CHAPTER IV

MATERIALS AND METHODS

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Experiment I :

Male patients of age between 35 to 55 years with perfect control of diabetes by treatment with diet alone, sulfonylureas, biguanides or combination of sulfonylurea and biguanide for atleast past one year were selected for this study from the Diabetic clinic, J.J.Group of Hospitals, Bombay-8. Patients having diabetes for more than five years were excluded from the study. Freshly diagnosed cases and normal subjects were also included for comparison. Each patient was interviewed before selection and thorough medical examination including blood pressure measurement, haemoglobin level, electrocardiogram etc. was conducted. Data regarding past history of cardiovascular disease (if any), fundoscopy, latest report of glucose tolerance test, urine examination report and reports of other abnormal laboratory tests if any were collected. Blood pressure readings of 140/90 mm/Hg or over were taken to indicate hypertension. Diagnosis of ischaemic heart disease was based on ECG abnormalities. This was done with the help of medical staff of the

diabetic clinic. ECG abnormalities have been divided in three categories (1) Presence of abnormal Q or QS to indicate myocardial infarction. (2) Presence of inverted T wave in significant leads to indicate myocardial ischaemia and (3) Nonspecific ST segment changes in the absence of 1 and 2 with history of chest pain to indicate evidence of coronary artery disease. Diagnosis of diabetes was based on finding of blood sugar readings : fasting - 131 mg% or above. Two hours reading after 100 g glucose - 141 mg% or above. Complete record of patient's particulars like age, weight, occupation, type of fat being consumed, nature and extent of smoking, tobacco and alcohol consumption, present and past treatment of the disease with dose of the drug and duration etc. were also collected in a specially designed proforma (vide Appendix 'A'). Fasting and post prandial blood sugar levels were checked before selecting every patient and the fasting blood sugar was once again estimated on the day of sample collection. Patients were catagorized as freshly detected diabetics (having no control of blood sugar), tolbutamide treated and those treated with chlorpropamide, phenformin and with combination of sulfonylurea

and biguanide. Normal subjects were also included.

Quantitative estimations of different lipoproteins by cellogel electrophoresis technique, triglycerides, cholesterol and phospholipids were done on the sera of these patients. Triglycerides were estimated by modified method of Carlson (1965). Serum cholesterol was estimated by modified method of Abell et al (1952). Lipid phosphorus was estimated by Fiske and Subbarao's (1925) method after digestion of the serum lipid extract with sulfuric acid. All techniques are described as below :

Blood samples were drawn after 16 hours fast. 10 ml of blood was collected from each subject in a sterile glass tube and the serum was separated.

1. Lipoprotein electrophoresis :

Cellogel (R) strips of the size 2.5 cm x 14 cm were purchased from Reeve angel Scientific Ltd., Life Science Supplies Division, London.

Reagents :

1. Buffer solution (0.04 M) : 8.24 gm of sodium veronal was dissolved in glass distilled water and the volume was made up to one litre with the same. Buffer was prepared fresh every time.

2. Dye solution : 50 mg of Sudan black B (E.Merck) was dissolved in 30 ml of alcohol with constant stirring and 35 ml of 5 percent NaOH was added to it. After thorough mixing the solution was poured in a suitable tank. Solution was prepared fresh every time.

Procedure :

Strips were immersed in a suitable tank containing sodium veronal (0.04 M) buffer by first placing each strip on the surface of the liquid allowing the buffer to displace air through cellogel structure and then immersing it completely in the buffer. After 10 minutes, these strips were removed and blotted between two sheets of chromatography paper and then evenly spaced on the bridge of suitable length parallel to the sides and with the porous and dull side of the strip facing upwards. The strips were kept taut without sagging. Fresh serum sample was applied on the strip with an applicator made up of small fine glass capillary having narrow elongated tip with one end attached to the rubber tubing plugged with glass rod (Rubber tubing was used to adjust pressure and control flow while applying the sample). Sample

application was evenly done with an applicator in the form of narrow long streak across the width of the strips on the bridge. The ends of the strips were dipped in tanks filled with 0.04 M. sodium veronal buffer. Care was taken to keep the buffer at the same level in both compartments to prevent siphoning taking place. Electrophoresis was carried out at a constant voltage of 200 V for 35 minutes (Current was passed for 10 minutes before the application). Strips were removed after completion of the run and immediately placed in the dye solution. After 3 hours, they were washed with running tap water and preserved in 5 percent formalin. Scanning was done on reflectance microdensitometer at Maharashtra Water Pollution Research Centre, Bombay.

