CHAPTER - III

METHODS AND MATERIALS

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Human Studies :

The normal controls were selected from the University staffs and students. The patients were taken from different medical colleges and hospitals in Calcutta. The hypothyroid and hyperthyroid conditions / status were confirmed by the estimation of Thyroxine (T_4), Triiodothyronine (T_3) and thyroid stimulating hormone (TSH) in the blood.

The human subjects were grouped as follows :

A) Without any treatment (between 20 to 40 years) :

1. Normal control (10 male and 10 female)
2. Hypothyroid (10 male, 10 female)
3. Hyperthyroid (10 male, 10 female)

B) With treatment of vitamin A 200000 I.U. orally (singel oral dose of 4 tablets of Arovit, Roche) (All were female between 20 - 40 years) :

1. Normal (12 in each group)
2. Hypothyroid (12)
3. Hyperthyroid (12)

C) With the treatment of Beta-carotene (single dose of Beta-carotene 120 mg orally, E. Merck) (All were female between 20 - 40 years; number of subjects 10 in each group) :

1. Normal
2. Hypothyroid
3. Hyperthyroid

D) With the treatment of all-trans retinoic acid (single oral dose 100 mg all-trans retinoic acid, Sigma) All were male between 20 - 40 years. Number of subjects 7 in each group):

1. Normal
2. Hypothyroid
3. Hyperthyroid

Animal Studies :

Protocol :

For the convenience of comparison, all the animal experiments were done in male albino rats weighing between 50 - 70 gms. The number in each group varied according to the experimental design. The animals were kept in identical laboratory condition. The temperature was maintained at 20°C in the animal room and the humidity was maintained at 70%. The rats were kept in natural day-light with 13 hours and 11 hours light-dark cycle. Each of the animals was isolated in separate compartment of the cage. The cages

were made of steel wire net and each cage had had enough space and it was so designed that it could be divided into three minor - mini-compartments with the help of two separators. Each rat was kept in each minor-mini-compartment. Every day each rat was given a bottle full of tap water except after thyroidectomy when 1% calcium lactate was given in the bottles instead of plain water; this prevented the development of Tetany in thyro-parathyroidectomized rats. The bottles were fitted with suitable glass tube. The bottles were kept upside down from the roof of the cage and the glass tubes were allowed to project through the wire net roof. The rats sucked the water from the projected end of the tube.

The animals were fed with one of the two types of diet mentioned below :

Diet No. I (Vitamin A poor diet) :

1.	Bengal gram powder - -	35 %
2.	Wheat flour - - -	35 %
3.	Casein (vitamin A free) -	15 %
4.	Coconut oil - - -	5 %
5.	Dry yeast - - - -	5 %
6.	Salt mixture (vide infra) -	5 %
7.	Retinol - - - -	30 I.U./100 gm.
8.	Water ad lib. - -	

Diet No. II (Vitamin A rich diet) :

1. Bengal gram powder - - - -	35 %
2. Wheat flour - - - - -	35 %
3. Casein - - - - - -	15 %
4. Coconut oil - - - - -	5 %
5. Dry yeast - - - - - -	5 %
6. Salt mixture (vide infra) - -	5%
7. Retinol - - - - - -	300 I.U./100 g. of food.
8. Water ad libitum	

Hawk - Oser Salt Mixture No. 4 (Hawk-Oser, 1931) :

Calcium Citrate - - - -	308.2 gm.
$Ca(H_2PO_4)_2, H_2O$ - - - -	112.8 gm.
K_2HPO_4 - - - - -	218.7 gm.
KCl - - - - - -	124.7 gm.
NaCl - - - - - -	77.0 gm.
$CaCO_3$ - - - - - -	68.5 gm.
$3MgCO_3, Mg(OH), 3H_2O$ - - -	35.1 gm.
$MgSO_4$ (Anhydrous) - - -	38.3 gm.

Added 16.7 gm of the following salt mixture :

$FeNH_4$ citrate U.S.P. - - -	91.36 gm.
$CuSO_4, 5H_2O$ - - - - -	5.97 gm.
NaF - - - - -	0.76 gm.
$MnSO_4, 2H_2O$ - - - - -	1.07 gm.
$KAl(SO_4)_2, 12H_2O$ - - -	0.54 gm.
KI - - - - -	0.24 gm.
$ZnSO_4, H_2O$ - - - - -	0.06 gm.
Total :	100.00 gm.

In this present work estimation of retinol, carotene and retinoic acid estimations were done in different tissues in different experimental conditions as follows :

1. Serum retinol was estimated by the methods described earlier by Kimble (1939).

2. Tissue retinol and retinol in subcellular fractions of liver were determined by the previous method (Kimble, 1939; Freed, 1966) after extraction from the tissue.

