CHAPTER TWO

MATERIALS AND METHODS

MATERIAL

About forty mature male and female squirrels (Funambulus Palmarum L.) were used for the present investigation. All the animals were trapped in the latter part of the summer and in early winter. Mature males and females were identified with the help of presence of sperms in the seminiferous tubules of testes and presence of Graafian follicles in the ovaries, respectively. Such mature squirrels were utilized for the further work without delay. The salivary glands: submaxillary, sublingual and parotid were dissected out and studied histologically and histochemically to demonstrate the sex dimorphism if any. The work on squirrel was done in the following direction:

1) A histological study of salivary glands in adult male and female squirrels,
2) A study of convoluted granular tubules,
3) A study of mucous and seromucous cells,
4) A study of serous cells and duct cells,
5) A study of myoepithelial cells.

METHODS

As outlined in the introductory chapter, the studies on salivary glands were planned to find out the sex

dimorphism in these organs of squirrel.

For the histological study haematoxylin-eosin (H-E) technique was selected where-as to study the convoluted granular tubules iron haematoxylin method was employed. To study the mucous and seromucous cells of the salivary glands PAS and Alcian Blue at pH 2.5 techniques were used. And to study the serous cells and duct cells histochemistry of esterase is carried out, as this enzyme is rich in these cells in other species (Burstone, 1956; Yoshimura et al., 1969; Pillai, 1974; Orstavik and Glenner, 1978). Myoepithelial cells could not be revealed with haematoxylin-eosin technique but they are rich in alkaline phosphatase and adenosine triphosphatase (Silver, 1954; Shear, 1964; Bogart, 1968; Garret and Harrison, 1970; Ohanian, 1973). So, to study their distribution histochemistry of alkaline phosphatase is carried out.

Salivary glands of four male and four female squirrels were fixed in 10% neutral formalin for 24 hours at room temperature, salivary glands of other 8 male and 8 female squirrels were in calcium acetate formalin (CAF) at $4^{o}C$ for 24 hours. After prolonged fixation the tissues were washed in running tap water, dehydrated in ascending series of ethanol, cleared in xyline, and embeded 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 albumenized glass slides.

The neutral formalin fixed sections of each tissue were routinely stained by Haematoxylin-eosin (H-E) and Iron-haematoxylin for histological observations.

The calcium acetate formalin (CAF) fixed sections of each tissue were subjected to two histochemical techniques. The periodic Acid Schiff (PAS) method was used for the identification of mucosubstances possessing vicinal hydroxyl group (Mowry, 1963). Presence of acid mucosubstances were 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).

The following two histochemical techniques are used for the identification of the above mucosubstances:-

Periodic Acid Schiff Reaction (PAS)

(McManus, 1946; Hotchkiss, 1948)

1) After dewaxing and hydration, sections were brought to distilled water,
2) Oxidized with 0.5% periodic acid for 10 min.
3) Washed with distilled water,
4) Treated with Schiff's reagent for 10 min.,

5) Rinsed three times (total 6 min.) with 0.5% sodium meta-bi-sulphite,
6) Washed in distilled water, followed by alcoholic dehydration, cleared in xylene and mounted in Canada balsam.

Result: Periodate reactive, mucosubstances stain pink-magenta.

Alcian Blue (AB) at pH 2.5 (Mowry, 1956)

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 min.
4) Rinsed in 3% acetic acid.
5) Washed in running water for 5 min.
6) Dehydrated, cleared and mounted as usual.

Result: Acidic mucosubstances stain blue.

Histochemically it has been shown that an enzyme esterase is rich in amount in serous cells or special serous cells and ductal elements of mouse and rat salivary glands (Pillai, 1974; Doonon et al., 1978). To study their distribution and nature both in male and female squirrel salivary glands histochemistry of enzyme esterase is carried out. In

the salivary glands besides the acinars and ductal elements, there are myopithelial cells and intralobular duct. To see the difference between the myoepithelial cells of male and female salivary glands of squirrel, histochemistry of alkaline phosphatase is carried out.

For the histochemical demonstration of esterase and alkaline phosphatase, 8 male and 8 female squirrels were sacrificed and the salivary glands 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 acacia which was previously chilled to 4°C (Holt, 1959; Hayashi and Fishman, 1961). The tissues were kept in Holt's gum sucrose exactly for 24 hours at 4°C. At the end of the 24 hours, sections were cut at 6 to 7 μ on a Lipshaw microtome, sections were collected in distilled water cooled to 4°C and free floating sections were selected for further treatment. Esterase was demonstrated by employing Indoxyl acetate method and Alkaline phosphatase was demonstrated by using Naphthol AS-MX phosphate method.

Indoxyl acetate method for esterase

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

(1) Solutions: 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.

(2) Technique

Sections were transferred into incubation cup which was filled with substrate solutions and then the incubation cups were kept at 37°C, for 15 to 20 minutes. Incubated sections were washed in cold distilled water for 1 minute. If the reaction product was formed, which was demonstrated by blue precipitates of indigo, sections were taken on clean albuminized slides and blotted with Whatman No. 1 filter paper with face down, and then mounted in polyvinylpyrrolidone.

Naphthol AS-MX phosphate method for alkaline phosphatase

(Burstone, 1958 modified by Barka, 1962)

Similar sections, as were used for the enzyme esterase were utilised in this method.

(1) Solutions: The solutions were prepared as follows:

a) Veronal acetate buffer, pH 9.2

b) Substrate solution was prepared as follows:

5 mg naphthol AS-MX phosphate (sigma) was dissolved in 0.5 ml of N,N dimethyl formamide, 25 ml veronal acetate buffer pH 9.2 was added and to this solution fast blue RR (C.I. No. 37155, Dajac) 25 mg was added and then mixture was filtered.

(2) Technique

Sections were transferred into incubation medium in embryological cups and they were kept at room temperature, for 30 minutes. The incubated sections were washed in cold distilled water, mounted in 1% aqueous polyvinyl pyrolidone and observed under microscope. Alkaline phosphatases hydrolyses naphthol AS-MX phosphate and the released naphthol combined with fast red RR to produce red coloured precipitate.