CHAPTER - 1

EXPERIMENTAL PROTOCOL, MATERIAL AND METHODS

Adult female albino rats weighing between 140 ± 20 gms, served as experimental animals. Rats were maintained on balanced diet and water *ad libitum*. The stages of estrous cycle were confirmed by observing vaginal lavages daily at 09:00 am. Only those animals which had normal 4-day estrous cycles were utilized for this study.

EXPERIMENTAL SET-UPS

For the study of normal cyclic variations, only those females which had normal 4-day estrous cycles were sacrificed at 09:00 am at each stage.

OVARIECTOMY / OvX

Only those females which were in diestrous stage were bilaterally ovariectomized or sham operated under mild ether anaesthesia. Following three post ovariectomized intervals were selected to study effects of ovariectomy on submandibular gland metabolism i.e. 24, 48 and 72 hours (H).

REPLACEMENT WITH 17β–ESTRADIOL (E_2)

To study this effect the experimental animals were divided into 4 groups of ten female rats each, as follows :-

1. 48 H post operated females. Ovariectomy was perfomed during the diestrous stage of estrous cycle.
2. 48 H sham operated females. The sham operation was perfomed during the diestrous stage of estrous cycle.

3. 48 H ovariectomized females injected intramuscularly (i.m.) with 0.1 ml of vehicle (propylene glycol) only.
4. 48 H ovariectomized controls injected i.m. with 0.1 ml of 17β–estradiol (E_2) solution. Three different doses of 17β–estradiol (in 0.1 ml of propylene glycol) were administered individually *viz.*- 5 (D-1), 10 (D-2) and 15 (D-3) μg/animal intramuscularly as a single injection at 09:00 am. The animals were sacrificed at the end of 1, 2 and 4 H post injection intervals.

SIMULTANEOUS REPLACEMENT WITH 17β–ESTRADIOL AND PROGESTERONE

The animals were divided into 4 groups of ten animals each as follows :-
1. 48 H post-operative females. Ovariectomy was perfomed on the day of diestrous stage of estrous cycle.
2. 48 H sham operated females. The sham operation was perfomed at diestrous stage of estrous cycle.
3. 48 H ovariectomized controls, injected with 0.5 ml of vehicle alone.
4. 48 H ovariectomized females injected with three different doses of estradiol i.e. 5, 10 and 15 μg simultaneously with 2 mg of progesterone in each case. Single intramuscular injection of hormonal mixture in 0.5 ml propylene glycol was administered to each animal. All the hormone replaced as well as control females were sacrificed after two H of injection.

PARAMETERS AND METHODOLOGY OF ESTIMATIONS/ASSAYS:-

Blood samples were collected from the nictitating membrane part of an eye in anti-coagulant coated capillary tubes so as to obtain plasma for glucose estimation. Later, the animals were sacrificed under mild ether anaesthesia taking maximum care to avoid any stress during handling. The submandibular glands were excised and freed of connective tissue. Submandibular glands were weighed accurately upto 0.01 mg on single pan electric Mettler balance.

GLUCOSE :- Plasma glucose level was estimated by the method of Winckers and Jacob (1971). The glucose concentration was expressed as mg/100 ml of plasma.

GLYCOGEN:- The glycogen concentration was estimated by employing the method of Seifter *et al.* (1950). One of the glands was quickly immersed in 2 ml of 30% KOH in pre-weighed test-tubes. Glycogen was precipitated with 95% ethanol. Re-suspended precipitate was suitably diluted and treated with anthrone reagent. Colour intensity was read colorimetrically at 620 nm. Glycogen concentration was expressed as mg/100 mg wet tissue weight.

PROTEIN :- The protein concentration was estimated by employing the method of Lowry *et al.* (1951). Tissue of known weight was homogenized in a pre-chilled mortar and diluted to an approximate concentration of 20 mg/ml with chilled glass distilled water. The colour produced 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) as an extractant and measured gravimetrically 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 a photoelectric colorimeter. Cholesterol concentration was expressed as mg/100 mg tissue.

SIALIC ACID :- The glandular tissue was hydrolysed by weak (0.5 and 0.1 N) sulphuric acid. From the hydrolysate total sialic acid was eluted successively over Dowex-2 and 50 columns and assayed colorimetrically as per the method of Svennerholm (1958), and expressed as μg sialic acid / 100 gm fresh tissue weight.

GLYCOGEN PHOSPHORYLASE :- (E.C. 2.4.1.1) Total phosporylase activity was assayed by the method of Cahill *et al.* (1957), using glucose-1-phosphate as the substrate. The inorganic phosphate released was measured as per the method of Fiske and Subbaraw (1925). Enzyme activity was expressed as μmoles phosphate realeased / mg protein / 60 minutes.

SUCCINATE DEHYDROGENASE :- (E.C. 11.3.99.1) Enzyme activity was assayed as per the method of Kun and Abood (1949) using Iodonitro-triphenyl-tetrazolium salt (INT) as the hydrogen acceptor. The formfoazan formed was extracted in 7 ml of acetone and colour intensity was read with blue filter at 420 nm on a colorimeter. Enzyme activity was expressed as μg formazan formed / mg protein / 60 minutes.

TOTAL ATPase (E.C. 3.6.1.3) AND Na^+-K^+ ATPase (E.C. 3.6.1.37) :- ATPase activities were estimated by the method described by Umbreit *et al.* (1957). Adenosine-5' triphosphate (sodium salt) was used as the substrate. Ouabain was used as the specific inhibitor of Na^+-K^+ ATPase. Enzyme activity was terminated with 6% TCA. Inorganic phosphate released was estimated by the method of Fiske and Subbaraw (1925). The colour intensity was read at 660 nm on a Klett-Summerson colorimeter and ATPase activity was expressed in terms of μmoles phosphate released / mg protein / 60 minutes.

CYCLIC AMP-SPECIFIC PHOSPHODIESTERASE :- (E.C. 3.1.4.17) c.AMP-specific phosphodiesterase (PDE) activity level was measured employing the method described by Butcher and Sutherland (1962). c.AMP was used as substrate. Aliquots of the reaction mixtures were treated with vipera venom and were then analysed for inorganic phosphate by the method of Fiske and Subbaraw (1925) and colour developed was read at 720 nm on colorimeter. Specific activity was expressed in terms of μmoles phosphate released / mg protein / 60 minutes.

HISTOCHEMICAL LOCALIZATION OF STEROID DEHYDROGENASE :-

The left submandibular gland from each rat was quickly excised after decapitation under mild ether anaesthesia and transfered to a cryostat microtome maintained at -20°C. Fresh frozen sections of 15 μ thickness were taken on a clean slide and finger thawed. 17β-hydroxysteroid dehydrogenase was localized employing the method of Kellogg and Glenner (1960), using testosterone and estradiol as substrates. 3β-hydroxysteroid dehydrogenase was localized according to the method of Wattenberg (1958) using dehydroepiandrosterone and pregnenolone as the substrates while 3α-hydroxysteroid dehydrogenase was demonstrated histochemically as per the method of Balough (1966) using androsterone as the substrate. Incubation of the sections was carried out at 37°C and NAD was used as a hydrogen acceptor. Stained sections were washed thoroughly in distilled water and post fixed in 10% neutral formalin for 15 minutes, washed again in distilled water, mounted in glycerine jelly. Control sections for the enzyme were incubated in media devoid of the respective substrates.

STATISTICAL ANALYSIS

For each of the parameters studied eight to ten replicates were assayed. The mean values and standard error of mean were calculated and the level of stastical significance was determined by applying student's *t* test. The levels of significance have been calculated with reference to those obtained during diestrous stage.