CHAPTER-1

EXPERIMENTAL PROTOCOL, MATERIAL AND METHODS

Adult female rats (*Rattus norvegicus albinus*). weighing between 140 $\pm$ 20 g served as experimental animals. Rats were maintained on balanced diet and water *ad libitum*. The phases of estrous cycle were determined by examining vaginal lavages under light microscope daily at 9.00 AM. Only those rats which had normal 4-day estrous cycles were utilized for this study.

EXPERIMENTAL DESIGN

I. NORMAL ESTROUS CYCLE:- For the study of normal cyclic variations, only those females which had normal 4-day estrous cycle were sacrificed at 9.00 AM at each phase (Group of ten animals at each phase).

II. OVARIECTOMY (OVX):- Only diestrous females were bilaterally ovariectomized or sham-operated under mild ether anaesthesia. Following three post-operative intervals were selected to study the effects of OVX on liver viz.- 24, 48 and 72 h.

III.REPLACEMENT THERAPY:- To study the effect of 17B-estradiol (E_2) administration the experimental animals were divided as follows:-

Treatment	**Number of Groups** (10 animals/group)
a.. Normal intact female during diestrous phase	1
b.. 48 hr post OVX	1
c.. 48 h sham-operated	1
d.. 48 h OVX + 0.1 ml vehicle only	3- one each per time interval
e.. 48 h OVX + 5 μg E_2 (D_1)	3- one each per time interval
f.. 48 h OVX + 10 μg E_2 (D_2)	3- one each per time interval
g.. 48 h OVX + 15 μg E_2 (D_3)	3- one each per time interval

Single intramuscular (im) injection of 0.1 ml propylene glycol containing 5, 10 and 15 μg E_2 per animal was given. These animals were sacrificed at 1, 2 and 4 h post-injection intervals.

IV.SIMULTANEOUS REPLACEMENT WITH 17B-ESTRADIOL AND PROGESTERONE (P):- For this regime experimental animals were divided as follows:-

Treatment	Number of Groups (10 animals/group)
a.. Normal intact females during diestrous phase	1
b.. 48 h post OVX	1
c.. 48 h sham-operated	1
d.. 48 h OVX + 0.5 ml vehicle only	1
e.. 48 h OVX + 2 mg P+ 5 μg E_2 (CD_1)	1
f.. 48 h OVX + 2 mg P + 10 μg E_2 (CD_2)	1
g.. 48 h OVX + 2 mg P + 15 μg E_2 (CD_3)	1

Single i.m. injection of 0.5 ml propylene glycol containing above mentioned doses. These animals of group d to g were sacrificed at 2 h post injection interval.

PARAMETERS AND METHODOLOGY OF ESTIMATION/ASSAY

Blood samples were collected by the nictitating membrane puncturing with slightly jagged end of anticoagulant coated capillary tubes to obtained plasma for estimating glucose, lipid, cholesterol and phospholipids. Later, the animals were sacrificed under mild ether anaesthesia taking maximum care to avoid any stress during handling. Hepatic tissue was quickly removed, trimmed free of adherent connective tissue, blotted free of blood and weighed accurately upto 0.01 mg on single pan electronic balance. Spigelian and the right lobes (nomenclature according to Green, 1959) were dealt with separately for different estimations.

GLUCOSE:- Plasma glucose level was estimated by the method of Wincker's and Jacobs (1971) using Orthotoluidine reagent. Glucose concentration was expressed as mg/100 ml plasma.

GLYCOGEN:- The glycogen concentration was estimated by employing the method of Seifter *et al.* (1950). Pieces of tissue were quickly immersed in 2 ml of 30% KOH in pre weighed test tubes. Weight of the fresh tissue taken could be calculated by difference. Test tube were kept in boiling waterbath for 10 minutes for extraction. Then test tubes were cooled. 2.5 ml of ethanol (95%) was added. The test tubes were kept again in boiling water bath till just bubbling occurred and then cooled in refrigerator for 30 minutes, precipitate was obtained by centrifugation at 3000 rpm for 30 minutes. Supernatant was removed by decanation. Precipitate was resuspended in 2.5 ml of 95% ethanol and centrifuged at 3000 rpm for 30 minutes. Final precipitate was resuspended in distilled water and suitably diluted. 1 ml aliquots were treated with 4 ml anthrone reagent (95% H_2SO_4) and transferred to boiling water bath for 4 minutes. Thereafter the testtubes were cooled immediately in ice bath. Colour intensity was read colorimetrically at 620 nm. Glycogen concentration was expressed as mg/100 mg of fresh tissue weight.

TOTAL PROTEIN:- Tissue of known weight was homogenized in pre-chilled mortar with cold glass distilled water and diluted to approximate concentration of 20 mg/ml. The total protein concentration was estimated by the method of Lowry et al. (1951) using Folin-ciacalteau reagent. The colour produce was read at 720 nm on a colorimeter and protein concentration was expressed in terms of mg/100 mg of fresh tissue weight.

LIPIDS:- Total lipid concentration was estimated by employing the method of Folch et al. (1957) using a mixture of Chloroform-methanol (2:1 v/v). Tissues was crushed in the extractant at room temperature, allowed to stand for 6-8 h with frequent stirring. The upper aqueous layer was separated and treated at least twice with extractant. The lower layer from each time were collected and pooled extract was utilized to estimate total lipid concentration gravimetrically. The total lipid concentration was expressed as mg/100 mg fresh tissue weight.

CHOLESTEROL:- Cholesterol concentration was estimated by the method of Crawford (1958) using alcohol ether mixture (3:1 v/v). $FeCl_3$ was used as the colour reagent. Colour intensity was read at 540 nm on photoelectric colorimeter. Cholesterol concentration was expressed as mg/100 mg of fresh tissue weight or as % of total lipid content.

