CHAPTER II

METHODS AND MATERIALS
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One of the chief aims of these investigations was, as stated in the introduction, to get systematic data on the dietary intake and milk constitution of vitamins in lactating women in this region, and to determine the extent to which the latter varies from the former.

The vitamins studied were: ascorbic acid, nicotinic acid, riboflavin, thiamine, pantothenic acid, cyanocobalamin, biotin, pyridoxine, and folic acid.

The investigations were conducted in five stages concerned with different aspects of the problem and they are indicated below:

The first was a preliminary study in which data were obtained on the composition of breast milk with regard to certain vitamins at different stages of lactation and in different socio-economic groups.

Next, determinations were made of dietary intake of lactating women and their milk composition with regard to certain vitamins. The relation between the dietary intake and milk constitution of these vitamins was studied.

This was followed by a longitudinal study in which investigations were made of the effects of oral vitamin supplementation in
progressively increasing doses on the vitamin constitution of milk in subjects of very poor nutritional status judged by dietary intake.

Additional studies were also made of variations in the composition of milk with the progress of lactation and the effects of dietary vitamin supplementation on milk yield.

Finally, studies were made on some of the milk enzymes, their partial purification and characterization, their relation to vitamin levels in milk and diet and stage of lactation.

Subjects

A total of 318 subjects of apparently normal health and ranging in age from 16 to 33 years were used in the conduct of the first three experiments. Blood examinations were made in the case of a few subjects (20 cases) of very low nutritional status and no gross abnormalities were found, the values obtained as compared to normal values being shown below:

<table>
<thead>
<tr>
<th>Present subjects</th>
<th>Normal range (Ref.197)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean value</td>
</tr>
<tr>
<td>Haemoglobin content (g%)</td>
<td>14.2</td>
</tr>
<tr>
<td>RBC count (Millions/ccm)</td>
<td>4.1</td>
</tr>
<tr>
<td>WBC count (ccm)</td>
<td>5265</td>
</tr>
<tr>
<td>Erythrocyte sedimentation rate (mm/hr)</td>
<td>8.2</td>
</tr>
<tr>
<td>Packed cell volume (%)</td>
<td>41.50</td>
</tr>
</tbody>
</table>
Collection of milk samples

For studies on the composition of milk, the samples were collected on three consecutive days in sterilised test tubes at about 3 p.m. each day by manual expression. Equal aliquots from both breasts were taken to make a sample. The samples were brought to the laboratory in the thermos flasks packed with ice.

Although considerable diurnal variation in the composition of milk particularly with regard to fat content have been demonstrated (160,163,164), it was not possible to collect 24-hour samples on account of obvious difficulties. However, the effects of such variations were sought to be minimised by collecting the samples at a specified time for all subjects. The variation in composition between right and left breasts was sought to be controlled by taking equal aliquots from both breasts, and that between fore and after milk, by taking the fore milk in all cases, the subjects having been asked not to nurse the infants for at least two hours before the collection of the sample.

For the estimation of ascorbic acid and enzyme activity, a portion of the sample was used immediately on arrival in the laboratory. For other estimations, the samples were stored at 0° to -4° till the analyses were made.

Milk yield

For the determination of milk yield, the 24-hour milk intake
of the infant was taken as the total yield of milk and was determined by weighing the infant before and after each feed.

The social worker spent from the first feed in the morning till the first feed next morning, i.e., whole day and night, at the home of the subject and weighed the infant before and after each nursing, long or short.

Collection and analysis of food

The whole day diet of the subject was obtained on three consecutive days by making collections of matched amounts of all the foodstuffs consumed by the subject including snacks, beverages, and dietary supplements in the form of vitamins, minerals, etc.

The collection of 24-hour food intake was made by the social worker who was present at every meal taken by the subject and caused equal helping of whatever food the subject took to be put on an additional plate, using appropriate measures, such as cup or spoon. The food thus collected was taken immediately after each meal to the laboratory in tiffin-carriers and stored in the cold room. The collections were pooled together after the last meal at night (at about 8 p.m) and a homogenate prepared for the different estimations according to the procedures to be described below. The subjects were requested to follow their usual patterns of eating, and are believed to have done so.
Preparation of the homogenate

For the determination of cyanocobalamin, foodstuffs containing milk, milk products and other known sources of cyanocobalamin in the diet were mixed thoroughly together and a small portion of the same (15 g) removed.

The other foodstuffs were weighed individually, mixed, and to this was returned the remaining portion of the above mixture. This was homogenized and the homogenate stored at 4°C to 6°C till it was taken up for analysis except for the estimation of ascorbic acid for which a portion of the homogenate was used immediately. Different aliquots of the homogenate were used for the different determinations.

