CHAPTER II

MATERIALS AND METHODS

MATERIALS AND METHODS

As stated earlier, the object of these investigations was to determine the extent of variation in the excretion of nitrogenous constituents in urine, particularly, creatinine, in relation to age, sex, plane of nutrition and activity level.

Additional investigations were carried out during starvation, pregnancy and lactation and in severe protein-calorie malnutrition. The data obtained were sought to be correlated with basal metabolic measurements and estimates of food intake in one group each of adult men and women.

Different groups of adult men were investigated in order to study the effects of variations in age (elderly), activity level (military personnel and postgraduate students and white collar workers) and plane of nutrition (Class IV unskilled employees of the University ~~employees~~). The studies on women included postgraduate students elderly and pregnant parturient and lactating women. The studies on starvation were made possible by individuals fasting for religious reasons for 8 days and included because of ~~its~~ The expected influence of fasting on nitrogen metabolism. Groups of children and adolescents in low and high income groups were investigated in order to study the effects of age and the overall plane of nutrition. These studies were extended to children suffering from severe malnutrition.

Comparative data were obtained to the extent possible on subjects belonging to the low and high income groups in urban and rural Baroda (Gujarat) and urban Trivandrum (Kerala) where dietary patterns are different from those prevailing in Baroda.

A total of 713 subjects were used in the investigations as shown in Tables 12, 13 and 14. In all cases, height and body weights were recorded. Detailed records of food intake were obtained for some of the groups.

Twenty four hour collections of urine were made whereever possible. The reliability of the collections was sought to be ascertained by scrutinising the value for a number of parameters such as density, volume, nitrogen and where 24h-72h collections were made, the extent of diurnal and day to day variation. Urine nitrogen was used as a rough check on the assumption that in adults maintaining constant body weight, the nitrogen in urine must be roughly equal to the difference between food nitrogen and estimates of fecal and sweat nitrogen. Fecal nitrogen was taken as 20% of food nitrogen on the basis of studies reviewed by Hegsted (1964) and those reported from Hyderabad and previously carried out in this laboratory (Rajalakshmi and Ramakrishnan, 1969). In the case of adult men, dermal losses were taken as approximately 1 g (about 0.6 g per sq.m.) according to Mitchell and Edman (1962).

A few instances of how these checks were applied are shown in Table 15.

Table 12 : Details of the studies on adult men and women.

age (yrs)	description	no.of subjects	period of urine collection
	Adult men		
24	military personnel	22	24 h for 3 consecutive days
22	postgraduate students	23	24 h, for 2 consecutive days
33	white collar workers	6	
67	elderly professional class	7	
30	unskilled workers in the university	6	24 h
	Adult women		
	High income group		
21	postgraduate students	15	24 h, for 2 consecutive days
32	housewives	5	
60	elderly	6	
	Low income group		
26	non-pregnant and non-lactating, Baroda	22	24 h
28	pregnant women, Baroda :		
28	I trimester	28	24 h
23	II trimester	55	24 h
24	III trimester	57	24 h
24	parturient	54	24 h
25	lactating	35	24 h
31	non-pregnant and non-lactating, Trivandrum	6	8-20 h
24	pregnant, Trivandrum	13	8 or 24 h
22	parturient, Trivandrum	8	8 h

Table 13 : Details of the studies on children and adolescents.

age (yrs)	description	no. of subjects		period of urine collection
		M	F	
	Baroda			
4 - 7	urban and rural poor	21	32	6-8 h
	urban upper class	10	4	4 or 24 h
	Trivandrum			
	PCM (on admission)	26 +	30	casual
	after rehabilitation	9 +	6	
8 - 10	Baroda			
	urban and rural poor	11	10	6 or 24 h
	urban upper class	-	14	
11 - 12	Baroda			
	urban and rural poor	14	20	6 or 24 h for 2-3 consecutive days in the case of males and 24 h in the case of females.
	urban upper class	6	21	24 h
13 - 15	Baroda			
	urban and rural poor	15	20	24 h for 2-3 consecutive days in the case of males and 24 h in the case of females.
	urban upper class	15	29	24 h
	urban poor (TVM)	16	16	6 h

