<td>2</td>
<td>Casein (86 % protein)</td>
<td>11.627</td>
</tr>
<tr>
<td>3</td>
<td>Oil</td>
<td>7.000</td>
</tr>
<tr>
<td>4</td>
<td>Fiber(cellulose)</td>
<td>5.000</td>
</tr>
<tr>
<td>6</td>
<td>Vitamin mix (AIN-93G-MX)*</td>
<td>1.000</td>
</tr>
<tr>
<td>7</td>
<td>L-cystine</td>
<td>0.300</td>
</tr>
<tr>
<td>8</td>
<td>Choline Bitartrate (41.1% choline)</td>
<td>0.250</td>
</tr>
<tr>
<td>9</td>
<td>Tert-butylhydroquinone</td>
<td>0.001</td>
</tr>
<tr>
<td>10</td>
<td>MMI (0.1 g/ 100 g diet)</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>Vitamin C (0.25g/ 100 g diet)</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>Vitamin E (0.25g/1000 g diet)</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>Vitamin A (8mg/100 g diet)</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>β-Carotene (2.4mg/ 100g diet)</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>100.0</td>
</tr>
</tbody>
</table>

- Note: Methimazole and antioxidants added in the experimental diets and mixed well before feeding.
- Group – I Control diet
- Group – II Control diet + MMI (1 g/ kg diet)*
- Group – III Control diet + MMI (1 g/ kg diet) + Vitamin C* (2.5 g/kg diet).
- Group – IV Control diet + MMI (1 g/ kg diet) + Vitamin E** (2.5 g/kg diet).
- Group – V Control diet + MMI (1 g/ kg diet) + Vitamin A*** (80 mg/kg diet).
- Group – VI Control diet + MMI (1 g/ kg diet) + β-Carotene*** (24 mg/kg diet).
- * Source: AIN 93 G.(added ten times higher than the RDA to the vitaminized oils).
Food intake

The animals were served known amount of diet daily, 8-10 ml of water was added to every 10 g of diet to make it edible. The left over diet was removed everyday, collected in brown paper bags and dried in an oven at 60°C temperature to make it moisture free. After drying it was weighed to calculate the actual food intake.

Food consumed g per week = Food given – (left over in the bowl + spilled food)

Autopsy procedure

At the end of each experimental period, the animals (fasted for 12 hour) were weighed and sacrificed under mild anesthesia. Blood was collected from the heart directly into cleaned, dried and heparinised tubes and was used for estimation of haemoglobin and glucose content. The remaining blood was collected in a clean tube and allowed to clot. The clot was removed and the remaining serum was centrifuged under refrigeration for obtaining clear serum, which was stored at −20°C temperature until it was analyzed.

Tissue processing

Tissues were excised and blotted free of blood and tissue fluids on a filter paper, cleaned off extraneous tissue and weighed on a previously weighed aluminum foil. A known amount of liver was homogenized in a glass-teflon homogenizing tube with cold phosphate buffer (pH 8.0) using a Eltek Homogenizer, Electro Craft Bombay, and volume was made up to obtain 10 % (w/v). The tissue homogenate was used for liver protein and malondialdehyde (MDA) estimation. Thyroid tissue were stored in formaline solution and used for histopathological study. Histopathological examination of the thyroid glands was carried out at the Department of Anatomy, College of Veterinary science and Animal Husbandry, AAU, Anand. The method used was as given by Humason,(1979).
Parameters studied

The parameters (common for all the 3 experiments) analyzed and recorded during the entire period of study are as follows:

<table>
<thead>
<tr>
<th>Physical</th>
<th>Biochemical analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Body weight</td>
<td>I. Blood</td>
</tr>
<tr>
<td>2. Food intake</td>
<td>1. Haemoglobin</td>
</tr>
<tr>
<td>3. Protein intake</td>
<td>2. Blood glucose</td>
</tr>
<tr>
<td>4. Iodine intake</td>
<td>II. Serum</td>
</tr>
<tr>
<td>5. Liver fresh weight</td>
<td>1. Total cholesterol</td>
</tr>
<tr>
<td>6. Kidney fresh weight</td>
<td>2. Triglyceride</td>
</tr>
<tr>
<td>7. Thyroid fresh weight</td>
<td>3. HDL</td>
</tr>
<tr>
<td>8. Liver body weight Ratio</td>
<td>6. LDH (LDH, EC: 1.1.1.27) **</td>
</tr>
<tr>
<td>10. Thyroid body weight Ratio</td>
<td>8. ALP (ALP, EC: 3.1.3.1) **</td>
</tr>
<tr>
<td></td>
<td>9. Thyroxine (T4)</td>
</tr>
<tr>
<td></td>
<td>10. Triiodothyronine (T3)</td>
</tr>
<tr>
<td></td>
<td>11. Thyrotropin (TSH)</td>
</tr>
<tr>
<td></td>
<td>12. Vitamin A *</td>
</tr>
<tr>
<td></td>
<td>13. Vitamin C *</td>
</tr>
<tr>
<td></td>
<td>14. Vitamin E *</td>
</tr>
<tr>
<td></td>
<td>III. Liver</td>
</tr>
<tr>
<td></td>
<td>1. Liver moisture</td>
</tr>
<tr>
<td></td>
<td>2. Liver protein</td>
</tr>
<tr>
<td></td>
<td>3. Liver MDA</td>
</tr>
<tr>
<td></td>
<td>4. Vitamin A *</td>
</tr>
<tr>
<td></td>
<td>5. Vitamin C *</td>
</tr>
<tr>
<td></td>
<td>6. Vitamin E *</td>
</tr>
<tr>
<td></td>
<td>IV. Kidney</td>
</tr>
<tr>
<td></td>
<td>1. Kidney moisture</td>
</tr>
<tr>
<td></td>
<td>V. Thyroid</td>
</tr>
<tr>
<td></td>
<td>1. Histopathology</td>
</tr>
</tbody>
</table>

* Serum and Liver Vitamin A, C, E were estimated in experiment-3.
** Serum LDH and ALP enzyme activity were estimated in experiment -1 and 2.
PART-III

1. Iodine status in school children (6-12 year old).

2. Iodine status in adult subjects (normal subjects and subjects suffering from hypothyroidism).

1. Iodine status in school children

Area of study

This part of the study was carried out among school children (6-12 years) in the semi-urban area of Vallabh Vidyanagar. (Figure-11 shows the experimental details more clearly)

Sample size

Considering a goiter prevalence of 20 %, 95 % confidence limit, 20 % relative precision of estimate, the sample size required was calculated as follows:

Anticipated prevalence of goiter (p) = level of significance (Z) 95 % i.e. 1.96, relative precision i.e. 20 % (L= allowable error 20 %), q = 1- P

Formula for sample size calculation (N) = \( \frac{(Z)^2 \times p \times q}{(L)^2} \)

That is sample size (N) = \( \frac{(1.96)^2 \times 0.20 \times 0.80}{(0.20)^2} \approx 1600 \)

Sampling design

Out of the prevailing nine schools in Vallabh Vidyanagar, three schools were randomly selected for the study. From these, a total 1596 children, 6-12 years old, consisting of 818 boys and 778 girls were covered for prevalence of clinical signs (by palpation) of iodine deficiency disorder.