About 50 g of the homogenate were dried in an electric oven at 60°C for 8 hours. Appropriate aliquots were used for the determinations of fat, protein, and pantotthenic acid content.

Chemicals

The chemicals used in the investigations were obtained from the following sources:

Bacto-peptone, yeast extract and dextrose from Difco Laboratories; thiamine hydrochloride, riboflavin, nicotinic acid, pyridoxine, folic acid, ascorbic acid, inositol, and 2,4-dinitrophenyl hydrazine, from E. Merck & Co.; Calcium pantothenate and biotin, from F. Hoffmann La Roche & Co. Ltd.; Para-aminobenzoic acid, from Ward, Blankinsop & Co. Ltd; Pyridoxal hydrochloride, from Penick & Co.; activated charcoal, from the British Drug Houses, Ltd.
METHODS OF ANALYSIS

Protein. To estimate the protein content of the diet, 0.1 g of the dried homogenate were digested with 5 ml of concentrated H2SO4 and the nitrogen content determined by the micro-kjeldahl procedure, the value obtained being multiplied by 6.25.

Fat. To estimate the fat content of the diet, a known amount of the dried homogenate was extracted with ether in Soxhlet apparatus for 8 hours. The amount of fat was estimated from the difference in weight.

Ascorbic acid

Milk. To 1 ml of milk were added 9 ml of 7% trichloroacetic acid and 200 mg of activated charcoal, washed previously with acid. The mixture was shaken vigorously for 2 minutes, allowed to stand at room temperature for 10 minutes, and filtered. The filtrate obtained was used for the determination of total ascorbic acid by the method of Roe and Kesther (198).

Diet. 10 to 15 g of the homogenate were suspended in 7% trichloroacetic acid, made to volume and filtered. A measured portion of the filtrate was treated with 200 mg of acid washed activated charcoal as described for milk sample and filtered. This filtrate was diluted suitably so as to yield about 5 mg of ascorbic acid per ml. The vitamin was determined by the method used for milk.

Nicotinic acid

Milk. To 2 ml of milk were added 4 ml of 1N H2SO4 and the mixture
autoclaved at 15 lb pressure for 20 minutes. After cooling, the mixture was adjusted to pH 4.6, filtered, and the filtrate adjusted to pH 6.8-7.0. This was diluted suitably so as to yield about 0.1 mcg/ml of nicotinic acid. The vitamin was assayed microbiologically using _Larabinosus_ as the test organism according to the procedure in the "Methods of Vitamin Assay" (199).

**Diet**

The reaction mixture for the enzymatic liberation of nicotinic acid, riboflavin, and thiamine consisted of 10 to 15 g of the homogenate, 30 ml of 0.05M acetate buffer, pH 4.6, 500 mg each of taka-diastase and papain. This was incubated at 37° for 24 hours under toluene. After incubation, the reaction mixture was steamed for 5 minutes, cooled, and filtered. The filtrate was adjusted to pH 6.8-7.0, diluted suitably, and its nicotinic acid determined as in the case of milk.

**Riboflavin**

To 2 ml of milk were added 4 ml of 0.1 N HCl and the mixture autoclaved at 15 lb pressure for 20 minutes. After cooling, the mixture was adjusted to pH 4.6 and filtered. For the total removal of fatty material, the filtrate was treated with peroxide free diethyl ether, adjusted to pH 6.3-7.0 and diluted, so that the concentration of riboflavin in the solution was about 0.05 mcg/ml. Riboflavin was assayed microbiologically using _L. casei_ as the test organism according to the procedure described in the "Methods of Vitamin Assay" (199).
Diet A portion of the enzymatic hydrolysate prepared for the estimation of nicotinic acid was treated with pesticide free di-ethyl ether. This was adjusted to pH 6.8-7.0, diluted suitably and riboflavin determined as in the case of milk. Corrections were made against a blank for the vitamins liberated from the enzymes used.

Thiamine

Milk To 2 ml of milk were added 2 ml of 1 M HCl and the mixture steamed for 20 to 30 minutes. After cooling, the mixture was adjusted to pH 4.6, and incubated with 50 mg each of takadiastase and papain at 37°C for 24 hours under toluene. After incubation, the mixture was steamed for 5 minutes, cooled and filtered. The filtrate was adjusted to pH 6.8-7.0, and diluted so as to contain about 0.025 mg/ml. The thiamine content of the filtrate was estimated using *L. fermentii* as a test organism according to the procedure described by Barton-Wright (200). To correct the effect of growth-promoting factors other than thiamine, about half of the filtrate was freed from thiamine by treating with sodium sulphite and used as a blank.

