C H A P T E R II

MATERIALS AND METHODS

CHAPTER II

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As stated earlier, the object of these investigations was to formulate and evaluate diets suitable for pre-school children. Different aspects of these studies were carried out as shown below :-

(1) Collection of data on the dietary intake of pre-school children in order to identify the extent of deficiencies of different nutrients in the diets consumed.

(2) Formulation of diets which would correct the above deficiencies.

(3) Chemical and biological evaluation of the formulations.

(4) Sensory evaluation of the diets formulated to ascertain their acceptability to pre-school children.

(5) Field trials :- Organization of a rural play centre in which pre-school children were fed the diets formulated and an assessment made of the effects of the same on physical, clinical and biochemical status.

(6) Comparison of the fed children with the apparently well nourished upper class children.

Dietary intake :-

Data were obtained on the dietary intake of pre-school children in the village of Raipura where the field centre was organized. Aliquot portions of all the foods consumed by six subjects aged two to five years were collected over

a six day period by field workers who were at the homes of the children from the first meal in the morning to the last meal at night. From data obtained on the quantities of raw ingredients used for cooking, and quantities of cooked foods, it was possible, to calculate the amount of raw ingredients in the portion consumed by the subjects using the recipe method (Rajalakshmi,... 1965a).

As the cooking practices in these homes were very similar, a diet survey was done by oral questionnaire method(a specimen of the form used is given in Appendix I) on eighteen more children. The mothers were questioned on how much of the different foods the child ate at different times of the day (they were asked to show the approximate quantity) and food intake calculated from tables prepared for raw food equivalents of cooked foods. As the diets were monotonous (dal, chapatie (home made unleavened bread), vegetable, rice, khichiri, kadi and tea), and standard cooking practices were used in most households, it was possible to do this without much difficulty.

Formulation of diets :-

The data obtained (which will be presented in the next chapter) showed the diets to be deficient in calories, protein, calcium, vitamin A and riboflavin as calculated from food tables (Aykroyd, Gopalan and Balasubramanian,1966).

The dietary formulations were aimed at the correction of the above deficiencies.

(1) Calorie intake was increased by increasing the quantity of foods consumed by making the foods more suitable for young children. Also, it was expected that improving the nutritional quality of food would result in better appetite. Procedures which would make the foods more suitable for young children from the point of view of texture, taste, flavour and digestibility were worked out. A gruel or conjee made of sprouted and roasted grains and a steamed food prepared from fermented batter were found suitable for breakfast and lunch respectively. Addition of groundnut meal to the breakfast gruel and potato to the lunch also served to increase calorie intake.

(2) As cereals are deficient in lysine and have a relatively low protein content (Ramachandran and Phansalkar, 1956), their protein quality was sought to be improved by the use of addition of legumes. The protein in the diet was sought to be made more adequate qualitatively and quantitatively by appropriate combinations of wheat (Triticum aestivum) and bengal gram (Cicer arietinum) with or without added groundnuts (Arachis hypogea).

(3) As growing children require more calcium to meet the demands of a growing skeleton, and as milk, which is by far the best source of calcium, is not available in adequate

quantities, the calcium content of the diet was increased, by incorporating lime powder (a mixture of CaO, $Ca(OH)_2$ and $CaCO_3$), costing practically nothing, in acid foods so as to achieve an increase in calcium content without an adverse effect on either taste or vitamin content. This was done only during the 1966-67 session.

(4) As foods of animal origin, which are the best sources of riboflavin are lacking in common diets, the riboflavin content was increased by the use of sprouted and fermented foods and by regular inclusion of leafy vegetables, which are known to be good sources of the vitamin.

(5) Vitamin C content was increased by the inclusion of leafy vegetables. In addition, a sweetened infusion was prepared from drumstick leaves (Moringa oleifera) and given as an additional source of vitamin C during 1965-66 session.