Anthropometric measurement on every 4\textsuperscript{th} child (n=402) was conducted in terms of height and weight. Method used was as given by WHO (1995). In addition, salt samples used for cooking were collected from the households and
Figure-11: Experimental design for school survey

Schools of Vallabh Vidyanagar town (n=9)

Three schools selected randomly

6-12 year old children from each school were selected (n=1596, B-818 and G-778).

Clinical examination of IDD (n=1596)

Iodine content of household salt, Anthropometric measurements and urinary iodine content on every 4th children (n=402)

Subjects with positive clinical iodine deficiency (n=96, 47 m and 49 f)

Demographic survey on SES

Awareness of mothers on IDD, use of I.salt, type of salt purchase

Knowledge of shop keepers on selin, storage and packing of salt

Iodine content of salt consumed by H.H.

Urinary iodine estimation of IDD subjects
tested for iodine content by the titration method. Urinary iodine excretion level was also conducted on these children.

From the 1596 children studied for clinical prevalence of IDD 96 children (46 boys and 50 girls) with positive clinical signs of iodine deficiency disorder (as per the standard given by WHO, 2001) were enrolled.

(i) The household of children suffering from clinical iodine deficiency disorder \(n=96\) was covered first. Demographic and socioeconomic particulars were collected from the households of these children using the questionnaire method (Appendix-1). Frequency of consumption of goitrogenic food was also collected (Appendix-I). Awareness on iodine deficiency disorder and use of iodized salt was collected from the mothers of children with positive clinical signs of IDD. (Appendix-II).

(ii) Estimation of urinary iodine level was carried out from these 96 clinically identified cases of goitre for confirmation of iodine deficiency in them using the method of Dunn (1993).

Selection of shops for awareness study

The particulars such as availability and extent of sale of iodized salt in the shops were assessed in 46 shops in the surrounding areas of each school, which was surveyed. Using a questionnaire (Appendix –III) through interview method collected the knowledge levels of the shopkeepers on the selling, storage and packing of iodized and non-iodized salt.

2. Iodine status in the adult subjects

Area of study

In the present study investigations were carried out to explore the effect of iodine deficiency in human beings. Seventy nine (79) adult subjects (30 normal and 49 suffering from hypothyroidism) aged 30-70 years were studied from Anand and Vallabh Vidyanagar.
Sampling design

Purposive sampling method for carried out for subject selection in this study. Fasting blood and urine samples were collected from a total of 79 subjects for biochemical analysis. In addition a questionnaire was also filled up (Appendix-IV) from the subjects to assess the knowledge on IDD and the consumption pattern of goitrogenic foods (Appendix IV).

Parameters studied were as follows:

(i) Serum T3, T4 and TSH levels.

(ii) Urinary iodine excretion.

(iii) KAP on salt consumption (Appendix-IV).

(iv) Consumption pattern of goitrogenic foods (Appendix-IV).
METHODS USED IN THE EXPERIMENTAL STUDY ARE DESCRIBED BELOW

PART-1: Water food and salt analysis

(1) Iodine content from water (Electrode method)

Iodine content of water samples was estimated using the Model 9653 Ion plus Series Iodide Electrode. The method described by Thermo Orion (USA) in the instruction manual supplied along with iodide electrode was followed. Different standards of iodide were prepared by using the iodide stock (1000 ppm) supplied by Thermo Orion USA for calibration of the instrument as per the procedure given.

For this 50 ml of standard iodide solution of stock concentration was pipetted out into a polythene plastic beaker and to this added 1 ml of 5 Molar NaNO₃ (Ionic Strength Adjusstor, USA). The electrodes were placed into the beaker with continuous stirring and then after obtaining a stable reading, the instrument was calibrated for different concentrations of iodide.

50 ml of water sample was taken in a polyethylene beaker and added 1 ml of adjustor, then the iodine content was directly measured with the help of the iodide ion plus specific electrode (Orion USA) by using Orion 920A pH / MV meter. All the iodide measurement were carried out at constant temperature with continuous stirring using a magnetic stirrer. All the samples, standards and reagents were stored in plastic containers.

(2) Iodine content from food (colorimetric method)

Food iodine was measured using a semi-automatic method for the determination of total iodine in food described by Moxon and Dixon (1980).

Accurately 1 g of food sample containing iodine not exceeding 1 µg was taken in a clean, dry crucible (for samples with a high water content and a low iodine level, up to 10 g can be taken, provided that a white ash is obtained). To this added 1ml of 30% potassium carbonate solution and then 1ml of (10%) zinc sulphate solution. Slurried the mixture with a glass rod and washed any residue
left on the rod back into the crucible with a jet of distilled water. Next the crucibles were placed in a drying cabinet at 95°C until dry, preferably overnight. Covered the crucible with a lid and placed it in a muffle furnace at 100°C. Then the temperature was raised evenly to 550°C in approximately 90 minutes and this was maintained for 1 hour. Crucibles were then immediately removed and allowed to cool to room temperature.

One ml of 10 % zinc sulphate solution was added and slurried the charred residue. Repeated the drying and ashing procedure as before. The cooled ash was transferred (normally white or gray in color), to a centrifuge tube with 50 ± 0.5 ml of distilled water and tubes were spun at 50 Hz for 5 min. Decanted about half of the solution and stored in a clean polyethylene container before analysis. Three reagent blanks were also taken through the whole procedure.

Pipetted out 4 ml of each standard, sample and reagent blank solution into its respective 15X1.5cm test-tube and to this added 1 ml of distilled water, 1 ml of potassium thiocyanate (0.023%) solution and 2 ml of ammonium iron (III) sulphate reagent. The orange coloured solution in the tubes were mixed well on a vortex mixer. Exactly after a 90-second interval between each tube added 1 ml of sodium nitrite solution (2.07 %) and again mixed on a vortex mixer. Allowed to stand for 20 minutes. Measured the color at 450nm on a spectrophotometer, exactly at 90-second intervals between each tube. All the solutions are to be maintained at the same temperature in this estimation.

Calculation

A standard curve was calibrated from the absorbance values of the sample and blank solutions and iodine content calculated in ng /ml, from the calibration graph. Then iodine content of the samples were calculated using the following equation

Iodine content (µg/100 g) =[(A-B) X5] /W

59
Where A was the iodine content of the sample solution (ng/ml), B was the mean iodine content of the blank solution (ng/ml) and W was the amount of sample in grams.

(3) Iodine content from salt (Titrmetric method)

The iodine content of salt samples was measured using an iodometric titration, as described by DeMaeyer et al (1979).

10 g of the salt sample was weighed and transferred into a 250 ml Erlenmeyer flask with stopper. Approximately 30 ml of water was added, swirled to dissolve salt samples completely, and volume was made to 50 ml, with distilled water. 1 ml of 2N H$_2$SO$_4$ and 5 ml of 10 % KI were added. The solution should turn yellow if iodine (as iodate in salt) is present. Stoppered the flask and placed in the dark (cup-board or drawer) for 10 minutes. The flask was removed and it was titrated with (0.005M) Na$_2$S$_2$O$_3$ solution from the burette until the solution turned pale yellow, then 1-2 ml of starch indicator solution (solution in the flask should turn dark purple) was added and continued the titration until the solution became pink and finally colorless.

