CHAPTER TWO

MATERIAL AND METHODS

A) MATERIAL

Norwegian rats were rared in the departmental animal house. These were supplied with Gold Mohur (Lipton India Limited, Banglore) food about 20 gms. daily, but water was supplied ad libitum.

For experimental purpose, only the male rats weighing about 200 to 225 gms were used. About 80 rats were used for experimental purpose. They were fasted overnight and diabetes was induced by intraperitoneal injection of alloxan monohydrate [40 mg/250 gm body weight in 1.0 ml of saline (0.9 %)]. Controls received 1.0 ml saline.

12 hours after the alloxan administration, six rats were fasted for 12 hrs, along with controls three of them received food after 12 hrs of fasting for half an hour as well as control were also fed for half an hour then all the twelve rats were killed by cervical dislocation and their salivary glands were pulled, weighed and then used for the estimation of enzyme activities like esterase, lipase and proteins. Blood was collected from sinus venosus in heparin coated syringe and glucose was estimated using Folin Wu method.

36 hours after the induction of diabetes, six rats were fasted for 12 hrs and three of them received food for 1/2 hr. After this, all of them were killed and used for further work.

60 hours after the induction of diabetes, six rats were

fasted for 12 hrs and three of them received food for 1/2 hr. and all of them were killed and used for further work.

132 hours after alloxan administration, six rats were fasted for 12 hours and three of them received food for 1/2 hr and all of them were killed and used for further experimental work.

Alloxan monohydrate (No.50-71-5 Batch No.1125) p-nitrophenyl acetate (PNPA no. N.8130 Batch No.100F-5068) were purchased from Sigma Chemical Company, (St.Louis, Mo., USA). All other chemicals were of reagent grades.

B) METHODS

a) Determination of Glucose By Folin-Wu Method : (Hawk, 1965)

The majority of the methods for the determination of blood glucose are based upon the ability of the glucose in hot alkaline solution to reduce certain metallic ions, of which the cupric and ferricyanide ions are almost commonly used. It has long been known that there are reducing substances other than glucose present in the blood, and that these may occur in sufficient amount to increase considerably the "apparent" glucose value.

i) Reagents :

1. Standard glucose solution (0.1 mg/ml)
2. Alkaline $CuSO_4$
3. Phosphomolybdic acid,
4. 10 % sodium tungstate solution,

5. 2/3 N H_2SO_4,

6. Heparin (20 mg/100 ml) an anticoagulant.

ii) Preparation of Unknown :

2 ml of blood was transferred in calibrated test tube and to this tube was added 6 ml of distilled water, 1 ml of sodium tungstate and 1 ml of 2/3 N H_2SO_4, mixed well and allowed to stand for 10 minutes so that all the protein gets precipitated. Then the mixture was filtered and the filterate was used as unknown.

iii) Procedure :

For the assay of glucose, 3-Folin Wu tubes were taken. In the first Folin-Wu tube, 2 ml. distilled water; in the 2nd tube, 2 ml standard; and in the 3rd tube, 2 ml. of unknown sample were taken. To all these tubes, 2 ml. of alkaline $CuSo_4$ was added and immediately, all the tubes were transferred to a rapidly boiling water bath and heated for 8 minutes; cooled all the tubes under running tap water without shaking. To each tube was added 2 ml of phosphomolybdic acid reagent and after about 1 minute, diluted with distilled water upto the 25 ml mark. By inserting blank set, 100 % transmittance (zero density) and the readings were taken at 420 mμ.

iv) Calculations :

$$\frac{\text{Density of Unknown}}{\text{Density of Standard}} \times \text{mg glucose in standard} \times \frac{100}{0.2} = \text{mg glucose/100 ml of blood.}$$

b) Esterase : (Stotz, 1955)

This enzyme catalyses the hydrolysis of lower fatty acid esters. A variety of essay methods are available to measure the esterase activity. In calorimetric method, esters give rise to a coloured base, p-nitrophenyl acetate, is hydrolysed to p-nitrophenol (Stotz, 1955). The development of the characteristic colour of p-nitrophenol by the enzymatic hydrolysis of its colourless esters furnishes a delicate test of esterase activity. However, because of low solubility of esters of long chain fatty acids, the method is applicable preferentially to esterases.

i) Reagents :

1. Phosphate buffer (M/15, pH 7.00)
2. 0.001 M-p-nitrophenyl acetate [(PNPA) substrate]
   63 mg of p-nitrophenyl acetate (PNPA) was dissolved in 10 ml of methanol (stock soln.)

