CHAPTER TWO

MATERIAL AND METHOD

MATERIAL

Sixty albino rats, 30 males and 30 females, weighing about 250 gms of 6 months age were used for the present study. The rats were maintained in the laboratory in individual cages. The rats were fed Gold Mohur rat feed (Hindustan Lever Ltd., Bombay) and water ad libitum.

Fifteen male and female rats were utilized to study the gross anatomy of male and female Brunner's glands. Ten rats, 5 each of male and female, were used to study the normal histology and histochemistry of the Brunner's glands. The remaining 10 each of male and female rats were used to study the effect of hormone on Brunner's gland. They were operated under ether anesthesia. The male rats were testectomised and females were overiectomised. Thirty days after operation five males and five females were utilized to study the effect of testectomy and overiectomy on the Brunner's glands histologically and histochemically. The remaining operated male and female rats were injected with respective sex hormone in corn oil, 4 mg testosterone (sigma) was injected in male rat per 100 gm of body weight for three days and 2 mg of Estradiol (sigma) was injected in female rat per 100 gm of body weight for three days. On the fourth day the rats were sacrificed to study the effect of testosterone and estradiol on the Brunner's glands.

METHOD

To study the anatomy, histology, histochemistry and effect of hormones on Brunner's glands the following methods were used. The study involved comparative anatomical, histological and histochemical observations of normal, operated and hormone-treated rat duodenums containing Brunner's glands.

Gross Anatomy

Pyloroduodenal junctions of male and female rats were processed according to method used by Landboe-Christensen (1944a). It involves washing of pyloroduodenal junctions which are split opened and pinned into a small wax tray, in cold running water, till the Brunner's glands stopped secreting mucus. The tissues were cleared in 3% Acetic acid, washed again in water for one day, and stained for one day in a 1% solution of Haematoxylin. The extra stain was removed in 3% Acetic acid. The muscularis externa and mucosa were carefully removed so that Brunner's glands could be observed directly.

Histology and Histochemistry

Histology

To study the histological structure of the Brunner's

glands, duodenum of normal, operated and hormone-treated male and female rats were removed and fixed in 10% neutral formalin for 24 hours at room temperature. The tissues were washed in running tap water for 24 hours, dehydrated through alcohol grades, cleared in xylene and embedded in paraffin. The sections were cut at a thickness of 7 μ on a rotary microtome. The sections were collected in water bath and mounted on albuminized glass slides.

The sections of each tissue were routinely stained by Haematoxylin-Eosin (H-E) method. The remaining sections of each tissue were subjected to histochemical techniques.

Histochemistry

i) Periodic Acid Schiff Reaction (PAS)

(McManus, 1946; Hotchkiss, 1948)

The periodic acid Schiff (PAS) method was used for the identification of mucosubstances possessing vicinal hydroxyl group (Mowry, 1963).

1) After dewaxing and hydration, sections were brought to distilled water.

2) Sections were oxidized with 0.5% periodic acid for

10 minutes.

3) Sections were washed with distilled water, and
4) treated with Schiff's reagent for 10 minutes,
5) rinsed three times (total 6 minutes) in 0.5% sodium meta-bi-sulphite,
6) washed in distilled water, followed by alcoholic dehydration,
7) cleaned in xylene and mounted in D.P.X.

RESULT

Periodate reactive mucosubstances stain pink-magenta.

ii) Alcian Blue (AB) at pH 2.5

(Mowry, 1956)

Presence of acid mucosubstances was studied by staining sections with Alcian Blue (AB) at pH 2.5. AB at pH 2.5 stains both sulfated and nonsulfated mucosubstances (Mowry, 1963).

1) After dewaxing and hydration, sections were brought to distilled water,
2) Rinsed in 3% Acetic acid,
3) Stained with AB (1% AB in 3% Acetic acid pH 2.5) for 30 minutes,

4) Rinsed in 3% Acetic acid,

5) Washed in running water for 5 minutes,

6) Dehydrated, cleared and mounted as usual.