The level of thiosulphate was recorded in the burette and converted this volume to parts per million (ppm) of iodine using the conversion table. Analysis time was approximately 20 minutes per sample.

PART-II: Animal experiments 1,2, & 3

(A) Blood

(i) Haemoglobin

Haemoglobin content of the blood was estimated by the Cyanmethaemoglobin method using the Hemocor–D kit supplied by Crest Biosystems, Goa, India.

Twenty microlitres of blood was added to 5 ml of Hemocor–D Reagent (ready to use haemoglobin diluting reagent), mixed thoroughly and incubated at
room temperature (25°C) for at least 3 minutes. The colour developed was read at 540 nm against a blank that consisted of 5 ml Hemocor – D reagent.

**Calculation:** Haemoglobin g% = Sample OD x 36.8

(ii) **Blood glucose**

Glucose content of blood was determined by the glucose oxidase method given by Huggett and Nixon (1957).

Hundred microlitres of blood was added to 1 ml 0.05 M sodium hydroxide. To this 1.0 ml of zinc sulphate was added, mixed well, centrifuged at 3000 rpm for 10 minutes and supernatant collected. Two hundred microlitres of the supernatant was taken and 4.0 ml of enzyme dye reagent was added. Incubation was carried out at 37°C for 30 minutes. Colour developed was read at 430 nm against the blank.

**Blank:** Taken 1.1 ml of NaOH, and 1ml of ZnSO₄, mixed well and treated as for sample.

**Standard:** One Hundred and two hundred microlitres of glucose standard solution were taken (100 mg%) and final volume was made up to 1.1 ml with NaOH, then 1.0 ml of ZnSO₄ was added and treated as for sample.

**Calculation**

Glucose concentration mg % = Std. Conc./Std.OD X Sample OD

(B) **Serum analysis**

(1) **Triiodothyronine (T₃)**

The triiodothyronine (T₃) level of serum was estimated by the Enzyme Immunoassay (EIA) kit supplied by Mono bind, Inc. (U.S.A.). The assay is based on a competitive reaction principle, The assay was performed in two steps.
Step-1 Immunological

100 micro liters of serum was added to tubes coated with anti- $T_3$ antibodies. 200 micro liters of enzyme conjugate was added and allowed to react. The tubes were incubated for 1 hour at 37°C. Then the tubes were repeatedly washed with wash solution.

Step: 2 Enzymatic

To the tubes 200 microlitres substrates was added and incubated for 20 minutes at room temperature, on adding 800 microlitres stopping reagent a yellow color developed which was read at 405 nm.

Standard: (Range of standard series 0-750 ng per dl ) The standard supplied was treated as for sample.

Calculation: A standard curve was prepared by plotting the standard absorbance against the standard concentration. The sample absorbance was read off the concentration of triiodothyronine on the curve and expressed as ng per dl of serum.

(ii) Serum thyroxine ($T_4$)

Thyroxine ($T_4$) levels of serum was estimated by the Enzyme Immunoassay (EIS) kit supplied by Mono bind, Inc. (U.S.A.). The assay is based on a competitive reaction principle. The assay was performed in two steps.

Step-1 Immunological

50 microlitres of serum was added to tubes coated with anti- $T_4$ antibodies. 200 micro liters of enzyme conjugate was added and allowed to react. The tubes were incubated for 1 hour at 37°C. Then the tubes were repeatedly washed with wash solution.
Step - 2 Enzymatic

To the tubes 200 microlitres substrate was added and incubated for 20 minutes at room temperature, on adding 800 microlitres stopping reagent a yellow color developed which was read at 405 nm.

Standard: (Range of standard series 0-25 μg per dl) The standard supplied was treated the same as above.

Calculation: A standard curve was prepared by plotting the standard absorbance against the standard concentration. The sample absorbance was read off the concentration of Thyroxine on the curve and expressed as μg per dl of serum.

(iii) Serum thyrotropin (TSH)

Serum thyrotropin levels were estimated by the Enzyme Immunoassay (EIS) kit supplied by Mono bind, Inc. (U.S.A.). The assay was based on a competitive reaction principle. The assay was performed in two steps.

Step -1 Immunological

100 microlitres of serum was added to tubes coated with anti-TSH antibodies. 100 micro liters of enzyme conjugate was added and allowed to react. The tubes were incubated for 1 hour at 37°C. Then the tubes were repeatedly washed with wash solution.

Step- 2 Enzymatic

To the tubes 200 microlitres of substrate was added and incubated for 20 minutes at room temperature. On adding 800 microlitres stopping reagent a yellow color developed was which read at 405 nm.

Standard: Range of standard series 0-40 μIU per ml supplied was treated the same as above.
Calculation: A standard curve was prepared by plotting the standard absorbance against the standard concentration. The sample absorbance was read off the concentration of thyrotropin on the curve and expressed as μIU per ml of serum.

(iv) Serum total protein

Serum total protein was estimated by the Biuret method given by Reinhold (1953) using the standard kit (Everzem, provided by Eve's Inn Diagnostic, Baroda) with a little modification.

Three clean dry test tubes were taken and labeled as blank (B), standard (S) and test (T). 1 ml of Biuret reagent and 2 ml of distilled water were added to all the three test tubes. Exactly 0.05 ml serum was added to test (T), while 0.05 ml of 6 g% standard protein solution was added to standard (S) and 0.05 ml distilled water was added to blank (B). The contents were mixed well and the tubes were kept in the water bath for incubation at 37°C for 10 minutes. Absorbance of standard (S) and test (T) was measured against blank (B) on a spectrophotometer at 555 nm.

Standard: 6 g% protein standard provided in the kit was used as the standard.

Calculation

\[
\text{Plasma total protein (g%) = } \frac{\text{Conc. of Std.}}{\text{O.D. of Std.}} \times \text{O.D. of sample}
\]

(v) Alkaline phosphatase

Determination of alkaline phosphatase activity in serum was carried out by using the kit supplied by Crest Biosystems, Goa, India.

Four clean dry test tubes were labeled as blank (B), standard (S), control (C) and test (T). 1.05 ml of distilled water was added to the blank (B) and 1 ml of distilled water to all the remaining three test tubes. 1 ml of Buffer reagent and 0.1 ml of substrate reagent were added to all the four test tubes. The contents were mixed well, and allowed to stand in a water bath at 37°C for 3 minutes and 0.05
ml serum free from haemolysis was added to test (T) and 0.05 ml of 10 mg% phenol standard to standard (S). The contents were mixed well after each addition and allowed to stand at 37°C for 15 minutes. 1 ml of colour reagent was added in all the four test tubes and 0.05 ml of serum sample to control (C). Mixed the contents well. The absorbance of the blank (B), standard (S), control (C) and test (T) were measured against distilled water.

