CHAPTER II

MATERIALS AND METHODS

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As mentioned earlier, the present studies were concerned with the changes in the size and chemical make up of selected bones during maturation and the impact of malnutrition on these changes in relation to age, severity and type of nutritional stress.

The bones investigated were the pelvis, femur, tarsus and mandible. Pelvic and femur bones were chosen as representatives of long bones. Femur is a typical long bone whereas pelvic bone is a compound bone in the sense it is composed of ilium, ischium and pubis. Mandible differs from other bones in its early mode of development as a membranous (flat) bone. So it was included in the studies as a representative of membrane bone. Tarsus is composed of 8 short bones which are separable in early stages but are fused in later stages. It was taken to represent the short bone (Crouch, 1967; Rowett, 1960; Wells, 1964).

The experiments concerned with the size and chemical make-up of these bones are indicated below:

Experiment 1: The pattern of changes during growth and maturation.

Experiment 2: The effects of different degrees of undernutrition during the suckling period.

Experiment 3: The effects of deficiencies of protein and food energy during the postweaning period.

Experiment 4: The effects of maternal vitamin A deficiency during gestation and lactation on the bones of the progeny.

Experiment 5: The effects of vitamin A deficiency during the postweaning period.

Experiment 1: The pattern of changes in the composition of selected bones during growth and maturation:

Groups of animals (males and females) were killed as soon as possible after birth and at 1,2,3,5,9,12 and 26 weeks of age. The controls in experiment two, three and five were used for 3,9 and 12 weeks. The animals killed at each age are believed to be representative of their age groups for body weight.

Experiment 2: The effects of different degrees of undernutrition during the suckling period on the composition of selected bones:

In this experiment, undernutrition was induced by either feeding the mother a low protein diet (5% protein diet - LP) after partus or by increasing the litter size from 8 to 16 (LL) and feeding the normal diet (20% protein diet - HP). Pregnant rats were obtained from the stock colony. Pups born the same day were pooled together and

assigned to different mothers in litters of 8 or 16. The mothers assigned standard size litters (8) were fed diets containing either 20% (G^+L^+) or 5% protein (G^+L^-) in the form of casein. The mothers assigned large litters (LL) were fed the high protein diet. The three groups are referred to as G^+L^+, G^+L^- and LL, respectively indicating maternal protein status during gestation (G) and lactation (L) and a large litter size (LL).

Experiment 3: The effects of deficiencies of protein and food energy after weaning:

In order to study the effects of postweaning deficiencies of food energy and protein, weanling rats were derived from litters nursed by mothers fed high protein diet. The animals were divided into three groups and fed either a low protein diet (LP) or a high protein diet (HP - control), or the latter in restricted amounts (HP-R). The degree of restriction was such as to get a group matched for body weight with the low protein animals.

Experiment 4: The effects of maternal vitamin A deficiency during gestation and lactation on the bones of the progeny:

Vitamin A deficiency was induced by feeding the mother a diet low in vitamin A from a month before mating until

partus and a diet devoid of vitamin A thereafter. This procedure was effective in previous studies in inducing vitamin A deficiency without resulting in resorption of the fetuses or high mortality after birth. In order to ensure that the effects are not due to the resulting growth retardation, an additional control group was used with a comparable degree of growth retardation achieved by increasing litter size to 16 and feeding normal diet.

Experiment 5: The effects of vitamin A deficiency during the postweaning period:

Groups of normally-reared weanling rats weighing 42-45 g were fed 20% protein diet (HP) with and without vitamin A or the former in restricted amounts. Again the degree of restriction was such as to get a group matched for weight with the vitamin A deficient animals.

In all the experiments water was provided ad lib. and so was food except when required otherwise by the experimental procedure. Body weights were recorded once a week. Food intake was recorded daily in the case of the studies carried out after weaning.

Animals used in this study were albino rats of Charles Foster strain maintained in the stock colony of the department animal house.

The composition of the diets used in these investigations is given in Tables 7 and 8.

Edible casein from buffalo milk, obtained from the Amul Dairy, Anand, was washed first with alcohol and then washed free of alcohol with tap water and finally with distilled water and dried. The amount to be included in the diet was decided on the basis of its nitrogen content determined by the microkjeldahl method.

Commercially available sago prepared from tapioca flour (Manihot utilissima) was ground and used as starch source as it contains only 0.2% protein and no more than traces of vitamins and minerals. As tapioca flour is processed to some extent during the preparation of sago, the starch in the same is believed to be readily available (Booher et al., 1951). This practice has been followed in this laboratory for the past several years.