Table 14 : Parameters measured in different groups.

groups	parameters measured
all groups	nitrogen, urea, ammonia, creatinine, uric acid in urine.
	additional
military personnel postgraduate men 11-15 years old boys (LIG)	amino-nitrogen in urine
postgraduate men and women military personnel elderly men and women (HIG)	food intake
postgraduate men and women	basal metabolism thermogenesis following meal diurnal variation in nitrogenous constituents in urine
postgraduate men and women elderly men and women (HIG) 11-12 and 13-15 year old boys (LIG)	day to day variation in urinary nitrogenous constituents.

Table 15 : The application of criteria used for ascertaining the reliability of urine collections to data on selected subjects.

subject	weight (kg)	height (cm)	S.A. (sq m)	food-N		urinary excretion						Remarks
						volume (ml)		nitrogen (g)		creatinine (mg)		
days →				1	2	1	2	1	2	1	2	
1	2	3	4	5	6	7	8	9	10	11	12	13
MEN :												
1. SBL	53	176	1.65	6.9	8.0	690	730	5.5	7.2	1000	984	collection considered reliable in view of the consistency of the values for urine volume and creatinine and the broad agreement between nitrogen intake and excretion.
2. SAM	38	162	1.37	10.2	10.0	1230	1470	6.4	6.5	812	712	collection considered reliable inspite of the relatively low value for creatinine in view of the consistency in values for volume and nitrogen.
3. NA	48	157	1.45	13.8	11.8	970	940	8.2	7.9	1273	1103	creatinine excretion more in relation to Body size, but urine volume and nitrogen were as expected.

contd..

Table 15 : contd.

1	2	3	4	5	6	7	8	9	10	11	12	13
4. CRJ	51	172	1.59	12	9.6	750	390	8.2	4.4	1150	823	The second day collection was considered as unreliable on the basis of volume nitrogen.
5. SG	45	149	1.36	7.2	6.1	920	595	5.1	5.0	502	588	creatinine low but consistent inspite of large differences in urine volume.
6. RJ	50	152	1.43	7.0	7.2	640	600	6.9	7.4	524	594	Creatinine excretion low but consistent.
7. SGO	43	153	1.36	5.4	6.7	270	510	2.8	5.6	468	563	first day collection considered unreliable on the basis of variations in nitrogen and volume.

* age ranged from 20-22 years.

Food nitrogen was calculated from the protein content of the diet as indicated by dietary records using the recipe method. This approach was considered fairly reliable on the basis of previous studies in this laboratory, in which aliquots of all the foods consumed were collected,weighed, homogenized and analysed for nitrogen and other constituents and their nutritive value calculated from the 'recipe' method, the 'recipe' having been obtained by the measurement of all the raw ingredients used for cooking and the cooked product (Sail, 1970, Ramachandran, 1968; Subbulakshmi, 1970). The reliability of the record was checked by comparing the energy value of the diet reportedly consumed and the energy requirements of the subject. On the whole, the intake was found to be in broad agreement with expected requirement, as in the examples given below :

age	32 years	weight	60 kg	
sex	male	height	160 cm	
		surface area	1.61	sq.m.
basal metabolism expected			1350	Calories
energy needed for activity			750	Calories
total energy needed			2100	Calories
average energy intake/day (over a period of one week)			2000	Calories

Where appreciable discrepancy was found, it was traced to recent changes in weight and food intake as in the following example :

age 22 years	weight 54 kg		
sex male	height 175 cm		
	surface area	1.65	sq.m.
total energy needed		2500	Calories
reported energy intake		3250	Calories

The subject, a postgraduate student residing in a hostel, reported on questioning that till a fortnight prior to the period of record, he had been eating 'free Lance' and been mostly skipping the evening meal and that he had rejoined the regular 'mess' at the time of the study. He had lost weight previously and was now gaining weight.

age 22 years	weight 58 kg		
sex male	height 175 cm		
	surface area	1.70	sq.m.
total energy needed		2400	Calories
reported energy intake		2200	Calories
weight loss during study		1	kg

The subject reported a loss of 4 kg prior to the period of study and attributed the same to tension and sleeplessness.

