CHAPTER II

MATERIALS AND METHODS

MATERIALS AND METHODS

As stated earlier, the present studies were designed to investigate the effects of nutritional deficiencies with special reference to undernutrition during the neonatal period and both undernutrition and protein deficiency during the postweaning period. Additional studies were undertaken on the skeletal status of rats fed cereal-based diets varying in protein quality and in overall nutritive value on the development and composition of the bone.

Different experiments concerned with the above aspects were carried out. Details of these experiments are summarized in Table 11.

Animals

Albino rats were used in the investigations. The animals used in the different groups were matched for age, sex and body weight. Unless otherwise stated, weanling rats weighing from 40-55 g were used.

Diets

For the studies on the effects of neonatal undernutrition (experiment I) the mothers were fed the stock diet (Table 12). For the studies on the effects of undernutrition during the postweaning period (experiment II) a modified Sherman's diet shown in Table 13 was used. For

Table 11 : Details of the experiments carried out.

experiment	experimental manipulation	diets used	mode of feeding	no. of groups	no. of animals in each group	age at start (weeks)	period of treatment (weeks)
I	neonatal undernutrition	stock diet (Table 12)	food intake of experimentals restricted by increasing litter size to 16	2	8 or 16	soon after birth	3
II	postweaning undernutrition	modified Sherman's diet (Table 13)	experimentals fed 50% of *ad lib.* food intake of controls.	3	8	4	16
III	postweaning protein deficiency	low and high protein diets (Table 14)	*ad lib.*	2	8	4	10
IV	postweaning protein deficiency as compared to calorie undernutrition.	low and high protein diets (Table 14)	undernourished animals given 66.7 and 33.3% of the *ad lib.* food intake of HP animals.	4	6	12	10
V	variations in dietary protein content.	5,8,10,15 and 20% protein (casein) diets (Table 14).	*ad lib.*	5	8	4	10

contd.

Table 11 : contd.

experiment	experimental manipulation	diets used	mode of feeding	no. of groups	no. of animals in each group	age at start (weeks)	period of treatment (weeks)
VI	variations in dietary protein quality	kodri (Paspalum scorbiculatum L.) alone or with lysine added and 7.3% casein diet.	ad lib.	3	8	4	10
VII	variations in nutritive quality of diet.	maize (Zea mays) and wheat (Triticum aestivum) alone, or with gram and greens added and cereal-gram-greens mixtures, Modern bread and 10% casein diet.	ad lib.	9	5-8	4	8

studies involving variation in protein content, a diet of semipurified ingredients with casein as the sole protein source was used (Table 14).

Table 12 : Composition of stock diet.

ingredient	amount (g)
wheat flour (Triticum aestivum)	35
bajra flour (Pennisetum typhoideum)	10
bengal gram flour (Cicer arietinum)	11
milk powder	21
sprouted legumes* (equal amounts of greengram (Phaseolus aureus, Roxb.) and cowpeas (Vigna catiang)).	16
groundnut oil	7
dark green fresh leafy vegetables	6-8
crude common salt	1

* in terms of dry legumes.

Table 13 : Composition of Sherman's* diet (modified)

ingredient	amount (g)
wheat flour	60
milk powder	40
groundnut oil	5
crude common salt	1

Two to three drops of shark liver oil were given orally to all animals once a week providing 70-100 mcg of vitamin A.

* Sherman (1949).

Table 14 : Composition of the diets used for variations in dietary protein content.

ingredient	amount (g)
vitamin free casein* + sago flour	87
vitamin mixture	2
salt mixture	4
groundnut oil	7
shark liver oil	2-3 drops per week per rat providing 70-100 mcg of vitamin A.

* amount adjusted so as to provide protein at the required level.

Edible casein was washed first with alcohol and then washed free of the latter with tap water and finally with distilled water. The washed casein was dried and used. Each lot was analysed for nitrogen content by the microkjeldahl method and protein content calculated therefrom.

Commercially available sago prepared from tapioca flour (Manihot utilissima) was ground and used in place of starch as it proved more suitable than commercially available starch. It contains only 0.2 per cent protein and no more than traces of vitamins and minerals. Tapioca flour is processed to some extent during the preparation of sago and the starch in the

same is believed to be readily available (Booher, Behan and McCance, 1951).

The diets used for the studies on variations in protein quality in experiments VI and VII are indicated in Tables 15 and 16.