The vitamin mixture used was formulated previously in this laboratory on the basis of the allowances suggested by Brown and Sturtevant (1949), recommendations made by NAS-NRC (1962) and reviewed by Mitchell (1964). The composition of the same is given in Table 9. The vitamin mixture was stored in dark brown bottles at 3-4°.

Table-7: Composition of the Stock Diet

Ingredients		Amount (g)
Wheat flour (*Triticum vulgare*)	..	350
Bajra flour (*Pennisetum typhoides*)	..	100
Bengalgram flour (*Cicer arietinum*)	..	110
Milk powder	..	210
Sprouted legumes*	..	160
Groundnut oil	..	70
Dark green leafy vegetables**	..	60-80
Crude common salt	..	10
Calcium carbonate	..	10
Vitamin mixture	..	20

* - Mixture of greengram (*Phaseolus radiatns*) and cow peas (*Vigna sinensis*)

** - Mostly fenugreek (*Trigonella foenum graecum*) and spinach (*Basella rubra*)

Table-8: Composition of Low and High Protein Diet

	% Dietary Protein	
	5%*	20%**
Washed Casein (g) ..	6	24
Vitamin mixture (g) ..	2	2
Salt mixture (g) ..	4	4
Groundnut oil (g) ..	7	7
Sucrose (g) ..	18	-
Sago (g) ..	63	63

* - Used for inducing maternal as well as postweaning protein deficiency.

** - Standard diet for the control.

Table-9: Composition of the Vitamin Mixture

	Amount/Kg of the diet
Thiamine hydrochloride (mg) ..	1.5
Riboflavin (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-aminobenzoic acid (mg) ..	10.0
Folic acid (mg) ..	1.0
Cyanocobalamin (mcg) ..	5.0
Biotin (mcg) ..	1.0
Vitamin A acetate (mcg)† ..	1720.0
Cholecalciferol (mcg) ..	7.5
Powdered sugar approximately 19 g so as to make a total of 20 g.	

† - Omitted in vitamin A deficient diet.

The salt mixture used was Hawk-Oser salt mixture No.3 (Hawk et al., 1954), the composition of which is given in Table-10. The salt mixture was prepared in bulk and stored in air tight bottles.

The diets were prepared once a week and the vitamin mixture and groundnut oil added at the time of feeding.

Chemicals:

The chemicals used in the experiments were of research grade purity and obtained either from the British Drug House or Sarabhai Chemicals or E.Merck. Water was glass distilled before use. The reagents used for various estimations are given in Table-11.

Morphological Measurements:

Measurements of bone length and width as indicated in Figure 4-e were made using Vernier calipers.

Chemical estimations:

Moisture: The bone was dissected free of all soft tissue as quickly as possible after slaughter and weighed immediately to get the fresh weight of the bone. In the case of mandible the teeth were removed before weighing. The bone was then dried in a hot air oven at 60° until constant weight was obtained. The difference between moisture free weight and fresh weight was taken as moisture content. Although moisture

Table-10: Composition of the salt mixture

		Standard (g)
Salt mixture A†	..	16.7
Calcium citrate	..	308.2
$Ca(H_2PO_4)_2H_2O$	..	112.8
K_2HPO_4	..	218.7
KCl	..	124.7
NaCl	..	77.0
Calcium carbonate	..	68.5
$MgSo_4$ (anhydrous)	..	38.3
$3\ MgCO_3 \cdot Mg(OH)_2,\ 3H_2O$	..	35.1

† Salt Mixture A

		(g)
$FeNH_4$ citrate, USP	..	91.41
$CuSO_4 \cdot 5H_2O$	..	5.98
NaF	..	0.76
$MgSO_4 \cdot 2H_2O$	..	1.07
$KAl\ (SO_4),\ 12H_2O$	..	0.54
KI	..	0.24
		100.00

		Per 100g diet
Amount of Salt mixture (g)	..	4.0
Calcium (mg)	..	440
Phosphorous (mg)	..	267
Ca:P ratio	..	1.65
Sodium (mg)	..	121
Potassium (mg)	..	654