Even in the case of groups where detailed records of food intake were not available it was possible to make a guestimate of the same on the basis of previous studies on similar groups of subjects. Food intake has been very carefully monitored in

previous studies in this laboratory on young children (Ramachandran, 1968), school boys (Sail, 1970), pregnant and lactating women (Subbulakshmi, 1970) and adolescents (Rajalakshmi and Ramakrishnan, 1969b).

This approach is subject to limitations but nevertheless the checks used may serve as a guide to judge the reliability of the collections. In cases where the same was suspected the subject was questioned again about missed collections, if any. Taken along with other information including day to day variation, urine volume etc. the approach was found to be useful.

The validity of using 24h collections and shorter collection periods has been pointed out by several investigators (Arroyave, 1962; Viteri, Alvarado and Alleyne, 1971; Viteri, 1972). All the same, this was reinvestigated in some cases by investigating the day to day variation using 24h collections for 7 consecutive days (Table 16) and comparing the values derived from shorter collection periods with 24h collections (Table 17).

Subjects classified as belonging to the low income groups had family incomes of less than Rs. 500 per month. In most cases this happened to be an income of much less than Rs. 60-70 per capita per month. Subjects classified as belonging to high income groups had incomes of more than Rs.1500 per month, often much more than this. The per capita income was more than Rs.400-500.

Table 16 : Day to day variations in nitrogenous constituents of urine in an adult male (height, 167 cm; weight 52 kg).

days	volume (ml)	creatinine (mg)	total nitrogen (g)	nitrogen (g) excreted per day as				
				creatinine	uric acid	urea	ammonia	undetermined
1	860	1152	8.3	0.43	0.10	5.6	0.53	1.73
2	1050	1260	8.8	0.47	0.14	6.0	0.67	1.54
3	1650	1353	8.4	0.50	0.15	5.9	0.69	1.20
4	1700	1360	9.4	0.50	0.14	6.6	0.78	1.39
5	1790	1253	9.1	0.47	0.16	6.3	0.78	1.37
6	1450	1515	7.8	0.56	0.17	5.4	0.76	0.98
7	1580	1564	9.1	0.58	0.21	6.5	0.65	1.21
mean	1440	1351	8.7	0.50	0.15	6.03	0.69	1.35
S.D.	351	147	0.55	0.055	0.032	0.47	0.09	0.245
C.V.	24	11	6	11	21	8	13	18

* This was done by the author on himself to make quite sure of the reliability of the collection so that the data could serve as a basis for interpreting other values.

S.D : Standard deviation.

C.V : Coefficient of variation.

Table 17 : Comparative data on creatinine excretion in shorter collection periods.

subject	age and sex	creatinine (mg) excretion						
		collection periods (hrs)		analysed values for periods		extrapolated values for 24 hrs		analysed value for 24 hrs
		1	2	1	2	1	2	
children :								
1	3.8	4	20	26	115	153	138	140
[illegible]	[illegible]	4	20	24	113	144	136	137
2	4 F	6	18	31	98	124	131	129
3	7 F	6	18	33	99	132	132	132
4	9 M	6	18	93	279	372	372	372
adults :								
1		4	20	226	1152	1336	1382	1378
2		4	20	180	986	1080	1183	1176
3		4	20	172	792	1032	950	964
4		4	20	188	782	1128	938	970
adults :								
1		8	16	452	973	1356	1459	1378
2		8	16	361	814	1083	1221	1176
3		8	16	345	627	1035	940	964
4		8	16	377	664	1134	996	970
adults :								
day 1		12	12	768	528	1536	1056	1152
2		12	12	550	737	1650	1474	1260
3		12	12	768	608	1536	1216	1353
4		12	12	665	660	1330	1320	1360
5		12	12	697	627	1394	1254	1253
6		12	12	888	627	1776	1254	1515