Diet The enzymatic hydrolysate prepared for nicotinic acid was diluted so as to yield a thiamine concentration of about 0.025 mg/ml. The vitamin was assayed using the procedure described for milk.
Pantothenic acid

**Milk**
To 1 ml of milk were added 0.2 ml of 0.1M NaHCO_3 buffer, pH 7.2, and 0.10 ml of enzyme prepared from acetone-dried powder of chicken liver. The reaction mixture was incubated at 37° for 4 hours. After incubation, the mixture was steamed for five minutes, cooled, adjusted to pH 4.6, and filtered. The filtrate was adjusted to pH 6.3-7.0 and diluted so as to yield a pantothenic acid concentration of about 0.02 mcg/ml. Pantothenic acid in the filtrate was determined microbiologically using *Larabinose* as a test organism according to the procedure described in "Methods of Vitamin Assay" (199). Corrections were made against a blank for the pantothenic acid liberated from the enzymes used.

**Diet**
About 1 g of the dry homogenate was suspended in 20 ml of 0.1M NaHCO_3 buffer, pH 7.2. The reaction mixture was treated as in the case of milk and the pantothenic acid content determined by the procedure described above.

**Cyanocobalamin**

**Milk**
To 2 ml of milk were added 0.2 ml of 0.05M KCN and 0.1 ml of sodium metabisulphite (prepared by dissolving 180 mg each of 100 mg of KH_2PO_4 and sodium metabisulphite in 10 ml water). The mixture was autoclaved at 15 lbs pressure for 20 minutes. After cooling, the mixture was adjusted to pH 4.6, and filtered. The filtrate-
The filtrate was adjusted to pH 6.5 and diluted so as to yield a cyanocobalamin concentration of about 0.02 μg/ml. The cyanocobalamin was estimated microbiologically using L. leichmannii as a test organism according to the procedure described in USP XIV (201). No corrections were made for the possible growth response due to other substances, such as deoxyribosides of thymine, hypoxanthine, etc. in milk.

Diet As mentioned earlier, foodstuffs containing known sources of cyanocobalamin (mostly milk and milk products in the subjects studied) were mixed thoroughly, weighed, and a small portion (about 15 g) of the same used for the estimation which was made as in the case of milk.

Biotin

Milk To 2 ml of milk were added 4 ml of 3N H₂SO₄ and the mixture autoclaved at 15 lb pressure for 1½ hours. After cooling, the mixture was adjusted to pH 4.6 and filtered. The pH of the filtrate was again adjusted to 6.8–7.0 and the filtrate diluted so as to contain 0.2 μg of biotin per ml. The biotin content was determined microbiologically using L. arabinosus as a test organism according to the procedure described in "Methods of vitamin assay". (199).

Diet About 5 g of the homogenate were weighed and mixed with 50 ml of 3N H₂SO₄ to constitute the reaction mixture. The remaining procedure was the same as that described for milk.
**Pyridoxine**

**Milk**  To 2 ml of milk were added 5 ml of 0.05 N \( \text{H}_2\text{SO}_4 \) and the mixture autoclaved at 15 lb pressure for 1 hour. After cooling, the mixture was adjusted to pH 4.6 and filtered. The filtrate was diluted so as to contain 0.01 mg/ml of pyridoxine. The vitamin was assayed microbiologically using *S. Carlsbergensis* as a test organism, according to the procedure described in "Methods of Vitamin Assay" (193).

**Dist**  About 5 g of homogenate were weighed and suspended in 25 ml of 0.05N \( \text{H}_2\text{SO}_4 \). The remaining procedure was the same as that described for milk.

**Folic acid**

**Milk**  To 2 ml of milk were added 2 ml of phosphate buffer, pH 7.5, and 15 mg of acetone-dried powder of chicken pancreas. The reaction mixture was incubated at 37°C for 24 hours, under toluene. After incubation, the mixture was steamed for five minutes, cooled, adjusted to pH 4.6, and filtered through Whatman filter paper No. 42. The resulting filtrate was adjusted to pH 6.8-7.0 and diluted, so that the final concentration of vitamin was about 0.2 mcg/ml. Folic acid was estimated microbiologically using *S. faecalis R.* as a test organism according to the procedure described in AOAC (202). Corrections were made against an enzymatic blank for the vitamin liberated from the enzymes used.
Diet About 10 g of homogenate were weighed, suspended in 0.1 M 25 ml of phosphate buffer, pH 7.5 and to this were added 200 mg of acetone-dried powder of chicken pancreas to constitute a reaction mixture. Folic acid was estimated by the procedure described for milk.

Enzyme Assays

These are described in Chapter VI.

(N.B. Except where otherwise specified, Whatman filter paper No.1 was used for filtration.)