2. Triglycerides :

Triglycerides were estimated from the serum by the method described by Carlson (1965). Two ml of serum was taken and serum lipids were extracted in chloroform methanol mixture (2:1) and purified by the method described by Folch et al (1957). This was followed by removal of phospholipids from the extract with silicic acid, hydrolysis of the triglycerides to

fatty acids and glycerol and determination of the glycerol moiety of the triglycerides.

Reagents :

1. Organic solvents : Reagent grade chloroform, methanol and diethyl ether were redistilled in an all glass apparatus before use. The diethyl ether was stored over sodium hydroxide and used within two days after distillation.

2. Silicic acid : Silicic acid from Mallinckrodt (100 mesh) was used after over night activation at 100°C.

3. Ethanolic potassium hydroxide : One volume of 2.5 percent potassium hydroxide was mixed with 19 volumes of absolute ethanol (Aldehyde free).

4. 0.02 M sodium periodate.

5. 0.2 M sodium arsenite.

6. 0.7 M sulfuric acid (Swastik).

7. Chromotropic acid : 1 gm of chromotropic acid (4,5-dihydroxy naphthalene-2, 7-disulphonic acid disodium salt), Eastman - Kodak, was dissolved in 100 ml of distilled water filtered and added to a

300 ml of concentrated sulfuric acid and 150 ml of distilled water. The reagent was stored in dark bottle and prepared fresh every week. Each batch of chromotropic acid was tested for blank values.

8. Triglyceride standard : Tripalmitin (BDH) was purified by chromatography on silicic acid column followed by recrystallization. Purity was checked by thin layer chromatography, using a system of 1 percent acetic acid and 25 percent diethyl ether in light petroleum ether. Two working standard solutions were prepared in chloroform to contain 0.2 and 0.1 micromoles of tripalmitin/ml.

Procedure :

To the purified lipid extract of serum, about a spoonful (approximately 500 mg) of silicic acid was added in a glass stoppered tube. After vigorous shaking, tubes were centrifuged for few minutes at 3500 r.p.m. Suitable aliquots of extracts along with the standard and the blank in duplicates were evaporated in glass stoppered tubes on a water bath adjusted to 60°–80°C. One ml of ethanolic KOH was added, the tubes stoppered and the triglycerides hydrolysed for 30 minutes at 60°C. After cooling, 0.8 ml of 0.7 M

sulfuric acid and 4 ml of diethyl ether were added and mixed by vigorous shaking. From the lower water phase, 0.3 ml was pipetted into test tubes and 0.1 ml of 0.02 M sodium periodate added. After 10 minutes 0.1 ml of 0.2 M sodium arsenite was added and the tubes shaken. 2.8 ml of chromotropic acid reagent was added after 5 minutes in dark room and the tubes were heated in a boiling water bath for 30 minutes. The colour was read at 570 u or on Leitz using filter 'D'.

Serum cholesterol :

Modified method of Abell et al (1952) was followed for cholesterol estimation.

Reagents :

1. Absolute ethyl alcohol
2. Acetic acid (BDH, AR)
3. Sulphuric acid (reagent grade)
4. Acetic anhydride (BDH, AR)
5. Standard cholesterol (recrystallized)
6. Petroleum ether (Burma Shell Co.)
7. Ethyl ether (Alembic)
8. Modified Liebermann - Burchard reagent.

To 20 volumes of chilled acetic anhydride in a glass stoppered container, one volume of concentrated sulphuric acid was added with gentle shaking and the mixture was cooled for some time. Then 10 volumes of acetic acid were added to the mixture and allowed to come to room temperature before use. The reagent was made fresh whenever required.

Procedure :

Total cholesterol from serum was extracted by adding 0.5 ml of serum to 9.5 ml of alcohol ether mixture (3:1) in a centrifuge tube. After thorough mixing followed by centrifugation, suitable aliquots of extracts were evaporated and treated with 6 ml of Liebermann - Burchard reagent. The intensity of the colour was read on Leitz (filter 'B') after 30 minutes. Standard graph was plotted using concentrations ranging from 100 to 500 micrograms.

Serum lipid phosphorus :

Serum lipids were quantitatively extracted with alcohol-ether mixture (3:1) and the lipid phosphorus after conversion into inorganic phosphate by acid digestion with 5 N H_2SO_4 was estimated by the method of Fiske and Subbarao (1925).

Reagents :

1. Ammonium molybdate No.2 (2.5 percent)

   Ammonium molybdate 12.5 gm

   Sulphuric acid (10 N) 150 ml

   Distilled water to make 500 ml.

2. 1,2,4-amino-naphthol-sulphonic acid

   Sodium bisulphite (15 percent) 195 ml

   Sodium sulphite (20 percent) 5 ml

   1,2,4-amino-naphthol-sulphonic acid 0.5 gm.