The working substrate solution was prepared by diluting 0.1 ml of the stock solution to 10 ml.

ii) Preparation of Sample :

After sacrificing the rats, salivary glands such as submandibular, sublingual and parotid were pulled out and weighed. Then each gland was carried out in a glass morter for homogenisation. The glass morter and pestle were well washed, rinsed in distilled water, dried and kept in the ice box of refrigerator for nearly 5 hours before homogenization.

Homogenization was carried out in such a chilled glass morter and pestle. Such a homogenization has two advantages :

1. During homogenization, no loss of enzyme activity occurs as temperature due to friction of the morter and pestle does not exceed beyond 12°C at the end of the homogenization.

2. During homogenization, the tissues (salivary glands) were first crushed at the bottom of the morter, these instantaneously freezed and then gradually thawed, which helped in breaking the lysosomes. When the tissue was thawing, 2 ml of chilled distilled water was added and the homogenization was carried out completely. When a perfectly uniform suspension was formed, chilled distilled water was added and filtered. Throughout the work, the concentration of homogenization of the tissues (salivary glands) was kept constant, i.e. 1mg/ml.

iii) Enzymatic Reaction :

For the assay, 3 test tubes were taken and labelled. In each test tube was added 5 ml of chilled distilled water, 2 ml of phosphate buffer (M/15, pH 7.0), 1 ml of enzyme sample and 2 ml of working substrate solution. The tubes were shaken vigorously and readings of the assayed tubes were

taken at '0' hour as reference control. The tubes were again vigorously shaken and kept for 20 minutes for incubation at 25°C, readings were taken at 400 mμ. Both control and incubated mixtures were measured against distilled water as blank. Readings at 'zero' hour were treated as control.

iv) Standard Curve for Esterase :

A series of solutions were prepared containing 0.1 to 0.7 micromoles of p-nitrophenol per millilitre in 1 ml of the p-nitrophenol standards, 9 ml of M/15 phosphate buffer was added. The colorimetric readings were performed immediately with a 400 mμ filter and were plotted on the logarithmic scale of a semilogarithmic paper against the conc. of p-nitrophenol in micromoles/millilitre.

v) Calculations :

The optical density was converted into micromoles of p-nitrophenol from the calibrated p-nitrophenol standard curve. The esterase activity was expressed in p-nitrophenol μ mol./gm. wet weight of the tissue and specific enzyme activity was expressed as p-nitrophenol μ mol/mg of protein p-nitrophenol μ mole were calculated by using the following formula :

$$\text{Enzyme activity} = \frac{\mu \text{ mole p-nitrophenol from graph} \times \text{dilution}}{\text{wt. in gms.}} = \mu \text{ mole p-nitrophenol/gm.}$$

Specific enzyme activity was calculated /mg of protein.

c) Lipase : (Arnold and Kramer, 1965)

Assay of lipase in the presence of large amounts of other esterases requires a relatively specific system and activation of lipase by bile salts is especially important in making the distinction. Tri-butyrin Tween and Olive oil are the natural substrates but they are relatively non-specific and, therefore, unsuitable as substrates. Phenol and napthol esters are sensitive (Bergmeryer, 1965), but they are sensitive to esterases. Earlier, sodium taurocholate was used as an activator, laurate as a substrate. Sodium taurocholate as activator was used but it was recently noted that taurocholate was not a reliable activator and the substitution of the 2-napthyl myristate for 2-napthyllaurate and sodium cholate for sodium taurocholate resulted as a useful method for the lipase determination. The slight hydrolysis that occurs is due to esterase, while in the presence of cholate, the hydrolysis is almost entirely due to lipase and very little to esterase. The difference corresponds to the lipase activity. The molecules of 2-napthol are coupled with tetrazotized O-dianisidine to give a purple A2O-dye which is determined calorimetrically.

i) Reagents :

For the biochemical assay, the following chemicals were required :

1. Tris buffer (0.4 M, pH 7.4),
2. Sodium cholate (0.232 M)
3. Substrate solution (1.4×10^{-2} M-2 napthyl myristate)

4. Tetrazotized O-dianisidine (4 mg/ml)
5. 2-Napthol standard solution (7×10^{-4} M 2-napthol)
6. Ethyleneglycol monoethyl ether solution (0.2 ml/ml).

ii) Enzymatic Reaction :

Each determination requires 6 test tubes ; 3 samples with cholate and 3 samples without cholate. Pipetted successively into test tubes :

	with cholate	without cholate
Tris buffer	2.0 ml	2.0 ml
Sodium cholate	0.5 ml	-
Distilled water	1.3 ml	1.8 ml
Sample	0.2 ml	0.2 ml
Incubated at 37.5°C for 10 min. and added 2-napthyl myristate substrate solution	0.2 ml	0.2 ml
Mixed and incubated for 5 hr at 37.5°C and then added with pipette tetrazotised O-diniside solution	1.0 ml	1.0 ml
After 3 minutes added glacial acetic acid	3.0 ml	3.0 ml

Mixed well and read optical densities against water.