FIGURE: 4-e

Measurements of Length and Width

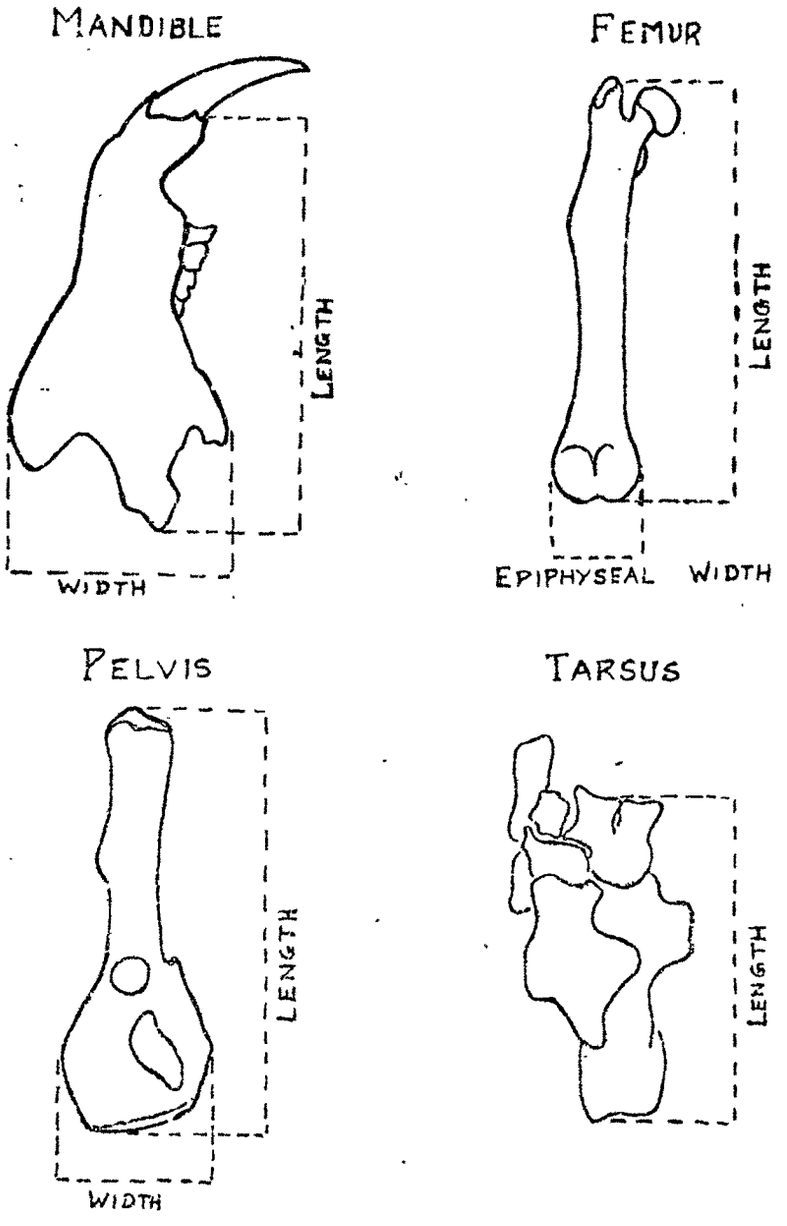

Table-11: Reagents used and the methods of preparation

Reagent	Method of Preparation
1. Acetylacetone reagent	: One ml of redistilled acetylacetone dissolved in 50 ml of 0.5N sodium carbonate solution. The reagent prepared fresh before each estimation.
2. Aminonaphtholsulphonic acid	: 195 ml of 15% sodium bisulphite solution taken in a glass stoppered cylinder and 0.5 ml of aminonaphtholsulphonic acid and 5 ml of 20% sodium sulphite added, the contents shaken well and dissolved and the solution transferred to a brown glass bottle: stored at 0-4°.
3. Ammonium molybdate	: 25 g of ammonium molybdate dissolved in 200 ml of distilled water, 300 ml of 10N sulphuric acid added and the final volume made up to 1000 ml.
4. Ammonia solution	: 4 ml of 25% ammonium hydroxide (25% v/v) made up to 100 ml with distilled water.
5. Bromocresol green solution	: 100 mg of bromocresol green dissolved in 10 ml of distilled alcohol.
6. 2M Calcium chloride solution	: 22.2 g of anhydrous calcium chloride dissolved in water so as to make a final volume of 100 ml.
7. Carbazole reagent	: 125 mg of carbazole dissolved in methanol so as to get a final volume of 100 ml and stored at 4° in the dark. The reagent is stable for 12 weeks.