Subjects in the different age groups in the two categories were chosen as follows :

(1) Pre-school children from a private nursery school catering to upper class families and from a Balwadi catering to low income groups and from families belonging to class IV employees (unskilled workers) in the university.

(2) School age boys and girls from municipal schools catering to the poor and private schools catering to upper class families.

(3) Adults :

(a) Personnel from the Military school for Electrical and Mechanical Engineers, Baroda.

(b) postgraduate students from the department of biochemistry, M.S. University, Baroda.

(c) white collar workers of university

(d) elderly men and women from private homes

(e) class IV workers of university

(f) non-pregnant and non-lactating women, pregnant, parturient and lactating women from hospitals and private homes.

The studies in Trivandrum were carried out on :

(a) boys and girls aged 13 to 15 years in a school catering to the urban poor.

(b) young children admitted for severe protein-calorie malnutrition at the nutrition rehabilitation centre in the medical college hospital, Trivandrum (Kerala).

(c) malnourished children treated in the above centre subjected to varying degrees and periods of rehabilitation.

(d) pregnant and parturient women admitted ~~in~~ to the maternity ward of above hospital and non-pregnant, non-lactating women of similar status.

Studies on thermogenesis following a meal:

As the values obtained for basal metabolism and creatinine were lower than those reported in the west even in the upper class, additional studies were made of the thermogenesis following a meal to ascertain whether this is also similarly influenced. In this connection, no studies on this aspect appear to have been carried out either in this country or in other 'developing countries' barring the studies of Ashworth (Ashworth, 1969; Brooke and Ashworth, 1972) on malnourished children.

The studies were carried out on post graduate students aged 20-24 yrs in the department. In the first series of investigations determinations were made of oxygen consumption under nearly basal conditions. The subjects came to the laboratory in the postabsorptive state soon after getting up in the morning and were asked to rest in the bed for 30 minutes,

before the first measurement was made. They were then given a standard breakfast and repeat measurements made at 1 and 2h thereafter. Only initial and the 2h measurements were made in 6 subjects and 8 controls who were not given breakfast.

In the second series of investigations some of the subjects in the above study came to the laboratory at about 12 noon and the first measurement made after a rest interval of 30 minutes. The subjects then had their usual lunch and repeat measurements were made every hour for 4 to 5h.

In the third series of investigations the conditions were the same as those in the first, but serial measurements were made on a fresh group of subjects every hour for 4 hours.

During the intervals between measurements, the subjects were housed in the same room or in an adjacent room (temperature 28-31°) and spent the time in light reading or conversation. Oxygen consumption was measured using Benedict-Roth type apparatus (Warren E, Collins, Inc, Boston, Mass, USA) with the subject in recumbant position.

In the first series of investigations the measurements were made on two consecutive days and most of the measurements were made in duplicate. Since duplicate measurements gave fairly comparable values only one measurement was made at each point in the subsequent studies.

Animal experiments

Additional investigations were carried out on rats on the effects of varying the plane of nutrition with regard to protein and calories on energy and protein metabolism.

In experiment I groups of weanling rats were fed ad lib for 4-6 weeks 0, 4, 5, or 20% protein as casein. ~~An~~ Two additional groups ~~was~~ were fed ~~the~~ 20% diet in quantities restricted to 50% and 67% of the voluntary intakes of the control animals. In experiment II they were fed for 8 weeks diets providing 20%, 10% or 5% protein as casein.