E_1 = Mean of optical densities of the solutions with cholate.

E_2 = Mean of optical denisites of the solutions without cholate.

iii) 2-Napthol Standard Curve :

Pipetted into test tubes 0 to 1 ml (0, 0.1, 0.2 .. 1.0 ml)

of 2-napthol standard solution and made the volume of each test tube 1 ml with glycol ether solution. The solutions contained 0.0 to 7 μ moles 2-napthol/ml. Then added to each test tube 2.0 ml of tris buffer (0.4 M pH 7.4), 0.2 ml blood serum and 1 ml of distilled water. Mixed and added 1 ml of tetrazotized O-dianisidine solution to each test tube. Optical densities were measured on calorimeter and the standard curve was plotted by taking optical densities (ordinate) against μ moles 2-napthol (abscissa). Since cholate increases the colour intensity, it is necessary to prepare two calibration curves, one with and one without cholate.

iv) Calculations :

1 unit is that amount which splits 1 μ mole substrate/minute. 1/1000 units is one milli-unit. At a higher temperature (37.5°C), it is possible to calculate the lipase unit from one or five hour values. The μ moles 2-napthanol formed is measured from the standard curves C1 and C2 corresponding to E1 (with cholate) and E2 (without cholate).

$\Delta C = C1 - C2$ is the amount of napthyl myristate split in five hours by 0.2 ml sample. For one hour incubation :

Milliunits of Lipase = $\Delta C \times 5 \times 1/60 \times 1000/ml$

= $\Delta C \times 83$/milliunits/ml for five hours, $\Delta C \times 16.7$ milliunits/ml.

C) Determination of Protein by Lowry's Method (Lowry et al., 1951)

The greatest asset of this method is its sensitivity :

i) Chemicals :

1. Reagent A : 2 % of Na_2CO_3 in 0.1N NaOH.
2. Reagent B : 0.5 % $CuSO_4.5H_2O$ in 1 % sodium tartarate.
3. Reagent C : 50 ml reagent A + 1 ml reagent B
4. Reagent D : 1 part phenol reagent + 2 parts dist. H_2O.
5. Bovine Serum Albumin (Sigma Chemical Company, St. Louis, MO; USA) : 13 mg in 250 ml.

ii) Procedure :

For this method, 4 test tubes were taken. In the first test tube was added 0.5 ml standard protein (13 mg/250 ml) and in the remaining each test tube was added 0.5 ml. sample. To these test tubes was added 5 ml of reagent C. After waiting for 15 min. at room temperature, added 0.5 ml of reagent D (phenol reagent). Made to stand for 30 min. and readings were taken at 660 mμ.

iii) Calculations :

$$\frac{\text{Amount of standard}}{\text{O.D. of standard}} \times \frac{\text{O.D. of unknown}}{\text{Amount of Unknown}} \times 1000 = \text{mg protein/gm.}$$

e) Statistical Methods : (Fisher, 1936; Snedecor, 1946 and Wilks, 1949)

1. $\bar{X}$ = arithmetic mean of X, independent variable.
2. S.D.= Standard Deviation $\sqrt{\frac{\leq x^2}{N}}$ (N = Number of variables)
3. S.E.= Standard Error for a mean of N variables.

$$S.E.= \frac{S.D.}{\sqrt{N}}$$

4. t - Student test $= \dfrac{\bar{X}_1 - \bar{X}_2}{\sqrt{\dfrac{S_1^2}{N_1} + \dfrac{S_2^2}{N_2}}}$

5. df = degree of freedom for a difference of two means of N_1 and N_2 variables.

6. Significance : The probability P_1 of obtaining t-value at least as great as the calculated one for a given number of df is found in Fisher's Tables

The p-values are signified according to the following conventions :

$P > 0.05$ The difference is said to be non-significant.

$P < 0.05$ The difference is said to be significant.

$P < 0.01$ The difference is said to be highly significant.