RESULT

Acidic mucosubstances stain blue.

iii) Malt diastase

Digestion of glycogen is commonly performed with malt diastase. The use of enzymes in the histochemistry of glycogen has been reviewed by Hale (1957).

1) Rinse deparaffined sections in alcohol and in distilled water.

2) Treat the sections for 1 hour at room temperature with diastase. Diastase solution (Lillie, 1940) is prepared as follows: 0.1% diastase in 0.02 M phosphate buffer, pH 6, containing 0.8% NaCl.

3) Rinse in distilled water and proceed with the method for demonstration of glycogen.

One hour digestion with diastase removes all glycogen from the sections.

iv) Pepsin digestion

Lillie, 1947

Pepsin (1:1000) solution is prepared by dissolving 0.1 gm of pepsin in 100 ml of 0.1 N HCl.

1) Deparaffined and hydrated sections are rinsed in distilled water.

2) Then keep the sections in pepsin solution from 1/2, 1, 2, ... to 16 hours of incubation at 37°C.

3) Wash the sections under tap water and proceed for staining.

v) Enzyme Histochemistry

For the histochemical demonstration of Esterase and β-glucuronidase, normal, operated and hormone-treated male and female rats were sacrificed and the duodenum were fixed in 1% calcium chloride in 5% formalin for 24 hours at 4°C. Tissues were then transferred to Holt's 0.88 M sucrose solution containing 1% gum accacia, which was previously chilled to 4°C (Holt, 1959; Hayashi and Fishman, 1961). The tissues were kept in gum sucrose exactly for 24 hours at 4°C and then sections were cut at 6 to 7 μ on Lipshaw microtome. The sections were collected in distilled water cooled to 4°C. Free floating sections were selected for further treatment.

a) Post-coupling Technique Employing Naphthol AS-BI-β-D Glucosiduronic Acid Substrate

(Fishman and Goldman, 1963)

The stock substrate solution of Naphthol AS-BI-β-D-Glucosiduronic acid (Sigma), 2×10^{-4} M was prepared by dissolving 11 mg of the substrate in 0.1 ml of 0.05 M $NaHCO_3$ and then diluting to 100 ml with 0.1 M acetate buffer pH 4.5. The stock solution was preserved in the refrigerator at $4^{\circ}C$. The stock solution was diluted with acetate buffer to obtain solution 1×10^{-4} M. The selected sections were incubated at $37^{\circ}C$ for 60 to 90 minutes.

The incubated sections were washed in cold distilled water for one minute and then coupled with fast garnet GBC (Sigma) diazonium salt. It readily couples with liberated naphtol AS-BI, and leaves no background staining so that stained product can easily be observed under microscope. The coupling solution was prepared by dissolving 0.2 gm of the salt in 100 c.c. of 0.01 M phosphate buffer at pH 7.4 and then filtered.

The sections were transferred to cold distilled water, washed well and mounted in 1% aqueous polyvinyl pyrolidone on clean glass slides.

b) Indoxyl Acetate Method for Esterase

(Pearson and Gross, 1959; Holt and Withers, 1958; Pearson and Defendi, 1957)

The following solutions were prepared:

a) 0.1 M Tris buffer pH 7.2,

b) 0.05 M Potassium ferrocynide,

c) 0.05 M Potassium ferricynide,

d) substrate solution was prepared as follows:- 5-bromoindoxyl acetate (Sigma) 10 mg was dissolved in 2 ml ethanol, to this were added 15 ml Tris buffer, 5 ml potassium ferrocynide and 5 ml Potassium ferricynide and the resultant mixture was filtered.

Sections were transferred into incubation cups filled with substrate solution and incubated at 37°C, for 15 to 20 minutes. Incubated sections were washed in cold distilled water for one minute. If the reaction product was formed, which was demonstrated by blue precipitate of Indigo, sections were taken on clean albuminized slides and blotted with Whatman No. 1 Filter paper with face down and then mounted in 1% Polyvinyl pyrolidone.