3. Retinoic acid was also estimated by colorimetric method where feasible (Wang et al, 1978).

4. Serum carotene was estimated by previous method (Kaser and Stekol, 1943).

5. Separation of retinol, retinal and retinoic acid was done by chromatography and their estimation when needed in low quantity were done by the earlier methods (Deshmukh et al, 1965; Futterman and Sasland, 1961).

Experimental protocol :

Human experiment - I :

Serum carotene and retinol were estimated in fasting condition in normal control, hypothyroid and hyperthyroid subjects.

Human experiment - II :

Serum carotene was estimated in some of the subjects 24 hours, 72 hours and 120 hours after oral carotene administration.

Human experiment - III :

Serum retinol was estimated in some of these subjects 24 hours, 72 hours and 120 hours after oral dose of vitamin A.

Human experiment - IV :

Serum retinol and carotene were estimated in some of these subjects 24 hours after oral retinoic acid administration.

N.B. Number of subjects or animals under experiments was mentioned in the Table of the Results.

These human subjects were not allowed milk, liver, cod liver oil or other food or drugs containing vitamin A or carotene for fifteen days before and 5 days during experiments. Carotene, retinol and retinoic acid were fed with equal quantity of bread and butter after over night fasting at 10.00 A.M. in the morning. The control subjects were given the same amount of bread and butter.

Animal experiments :

In animal experiment all the animals taken were male albino rats. One third of these were kept as control. One third were made hypothyroid and one third were made hyperthyroid by the method as described by Zarrow et al, 1964.

Animal Experiment - I : Absorption study of retinol in control, hypothyroid and hyperthyroid animals (on diet I).

Animal Experiment - II : Absorption study of carotene in control, hypothyroid and hyperthyroid animals (on diet I).

Animal Experiment - III : Absorption study of retinal in control, hypothyroid and hyperthyroid animals (on diet I).

Animal Experiment - IV : Absorption study of retinoic acid in control, hypothyroid and hyperthyroid animals (on diet I).

Animal Experiment - V : Retinal oxidase and reductase in liver of control, hypothyroid and hyperthyroid animals (on diet I).

Animal Experiment - VI : Study of growth in rats under influence of vitamin A acid in different experimental conditions (on diet II).

Animal Experiment - VII : Vitamin A in sub-cellular fractions of liver of different experimental conditions (on diet II).

Animal Experiment - VIII : Vitamin A in different tissues in different experimental conditions (on diet II).

Before the start of the experiments, the animals were acclimatized in the laboratory for 10 days. All the animal experiments were started after over night fasting and estimation of the vitamin A and allied compounds were done in the serum and tissues after sacrifice on the next day.

Feeding of carotene, retinol, retinal, and retinoic acid were done by feeding needle after over night fasting. Then usual food and water were allowed.

Hypothyroidism of animals :

1. Thyroidectomy in the rat (Zarrow et al, 1964):

The albino rat was anaesthetized with ether in a jar and placed on an operating board. Anaesthesia was continued with ether vapor from an ether soaked pledget of cotton wool. Midventral incision was made in the neck, the skin, fascia and submaxillary glands

were retracted to expose the sternomastoid and sternohyoid muscle. The muscles were carefully separated from the midventral line to expose the trachea and thyroid gland. Fine forceps were used for disceting the thyroid gland which was bilobed in rats with iridectomy scissors holding the horizontal plane and both the lobes were removed with the isthmus. Delicate approach was necessary to remove the lobes with the isthmus. Muscles were allowed to return the normal position. Incisions were closed with sutures.

Following surgery, the animals were placed on 1% calcium lactate drinking solution.

2. Hypothyroid by propylthiouracil :

In another group of animals, hypothyroid condition was produced by oral administration of propylthiouracil (0.1%) with drinking water (Zarrow et al, 1964) for 21 days.

Hyperthyroidism in animals :

Hyperthyroid conditions in rats were produced by administration of thyroxine - 20 μg I.P. daily for 21 days (Zarrow et al, 1964).

Estimation of carotene and vitamin A in serum (Kimble, 1939; Freed, 1966) :

Carotene :

To 3 ml of serum, equal volume of 95% of ethanol was added slowly with shaking followed by 6 ml of petroleum ether (B.P. 40-60°C). It was stoppered lightly and was shaken for ten minutes. Then it was centrifuged slowly for one minute. The petroleum ether layer was taken and the light absorption of the solution was measured at 440 mμ setting the photoelectric colorimeter at 100 percent transmittant with petroleum ether. A calibration curve was made by measuring the absorption of standard solution of beta-carotene in petroleum ether containing 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, 2.2, 2.4 μg per ml. Calculation of beta-carotene was done from the standard curve.