(6) Dilute butter milk prepared from 2-3g of skim milk powder was included. The amount used was consistent with the amount of milk (20-30g) that would be available at home were the child fed.

Organoleptic evaluation of the diets :-

The recipes based on the above formulations were standardized on the basis of : (a) the acceptance of the

foods by children; (b) the ease with which they could be prepared in rural homes and on a large scale in play centres and nursery schools; (c) their adequacy from the nutritional standpoint; and (d) cost. They were subjected to sensory evaluation by pre-school children in a nursery school.

Chemical and biological evaluation :-

(1) Protein :- Wheat and bengal gram were chosen for the cereal-legume combination to be used. Both were available in fair-price shops at the time of the investigations and were found suitable from the nutritional and organoleptic standpoint. Previous studies in the laboratory showed that the addition of bengal gram to wheat was as effective as that of skim-milk powder or lysine in improving the quality of wheat protein (Tambe, 1965). Further studies were carried out to decide the proportions in which wheat and bengal gram should be combined for maximum improvement and on the effects of adding groundnut with or without skim-milk powder to wheat, bengal gram mixtures. It was considered desirable to add groundnut to the wheat, bengal gram mixture used for preparing the breakfast gruel in order to increase its protein and fat content. Since groundnut is deficient in methionine it was considered desirable to investigate whether such addition affects adversely the protein quality of the mixture and whether the addition of skim milk powder can prevent it.

In experiment I, concerned with the evaluation of wheat-bengal gram mixtures, groups of albino rats were fed on wheat or bengal gram or the two mixed in different proportions.

In experiment II, concerned with the effects of adding groundnut and skim-milk powder, groups of rats were fed on a wheat- bengal gram mixture with or without the addition of groundnut and skim-milk powder.

(2) Calcium :- As the addition of lime water to non acid foods results in losses of vitamins (Pasricha and Rao, 1965; Rajalakshmi and Ramachandran, 1967) studies were made of the effects of adding lime powder in different amounts to sour foods with a pH of less than 5 on their taste, pH, thiamine and riboflavin content, and determinations made of the optimum amount that could be added without adversely affecting taste and vitamin content and without changing the pH to the alkaline range. A selected food (dhokla) in which such incorporation was possible was fed to rats with and without such incorporation and data obtained on the effects of such incorporation on calcium retention and calcium content of tibia and femur (experiment III) (Rajalakshmi and Ramachandran, 1967).

(3) Carotene :- Concurrent investigations carried out by other investigators on the availability of carotene in selected greens showed a fair availability of carotene in leaf greens

(Rajalakshmi and Chari, 1968). Carotene was provided mainly in the form of 30 g of leafy vegetables and frequent use of 30 g. of seasonably available fruits such as papaya (Carica papaya) or rock melon (Cucumis melo) and vegetables such as yellow pumpkin (Cucurbita maxima).

(4) Riboflavin :- Previous studies in this laboratory showed the beneficial effects of sprouting and fermentation on vitamin content and the effects of fermentation on the protein, thiamine and riboflavin status of rats. (Dhand, 1964; Rajalakshmi and Vanaja, 1967). The fermented foods used in the present studies were analysed for thiamine by the thiocrome method and riboflavin by fluorimetric method

(5) Vitamin C :- Previous studies showed that when chopped drumstick leaves are boiled for a few minutes in water and strained, the filtered infusion contains about 1 mg. of vitamin C per g. of leaf used (Rajalakshmi and Kothari, 1964). This was ascertained by repeated analysis. Vitamin C content of the infusion was determined by the method described by Roe (1954).

(6) Whole day diets :- On the basis of these experiments whole day diets were formulated. The breakfast and lunch formulated (omitting the fruit) were prepared as fed to children, dried at 60°C and powdered and fed to one group of rats. Other groups of rats were fed : (a) the home diet

similarly prepared and (b) a combination of the home diet and the play-centre diet representing the dietary intake of children receiving breakfast and lunch at the centre and other meals at home (experiment IV).