In both the experiments the animals were caged individually in ordinary galvanized iron cages. Weighed amounts of food were given and the left over food collected and carefully weighed. Body weights were measured once a week. The temperature of the animal house ranged from 25-33° during the period of study.

In experiment I urine, collected for three days, was analysed for nitrogen, creatinine, urea, ammonia and uric acid and the carcass for nitrogen, creatinine, moisture and fat.

In experiment II, nitrogen balance studies were carried out over a three day period and the urine analysed for creatinine, creatine and nitrogen.

The diets were composed as shown in Table 1~~3~~.

Table 1% ; The composition of the diets.

	% dietary protein				
	20	10	5	4	0
vitamin free casein (g)	24	12	6	4.8	-
vitamin mixture (g)	2	2	2	2	2
salt mixture (g)	4	4	4	4	4
groundnut oil (g)	7	7	7	7	7
sago (g)	63	75	81	82.2	87

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

Composition of vitamin mixture

vitamin	amount (mg) per kg diet	vitamin	amount (mg) per kg of diet
thiamin hydrochloride	1.5	inositol	200.0
riboflavin	2.5	p-amino benzoic acid	10.0
pyridoxine hydrochloride	1.0	folic acid	1.0
niacin	15.0	cyanocobalamin	0.005
calcium-d-panto-thenate	10.0	biotin	0.001
choline chloride	750.0	powdered sugar to make a total weight of 20 g	

contd...

Table 19 : contd.

Composition of salt mixture

salt	g/kg	salt	g/kg
calcium citrate	308.2	NaCl	77.0
$Ca(H_2PO_4)_2.H_2O$	112.8	$CaCO_3$	68.5
K_2HPO_4	218.7	$3MgCO_3.Mg(OH)_23H_2O$	35.1
KCl	124.7	$MgSO_4$ (anhydrous)	38.3
		salt mixture A*	16.7

* salt mixture A consists of ferrous ammonium citrate, 91.4 g; $CuSO_4.5H_2O$, 6.0 g; NaF, 0.76 g; $MnSO_4.2H_2O$, 1.07 g; $KAl_2(SO_4)_3.12H_2O$, 0.54 g; and KI, 0.24 g; $ZnSO_4$, 0.06 g.

Diets

Edible casein from Amul Dairy, Anand, Gujarat, was washed first with 50% alcohol and then with tap water. The washed casein was dried and used after analysis for nitrogen content by the micro-kjeldahl method and the calculation of protein content therefrom.

Commercially available sago prepared from tapioca flour (Manihot Utilissima) was ground and used in place of starch. This contains 0.2% protein and 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, Behan and McMeans, 1951).

The vitamin mixture used was formulated previously in this laboratory on the basis of the allowance suggested by Brown and Sturtevant (1949), the recommendations made by NAS-NRC (1962) and evidence reviewed by Mitchell (1964).

The salt mixture used was Hawk-Oser salt mixture no. 3 (Hawk et al, 1954). Both the mixtures were prepared in bulk and stored in air tight bottles. The vitamin mixture was kept in brown bottles and stored in the refrigerator. The diets to be fed were prepared once a week and the vitamin mixture and groundnut oil added at the time of feeding.

Collection of urine

In all the studies, urine was collected in brown glass bottles containing about 10 ml of toluene, and the samples were stored in cold condition at 4^{0} and analysed within 24 h.

In the rat experiments, urine was collected for 3 consecutive days in glass bottles containing 2 ml of toluene. Care was taken to avoid any food contamination during the collection.

In experiment 1, the animals were kept in plastic cages for urine collection. A hole was drilled at the bottom of the cage which was slightly tilted over an iron stand so that the urine would drip down through the hole to a glass funnel into bottle. The cages were washed with glass distilled water and the washings were added to the sample which was made up to a known volume.

In experiment 2, the animals were kept in stainless steel metabolic cages during the collection. The cages were washed as in the case of above experiment.