Table-11 (continuation)

Reagent	Method of Preparation
8. 5% Cetylpyridinium chloride	: 5 g of cetylpyridinium chloride dissolved in water so as to make a final volume of 100 ml.
9. 0.1% Cetylpyridinium chloride	: 175 mg of sodium chloride dissolved in water, 100 mg of cetylpyridinium chloride added and the volume made up to 100 ml.
10. 0.1M citrate buffer (pH 6.0)	: A. 58.8 g of sodium citrate. $2H_2O$ dissolved in 2 litres of distilled water. B. 19.2 g of citric acid dissolved in one litre of distilled water. 150 ml of citric acid solution (B) added to 2 litres of sodium citrate solution (A) and pH adjusted to 6.0 by small additions of either A or B as appropriate
11. Ehrlich's reagent for hydroxyproline	: A. 2 g p-dimethylaminobenzaldehyde dissolved in 3 ml 60% perchloric acid. Three volumes of(A) mixed with 13 volumes of analar isopropanol before use.
12. 6N Hydrochloric acid	: 50 ml of concentrated HCl (12N) diluted to 100 ml with water.
13. Mucopolysaccharide buffer (MPS buffer)	: 932 mg of KCl, 203 mg of $MgCl_2 \cdot 6H_2O$, 372 mg of EDTA, and 106 mg of L-cysteine-HCl dissolved in 75 ml of water, the pH adjusted to 8.0 with dilute KOH solution and the volume made up to 100 ml.

Table-11 (continuation)

Reagent	Method of Preparation
14. Oxalic acid	: 3 g of oxalic acid dissolved in 100 ml of water.
15. 0.01 N Oxalic acid	: 63 mg of oxalic acid dissolved in 100 ml of water.
16. Oxidant solution	: A. 7% (W/V) aqueous solution of chloramine - T (the sodium salt of p-toluene sulfonchloramide was prepared by dissolving 7 g of the salt in 100 ml water. B. An acetate/citrate buffer of pH 6.0 was prepared by dissolving 57 g of sodium acetate ($2\ H_2O$), 5.5 g of citric acid (H_2O) and 385 ml of isopropanol in water and made up to one litre with water. This solution was stable indefinitely. Solutions A and B were mixed in 1:4 proportion before use.
17. p-dimethylaminobenzaldehyde reagent	: 0.4 g of p-dimethylaminobenzaldehyde dissolved in 95 ml of methanol and 15 ml of concentrated hydrochloric acid added.
18. Sodium acetate	: Increasing amounts of sodium acetate dissolved in water till a saturated solution is obtained as seen by the undissolved salt.
19. 5% Sodium carbonate	: 5 g of anhydrous sodium carbonate dissolved in 100 ml of water.
20. 1 M Sodium chloride	: 5.845 g of sodium chloride dissolved in water to a final volume of 100 ml.

Table-11 (continuation)

Reagent	Method of Preparation
21. Sodium tetraborate reagent	: 954 mg of sodium tetraborate, 10 H_2O (Borax) dissolved in concentrated sulphuric acid and the final volume made up to 100 ml with the same.
22. Standard solution of $KMnO_4$ (0.01N)	: Stock solution:- 316.2 mg of $KMnO_4$ dissolved in 100 ml of distilled water.
	Working standard:- One ml of stock standard solution diluted to 10 ml with water and standardised by titrating against 0.01 N oxalic acid.
23. Uronic acid standard	: 40 mg of glucuronolactone dissolved in water saturated with benzoic acid and made up to 100 ml with the same to give a concentration of 400 mcg/ml. One ml of this was further diluted to 10 ml so as to result in a concentration of 40 mcg/ml.

content obtained by such a gravimetric procedure is not considered very reliable every attempt was made to ensure a reasonable degree of reliability and the resulting picture found to yield information of some interest.

Fat-free dry weight:

The whole bone was immersed in petroleum ether: distilled alcohol (2:1 v/v) at room temperature (25-30°) for 24 hours, wiped free of solvent with filter paper and dried at 60° until constant weight was obtained. The difference in the weights of the dry bone before and after treatment with solvent mixture was taken as fat content. The term 'fat' is used as triglycerides form the major lipid component of bone lipids; but the value includes lipids other than triglycerides and fatty acids.

Ash :

The fat-free dry bone was taken in previously ignited, cooled and weighed crucible, weighed, and ashed at 600° in a muffle furnace for about 8 to 9 hours till constant weight was obtained and the ash was pure white. The crucible was allowed to cool to room temperature, weighed and the ash content calculated from the difference in weights.