The details of the animal experiments carried out are summarized in Tables 6 and 7.

The conduct of the feeding trials :-

The co-operation of parents in the village of Raipura was obtained for organizing a play-cum-feeding centre. Voluntary donations from urban and rural people supplemented by a grant from the Panchayat (Village Council) enabled the villagers to construct temporary hutments and purchase of some toys.

The children came to the centre between 8 a.m. and 9 a.m. and were in the charge of an ayah and a nursery school teacher till about 1 p.m. The meals were prepared by a cook under the supervision of the teacher. They were given the diet shown in Table 8. The methods used in their preparation are shown in Table 9. Their cost and nutritive value are shown in Table 10. Conjee and dhokla were given ad libitum. Records were kept of their attendance and the food consumed by each child. The children who came to the centre to play, but did not eat at the centre, and some children who did not come to the centre served as controls. The age of the subjects was one to five years at start. None of the subjects

Table 6. Diets evaluated in the animal experiments.

Experiment	Evaluation of	Diets used*	Salt mixture used
I	protein value of wheat and bengal gram mixture in different proportions.	Wheat and bengal gram alone or in proportions of 8:1, 4:1, 2:1 and 1:1	Hawk-Oser salt mixture No. 3 (Hawk, Oser and Summerson, 1954).
II	effects of adding groundnut and skim milk powder on the protein value of wheat and bengal gram mixture.	Addition of groundnut to a mixture containing equal parts of wheat and bengal gram at 50% level with or without the addition of skim milk powder at about 8% level.	- do -
III	availability of calcium in lime incorporated in 'dhokla'.	Dried powder of 'Dhokla' prepared from wheat and bengal gram mixture in the ratio 1:1 with and without calcium incorporation.	Calcium salts as in Hawk-Oser salt mixture to one of the groups.
IV	growth and body composition of rats fed the diet formulated.	Diets fed to rats based on the diet provided to pre-school children. The diets used are shown in Tables 11 and 12.	None

* Five ml of groundnut oil were added to 100g of the diet in all the cases. Three drops of shark liver oil were given to each rat once a week in experiments 1, 2 and 3. No vitamin mixture was added as the studies were designed from the standpoint of practical nutrition. For the same reason no salt mixture other than crude common salt was used in experiments 3 and 4. Dehusked bengal gram was used in all the studies.

Table 7. Details of rat experiments described in Table 6

Experiment	No. of groups	No. of animals in each group	Period of treatment (weeks)	Parameters measured	Method used
I	6	8	8	Weight gain, PER, hemoglobin content,	Oxyhemoglobin method of Evelyn & Malloy (1938) for Hb estimation
				Calcium content of tibia and femur	Tibia and femur were ashed at 600-800°C in a muffle furnace and the calcium estimated by permanganate titration.
II	4	6	13	Weight gain, PER, hemoglobin content	
				Calcium content of femur	
III	3	8	4	Weight gain, hemoglobin content, calcium retention, calcium content of tibia and femur and radiological status	Radiographs taken using orwo Rapid, RF_2 film and X-ray of 40 KV, 80 mA with an exposure of 0.1 second from a distance of 44".
IV	3	6	13	Weight gain, hemoglobin content,	
				Performance on Hebb-Williams Maze	Rabinovitch and Rosvold (1951).
				brain enzymes:- L-glutamate-NAD-oxidoreductase (E.C., 1.4.1.2), L-glutamate-1-carboxylase (E.C., 4.1.1.14), 4-amino-butyrate-2-oxoglutarate aminotransferase (E.C.,2.6.1.19)	Rajalakshmi, Govindarajan and Ramakrishnan (1965).
				Liver enzymes :-	
				Xanthine oxidase	Luck (1963).
				Succinic dehydrogenase	Srinivasamoorthy and Swaminathan (1955).