Table 15 : Composition of the diets used in experiment VI.

ingredient	g per 100 g of diet		
	1	2	3
kodri (Paspalum scorbiculatum L.)	87.0	87.0	-
lysine	-	0.173	-
casein	-	-	8.0
sago	-	-	79.0
salt mixture	4.0	4.0	4.0
vitamin mixture	2.0	2.0	2.0
groundnut oil	7.0	7.0	7.0
protein in the diet	7.3	7.3	7.3

Two to three drops of shark liver oil were given orally to all animals once a week providing 70-100 mcg of vitamin A.

Table 16 : Composition of different diets used in experiment VII.

ingredient	amount (g)	
	unsupplemented	supplemented
cereal or millet*	75.0	60.0
dehusked bengal gram (Cicer arietinum)	-	15.0
groundnut oil	4.0	4.0
fresh fenugreek leaves (Trigonella foenum graecum)	-	20.0
crude common salt	1.5	1.5
spicespices**	1.5	1.5

* This was either wheat (Triticum aestivum), maize (Zea mays), rice (Oryza sativa), bajra (Pennisetum typhoideum), or jowar (Sorghum vulgore).

** Chili powder (0.60 g); turmeric powder (0.30 g) and cumin seeds (0.60 g).

The vitamin mixture used was formulated previously in this laboratory largely on the basis of recommendations made by Brown and Sturtevant (1949), and NAS-NRC (1962). The composition of the same is given in Table 17. The prepared mixture was stored in brown bottles in the cold room.

Table 17 : Composition of vitamin mixture.

vitamin	amount per kg. of the diet
thiamine hydrochloride (mg)	1.5
riboflavine (mg)	2.5
pyridoxine hydrochloride (mg)	1.0
niacin (mg)	15.0
calcium-d-pantothenate (mg)	10.0
choline chloride (mg)	750.0
inositol (mg)	200.0
para amino benzoic acid (mg)	10.0
folic acid (mg)	1.0
cyanocobalmin[a] (mcg)	5.0
biotin (mcg)	1.0
powdered sugar to make a total weight of 20 g.	

The salt mixture used was the Hawk-Oser salt mixture No. 3 (Hawk, Oser and Summerson, 1954). The composition of the same is given in Table 18. The mixture was prepared in bulk and stored in air tight bottles.

The diets to be fed were prepared once a week. The vitamin mixture and groundnut oil were added just before feeding.

Table 18 : Composition of salt mixture.

ingredient	amount (g)
salt mixture A*	16.7
dicalcium tricitrate, $4H_2O$	308.2
$Ca(H_2PO_4)_2 H_2O$	112.8
K_2HPO_4	218.7
KCl	124.7
NaCl	77.0
$CaCO_3$	68.5
$3MgCO_3 . Mg(OH)_2 . 3H_2O$	35.1
$MgSO_4$ (anhydrous)	38.3

* 100 g of salt mixture A contained : Ferric Ammonium Citrate, USP, 91.41 g; $CuSO_4 . 5H_2O$, 5.98 g; NaF, 0.76 g; $MnSO_4 . 2H_2O$, 1.07 g; $KAl(SO_4)_2 . 12H_2O$, 0.54 g; and KI, 0.24 g.

The millets, cereals, pulses, sago and groundnut oil were purchased in bulk from the local market. Casein and milk powder were purchased from Amul Dairy, Anand.

Mode of feeding

The animals were housed in galvanized iron cages individually except for the studies on neonatal undernutrition and water provided ad lib.. Food was provided ad lib. or in restricted amounts according to the requirements of the experiment.

Food intake was recorded daily and body weight once a week.

Chemicals

The chemicals used in the experiments were of research grade purity and were generally obtained from the British Drug House Ltd. or from E. Merck.

Parameters measured

In all the experiments determinations were made on the femur of length, wet weight, dry weight, fat-free dry weight, ash and calcium content.

The different experiments concerned with different aspects are briefly described below :

Experiment I

Since neonatal undernutrition is found to influence growth, physical and neuromotor development, studies were made of the effects of the same on bone development as judged by the composition of the femur. The same was induced by increasing litter size. Pups born on the same day were pooled as soon as possible after birth and assigned to foster mothers in litters of 8 or 16 till 3 weeks of age. The mothers were given food and water ad lib. throughout the lactation period.

Experiment II

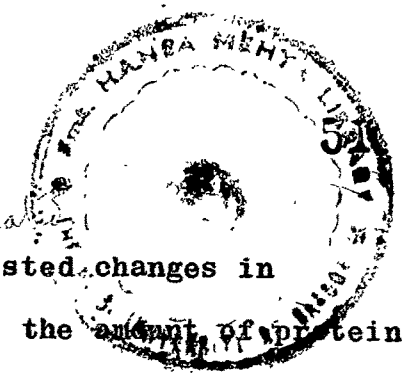
This was an extension of the studies on undernutrition to the immediate postweaning period. The food intake of the experimentals was restricted to 50% of the ad lib. intake of controls. At the end of 5 weeks, the former were divided into two groups and one group continued on the restricted diet and the other fed ad lib. for a further period of 11 weeks.