3. Sulphuric acid 5 N

4. Hydrogen peroxide 30 percent (BDH phosphorus free)

5. Standard phosphorus (Stock) 1 mg/ml.

6. Working standard phosphorus

   100 µg/ml (Stock diluted 1:10).

Procedure :

Serum lipids were extracted quantitatively with alcohol ether mixture as mentioned for cholesterol estimation. Suitable aliquots of the extract were evaporated and after cooling, digested with 2.5 ml of 5 N sulphuric acid on a sand bath till the contents were dark brown in colour. Two to three drops of hydrogen peroxide were added to clarify the mixture.

After 10 minutes, tubes were cooled, diluted with water and treated with 2.5 ml of ammonium molybdate and 1 ml of 1,2,4-amino-naphthol sulphonic acid reagent. The volume was made up to 25 ml and the tubes were kept in dark for 10 minutes. Reading were taken on Leitz (filter 'A'). The factor of 25 was used to convert lipid 'P' into phospholipid (Youngberg 1930).

Blood sugar :

Blood was taken in isotonic diluent (King 1964) and after precipitating proteins with sodium tungstate, the filtrate was used for estimation of blood sugar, using Somogyi's Copper and Nelson's arsenomolybdate reagents (Somogyi 1952; Nelson 1944).

Reagents :

1. Diluent

Sodium sulphate (anhydrous)	12.5 gms
Copper sulphate	6.25 gms
Distilled water to make	1 litre.

2. Somogyi's reagent

Copper sulphate	4 gms
Sodium carbonate	24 gms

Sodium bicarbonate	16 gms
Rochelle salt	12 gms
Sodium sulphate	18 gms
Distilled water	1 litre

3. Nelson's reagent

Ammonium molybdate	25 gms
Concentrated sulphuric acid	20 ml
Sodium arsenate	3 gms
Distilled water to make	500 ml

Reagent was kept in the incubator at 37°C for 36 to 48 hours.

4. Sodium tungstate 10 percent

5. Standard glucose (stock) 1 mg/ml
(Preservative - Benzoic acid)

6. Working standard : 1 ml of stock standard was diluted to 10 ml with distilled water.

Procedure :

0.1 ml of blood was added to 3.7 ml of diluent in a centrifuge tube followed by 0.2 ml of sodium tungstate. After centrifugation, 1 ml of filtrate was taken in Folin Wu tube and boiled in water bath

with 1 ml of Somogyi's reagent for 15 minutes and then treated with 1 ml of Nelson's reagent. The colour was read on Leitz (filter 'A') after making up the volume to 12.5 ml.

Experiment II :

Thirty six adult male albino rabbits of Haffkine bred strain weighing between 1.5 to 2 kg at the start of experiment were selected. Two separate sets of experiments were planned (A and B). Animals were divided into six groups (3 in each set of experiment) - each consisting of six animals - and caged individually after allocation into three groups*. In experiment A, group I was kept as control. Group II received 0.03 units/kg body weight/day of plain insulin subcutaneously with a microsyringe and group III received 50 mg/kg body weight/day of tolbutamide orally per day by forced feeding with the tube. Dose of insulin was standardized initially with six animals. Dose which gave maximum fall of 10 to 20 mg of blood sugar after insulin injection was selected for the experiment so that minimum degree of hypoglycaemia

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* All animals were fed ad libitum the standard stock diet consisting of lucern grass, green bengal gram, cereal bran and carrots.

was maintained and absolute effect of the drug could be studied. In experiment 'B' which consisted of group IV, V and VI, group IV received 0.7 gm of cholesterol/kg body weight/day in the form of thick suspension with ground nut oil orally by forced feeding and served as control for group IV and V which were treated with doses of insulin and tolbutamide respectively along with cholesterol in similar fashion as mentioned for experiment 'A'. Tolbutamide powder was supplied by Department of Chemotherapy, Haffkine Institute, Bombay and insulin was purchased from Boot's Laboratory, Bombay. Cholesterol was obtained from J.T.Bakers, Germany.

Treatment was carried out for three months during which sugar, phospholipids and cholesterol levels in the blood were studied at various intervals. At the end of twelve weeks, animals were sacrificed after 36 hours fasting and blood, liver and aorta were removed for lipid studies. Total lipids, cholesterol (free and total), triglycerides and phospholipids were estimated from liver, serum and aorta.

Sections of coiled aorta, heart, liver and kidney were processed for histopathological examination.

Analysis of liver :

The liver was removed quickly and washed with saline, blotted with filter paper and weighed immediately. A portion of the liver was taken for lipid analysis. Lipids were quantitatively extracted with chloroform and methanol (2:1) according to the procedure of Folch et al (1957) and the extracts were made to a known volume.