Table 8. Diet given at the rural play centre

Time	Foodstuff given in		Amount (g)	Weight of raw ingredients (g)	
	1965-66	1966-67			
Morning	Conjee with 10g. skim milk powder	Conjee without skim milk powder	200-300	Wheat Bengal gram Groundnut Jaggery	10-15 10-15 20-30 10-20
Midmorning	Drumstick leaf tea.	-	100	Drumstick leaves Jaggery	10 7
Noon	'Dhokla' without lime treatment	Lime treated Dhokla	150-200	Wheat Bengal gram Fat Lime powder (in 1966-67) Salt and seasoning	35-50 35-50 5-7.5 0.3-0.5
	Vegetable	Vegetable	40-50	Potatoes Leaf greens salt and seasoning	20-25 20-25*
	Dilute butter milk	Dilute butter milk	100-200	skim milk powder	2-4
	Fruits	-	30	Papaya or other fruits	30

* Pumpkin was occasionally used as an additional vegetable.

Table 9. Methods of preparation of the diet

Diet	Method
Conjee	Wheat is first soaked in water for about 6-12 hours and allowed to sprout for another 12-24 hours, dried, roasted slightly to develop malty flavour and then ground. Similarly the decorticated bengal gram is pre-soaked in water for an hour and roasted. Groundnut is roasted, skinned and ground. The meals of all the three are mixed in the ratio 1:1:2 and boiled in water for a few minutes to form a thick gruel. Jaggery is added and mixed well.
Drumstick leaf tea	An infusion is prepared by boiling 10g of chopped drumstick leaves for a few minutes and the filtered infusion sweetened with jaggery.
Dhokla	Coarsely ground wheat and bengal gram in the ratio 1:1 mixed with water and salt to form a thick batter and the same fermented overnight at room temperature. The fermented batter steamed with or without the addition of lime powder, cooled, cut into pieces and seasoned (Rajalakshmi, 1965b).
Vegetable	Fresh chopped leaves and cut potatoes were cooked together in water with salt, spices and oil added.
Butter milk	20g. curd prepared from 2g. skim-milk powder and diluted to 100g. of butter milk by adding water and churning.

Table 10. Cost and nutritive value of the diets fed at the baby centre.

	1965-66	1966-67
Cost*	Rs. 0.45	Rs. 0.40
Calories	620-850	560-790
Protein (g)	25-35	21-31
Fat (g)	12-17	12-17
Calcium (mg)	305-380	310-480
Iron (mg)	13-17	13-17
Vitamin A (I.U.)		
as carotene	1900-2400	1600-2200
as preformed vitamin	600	-
Thiamine (mg)	0.8-1.0	0.6-0.9
Riboflavin (mg)	0.7-1.0	0.5-0.7
Vitamin C (mg)	50-60	15-25

* Excluding the cost of skim milk powder.

Table 11. Composition of 100 g. of the diet* used in animal experiment IV.

	Whole day home diet I (g)	Diet at centre plus diet at home II (g)	Diet at centre alone III (g)
Cereals	72 (a)	40 (b)	33
Pulses	7 (c)	23 (d)	33
Groundnut	0.8	7	13
Leafy vegetables	0.2	1	1
Other vegetables	3	3	2
Sugar	10	15	10
Vegetable oils	4	4	3
Skim milk powder	0	4	5
Whole milk powder	3	3	0

* On dry weight basis.
(a) Bajra, wheat, rice, kodri (Paspulum scorbiculatum); (4:2:1:1).
(b) Wheat, bajra, rice, kodri;(3:1:1:1);
(c) Red gram;
(d) Bengal gram and red gram (7:1).

Table 12. Nutritive value of 100 g. of the above diet

	I	II	III
Calories	360	390	400
Protein (g)	10.5	14.0	15.5
Calcium (mg)	114	290	300
Iron (mg)	7.4	8.0	10.0
Vitamin A (I.U.)	220	1150	1590
Riboflavin (mg)	0.22	0.41	0.42

were breastfed at the time of study. The experimentals and controls were matched for initial age, sex, height, weight and food intake.