**Standard:** 10 mg% phenol provided in the kit was used as the standard.

**Calculation:** Alkaline Phosphatase activity (k A units) = \( \frac{\text{Abs.} \text{T} - \text{Abs.} \text{C}}{\text{Abs.} \text{S} - \text{Abs.} \text{B}} \times 10 \)

(vi) **LDH**

Determination of LDH activity in serum was carried out by the modified IFCC method, 1982, using the LDH kit supplied by Crest Biosystems, Goa, India.

Used a clean dry test tube, labeled it as test (T). To this added 1 ml of working reagent and incubated at 37°C temperature for 1 minute then added 0.02 ml serum sample, free from haemolysis. Mixed well and absorbance \( A_0 \) was read, initially after 1 minute and again after every one minute up to three minutes. Mean absorbance change per minute (\( \Delta A / \text{min} \)) was calculated.

**Calculations:** LDH activity in U/L = \( \Delta A / \text{min} \times 8095 \)

(vii) **Total cholesterol**

Serum total cholesterol was estimated by the CHOD / PAP method using the cholesterol kit supplied by Crest Biosystems, Goa, India.

Exactly 0.01 ml serum sample was mixed well with 1.0 ml of working reagent and incubated in a water bath at 37°C for 5 minutes or at room temperature (25°C) for 15 minutes. The colour developed was measured at 505 nm against a reagent blank within 30 minutes.
Blank: Working reagent 1 ml and 0.1 ml distilled water was mixed and treated as for sample.

Standard: 0.01 ml of 200 mg % cholesterol standard and 1 ml working reagent were mixed and treated as for the sample.

Calculation: Cholesterol mg% = \( \frac{\text{Sample O.D.}}{\text{Standard O.D.}} \times 200 \)

(viii) Serum triglycerides

Serum triglyceride was estimated by the GPO / PAP kit method, supplied by Crest Biosystems, Goa, India.

Exactly 0.01 ml serum sample was mixed well with 1.0 ml working reagent and incubated in the water bath at 37°C for 5 minutes or at room temperature (25°C) for 15 minutes. The colour developed was measured at 505 nm against a reagent blank within 60 minutes.

Blank: 1 ml working reagent was mixed with 0.01 ml distilled water and used as a blank and treated as for the sample.

Standard: 200 mg% triglycerides standard provided in the kit was used as the standard. Exactly 0.01 ml standard was mixed with 1 ml working reagent and treated as for the sample.

Calculation:

\[
\text{Triglycerides mg%} = \frac{\text{Sample O.D.}}{\text{Std.O.D.}} \times 200
\]

(ix) HDL Cholesterol

HDL cholesterol content of serum was estimated by PEG precipitation kit supplied by Crest Biosystems, Goa, India.
For precipitation of VLDL and LDL 0.1 ml precipitating reagent was pipetted into a clean dry tube, followed by 0.1 ml of sample. The contents were mixed well, incubated at room temperature for 5 minutes, and centrifuged at 4000 rpm. Then 0.05 ml of clear supernatant was mixed with 1 ml of working reagent (freshly prepared) and incubated in a water bath at 37°C for 5 minutes or at room temperature (25°C) for 15 minutes. The colour developed was read at 505nm against a reagent blank within 60 minutes.

**Blank**: 1 ml of working reagent and 0.05 ml distilled water were mixed and treated as for sample.

**Standard**: 0.05 ml 25 mg% HDL cholesterol standard and 1 ml of working reagent were mixed well and treated as for sample.

**Calculation**:

\[
\text{HDL cholesterol mg\%} = \frac{\text{Sample O.D.} \times 25 \times 2}{\text{Standard O.D.}}
\]

(2 is the dilution factor due to the deproteinisation step).

(x) **VLDL cholesterol**

VLDL cholesterol was calculated according to the formula given by Freidewald (1972).

\[
\text{VLDL cholesterol mg\%} = \frac{\text{Triglyceride}}{5}
\]

(xi) **LDL cholesterol**

LDL cholesterol was calculated according to the formula given by Freidewald (1972).

\[
\text{LDL Mg\%} = \text{Total Cholesterol} - (\text{VLDL} + \text{HDL})
\]
(xii) Serum vitamin E

Serum vitamin E was estimated by the method given by Emmerie and Engel (1938) modified by Desai (1986).

Exactly 0.2 ml of serum was pipetted out into a clean and dry test tube. To the tube, the same amount of 1% pyrogallol in ethanol was added. After equilibration for 2 minutes at 70° C, 0.05 ml of saturated KOH was added, followed by 2.0 ml of hexane. After 2.0 minutes of vigorous shaking followed by centrifugation at 1500 rpm for 5 minutes, the upper layer was carefully removed and a known amount evaporated to dryness under vacuum at 40° C.

The residue was dissolved in 1.0 ml of ethanol. The standards (1-4 µg) were also prepared in ethanol having 1.0 ml of final volume. To each tube added 0.2 ml of 0.2% bathophenanthroline reagent and the contents of the tubes were thoroughly mixed. The assay was processed very rapidly from this point onwards and unnecessary exposure to direct light was minimized. Added 0.2 ml of FeCl₃ reagent and vortex mixed. After 1 minute, 0.2 ml of orthophosphoric acid reagent was added thoroughly mixed and tubes were read at 536 nm against a blank containing all reagents except the sample or the standard.

**Calculation:** Serum vitamin E (mg%)

\[
\text{Serum vitamin E (mg\%)} = \frac{\text{Conc. of std.}}{\text{O.D. of std.}} \times \frac{\text{O.D. (s)}}{\text{Aliquot taken}} \times \frac{\text{Total volume of hexane}}{1.0} \times 100
\]

(xiii) Serum vitamin A

Serum vitamin A was estimated by the method given by Neeld and Pearson (1963).

Exactly 0.3 ml of serum and 0.3 ml of ethanol were mixed thoroughly and vitamin A was extracted with 2.0 ml of hexane. Mixture was centrifuged and 1.5 ml of supernatant was collected in a tube, evaporated to dryness under vacuum at 40° C and 20 microlitres of chloroform was added. Then 1.0 ml of chloroform-
TFA (2:1) reagent was added and the colour intensity was read at 620 nm within 30 seconds, against chloroform as the blank.

**Standard:** Standards were prepared from the working solution of retinol and treated as for sample.

**Calculation:** Serum vitamin A (µg %)

\[ \text{Serum vitamin A (µg %)} = \frac{\text{Conc. of std.} \times \text{O.D. of sample}}{\text{O.D. of STD.} \times \text{Aliquot taken}} \times \frac{\text{Total volume}}{0.2} \times 100 \]

(ivx) Serum vitamin C

Serum vitamin C was estimated by the method given by Roe and Kuether (1943) and Bessey et al. (1947).

Exactly 0.2 ml serum was mixed with 0.2 ml of chilled 10 % TCA, centrifuged at 5,000 rpm for 10 minutes, 0.2 ml of the supernatant was taken and volume made up to 0.5 ml with 5.0 % TCA and added 0.1 ml of 2,4-Dinitrophenylhydrazine-Thiourea - Copper (DTC) solution. The mixture was incubated at 37 °C for three hours, 0.75 ml of 65% H₂SO₄ added, mixed and was allowed to stand at room temperature for an additional 30 minutes. Absorbance was determined at 520 nm using blank.