NUCLEIC ACIDS:- Nucleic acid concentrations (DNA and RNA) were assayed spectrophotometrically as suggested by Schneider (1957) and are expressed as μg/mg fresh tissue weight.

GLYCOGEN PHOSPHORYLASE: (EC 2.4.1.1):- Glycogen phosphorylase activity was assayed by the method of Cahill *et al.* (1957) using glucose-1-phosphate (dipotassium salt) Sigma Chem Co.) as the substrate . Phosphorous concentration was measured from inorganic phosphate radicals released, as per the method of Fiske and Subbarow (1925). Enzyme activity was expressed as μg phosphate released/mg protein/60 minutes at 37°C.

GLUCOSE-6-PHOSPHATASE (EC 3.1.3.9) (G-6-Pase) :- G-6-Pase activity was assayed by the method of Harper (1963) and phosphorus released was measured by the classical method of Fiske and Subbarow (1925). Activity was expressed as μmoles of phosphate released/mg protein/60 minutes at 37°C.

SUCCINATE DEHYDROGENASE (EC 11.3.99.1) (SDH):- Enzyme activity was assayed as per the method of Kun and Abood (1949) using Iodonitro-triphenyl-tetrazolium salt (INT) as the hydrogen acceptor. The Formozan formed was extracted in 7 ml of acetone and the colour intensity was read at 420 nm (blue filter) colorimetrically. Enzyme activity was expressed as μg formozan formed/ mg protein/60 min at 37° C.

CYCLIC AMP-SPECIFIC PHOSPHODIESTARASE (EC 3.1.4.17) (cAMP-PDE):- c.AMP-PDE activity was measured by employing the method of Butcher and Sutherland (1962). cAMP was used as the substrate. 0.9 ml aliquots of the reaction mixture were incubated at 37°C for 20 mins and then 0.1 (1 mg/ml) of venom (*Vipera Russeli*) was added to each test tube and incubated again for 10 minutes. There after the reaction was stopped by adding 1 ml of 30% TCA solution and then assayed for inorganic phosphate by the method of Fiske and Subbarow (1925). Colour developed was read colorimetrically at 720 nm. Specific activity was expressed in terms of μg phosphate released/mg protein/60 min. at 37°C.

ADENOSINE TRIPHOSPHATASE (EC 3.6.1.3)(Total ATPase) :- ATPase activity was estimated by the method as described by Umbreit *et al.* (1957). Enzyme activity was terminated with 6% TCA. Inorganic phosphorus released was estimated by the method of Fiske and Subbarow (1925). The colour intensity was read at 660 nm on a Klett-Summerson colorimeter and ATPase activity was expressed in terms of μg phosphate released/mg protein/60 min. at 37°C.

5'-NUCLEOTIDASE (EC 3.1.3.5):- Enzyme activity was assayed by the method of Jaffri and Mustafa (1976). One ml chilled aqueous homogenate was centrifuged at 2500 rpm for 10 min. supernatant was used for estimation of enzyme activity and protein concentration. AMP was used as substrate and the reaction was stopped by adding 1 ml of 10% TCA solution and then assayed for inorganic phosphate by the method of Fiske and Subbarow (1925). Colour

developed was read at 660 nm colorimetrically. Enzyme activity was expressed in terms of μg phosphate released/mg protein/60 min. at 37°C.

SEPARATION AND QUANTIFICATION OF PHOSPHOLIPIDS BY USING THIN LAYER CHROMATOGRAPHY (TLC):-

Extraction of lipids:-

Weighed tissue was ground thoroughly with washed sand and chilled chloroform-methanol (2:1 v/v) mixture. It was left standing for 6-8 h at room temperature with frequent stirrings. The supernatant was then filtered out into a flask. The residue was extracted thrice with increasing proportion of methanol and finally with pure methanol. The pooled extract was reduced to dryness by evaporation at 55°C. The dried material was redissolved in a measured volume of chloroform-methanol (2:1 v/v) mixture and washed according to Folch et al. (1957) to free it from other contaminants.

Fractionation by thin layer chromatography (TLC):-

Phospholipid were fractionated by TLC according to the method of Stahl (1959). The samples were run on silica gel G plates, which were activated at 110°C for 60 min. and cooled. The sample in chloroform-methanol mixture were loaded (50 μl in each case) by micropipettes 2 cm from the bottom edge of the plate. The plates were developed in chromatographic chambers already saturated with solvent system. The solvent system-chloroform:methanol:ammonia (60:35:5)-was employed for fractionation. Reference channels were run simultaneously with samples to run up to 14 cm from the origin. After full run the plates were removed from the chambers and dried at room temperature. The spots were visualized by exposure to iodine vapours. The spots were identified and marked with reference to known compounds. Different fractions were scraped off the plates and collected quantitatively after complete sublimation of iodine. Phospholipid fractions were eluted with methanol and dried completely at 55-60°C. Phosphorous of individual fraction and original total phospholipid fraction was estimated by the method of Bartlett (1959) and concentration were expressed as mg/100 mg tissue or % of total lipid and of total phospholipids.

PLASMA LIPIDS:- Total lipids were estimated by gravimetric method of Folch et al (1957). Quantitative evaluation of phospholipids and cholesterol were carried out as per the method of Bartlett (1959) and Crawford (1958) respectively, and concentration expressed as mg/100 ml of plasma and % of total lipids in plasma.

STATISTICAL ANALYSIS:- The mean values, standard deviations and standard errors were calculated as per routine statistical methods. Student's 't' test was applied to determine the statistical significance and is expressed as p values.