The feeding programme was in operation from November, 1965 to April, 1966 and from October, 1966 to February, 1967. The following parameters were measured at the beginning and at the end of each experimental period:

Parameter measured	Method used
Weight	Weight was recorded without clothes and during empty stomach using a dietecto balance.
Height	Erect body length was taken with the subject's heels, buttocks, and upper back in contact with an upright board having an inlaid millimeter scale and a sliding horizontal that rests on vertex.
Urine creatinine	Alkaline picrate method (Hawk, Oser, and Summerson, 1954).
Urine thiamine	Thiochrome method (Methods of Vitamin Assay, 1951).
Urine riboflavin	Fluorimetric method (Methods of Vitamin Assay, 1951).
Urine vitamin C	Dinitrophenyl hydrazine method described by Roe and Kuether (1943).
Urine nitrogen	Microkjeldahl method. (Hawk, Oser and Summerson, 1954).
Clinical examination	ICMR schedule (1948).

The following parameters were measured at the beginning and the end in the 1965-66 session but not in the 1966-67 session.

Parameter measured	Method used
Blood hemoglobin	Oxyhemoglobin (Evelyn and Malloy, 1938).
Serum total protein	Biuret method (Reinhold,1953) as described by Varley, 1958) and further modified to a microscale.
Serum albumin	Paper electrophoresis and elution (Varley, 1958).
Serum carotene and vitamin E	Quaife, Scrimshaw, and Lowry, (1949).
Serum ascorbic acid	Lowry, Lopez and Bessey (1945).
Serum alkaline phosphatase (millimoles of p-nitrophenyl-phosphate Split per hour per litre.	Bessey, Lowry and Brock (1946).

In the second session (1966-67) radiological examination of the right hand (palm and wrist) and estimation of salivary amylase were made. The latter was done in the belief that it may be related to serum amylase which is found to change with nutritional status. The collection of saliva does not pose the problems involved in blood collection. Salivary amylase was estimated by the method of Bernfeld (1955)..

The subject was given a piece of candy (25 mg) and asked to retain it in the mouth for 1-2 minute in order to facilitate salivary secretion.

Saliva was collected in clean beakers and transferred to clean test tubes. The tubes were kept in the ice and the

samples were analysed within 4 hours. The samples were diluted 100 to 200 times with 0.02M phosphate buffer pH 6.9.

1 ml of diluted enzyme was incubated for 3 minutes at 30° with 1 ml of substrate solution (1g of soluble starch, in 100 ml of 0.02M phosphate buffer, pH 6.9, containing 0.0067M NaCl). The enzyme reaction was stopped by the addition of 2 ml of dinitrosalicylic acid reagent (1g of 3,5-dinitrosalicylic acid in 20 ml of 2N NaOH and 50 ml water, and 30g of sodium potassium tartrate made up to 100ml with distilled water). The tubes containing this mixture were heated for 5 minutes in boiling water, cooled and 20ml of water added. The optical density of the solutions were read in Klett Summerson Colorimeter using 540 filter. A blank was prepared in the same manner without enzyme. Maltose was used as standard.

Enzyme activity is expressed in terms of milligrams of maltose liberated in 3 minutes at 30°C by 1 ml of enzyme solution.

Comparative data on all the above parameters were also obtained on twenty subjects aged one to five belonging to the upper socio-economic group in Baroda.

Collection of samples for analysis :-

Blood samples (0.3 to 0.5 ml) were collected from the subjects in the fasting state by finger prick. For the

estimation of blood hemoglobin 10 cmm of the blood were immediately transferred to 4 ml of 1% ammonia solution and the tubes were kept in ice and the samples analysed within 4 hours. For other estimations the blood was collected in clean capillary tubes and the serum separated.