**Standards :** (2-8µg) were prepared in 0.5 ml of 5 %TCA and analyzed as for sample.

**Blank:** Consisted of 0.5 ml of 5 %TCA and 0.1 ml of DTC solution, which was incubated for 3 hour. 0.75 ml of 65 % H₂SO₄ was added and mixed.

Serum vitamin C (mg%)

\[ \text{Serum vitamin C (mg%)} = \frac{\text{Conc. of std.} \times \text{O.D. (s)}}{\text{O.D. of std} \times \text{Aliquot}} \times \frac{\text{Total volume}}{1000} \times 100 \]
(C) LIVER ANALYSIS

(i) Moisture

Moisture content of the sample was determined by the evaporation (loss on drying) method according to AOAC (1984).

In an aluminum foil of known weight, about 1 g of liver was weighed and dried completely in a hot air oven at 60°C until the weight remained constant, assuming that the loss in weight of the sample on drying is due to the loss of moisture only. The moisture and total solids content of the sample per 100 g of tissue on fresh weight basis were calculated.

Calculation: Moisture g% = \( \frac{(\text{Initial wt.} - \text{Final wt.})}{\text{Initial wt.}} \times 100 \)

(ii) Protein

Protein content of liver was determined by the method of Lowry et al. (1951).

Sample Preparation: 10% liver homogenate was prepared in the phosphate buffer (pH 8), by homogenizing at 3000 rpm for 2 minutes in a Remi Homogenizer.

0.2 ml of the homogenate was precipitated with 1 ml of 10% TCA, mixed and centrifuged at 5000 rpm for 10 minutes. The supernatant was decanted and the precipitate dissolved in 10 ml of 0.1 N NaOH. 0.2 ml of the diluted solution was used for protein estimation. Total volume was made upto 0.6 ml with distilled water. To this 3.0 ml of Lowry C reagent was added and allowed to stand for 10 minutes at room temperature. Later 0.3 ml of Lowry E diluted reagent (Folisch reagent) was added and cyclomixed. Exactly after 30 minutes the colour developed was read at 750 nm against blank.

Blank: 0.6 ml of distilled water was treated as for sample.
**Standard** (200 µg / ml): A standard series was prepared having 40, 80, 120 and 160 µg per tube and treated as for sample.

**Calculation**

\[
\text{Liver protein:g}\% = \frac{\text{Conc. of Std.} \times \text{O.D. of Std}}{\text{O.D. of Std} \times \text{aliquot taken}} \times \frac{\text{Vol. made up}}{1 \times 1000 \times 1000}
\]

(iii) **Liver lipid peroxidation**

Liver lipid peroxidation was estimated by the method given by Ohkawa et al (1979).

Exactly 1 ml of 10% liver homogenate was added to 2 ml of TCA-TBA-HCL reagent and mixed thoroughly using a vortex mixture. The mixture was then heated in a water bath at 100°C for 15 minutes. After cooling with chilled water, the flocculent precipitate was removed by centrifugation at 1000g for 10 minutes. Pink colour supernatant or the organic layer was taken and absorbance was read at 532 nm against the blank that contains all the reagents minus the lipid.

The malondialdehyde concentration of the sample was calculated using the extinction co-efficient of 1.56x10⁵ M⁻¹cm⁻¹

(iv) **Liver vitamin E**

Liver vitamin E was estimated by the method given by Emmerie and Engel (1986) modified by Desai (1986).

1.0 ml of 10% liver homogenate was added to 2.0 ml of 1% pyrogallol in ethanol. After equilibration for 2 minutes at 70°C, 0.3 ml of saturated KOH was added and the resultant mixture was incubated for a further 30 minutes. After cooling in the ice, 1.0 ml distilled water was added, followed by 3.0 ml of hexane. After vigorous mixing for 2 minutes, centrifuged at 1500 rpm for 5 minutes. The
upper layer was carefully removed, and a known amount was evaporated to dryness under vacuum at 40° C. The residue was dissolved in 1.0 ml ethanol.

The Standards (1-4 µg) were also prepared in ethanol having a final volume of 1.0 ml. To each tube was added 0.2 ml of 0.2% bathophenanthroline reagent and the contents of the tubes were thoroughly mixed. The assay was processed very rapidly from this point onwards and unnecessary exposure to direct light was minimized. Added 0.2 ml of FeCl₃ reagent and vortex mixed. After 1 minute, 0.2 ml of orthophosphoric acid reagent was added, thoroughly mixed and tubes were read at 536 nm against blank containing all reagents except the sample or the standard.

**Calculation: Vitamin E (µg/g)**

\[
\text{Vitamin E (µg/g)} = \text{Conc. of std.} \times \frac{\text{O.D. (s)}}{\text{O.D. of std.}} \times \text{Total volume of hexane} \times \frac{10}{\text{1.0}}
\]

**(v) Liver vitamin A**

Liver vitamin A was estimated using the method of Neeld and Pearson (1963).

Frozen liver samples (0.5-1.5g) were brought to room temperature and saponified for 45 minutes in a boiling water bath with 10 ml of 5 % KOH. Then the tube was cooled in ice and the volume was made up to 25.0 ml with 5% KOH.

5.0 ml of sample was taken in a tube to which 2.0 ml ethanol was added and mixed. Vitamin A was extracted with 3.0 ml of hexane. Mixture was centrifuged at 1500 rpm for 5 minutes. Two ml of supernatant was transferred to another tube and evaporated to dryness under vacuum at 40° C. Rest of the steps followed were similar to that for plasma vitamin A.

**Calculation: Vitamin A (µg/g)**

\[
\text{Vitamin A (µg/g)} = \text{Conc. of std.} \times \frac{\text{O.D. (s)}}{\text{O.D. of std.}} \times \text{Total volume of hexane} \times \frac{25}{\text{5.0 Liver wt.}}
\]
Liver vitamin C was assayed by the method given by Roe and Kuether (1943) and Bessey et al (1947).

One ml of 10 % liver homogenate was added to 2.0 ml of chilled 5 % TCA, mixed and centrifuged at 5,000 rpm for 10 minutes.

Exactly 0.2 ml of supernatant was taken and the volume was made up to 0.5 ml with 5% TCA. This was mixed with 0.1 ml of DTC solution and incubated for 3 hour at 37° C. 0.75 ml of ice-cold 65% H2SO4 was added and mixed well. The solution was allowed to stand at room temperature for an additional 30 minutes. Absorbance was determined at 520 nm against the blank consisting of 0.5 ml of 5 % TCA, 0.1 ml DTC and 0.75 ml of 65% H2SO4.

Standards (2-8 µg): were also prepared as above.

Calculation: Liver vitamin C mg/g tissue

\[
= \frac{\text{Conc. of std.} \times \text{O.D. (s)} \times \text{Total volume} \times 10}{\text{O.D. of std.} \times \text{Aliquot taken} \times 1000}
\]

KIDNEY ANALYSIS

MOISTURE

Moisture content of the sample was determined by the method given by AOAC (1984).