Preparation of the carcass

The animals were deeply anaesthetized with solvent ether so that they were practically dead, autoclaved for 15' at 15 lb pressure and cut into pieces according to the procedure of Mickelsen and Anderson (1959).

Moisture

Moisture content was determined from loss of weight during drying of the whole body carcass in a hot air oven regulated at 60^{o} till constant weights were obtained.

Fat-free dry weight

Fat was extracted with ether from the whole body carcass using Soxhlet's apparatus for 48h. Both fat extracted and fat-free body were weighed, the former, after evaporation of the ether.

The fat-free carcass was powdered in a metallic blendor and used for other estimations.

Details of procedure

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 bar that rests on a vortex.

Weight

Weight was taken without shoes, either on the Avery scale or bath room scales. In the latter case, the reliability of the measurements was checked using standard weights.

Surface area

This was obtained from nomograms (Boothby and Sandiford, 1921) on the basis of height and weight.

In the rat experiment (experiment 1) surface area was determined by a co-investigator directly from the skin and found to compare with values derived from the equation of Hill and Hill (1913) viz, surface area (sq.cm.) = $wt._{(g)}^{2/3} \times 10$

Basal metabolism

The subjects were brought to the laboratory in the post absorptive state soon after waking up in the morning and were asked to rest for 30 minutes before measurement was made. Oxygen consumption was measured using Benedict-Roth type apparatus (Warren E, Collins, Inc, Boston, Mass, USA) with the subject in recumbant position. Two measurements were made as a routine and a third measurement made in case of discrepancy between the first two.

Chemicals

The chemicals used in the experiments were of research grade purity and obtained either from the British Drug House Limited or Sarabhai Chemicals. The reagents used for various estimations are given in Table 19.

Table 19 : Reagents and standards used for various estimations.

Sr.No.	Name of the reagent/ standard	Method of preparation
1	borate buffer	to 57.21 g of sodium borate in 1.5 litre of distilled water, 100 ml 1N HCl is added and made upto 2 litres with distilled water.
2	boric acid 2%	20g of boric acid is dissolved in 500 ml of distilled water and 2-3 drops of mixed indicator is added (to give pink color) and made upto ~~volume~~ 1 litre.
3	copper phosphate suspension	one volume of cupric chloride solution with 2 volumes of trisodium phosphate solution and 2 volumes of borate buffer are mixed to get suspension. The suspension appears to keep well in the refrigerator for about one week.
4	creatinine standard	
	(a) stock	one gram of pure dry creatinine is dissolved in 0.1N HCl and made upto 1 litre with distilled water. This solution contains 1 mg of creatinine per ml and is stable over long periods.
	(b) working	one ml of stock standard is diluted to 10 ml to give concentration of 100 µg/ml.
5	cupric chloride	27.3g of cupric chloride dissolved ~~in 10 ml to give concentration of 1000 µg/ml~~. in one litre of water

contd..

Table 1g : contd.

Sr.No.	Name of the reagent/ standard	Method of preparation
6	mixed indicator	25 mg of bromocresol green and 75 mg methylred dissolved in 100 ml of alcohol.
7	phosphotungstic acid	10g sodium tungstate dissolved in 400 ml water, 40 ml of 85% phosphoric acid added and refluxed for 2 hs. in all glass apparatus. Cooled and diluted to 500 ml. This solution is stable indefinitely when stored in brown bottles and protected from contact with organic matter. Since the acidity of the reagent is important in the reaction, other phosphotungstic acid reagents may not be substituted indiscriminately.
8	picric acid 1%	1g of picric acid crystals dried between the folds of filter paper are taken in distilled water and heated till dissolved and diluted to 100 ml. Cooled and then filtered. Preserved in brown bottle.
9	potassium carbonate	900 g of potassium carbonate added to about 1 litre of water and dissolved by stirring.
10	potassium iodate 0.01N	0.3567mg of potassium iodate dried in an oven at 110° for one hour and dissolved in a litre of distilled water.

conti.