Estimation of Calcium :

Calcium was determined according to the method described in A.O.A.C. (1950). For this the calcium present in the

bone ash was precipitated as calcium oxalate in acidic medium (pH 4.5). The precipitate was treated with sulphuric acid and the oxalic acid released was titrated against standard potassium permanganate.

Bone ash was dissolved in glass distilled water with a few drops of concentrated nitric acid and the volume made up with water so that one ml of the sample contained approximately 2 mg of ash. One ml of this sample was taken in a centrifuge tube and additions were made of 2.0 ml of glass distilled water and 3-5 drops of bromocresol green. To this, saturated sodium acetate was added gradually till the solution became blue in colour. This was followed by the addition of 3% oxalic acid till the solution turned yellowish green. The tube was kept in a sand bath for 45 minutes at 80° to allow complete precipitation of calcium oxalate. The precipitate was allowed to settle overnight.

The sample was centrifuged the following day, the supernatant drained off and the tube inverted over a filter paper for 2-3 minutes so that any remaining supernatant was removed. The precipitate was washed with 3.0 ml of 1% ammonia solution, again centrifuged and the supernatant drained off. The whole procedure was repeated once again. The precipitate was dissolved in 3.0 ml of 1N sulphuric acid, kept at 80° for 3 minutes and titrated against standard potassium permanganate.

Calculation :

Calcium content was calculated on the basis that a titre reading of 1 ml of 0.01 N potassium permanganate represents 0.2 mg of calcium.

Estimation of phosphorus :

This was done according to the method described by Fiske and Subba Row (1925). In this method, the inorganic phosphorus is treated with an acid molybdate reagent which reacts with it to form phosphomolybdic acid. The hexavalent molybdenum of the phosphomolybdic acid is reduced by 1,24-aminonaphtholsulphonic acid to give a blue compound.

The sample solution prepared for the estimation of calcium was used for the estimation of phosphorus. 0.5 ml of the same was diluted to 10 ml with distilled water and 1 ml of the diluted sample taken in a test tube and diluted to 8.6 ml with distilled water. To this was added 1 ml of molybdate II reagent and 0.4 ml of aminonaphtholsulphonic acid solution (ANSA). The contents of the tube were mixed well on a vortex mixer and allowed to stand at 27-30^{o} for 30 minutes and the colour developed read at 660 nm against a blank containing 8.6 ml of distilled water, 1 ml of molybdate II and 0.4 ml of ANSA. A standard graph was obtained using different concentrations of standard phosphate solution.

water and the washings added to the tube. Approximately 20 mg of norite (activated charcoal) were added, the tube shaken well and centrifuged at 3000 rpm for 10 minutes. The supernatant obtained was used for the estimation of hexosamine.

Treatment of above supernatant for the estimation of hexosamine:

One ml of the superanatant was taken in a 3 ml graduated glass stoppered test tube. To this was added one drop of phenolphthalein followed by the addition of a minimum quantity of 0.5N sodium hydroxide so as to get a pink colour. Half normal hydrochloric acid was added gradually until the indicator colour just disappeared. One ml of acetylacetone reagent was added and the final volume made up to 3 ml by the addition of distilled water. The tubes were closed and heated in a boiling water bath for 15 minutes, cooled, and 2.5 ml of 95% ethanol added followed by the addition of 1 ml of Ehrlich's reagent. The pink colour developed after 30 minutes was read at 540 nm against a blank in which water replaced the sample.

Estimation of chondroitin sulphate:

This was done according to the method of Horowitz and Dorfman (1968). For the isolation and estimation of chondroitin sulphate, fresh bone was homogenised with the mucopolysaccharide (MPS) buffer described by Perlman et al,

(1964) except that phenol red was omitted. The mucopolysaccharide was isolated essentially by the method described by Horowitz and Dorfman (1968) but without the addition of carrier.