Procedure followed for determination of moisture in kidney tissue were similar to those adopted for liver tissue.

PART-III: Human experiment 1 and 2

Iodine status in school children (6-12 year old)

Clinical examination of goitre
Children in the age group of 6-12 years \( (n=1596) \) were examined for the presence of iodine deficiency such as goitre. Depending on the size of enlargement of thyroid gland it was graded as follows (WHO/ICCIDD/UNICEF, 2001).

**Grades of goitre**

<table>
<thead>
<tr>
<th>Goitre Grade</th>
<th>Stage of enlargement of thyroid gland</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade O</td>
<td>The thyroid gland was neither visible nor palpable.</td>
</tr>
<tr>
<td>Grade I</td>
<td>The thyroid gland was enlarged, not visible, but palpable and each lobe had a volume greater than the terminal phalanx of the thumb of the subject.</td>
</tr>
<tr>
<td>Grade II</td>
<td>The thyroid gland was enlarged and visible and palpation was not necessary.</td>
</tr>
</tbody>
</table>

(ii) **Anthropometry**

Anthropometric measurements such as height and weight of a sub-sample of children covered for clinical examination were recorded using standard equipment and procedures, to assess their nutritional status WHO (1995).

(iii) **Urinary iodine**

Urinary iodine excretion levels were estimated from urine samples of children and adult subjects by the wet digestion method based on the Sandell Kolthoff reaction as given by ICCIDD/UNICEF/WHO, (Dunn et al., 1993).

Mixed urine to suspend the sediment. Pipetted 250 µL of each urine sample into a test tube in duplicate. Pipetted each iodine standard into a test tube; volume was made upto 250 µL with distilled water. To each test tube (i.e., blank, standard and samples), 1ml of 1-M ammonium persulphate was added and vortexed. The volume in each tube was marked and kept in an oven (or Digital Block Digestor) at 100° C for 60 minutes. The test tubes were removed and allowed to cool at room temperature.
The volumes were made up to the mark with double distilled water. 2.5 ml of arsenious acid solution was added to each test tube and mixed by vortexing, and allowed to stand for 15 minutes.

300 \(\mu l\) of ceric ammonium sulphate solution was added to each test tube and mixed quickly. 30-second intervals were given between successive additions to the tubes.

They were allowed to stand at room temperature. Exactly 30 minutes after the addition of ceric ammonium sulphate to the first tube, the absorbance was read at 420nm. Similarly the absorbance of successive tubes were read at 30 second intervals. The time of addition to each tube was monitored using a stopwatch.

**Calculation:** A standard curve was constructed by plotting the iodine concentration (\(\mu g/L\)) of each standard against its optical density.

**Epidemiological criteria for assessing severity of IDD based on median urinary iodine levels as under:**

<table>
<thead>
<tr>
<th>Median values ((\mu g/L))</th>
<th>Severity of IDD</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;20</td>
<td>Severe IDD</td>
</tr>
<tr>
<td>20-49</td>
<td>Moderate IDD</td>
</tr>
<tr>
<td>50-99</td>
<td>Mild IDD</td>
</tr>
<tr>
<td>&gt;100</td>
<td>No deficiency</td>
</tr>
</tbody>
</table>
2. Iodine status in adult subjects (normal and hypothyroidism)

(i) Serum T₃, T₄, TSH and urinary iodine

Serum thyroid hormones and urinary iodine were estimated as described in the earlier section.

STATISTICAL ANALYSIS

The standard SPSS programme was run to analyze the data. All the data were tested for significance using the ANOVA and Least significant difference test among means of various parameters (Steel and Torrie, 1960). Difference between treatment at P < 0.05 were considered significant.

For population data statistical tests such as Chi-square, ANOVA with post hoc analysis were carried out. Frequency distribution and T-test was also done wherever required.
Reagents used in analytical procedure

All reagents used were of AR grade.

1. Acetic acid (glacial): As such.
2. Acetic acid (20%): 20 ml of acetic acid (CH₃COOH) was diluted to 100 ml with distilled water.
3. Acetone: Ethanol mixture (1:1) One volume of acetone (CH₃COCH₃) mixed with one volume of ethanol (C₂H₅OH).
4. Alcohol: As such.
5. ALP assay mixture: Consists of p-nitro phenyl phosphate and buffer.
6. Ammonium iron (III) sulphate reagent. Dissolved 77 g of ammonium iron (III) sulphate [NH₄Fe(SO₄)₂·12H₂O] in approximately 400ml of water. Added 167±1 ml of concentrated nitric acid (sp gr. 1.42) and diluted to 1 L, warming until all traces of the solid dissolved.
7. Ammonium persulfate (1.0 M): Dissolved 114.1 g H₂N₂O₈S₂ in H₂O; made up to 500 ml with H₂O. Stored away from light. Stable for at least one month.
8. Arsenious acid reagent (0.02 N): 0.986 g ASO₃ was dissolved in 10 ml of 0.5N NaOH in a beaker by heating. This was added to 850 ml of water and to this 20 ml HCL and 20.6 ml H₂SO₄ were added. The volume was then made up to 1 Liter.
9. Arsenious acid solution: In a 2000 ml Erlenmeyer flask, placed 20 g As₂O₃ and 50 g NaCl, then slowly added 400 ml 5 N H₂SO₄. Added water to about 1 litre, heated gently to dissolved, cooled to room temperature, diluted with water to 2 litres, filtered, stored in a dark bottle away from light at room temperature. The solution was stable for one month.
10. Ascorbic acid:
    Stock: 100 mg of ascorbic acid dissolved in 5.0 % TCA.
    Working: 1.0 ml of stock was diluted to 100 ml with 5.0 % TCA and treated with charcoal.
11. Bathophenanthroline (0.02%): Weighed exactly 20.0 mg of bathophenanthroline and dissolved in 100 ml of alcohol.
12. Biuret reagent:
Stock: 22.5 gm. of sodium potassium tartarate (COCOK.CH0H.CH0H.COONa.4H2O) was dissolved in about 200 ml. Of 0.2N NaOH followed by the addition of 7.5 gm. of copper sulphate (CuSO4.5H2O) and dissolved completely. To this added 2.5 gm. of potassium iodide (KI) and volume was made up to 500 ml. with 0.2 N NaOH solution. Stored in a brown bottle.

Working: 100 ml of stock was diluted to 500 ml. with 0.2 N NaOH. Added 2.5 gm of KI and dissolved completely.

13. Biuret stock: 8 gm % standard protein (Sigma U.S.A) was directly used for the standard series.

14. Boric acid (2%, prepared freshly): 2 g of boric acid dissolved in 80 ml distilled water and volume was made to 100 ml with distilled water.

15. Ceric ammonium sulphate solution: Dissolved 48 g of ceric ammonium sulphate in a 1 litre 3.5 N H2SO4. (The 3.5 N H2SO4 was made by slowly adding 97 ml concentrated (36 N) H2SO4 to about 800 ml deionized water (careful – this generates heat!), and when cooled, adjusting with deionized water to a final volume of 1 litre). Stored in a dark bottle away from light at room temperature. The solution was stable for one month.