Table 1g : contd.

Sr.No.	Name of the reagent/ standard	Method of preparation
11	potassium iodide	1g dissolved in 1 ml of water
12	sodium hydroxide 10%	10g of sodium hydroxide dissolved in distilled water and made upto 100 ml.
13	sodium hydroxide 50%	500g of sodium hydroxide dissolved in water and made upto 1000 ml.
14	sodium hydroxide 0.02N	80 mg of sodium hydroxide dissolved in 100 ml distilled water to give approximately 0.02N.
15	sodium hydroxide 1N	40g sodium hydroxide dissolved in one litre of water.
16	sodium carbonate 10%	100g of anhydrous sodium carbonate dissolved in distilled water and made upto one litre.
17	sodium thiosulfate	
	(a) stock 0.1N	49.6 g of sodium thiosulfate dissolved in 200 ml of water. 20 ml of amyl alcohol added to serve as stabilizing agent and made upto 2 litres with water.
	(b) working	prepared from stock by diluting with water.
18	starch indicator	1g of starch dissolved in 100 ml of saturated sodium chloride by heating over a steam bath and cooled overnight and the supernatant solution decanted.

conti...

Table 19 : contd.

Sr.No.	Name of the reagent/ standard	Method of preparation
19	sulfuric acid,0.02N	1N sulfuric acid prepared by diluting concentrated sulfuric acid by 36 times. 20 ml of 1N sulfuric acid diluted to one litre to give 0.02N sulfuric acid.
20	thymophthalein indicator	0.25 g of thymophthalein dissolved in 50 ml of 95% ethanol and made upto 100 ml with distilled water.
21	trisodium phosphate	64.5 g of trisodium phosphate dissolved in litre of distilled water.
22	urea standard	
	(a) stock 2%	2g of urea dissolved in 100 ml of distilled water.
	(b) working	10 ml of stock solution diluted to 100 ml to give a concentration of 2 mg/ml.
23	urease	one tablet of urease (BDH) dissolved in 50 ml of distilled water.

conti...

Table 19 : contd.

Sr.No.	Name of the reagent/ standard	Method of preparation
24	uric acid	
	(a) stock	0.5g lithium carbonate dissolved in 150 ml of hot water. 1 g of pure uric acid added and ~~to~~ swirled ~~to~~ the mixture till complete dissolution and transferred, quantitatively to one litre volumetric flask, using about 300 ml water. Then 25 ml of 40% formalin and 3 ml of glacial acetic acid are added. Diluted with water to a litre. This solution is stable for one year, if protected from light. It contains 1 mg uric acid per ml.
	(b) working	1 ml of stock diluted to 200 ml with distilled water. Stored in refrigerator and made fresh at least every ~~w~~ 2 weeks. This solution contains 0.5 mg% of uric acid.
25	oxalic acid, 0.02N	126 mg of oxalic acid dissolved in 100 ml of distilled water.

Biochemical assays :

Total nitrogen

This was done according to the microkjeldahl method described by Hawk, Oser and Summerson (1954). The sample (urine 5 ml) was placed in a 100 ml capacity kjeldahl flask. To this were added 10 ml of concentrated sulfuric acid and 0.1 g of copper sulfate and potassium sulfate mixture (1:1). The mixture was allowed to boil until it became light green or almost colorless and the boiling continued for another hour.

After cooling to room temperature (27 to 30^{o}) the contents of the flask were diluted with 20 ml of water, transferred to a 50 ml volumetric flask and made upto 50 ml. Ten ml of 2% boric acid containing 1-2 drops of mixed indicator were placed in a 100 ml conical flask. The flask was arranged so that the tip of the condenser outlet dipped below the surface of the solution. A known amount of the digested sample (0.25 to 5.0 ml) was quantitatively transferred to the chamber of the steam distillation apparatus, cleared of any contaminating ammonia by a blank distillation. Ten ml 50% sodium hydroxide were then added and the generation of steam in the boiler started. The sample was steam distilled until about 8-10 ml of distillate were obtained. The ammonia content of the distillate was determined by titrating with 0.02N sulfuric acid. Total nitrogen content was calculated using the following equation.