The fresh bone was finely ground using a pestle and mortar with approximately 8-10 ml of mucopolysaccharide buffer (MPS buffer) and the sample transferred to a test tube, kept in a boiling water bath for 3 minutes and cooled to 28-30°. The pH of the sample was adjusted to 5.5 with 0.5N hydrochloric acid, 5 mg of papain in 0.1 ml of water was added and the tube incubated at 60° for 18 hours. The enzyme was inactivated by heating the tube in a boiling water bath for 3 minutes. The tube was cooled to room temperature (28-30°) and the contents transferred to a centrifuging tube and centrifuged at 10,000 x g for 10 min. at 25° in a refrigerated centrifuge. The supernatant obtained was collected and dialysed for 24 hours against distilled water with frequent changes. The dialysed solution was collected and made up to 10 ml with water. Six ml of the dialysed solution were placed in another test tube, 0.2 ml of 1M sodium chloride and 0.5 ml of 5% cotylpyridinium chloride (CPC) added, the tube kept at 37° for one hour and centrifuged at 2000 rpm for 10 minutes at 28-30° in a clinical centrifuge. The precipitate obtained was washed twice with 5 ml of 0.1% cetylpyridinium chloride in 0.03 M sodium chloride and the MPS:CPC complex reprecipitated by the addition

of 9 volumes (4.5 ml) of ethanol:ether (2:1 v/v) mixture and 5.0 ml of solvent ether, dried overnight at 37^{o} in a hot air thermostat and dissolved in 10.0 ml of water. This solution was used for the estimation of uronic acid.

Estimation of uronic acid :

This was done according to the method of Bitter and Muir (1962). 0.5 ml of the above solution was made up to 1.0 ml with water. The sample was then kept at -10^{o} for 12 hours. 5 ml of chilled sodium tetraborate reagent was added and the tube placed in an ice:salt mixture and its contents gently mixed. The tube was then heated in a boiling water bath for 10 minutes, cooled to room temperature (28-30^{o}), treated with 0.2 ml of carbazole reagent, shaken and kept in a boiling water bath for another 15 min and cooled again. The colour intensity of this sample was measured at 540 nm against a blank made up to 1 ml of distilled water, 5.0 ml of sodium tetraborate reagent and 0.2 ml of carbazole reagent and treated in the same manner. A standard graph was obtained using different concentrations of glucuronolactone standard.

Estimation of hydroxyproline:

This was done in order to derive an estimate of the amount of collagen in bone. The hydrolysis and neutralisation of the sample was according to the 'direct acid method' of Pirschein and Shill (1966). The hydroxyproline in the sample was determined according to the method of Bergman and Loxley (1963).

Dry, fat-free bones were broken into small pieces and taken in test tubes. Ten ml of 6N hydrochloric acid were added and the tubes closed with glass stoppers and sealed with plaster of Paris. The samples were hydrolysed at 120° for 18 hours and cooled. The tubes were opened as described and the samples filtered through Whatman No. 1 filter paper.

One ml of the filtrate was treated with 6 ml of 5% sodium carbonate and 3 ml of the 0.1 M citrate buffer, pH6.0. The resulting neutralised solution was expected to be slightly acidic but was found to have no effect upon colour development. The neutralisation was carried out in 50 ml volumetric flasks in order to prevent the loss of material by splashing during the evolution of carbon dioxide.

Determination of hydroxyproline:

This was done according to the method of Bergman and Loxley (1963). One ml of neutralised solution to be analysed was pipetted into a clean, dry test tube. Two ml of isopropanol were gradually added with constant mixing followed by the addition of one ml of the exidant solution (chloromine-T). The mixture was allowed to stand for 4 minutes at room temperature. Thirteen ml of Ehrlich's reagent were added, the contents well mixed, the tubes heated for 25 minutes at 60° in a water bath and cooled for 2 or 3 min in running tap water. A reagent blank was included in the procedure substituting water for the hydroxyproline sample and the absorbance against the blank was measured within 30 min at 558 nm in a Beckman spectrophotometer.

Estimation of vitamin A :

Vitamin A was estimated from rat liver according to the method of Neeld and Pearson (1963). One gram of liver was minced and taken in 15 ml of 5% potassium hydroxide and kept in boiling water bath for 45 minutes. It was cooled to 30^{0} and the volume made up to 15 ml. To 2 ml of this, 10 ml. of ethanol and 2 ml of petroleum ether were added and shaken well for two minutes. Aliquotes (0.5 - 1.0 ml) of petroleum ether layer were taken in test tubes and evaporated to dryness in a vacuum dessicator. To the residue one ml of chloroform and 2 ml of trifluroacetic acid reagent were added and the colour developed was read at 620 nm in a spectrophotometer within 30 seconds against the blank. The standard graph was obtained by taking different concentrations of vitamin A acetate in the range of 5-20 I.U.