Liver cholesterol :

Suitable aliquots of extracts representing about 200 mg of liver were treated with alcoholic KOH for two hours in a boiling water bath and the nonsaponifiable matter was extracted with petroleum ether. After evaporating petroleum ether, cholesterol was estimated as described earlier.

Liver phospholipids :

Known aliquots of extracts representing approximately 100 mg of liver were evaporated and processed for the phospholipid estimation as described earlier. Liver fat was estimated gravimetrically.

The kidney and a piece of liver were preserved in formal saline for histopathological work.

Analysis of aorta :

Aorta was dissected from its point of origin to the point of bifurcation and washed with saline. It was opened and coiled and section was cut for histopathological examination and fixed immediately in formal saline. Rest was used for lipid studies. Lipids were extracted quantitatively from each specimen with chloroform-methanol (2:1) mixture and processed for the estimation of cholesterol and total lipids as described for liver.

Analysis of serum :

Serum phospholipids and cholesterol were estimated as described earlier.

Histopathological examination :

After the animals were sacrificed, a portion of the aorta, heart, liver and kidney were subjected to histopathological examination. Prior to this, an assessment of atherosclerotic involvement of aorta was done by grading the lesion as either 0; + ; ++ ; +++ ; or ++++ depending upon whether there is absence of plaque or presence of occasional fatty streaks or there are definite nonulcerated yellowish raised

plaques at several places or there are several such plaques with a few ulcerated ones or there are such plaques with ulcers and calcification or thrombus formation.

Experiment III :

For this experiment, liver and serum were removed from the same animals which were used for lipid studies. The method of grouping and treatment of the animals has been described earlier.

Livers were removed quickly, washed with saline, blotted, weighed and kept in a container of ice. Liver piece of known weight was immediately homogenised in 0.14 M KCl solution (1:10 w/v) in cold condition in potter-Elvejhem type homogeniser. Homogenate was centrifuged at 0-4°C for 30 minutes at 27,000 g and the supernatant cytosol fraction was used for enzyme assay. Enzyme assays were carried out according to the procedures described in Table 9.

Reagents :

Reagents were prepared in glass distilled water.

1.	Glycyl-Glycine buffer (0.1 M, pH 7.4)	
	158 mg of Glycyl - Glycine (Sigma)	100 ml
2.	Tris HCl Buffer (0.2 M, pH 8.0)	
	Tris (BDH)	1.21 gms in 100 ml
	pH adjusted with 0.2 M, HCl	
3.	Phosphate buffer (0.1 M, pH 7.4)	
	a) Disodium hydrogen phosphate (Na_2HPO_4) (BDH)	1.22 gms/100 ml
	b) Sodium dihydrogen phosphate (NaH_2PO_4 $2H_2O$) (BDH)	1.16 gms/100 ml
	81 ml of (a) was mixed with 19 ml of (b) and	
	pH was adjusted to 7.4.	
4.	Manganese chloride (BDH)	100 mg/10 ml
5.	Magnesium chloride (BDH)	0.1 M 203 mg/50 ml
6.	Glucose-6-Phosphate (NBC)	8 mg/10 ml
7.	Nicotinamide-adenine dinucleotide (NBC) (Reduced)	10 mg/ml
8.	Nicotinamide-adenine-dinucleotide phosphate (NBC)	10 mg/ml
9.	Coenzyme A (Sigma)	10 mg/ml
10.	Potassium citrate	162 mg/ml
11.	Sodium pyruvate	2.5 mg/ml
12.	L-malic acid	53.6 mg/ml

Enzyme methods

Enzyme	Assay mixture	Reference
Glucose-6-phosphate-dehydrogenase	Glycyl-glycine 60 u moles, Magnesium chloride 10 u moles. NADP 2.5b u moles, Glucose-6-phosphate- 2.0 u moles, 1 ml homogenate, pH 7.6	Kornberg and Horecker (1955)
Malic enzyme	Tris buffer 440 u moles. L-malic acid 20 u moles. Manganese chloride, 5 u moles. NADP 3 u moles, homogenate 1 ml, pH 7.5.	Olson et al (1948)
Citrate cleavage	Tris buffer 30 u moles, Magnesium chloride 20 u moles Mercapto ethanol 20 u moles, Potassium citrate 100 u moles. Coenzyme A 100 u moles, $NADH_2$ 35 u moles, homogenate 1 ml, pH 7.6.	Srere P.A. (1962)
Lactic dehydrogenase	Phosphate buffer 100 u moles, Sodium pyruvate 5 u moles, homogenate 1 ml pH 7.4.	Bergmayor et al (1965)

Results were analysed statistically by using student's 't' test. Findings were further checked on TDC-12 Computer. Difference was considered significant when $P<0.05$