1 ml of 0.02N sulfuric acid = 0.28 mg of nitrogen.

Creatinine

This was done according to the method described by Hawk, Oser and Summerson (1954). 0.2 ml of urine was taken in a tube and to this were added 10 ml of picric acid and 1 ml of 10% sodium hydroxide. The contents of the tube were mixed well on a vortex mixer and allowed to stand at room temperature for 30 minutes and the color developed read at 540 mμ against a blank containing 10 ml of picric acid and 1 ml of 10% sodium hydroxide after the contents were diluted to 50 ml with distilled water.

A standard graph was obtained using different concentrations of standard creatinine solutions.

Carcass creatinine was done according to the procedure described by Chinn (1966). Carcass powder weighing 100 mg was extracted with 10 ml of $\frac{2}{3}$N H_2SO_4. The extract was then autoclaved at 25 lb for 20 minutes to convert creatine into creatinine which was analysed by the method described above.

Uric acid

This was done according to the method described by Bauer et al (1968). One ml of urine sample was diluted to 100 ml with distilled water and 5 ml of the diluted sample was taken in a test tube. To this was added 1 ml of 10% sodium carbonate and the tube kept at room temperature for 10 minutes after mixing the

contents of the tube on a vortex mixer after which 1 ml of phosphotungstic acid was added and the contents mixed again. The tube was then allowed to stand at room temperature for 30 minutes and the color developed read at 660 mμ against a blank containing 5 ml of distilled water, 1 ml of 10% sodium carbonate and 1 ml of phosphotungstic acid. A standard graph was obtained using different concentrations of standard uric acid solution.

Urea nitrogen

This was done according to Vanslyke method described by Hawk, Oser and Summerson (1954). A known quantity of urine (0.5 ml) was taken, 5 ml of urease enzyme added and the tube allowed to stand at room temperature for 3 hrs. To this was added one drop of caprylic alcohol and 10 ml of saturated potassium carbonate and the tube was immediately closed with the rubber cork. Then aerated the contents into the other tube containing 25 ml of 2% boric acid colored by mixed indicator. The aeration is carried out for about 1 hr. The total ammonia nitrogen content of the sample was determined by titrating with 0.02N sulfuric acid. The nitrogen was calculated using the following equation.

1 ml of 0.02N sulfuric acid = 0.28 mg nitrogen.

This gives the value for nitrogen derived from urea and ammonia.

The above procedure is followed for the determination of ammonia nitrogen. In this case 5 ml of urine sample is used without enzyme.

Urea nitrogen = total urea and ammonia nitrogen - ammonia-N

Amino nitrogen

This was done according to the method described by Albanese and Irby (1944).

Fifteen ml of urine were taken in a 50 ml volumetric flask and to this were added 4 drops of thymolphthalein indicator. This was titrated with 1N sodium hydroxide until the appearance of a faint green or blue color. To this was added 30 ml of copper phosphate suspension from a graduated cylinder and the solution was made up to a volume of 50 ml with distilled water. The contents were mixed and allowed to stand at room temperature for 5' and then filtered through Whatman No. 12 filter paper. Ten ml of the filtrate was acidified with 0.5 ml glacial acetic acid. To this was added 1 ml of freshly prepared potassium iodide solution followed by 4-5 drops of starch solution. The amino nitrogen content of the sample was determined by titrating with 0.01N sodium thiosulfate. The amino nitrogen content was calculated using the following equation.

1 ml of 0.01N thiosulfate = 0.28 mg of amino nitrogen.