16. Ceric ammonium sulphate (0.03N): 48.6 ml of concentrated H2SO4 was added to about 600 ml of water and then 20 g of ceric ammonium sulphate dissolved in this solution. The total volume was made up to 1 liter with distilled water.

17. Charcoal: As such.

18. Chloroform: (CHCL3) As such.

19. Chloroform-Trifluoroacetic acid mixture: Chloroform and Trifluoroacetic acid was mixed in a ratio of 2:1.

20. Cholesterol standard: 200 mg % as such (from kit).

21. Colouring reagent (Ferric chloride color reagent): Stock: 10 g. of ferric chloride was dissolved in 100 ml. of acetic acid. Working: 1 ml. of stock was diluted to 100 ml. with concentrated sulfuric acid (prepared freshly).

22. Copper sulphate 1%: 1g Copper sulphate was dissolved in 100 ml of distilled water.
23. Cyanmeth regent: Ready to use.
24. Diethyl ether (CH₃CH₂)₂O. As such.
25. Digestion mixture: Copper sulphate and potassium sulphate mixture (1:1).
26. 2,4 Dinitrophenyl hydrazine-Thiourea-Coppersulphate (DTC) solution: 3.0 g of DNPH, 0.4 g thiourea and 0.05 g of copper sulphate was dissolved in 100 ml of 9 N H₂SO₄.
27. Ferric chloride (0.001 M): 13.5 mg of FeCl₃ was dissolved in 50 ml of alcohol.
28. Heparin (anticoagulant – 1 g%): 1000 mg of heparin dissolved in 100 ml distilled water.
29. Hexane (C₆H₁₄): As such.
30. Hydrochloric acid (0.1 N): 0.1 ml of HCl was diluted to 11.0 ml with distilled water.
31. Hydrochloric acid 1N: 1 ml concentrated HCL was diluted to 11 ml of distilled water.
32. Hydrochloric acid 0.25N: 50 ml of 1N HCL diluted to 150 ml of distilled water.
33. Lowry’s A: Dissolved 2% sodium carbonate (Na₂CO₃) in 0.1 N sodium hydroxide (NaOH).
34. Lowry’s B: 1% copper sulphate and 2% sodium potassium tartarate mixed in equal volume.
35. Lowry’s C (prepared freshly): 50 parts of Lowry’s A mixed with 1 part of Lowry’s B reagent.
36. Lowry’s E:
   Stock: Fohlich’s reagent was used as stock. Working: 1 ml of stock was diluted to 2 ml with distilled water (1:2).
37. Mixed Indicator: 25 mg of bromocresol green (C₂₁H₁₃Br₄O₆SNa) and 75 mg of methyl red (C₁₅H₁₇N₃O₂) in 100 ml of alcohol.
38. Orthophosphoric acid (0.001 M):
   Stock: 0.98 ml of orthophosphoric acid in 100 ml of alcohol (0.1 M). Working: 1.0 ml of stock was diluted to 100 ml with alcohol (0.001 M).
39. Phosphate buffer: (pH-8). 93.2 ml M/15 Na$_2$Hpo$_4$ and 6.8 ml M/15 KH$_2$PO$_4$.

40. Potassium carbonate solution (30% m/V): Dissolved 30 gm of potassium carbonate in distilled water and dilute to 100 ml.

41. Potassium carbonate wash solution (0.3% m/V). Dissolved 3 gm of potassium carbonate in distilled water and diluted to 1L.

42. Potassium chloride (KCl) (0.9%): 0.9 g of potassium chloride was dissolved in few ml of distilled water and volume were made to 100 ml with distilled water.

43. Potassium hydroxide (5%): 5.0 g of KOH dissolved in 100 ml of distilled water.

44. Potassium hydroxide (saturated): Dissolved sufficient amount of KOH in distilled water.

45. Potassium iodide (KI) 10%: Dissolved 100 g KI in 1000 ml Distilled water. Store in a cooled, dark place. This volume sufficient for 200 samples. Properly stored the solution is stable for 6 months.

46. Potassium iodide standard solution (4 gm/l): Dissolved 0.5232 g of potassium iodide, previously dried in desiccators, in distilled water and diluted the solution to 100 ml in a calibrated flask (stable for at least 1 month).

(i) Standard Iodide solution (40mg/l): Diluted 10 ml of the standard iodide solution (4g/l) to 1000 ml with distilled water in a calibrated flask (stable for at least 1 month).

(ii) Standard iodide solution (200 ng/ml). Diluted 5 ml of the standard iodide solution (40mg/l) to 1000 ml with distilled water in a calibrated flask. Stored the solution in a glass bottle away from light (stable for 1 month only).

Working: Into a series of 100 ml calibrated flasks, pipetted 10, 8, 6, 4, 2 and 0 ml of standard iodide solution (200ng ml/l). To each solution added an amount of 30% m/v potassium carbonate solution so that when diluted to 100 ml it contained the same alkalinity as that of the sample solution (usually 1ml of 30% m/v potassium carbonate solution). These all the
working standard; stored them in the glass bottles away from light and prepared them freshly at weekly intervals.

Standard iodine solution: 1 µg iodine/ml (7.9 µmol/l): Dissolved 0.168 mg of KI03 in deionized water to a final volume of 100 ml (1.68 mg KI03 contains 1.0 mg iodine; KI03 is preferred over KI because it is more stable, but KI has been used by some laboratories without apparent problems). It may be more convenient to make a more concentrated solution, e.g., 10 or 100 mg iodine/ml, then diluted to 1 µg /ml. Stored in a dark bottle. The solution is stable for months (useful standards are 20, 50, 100, 150, 200, and 300 µg /l).