Vitamin A :

To measure the vitamin A the petroleum ether extraction was evaporated to dryness on a water bath (40°C) in a stream of N_2, heating finally to 70°C for a few seconds. The residue was mixed with 0.5 ml of chloroform and the cell was placed in the photoelectric colorimeter equipped with 620 mμ filter, set at 100 percent transmittance with

4 ml of chloroform. Antimony trichloride reagent 3.5 ml was rapidly added to the unknown and the maximum extinction was measured. A calibration curve was made by conducting the test on pure solution of vitamin A (Retinol, Sigma) containing 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 μg per 0.5 ml of chloroform. Calculation was made from the calibration curve.

Antimony trichloride reagent :

25 g of Antimony trichloride ($SbCl_3$) (A.R., B.D.H.) was added to 100 ml of pure chloroform (Glaxo). Antimony trichloride was dissolved by warming and shaking for several hours and then filtered through Na_2SO_4 into a clean, dry, amber bottle with ground glass stopper. The solution was stored in the dark when not in use. Fresh solution was made if the whole was not used up within a month. The glass ware coming in contact with this reagent were always rinsed with chloroform, a mixture of ethanol and ether or dilute HCl before washing since SbOCl which forms is insoluble in water.

Separation of vitamin A alcohol, aldehyde and acid by column chromatography (Mahadevan et al, 1962; Deshmukh et al, 1964):

A mixture of these compounds dissolved in a small volume of light petroleum (B.R. 40-60°C) was passed through a 10% deactivated alumina column (1 cm x 5 cm); the retinene was first eluded with 20 ml of 2% (V/V) acetone in light petroleum and the

alcohol with 20 ml of 8% (V/V) ethanol in light petroleum. The retinoic acid which remain firmly adsorbed on the alumina, was then eluted out with 30% glacial acid in petroleum ether (V/V). The elutes were washed twice with water and evaporated to dryness in vacuum and redissolved in suitable solvent for estimation.

Separation of retinol, retinal and retinyl ester by chromatography (Deshmukh et al, 1965; Olson, 1961) :

Small quantity of petroleum ether (B.R. 40-60°C) with the mixture of the compounds was passed through the alumina column (1 cm x 5 cm). The retinyl esters were eluted by 100 ml of petroleum ether, which was followed by 40 ml of 1% (V/V) acetone in light petroleum to remove the aldehyde and 40 ml of 6% (V/V) acetone in light petroleum to remove the alcohol. The elute was washed with water twice and the evaporated to dryness in vacuum and then was redissolved in desired solvent for measurement.

Spectrophotometric measurements of retinol, retinal and retinoic acid (Mahadevan et al, 1962; Deshmukh and Ganguly,1967):

When the quantity of the compounds are smaller than 5 μg their measurements were done in spectrophotometer (systronics). A 3 ml solution was made with ethanol and was

measured in spectrophotometer with absorbtion maximum (lamda max) at 325 mμ 350 mμ and 380 mμ for retinol, retinoic acid and retinal respectively. Calculation was done from the standard curves.

Retinylesters were measured by the Carr Price method described elsewhere. Standard curve was done with vitamin A acetate

Estimation of vitamin A in tissue (Freed, 1966):

One gram of tissue was homogenized in 19 ml of distilled water. To it 1 ml of alcoholic KOH (one ml of 100% KOH dissolved in 10 ml of 95% alcohol) was added and boiled for five minutes. To it 20 ml of petroleum ether, 20 ml of alcohol and 20 ml of water were added and was shaken well and then allowed to settle. The upper layer of petroleum ether was taken. The procedure was repeated and the petroleum ether was evaporated to dryness in vacuum. The residue was mixed with 0.5 ml of chloroform and vitamin A was measured by Carr-Price Reagent.

Estimation of retinal after chromatographic separation (Futterman and Suslaw, 1961) :

Reagents :

Thiourea reagent : Thiourea was dissolved in glacial acetic acid and was filtered through glass wool.

Thiobarbituric Acid Reagent : 600 mg of thiobarbituric acid in 100 ml of absolute alcohol (ethanol). The solution was filtered and stored in a refrigerator.

Vitamin A aldehyde stock solution : 10 mg of all-trans-vitamin A aldehyde (Sigma) was dissolved in 100 ml of absolute alcohol. The solution was shielded from light with aluminium foil and stored in a refrigerator.