In case of vitamin C estimation 20 cmm of the serum were immediately transferred to 4% trichloroacetic acid solution using calibrated λ pipettes and then the sample was frozen and the estimation was carried out the next day. For carotene and vitamin E estimation, 60 cmm of the serum was immediately transferred to tubes and frozen. The analyses were carried out within two weeks. For the estimation of total serum protein, albumin, and alkaline phosphatase the serum samples were preserved at 4°C and the estimations carried out within a week.

Urine samples were collected for 4 hours from 9 a.m. to 1 p.m. everyday for 3 consecutive days at the beginning and at the end of the studies. The subjects were asked to void the urine in a porcelein dish and the urine immediately transferred to coloured bottles containing about one gram of oxalic acid and kept in ice. Aliquots were immediately transferred to 5% trichloroacetic acid for vitamin C estimation and to 0.3N hydrochloric acid for thiamine and riboflavin estimations. All the samples were then preserved in a frozen condition and the estimations were completed within one week.

Animal experiments :-

In the animal experiments albino rats reared and bred in the laboratory and weighing 40 to 50 g at start were used. Cushine's tail vein technique with some modification (Porter, 1959) was used for the collection of blood. For the balance studies with regard to calcium in experimentIII,the animals were transferred after three weeks of treatment to plastic cages kept slightly tilted over a stand so that the urine would drip down through the hole through a plastic funnel containing glass wool into a bottle containing little toluene. The tilt (15° from horizontal) was uniform in all cases. Weighed amounts of food were given and the left-over food collected carefully, dried and weighed. The feces were collected and similarly dried. The samples of urine for 6 days were pooled together, and concentrated by evaporation. Calcium was determined on diet, urine and feces samples after digestion with concentrated nitric acid as given in AOAC (1960).

Cerebrum :-

The animals were killed at the end of treatment by decapitation. The heads were plunged into powdered ice immediately after decapitation. The cerebrum was quickly removed, blotted on a filter paper and chilled in a watch glass kept in ice. The tissue was weighed and then minced.

A weighed portion of the tissue was homogenized in a Potter - Elevehjem homogenizer at 0°C and at 2000 r.p.m. for 30 seconds. A 10% sucrose (0.25 M) homogenate was prepared and used as such for the estimation of the activities of L-glutamate-1-carboxylase (E.C., 4.1.1.15) and 4-aminobutyrate-2-oxoglutarate aminotransferase (E.C., 2.6.1.19).

A 25% phosphate extract (0.02M phosphate buffer pH 7.0) was prepared and centrifuged at 5900 g. at 0°C in a servall refrigerated centrifuge. The supernatant obtained was used for the estimation of the activity of L-glutamate-NAD-oxidoreductase (E.C., 1.4.1.2). The activities of the enzymes were assayed using the method described by Rajalakshmi, Govindarajan and Ramakrishnan, (1965).

Liver :-

Soon after decapitation (and removal of the cerebrum in experiment IV) the liver was quickly removed, freed from adhering blood and chilled in a watch glass kept in ice. The tissue was weighed and then minced. A weighed portion of the tissue was homogenized with a grinding medium in a Potter Elevehjem homogenizer at 0°C for one minute at 2000 r.p.m. For xanthine oxidase, a 10% homogenate was prepared in 2% sodium fluoride. For succinic dehydrogenase, a 10% homogenate in 0.1M phosphate buffer, pH 7.4 was prepared.

Femur and tibia :-

The femur and tibia were removed from both sides, freed from adhering connective tissue and then weighed, after which they were dried in an electric even at 60°C to a constant weight. Fat was removed by immersing the bones in petroleum ether for 24 hours and the left tibia and femur were ashed at 600-800°C for 8 to 12 hours and the weight of ash and its calcium content determined. In experiment III the right tibia and femur were weighed, dried and freed of fat, and radiographs taken of the same. In the other experiments, the right and left femur were pooled together and ashed for analysis after the determination of fat free dry weight.