47. Potassium thiocyanate solution (0.023% fn/v). Dissolved 0.23 gm of potassium thiocyanate in water and diluted to 1 L.
48. Protein standard: 8.0 g % protein standard from Sigma Chemical Company, U.S.A.
49. Protein standard: 0.1 ml of protein standard (8.0 g % from Sigma Chemical Company, U.S.A.) was diluted to 10 ml with 0.1 N NaOH.
50. Pyrogallol (1 %): 1.0 g of pyrogallol dissolved in 100 ml of alcohol.
51. Pyrogallol (40µM): 5.04 mg of pyrogallol dissolved in 100 ml of 0.01 N HCL.
52. Retinol standard:
Stock: 1.0 mg of Retinol standard (from Sigma Chemical Company, U.S.A) was dissolved in 100 ml of alcohol.
Working: 2.0 ml of stock was diluted with 100 ml of alcohol.
Note: On the day of analysis, concentration of working standard was checked at 325 nm and calculated the exact concentration using E19 % = 1785.
53. Saline solution (0.9 %): 0.9 g of NaCl was dissolved in 100 ml distilled water.
54. Sodium carbonate (2%) : 2 g. sodium carbonate dissolved in few ml of distilled water and volume was made to 100 ml With distilled water.
55. Sodium carbonate-potassium chloride solution: 212 g of anhydrous Na2Co3 and 20 g of KClO2 was dissolved in water and volume made up to 1 Liter.
56. Sodium hydroxide (50 %): 50 g of NaOH was dissolved in few ml. of distilled water and volume made to 100 ml with distilled water.
57. Sodium hydroxide (0.1 N): 0.4 g of NaOH dissolved in 100 ml of distilled water.
58. Sodium hydroxide (0.2 N): 0.8 g of NaOH dissolved in 100 ml of distilled water.
59. Sodium hydroxide (0.02 N): 0.08 g of NaOH dissolved in 100 ml of distilled water.
60. Sodium iodide standard solution 0.1M:
   Stock: 7.87 ml of (no; 945306) sodium iodide standard was diluted to 1000 ml of distilled water.
   Working: 10 ml stock was diluted to 100 ml of distilled water.
61. Sodium nitrite solution (2.07% m/v): Dissolve 2.07 gm of sodium nitrite in water and diluted to 100ml (stable for 1 day only).
62. Sodium potassium tartarate (2%): 2 g of sodium potassium tartarate dissolved in few ml of distilled water and volume made to 100 ml with distilled water.
63. Sodium thiosulfate (Na$_2$S$_2$O$_3$) 0.005M: Dissolved 1.24 g Na$_2$S$_2$O$_3$.5H$_2$O in 1000 ml water, stored in a cool, dark place. (This volume was sufficient for 100-200 samples, depending on the iodine content of samples. The solution was stable for at least one month, if stored properly.)
64. Starch indicator solution: Prepared 100 ml of a saturated NaCl solution, by adding NaCl to approximately 80 ml water in a beaker, with stirring and / or heating, until no further solids dissolved. This solution was stable for at least 1 year. Weighed 1 g soluble starch into a 100 ml beaker; added 10 ml water, heat to dissolve. Added saturated NaCl solution to the hot starch solution to made up to 100 ml. Stored in a cool, dark place. (This volume was sufficient for 50 samples. This solution was stable for at least 1 month, and should be heated (not boiled) each day before use re-suspend any solids.)
65. Sulfuric acid (65 %): 65 ml of sulfuric acid was diluted to 100 ml with distilled water.
66. Sulfuric acid (85 %): 85 ml of sulfuric acid was diluted to 100 ml with distilled water.

67. Sulphuric acid (0.02 N): 0.2 ml of conc. H$_2$SO$_4$ was diluted to 360 ml with D/w.

68. Sulphuric acid (Conc.): as such.

69. Sulfuric acid (H$_2$SO$_4$) 2 N: Slowly added 6 ml concentrated sulfuric acid to 90 ml water. Made to 100 ml with water (this volume is sufficient for 100 samples and stable indefinitely).

70. 5 NH$_2$SO$_4$: Slowly added 139 ml concentrated (36 N) H$_2$SO$_4$ to about 700 ml deionized water (careful - this generates heat!). When cool, adjusted with deionized water to a final volume of 1 litre.

71. 60% sulfuric acid: 60 ml of concentrated sulfuric acid was diluted to 100 ml.

72. TBA: 375 mg dissolved in 100 ml 0.25N HCL

73. Tetra ethoxy propane (992 mg/ml fluca). stock: 1.655 ml tetra ethoxy propane was diluted to 10 ml of 40 % ethanol (1 Molar).

Working -A: 0.2 ml stock -1 was diluted to 10 ml of 40 % ethanol (µM/ml). Working -B: 0.2 ml working-A was diluted to 10 ml of 40 % ethanol (µM/ml). From this 250,500,750 and 1000 (µL were taken for standard series.

74. Thiobarbituric acid (TBA): 0.8 g of TBA (from Sigma Chemical Company, U.S.A) dissolved in 100 ml of distilled water.

75. Tocopherol standard:

Stock: 50 mg of α-tocopherol (from Sigma Chemical Company, U.S.A) was dissolved in 100 ml of alcohol.

Working: 2.0 ml of stock diluted to 100 ml with alcohol.

Note: On the day of analysis, concentration of working standard was checked at 292 nm and calculated the exact concentration using $E_{1%}^{1g} = 75.8$.

76. Trichloroacetic acid (5%): 5.0 g of TCA was dissolved in 100 ml of distilled water
77. Trichloroacetic acid (10%): 10.0 g of TCA was dissolved in 100 ml of distilled water.
78. Trichloroacetic acid 15 g w/v: TCA was dissolved in 100 ml 0.25N HCL.
79. TCA-TBA-HCL: 15 % w/v TCA, 0.375 % w/v TBA; 0.25N HCL. This solution may be mildly heated to assist in the dissolution of the thiobarbituric acid.
80. T₃ Human Serum Reference. Six vials of serum reference for triiodothyronine at concentrations of 0(A), 50(B), 100(C), 250(D), 500(E) and 750(F) ng/dl.
81 T₃ CT-Enzyme Conjugate: T₃-horseradish peroxidase (HRP) conjugate in a bovine albumin-stabilizing matrix.
82 T₃/T₄ Conjugate buffer: Buffer red dye preservative, and binding protein inhibitors.
83 T₃ Working Reagent: A = T₃ Enzyme Conjugate Solution. Diluted to T₃-Enzyme conjugate 1:11 with total T₃/T₄ conjugate buffer in a suitable container. For example, dilute 200 μl of conjugate with 2ml of buffer for 10 tubes.
84 T₃ Antibody Coated Tubes: Coated tubes with sheep anti T₃ serum.
85 T₄ Human serum References: Six vials of serum reference for thyroxine at concentrations of 0(A), 2.0(B), 5.0(C) 10.0(D), 15.0(E) and 25.0(F) μg/dl.
86. T₄ Enzyme Conjugate : T₄-horseradish peroxidase (HRP) conjugate in a bovine albumin-stabilizing matrix. A preservative has been added.
87 T₄ Working Reagent: A = T₄ Enzyme Conjugate 1:11 with total T₃/T₄ conjugate buffer in a suitable container. For example, dilute 200 μl of conjugate with 2ml of buffer for 10 tubes.
88 T₄ Antibody Coated Tubes: Coated tubes with sheep anti T₄ serum.
89 T₃, T₄, TSH Substrate A. One bottle containing tetramethylbenzidine (TMB) in buffer.
90 T₃, T₄, TSH Substrate B. Containing hydrogen peroxide (H₂O₂) in buffer.
91. T₃, T₄, TSH Stop solution Concentrate: Containing strong acid (1N HCL). Working: 6ml Stop solution concentrate diluted to 100ml with distilled water.
92. T₃, T₄, TSH Wash Solution Concentrate: Containing a surfactant in phosphate buffered saline. A preservative has been added. Diluted contents of wash concentrate to 1000 ml with distilled or deionized water in suitable storage container.

93. TSH (Thyrotropin) Calibrators: Seven vials references for TSH Antigen at level of 0(A), 0.5(B), 2.5(C), 5.0(D), 10(E), 20(F) and 40(G) μU/ml.

94. Enzyme-TSH antibody conjugate and Biotinylated monoclonal antibody: TSH Streptavidin Coated Tubes. Coated tube with streptavidin and packaged in to a Ziploc bag with a drying agent.

95. Zinc sulphate solution (10% m/v): Dissolved 10 gm of zinc sulphate (ZnSO₄. 7 H₂O) in water and diluted to 100 ml.