Vitamin A aldehyde standard solution : 2 ml of the stock solution was mixed with 2.5 ml of water and diluted to 25 ml of 90% ethanol. The solution is made each day.

All solutions were stored for 30 days except as noted.

Estimation of vitamin A aldehyde : To 3 ml of 90% ethanol containing vitamin A aldehyde approximately 2 to 20 μg, 1 ml of thiourea reagent and 1 ml of thiobarbituric acid reagent were added and mixed well. A reagent blank was prepared with 3 ml of 90% ethanol. Kept in the dark for 30 minutes for colour development. Absorbancy of the red solution was measured at 530 mμ in a spectrophotometer.

Calculation was made from the standard curve.

Estimation of retinoic acid after chromatographic separation (Jurkowitz, 1962):

The ultimate petroleum ether extract was evaporated to dryness in vacuum and mixed with 0.5 ml of chloroform. To it 3 drops of acetic anhydride and 3 ml of antimony trichloride in chloroform (25%, W/V) were added and after 30 minutes reading was taken in spectrocolorimeter at 570 mμ.

If the amount of retinoic acid is less than 5 μg, it was confirmed by spectroscopic measurement in alcohol described elsewhere. Standard curve was made with all-trans-retinoic acid (Sigma) similarly.

Preparation of subcellular fractions (Schmeider and Hogeboom, 1950):

Livers were chilled and forced through perforated plate to remove connective tissue. The pulp was weighed then homogenized in 9 volumes of cold 0.25 M sucrose solution (8.557 %; Sp.Gr. 1.0338). It was then centrifuged at 2000 r.p.m. (700 x g) for 10 minutes. This helped to sediment nuclei, R.B.C. and unbroken cells.

Mitochondria were sedimented in 10 minutes by centrifuged at 9200 r.p.m. (5000 x g) and washed twice at 20,800 r.p.m. (24,000 x g) .

The microsomes were sedimented in 60 minutes at 25,980 r.p.m. (57,000 x g) and washed twice at 50,740 r.p.m. (148,000xg)

for ten minutes.

After sedimentation of microsomes the supernatant was regarded as cytosol.

Preparation of mucosal cells and muscles of intestine and intestinal contents (Glover and Green, 1957):

The small intestines were immediately removed after decapitation and the contents washed out with cold physiological saline. The mucosal cells were then scraped off with the blunt end of the scalpel. Both the intestinal muscles (that part of the small intestine left after the removal of the mucosa) and the mucosa were collected in separate beakers previously placed in crushed ice and weighed homogenates of mucosa or muscles were taken to estimate the carotene or retinoids.

Preparation of tissue homogenate :

500 mg of fresh tissue was taken each time in all glass homogenizer. To it 9.5 ml of distilled water was added and homogenized for 10-20 minutes each time to get a good homogenate.

From the homogenate extraction and estimations of vitamin A, vitamin A esters, vitamin A aldehyde and vitamin A acid were done following the protocols stated above.

Preparation of vitamin A for oral administration in rats :

Synthetic vitamin A alcohol was dissolved in light petroleum(B.R. 40-60°C) together with the coconut oil so as to obtain a final concentration of 4 mg of the vitamin / ml of the carrier. The solvent (light petroleum) was evaporated under vacuum at 40-50°C, the last traces being removed with a gentle stream of N_2 (Mahadevan and Ganguly, 1961).

Preparation of beta-carotene for oral administration in rats :

Beta-carotene was dissolved in coconut oil (heated 40-50°C) by shaking vigorously for ten minutes so as to make a solution of 4 mg/ml. It was freshly prepared and used.

Preparation of retinoic acid for oral administration in rats :

Retinoic acid was dissolved in coconut oil (heated at 40-50°C) by shaking vigorously for ten minutes so as to make a solution of 2 mg/ml and always fresh preparations were used.

Enzyme Assay (Retinal oxidase and reductase) (Mahadevan et al, 1962):

The reaction mixture contained 0.5 ml of the homogenate and various cell fractions equivalent to 200 mg of wet liver, 0.5 ml of substrate (retinal) dispersion and 3 ml of phosphate buffer (Na_2HPO_4-$NaHPO_4$; 0.1 M, pH- 7.20). Water was added to

make the final volume 5 ml. Incubation was carried out for 60 minutes at 37°C. Afterwards reactions were stopped by addition of 5 ml of ethanol. The reaction mixture was extracted twice with 20 ml of petroleum ether (B.R. 40-60°C). The extracts were pooled and evaporated to dryness under reduced pressure in nitrogen atmosphare. The final products were subsequently separated and estimated by the methods described above.