CHAPTER II

METHODS AND MATERIALS

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As stated in the introduction, the purpose of these investigations was to study whether the quality of breast milk, and more specifically, its composition with regard to protein, fat, lactose and essential amino acids, varies with the dietary intake of the lactating mother. Additional studies were also made of variations in the composition of milk with the progress of lactation and the effects of dietary supplementation on the yield of milk.

The investigations were carried out in four stages concerned with different aspects of the problem and they are indicated below.

The first was a cross sectional study aimed at comparisons of the composition of milk with regard to fat, protein, lactose, and essential amino acids, at different stages of lactation and in different socioeconomic groups.

Next, determinations were made of the correlation between dietary and milk composition with regard to fat, protein, and essential amino acids.

These studies were followed by a longitudinal study in which investigations were made of the effects of dietary fat and protein supplementation, with and without other supplementation, on the fat, protein, and essential amino acid composition of milk.

Finally the presence in milk of certain enzymes involved in fat metabolism was investigated and one of them, viz., alkaline phosphatase was partially purified and characterised.

Subjects: A total of 367 subjects of apparantly normal health and ranging in age from 16 to 32 years were used in the conduct of the first three experiments. Blood examinations were made in the case of a few subjects of low nutritional status and no gross abnormalities were found, the range of values obtained being shown below.

	Present subjects		
	Mean value	Range	Normal range
Hemoglobin (g %)	14.2	9-16	12.4-17.01
R.B.C. count (millions/cmm)	4.1	3.7-4.6	4.2-6.37
W.B.C. count/cmm	5265	4000-8000	4000-11,000
Erythrocyte sedimentation rate (mm/hr)	8.2	1-10	0-20
Packed cell volume (%)	41.50	27-52	37-47

Collection of milk samples: The samples, made up of equal aliquots from both breasts, were collected on three consecutive days in sterilized test tubes at about 3 p.m. by

manual expression, and brought to the laboratory in thermos-flasks packed with ice.

Although considerable diurnal variation in the composition of milk particularly with regard to fat content have been demonstrated (Planchu and Rendu, 1911, Deem, 1931, Nims etal, 1932, Gunther and Stanier, 1951 and Hytten 1954), it was not possible to collect 24 hour samples on account of obvious difficulties. However, the effects of such variations were sought to be minimized 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 foremilk in all cases, the subjects having been asked not to nurse the infants for atleast 2 hours before the collection of the sample.

When enzyme activity was estimated, a portion of the sample was used immediately on arrival in the laboratory. For other estimations the sample was stored at 0° to 4°C till the analyses were made.

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.

Collection and analyses of food: The whole day diet of the subject was obtained on three consecutive days by

making collections of matched amounts of all the food stuffs consumed by the subject, including snacks, beverages, and dietary supplements, if any.

Preperation of the homogenate: After separate weighment of each foodstuff, all the food stuffs were thoroughly mixed and homogenised. A portion of the homogenised food was dried in an electric oven at 60° for 8 hours. The dried homogenate was used as needed for the different determinations.

Methods of analysis

Protein: Nitrogen was estimated by the micro-kjeldahl method.

Milk: To 0.1 ml of milk was added 1.0 ml of concentrated sulphuric acid. The mixture was digested and total nitrogen content estimated, the value obtained being multiplied by 6.38.

Diet: 0.1 gm of the dried homogenate was digested with 5 ml of concentrated sulphuric acid and the nitrogen content estimated, the value obtained being multiplied by 6.25.

Amino acids:

Milk: An alkaline hydrolysate was prepared for the estimation of tryptophan, and an acid hydrolysate, for that of the others.

Alkaline hydrolysate: To 1.0 ml of milk were added 10 ml of 5N. sodium hydroxide and the mixture autoclaved at 15 pounds pressure for 8 hours. The hydrolysate obtained was neutralised, diluted to a known volume, and filtered. Acid hydrolysate: To 2.5 ml of milk were added 20 ml of 2.5N. hydrochloric acid and the mixture autoclaved at 15 pounds for 8 hours. The hydrolysate obtained was freed from starchy and fatty materials by adjusting to pH 4.5, diluted to a known volume, and filtered. A known amount of filtrate was adjusted to pH 6.8 and diluted to a known volume.

The circular paper chromatography technique described by Giri et al (1950,1953) was used for the estimation of tryptophan. The microbiological assay technique described by Barton-wright (1952) was used for the estimation of the others, the test organism used in each case being indicated below:

leucine, isoleucine and valine	-	L.Arabinosus
arginine and threonine	-	S.Faecalis
histidine, phenylalanine, lysine and methionine	-	L-mesentroides

Diet: The dried homogenate was used for the preparation of acid and alkaline hydrolysates. Procedures for the preparation of the hydrolysates and the estimation of amino acids were the same as described for milk.

Fat:

Milk: Fat content was estimated according to the method of Chiba et al (1957). Five ml of milk were diluted to 10 ml with glass distilled water and centrifuged in tubes calibrated in mm for 30 minutes at 3000 r.p.m. The fat content was read off from the height of the column of fat layer seperating to the top.

To investigate the validity of the method, the fat layer was seperated by decantation in a few cases and dried in a watch glass in a vacuum desicator till a constant weight was obtained. The values obtained by the two methods were found to be in very close agreement.

The validity of the method used was further checked by comparing a few values with those obtained by Gerberg's method. The values obtained were found to agree well as can be seen from the following table.

Sample No	present method	Gerberg's method
	g per 100 ml	
1	4.10	4.00
2	3.90	4.10
3	4.20	4.10
4	3.75	3.80
5	4.00	4.00

The method used was prefered to Gerberg's method as sulphuric acid used in the latter would prevent the use of the lower nonfatty layer for other estimations. Further, the method used has the added advantage of requiring only a small sample.

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.

Lactose: Lactose in milk was estimated essentially according to the method of Plumel (1953).

0.1 ml of milk was diluted to 100 ml. To 1.0 ml of the diluted milk sample was added 1.0 ml of alkaline ferricyanide solution and the mixture kept in a boiling water bath for 15 minutes. The tubes were cooled under tap water and 1.0 ml of sodium fluoride added to each tube as a stabilizer for Prussian blue. This was followed by the addition of 2.0 ml of ferric alum solution in sulphuric acid and the volume made upto 20 ml using distilled water. The colour was read at 660 mμ, in photo-electric colorimeter. The standard with a range of 30 to 80 μg of lactose, and the blank containing water instead of lactose solution, were run along with each set.

In all the above determinations only distilled water was used in the reagents. pH adjustments were made with either N HCL or NaOH, using as indicators Bromocresol green and bromothymol blue respectively. Except where otherwise specified, Whatman filter paper No.1 was used for filtration.