Experiment III

Since protein deficiency has been found to influence the composition of the bone in both children and experimental animals, studies were made of the effects of a low protein diet on bone composition. Animals were fed ad lib. either a low protein (LP) or a high protein (HP) diet containing 5 or 20 per cent protein.

Experiment IV

Since protein deficiency appeared to have effects different from those of undernutrition, comparative studies were made of both in the same experiment. Groups of animals were fed either the LP or HP or the latter in restricted amounts representing 66.7% and 33.3% of the food intake of animals fed ad lib. In this experiment the animals were 12 weeks old at start.



Experiment V

Since the previous experiments suggested changes in bone composition with a low protein diet, the amount of protein in the diet was varied in order to determine the level of protein needed to prevent these changes. Groups of animals were fed 5, 8, 10, 15 or 20% protein in the form of casein for a period of 10 weeks.

Experiment VI

As ordinary cereal or millet-based diets are poor not only in protein content but also in protein quality, studies were made of the effects of variations in the latter by comparing animals fed a diet composed mainly of kodri (Paspalum scorbiculatum L.), a millet very deficient in lysine, or the same with lysine added. An additional group was fed an isonitrogenous diet with casein as protein source.

Experiment VII

This was an extension of the previous studies on protein quality. Wheat (Triticum aestivum) and maize (Zea mays) are commonly used food grains, and in the absence of animal foods, their nutritional improvement is best brought by the addition of legumes and leafy vegetables (Rajalakshmi, Sail, Ramchandran, Chandrasekaran and Subbulakshmi, 1972).

In 1969, "Modern bread" made of wheat flour fortified with lysine and other nutrients was introduced in the market with international assistance with the professed long term objective of improving the nutritional status of people subsisting on cereals. However, in this laboratory, diets based on foods locally available in rural areas, particularly appropriate combinations of cereals, legumes and greens, have been advocated (Rajalakshmi and Ramakrishnan, 1967; Rajalakshmi, et al., 1972; Rajalakshmi, Sail, Shah and Ambady, 1973). Studies were therefore undertaken on the comparative nutritive value of Modern bread as compared to such combinations as judged by several parameters including femur composition.

Chemical analysis

At the end of treatment the animals were killed by decapitation. The femur was removed and freed from adhering tissue. Wet weight was taken as soon as possible and length was measured with vernier calipers.

Moisture

The femurs were dried in a hot air oven maintained at 60^{o} till constant weights were obtained. The loss of weight was taken as moisture content.

Fat-free weight

Fat was extracted from dry femur by immersing it in petroleum ether in a staining jar at room temperature (25-30°) for 24 hours. The femur was wiped with filter paper and dried at 60° till constant weights were obtained. The weight loss during extraction with petroleum ether was taken as fat content.

Bone ash

Fat-free dry femur was taken in previously ignited, cooled and weighed crucibles, charred and then incinerated at 550-700° in a muffle furnace till it was completely ashed. The weight of the ash formed was then determined.

Calcium

Calcium content of the femur was estimated by the method described in A.O.A.C. (1950). The ash obtained by dry ashing was dissolved in a few drops of concentrated nitric acid and made up to a known volume with glass distilled water. One ml of the diluted solution was taken in a centrifuge tube to which were added two ml of glass distilled water and three to five drops of bromocresol green indicator. The pH was adjusted just to 4.8 to 5.0 (indicated by the appearance of blue colour) with saturated solution of sodium acetate and brought back to 4.4 to 4.6 with 3 per cent oxalic acid as indicated by the appearance of a distinct green shade. At

this pH calcium is completely precipitated as calcium oxalate. The tubes were kept for 45 minutes in a sand bath at 85^{o} to allow complete precipitation of calcium oxalate. The tubes were kept overnight to allow the precipitate to settle down. The following day the tubes were centrifuged for half an hour at 3000 rpm in a clinical centrifuge and the supernatant was drained off and the tubes inverted over a filter paper for 2-3 minutes. The precipitate was washed with 3 ml of 2% ammonia solution, again centrifuged and the supernatant drained off. This was repeated once again. The precipitate was dissolved in 3.0 ml of 1N H_2SO_4, kept at 80^{o} for 3 minutes and titrated against standard potassium permanganate. Calcium content was calculated from the amount of permanganate needed for titration.