CHAPTER TWO

MATERIAL AND METHODS

The present investigation was carried out on the leaf-nosed bats, H. fulvus fulvus since these bats possessed frontal sac gland in males and frontal papilla in females.

H. fulvus fulvus is a microchiropteran bat found hanging from the ceilings of caves, underground food cellars or old buildings, temples, mostly in the darker and cooler parts. These are small in size with mobile head and pinnae. The fur on the dorsal side is dark brown and that on the ventral side is brignt white or dirty white in colour. The colonies consist of about twenty to fourty individuals. The presence of the colony can be recognised by a characteristic odour or smell of faecal matter. They are very much sensitive and swiftly fly away even with a slight disturbance. These are insectivorous bats mainly feed on mosquitoes and flies. They leave their place late in the evening for feeding. These bats were collected from Ramling and other temples near Hatkanangale (Maharashtra, India) throughout the course of present investigation. The colony in this temple consisted of 20 to 40 specimens/bats. Males and females were found in the same colony irrespective of the season except at parturition. At the time of parturition and during lactation the females migrated to a old and delapidated building near the temple, some males also accompained with these females. Thus this bat exhibited a social structure in which species without overt sexual segregation at parturition is observed. These bats did not survive in captivity.

This species of bat was selected for the present investigation, since it experiences single sex-cycle in a year and the periodicity is absolutely strict. The sexual cycle in this species of the bat was studied by Patil(1968) and Vibhute (1980). The generalised reproductive pattern in this species of the bat is as follows :

1) The period of sexual quiescence in male from March to July.
2) The preparatory period of spermatogenesis in male from August to September.
3) Active breeding period from October to December.
4) Post breeding period in male from January to February

Collection :

The bats were collected every month from Ramling Temple at Hatkanangale. About four adult males and two adult females were collected every month from June 1986 to May 1987. Both from males and females the frontal sac glands and frontal gland papillae were dissected out and used to study histology and histological changes during the annual breeding cycle. The mucosubstances were studied histochemically in frontal sac gland of the male bats. For confirmation of the results the bats were collected again from June, 1987 to May, 1988. Since the gland was non-functional and rudimentary in the female bats throughout the sex cycle, only the histological observations are included in the present work.

METHODS

Histological :

The bats were sacrificed by decapitation, dissected out the testes, frontal sac gland of male bats and frontal gland

papilla of female bats and immediated fixed in Bouin's fluid, ~~for histological observations.~~ After fixation (24 hr), the tissues were well washed in running water, dehydrated through ethanol grades, cleared in xylene and embedded in paraffin wax. The sections were cut at 5-7 µm and were stained with Haematoxylin-Eosin (H-E) technique for the study of histology and histological variations occurring in the testes and glands during the annual breeding cycle of the bat.

Histochemical Methods for Mucosubstances :

The bats were sacrificed by decapitation and frontal sac glands were dissected out and immediately fixed in solution of 2% calcium acetate in 10% Formalin (CAF-fixative) at 4°C. After fixation for 24 hrs, the glands were well washed in running water, dehydrated through ethanol grades, cleared in xylene and embedded in paraffin wax. The sections were cut at 5-6 µm and stained with various histochemical methods described hereafter for the identification of mucosubstances.

The various histochemical techniques with their merits and demerits for the mucosubstance localization have been reviewed by Spicer (1963), Curran (1964), Barka and Anderson (1965), Lillie (1965), Thompson (1966) Spicer and Henson (1967), Spicer et al. (1967) and Pearse (1968).

For the present study the following series of techniques for visualization of mucosubstances in the frontal sac gland of the male H. fulvus fulvus were employed.

A) Neutral Mucosubstances :

i) Periodic Acid Schiff reaction (PAS) (Mc Manus, 1946; Hotchkiss, 1948) :

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

2) Oxidized with 0.5% periodic acid for 10 minutes

3) Washed with distilled water.

4) Treated with Schiff's reagent for 10 minutes.

5) Rinsed three times (total 6 minutes) with 0.5% sodium meta-bi-sulphite.

6) Washed in distilled water, followed by alcoholic dehydration, cleared in xylene and mounted in canda balsam/DPX.

Results :

Periodate reactive, hexose containing mucosubstances stain pink-magenta.

ii) Phenylhydrazine-PAS (Spicer, 1965; Spicer et al., 1967) :

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

2) Oxidized with 0.5% periodic acid for 10 minutes.

3) Followed by treatment with 5% phenylhydrazine for 30 minutes.

4) Washed with distilled water.

5) Immersed in Schiff's reagent for 10 minutes.

6) Rinsed three times (total 6 minutes) with 0.5% sodium meta-bi-sulphite.

7) Washed, dehydrated, cleared routinely and mounted in canda balsam/DPX.

Results :

Periodate reactive acid mucosubstances were selectively stained, periodate engendered dialdehydes are blocked.

iii) Diastase digestion-PAS technique for identification of glycogen (Lillie, 1954; Lison, 1960) :

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

2) Incubated for one hour at 37°C in the following medium : 0.1% malt diastase in 0.2 M phosphate buffer at pH 6.0

3) Washed in distilled water.

4) Processed as in A-i for PAS.

Results :

Loss of PAS reactivity or reduction in the staining intensity indicates the presence of glycogen.

B) Acid Mucosubstances :

i) Alcian blue (AB) at pH 2.5 (Mowry, 1956) :

1) After dewaxing hyeration, 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.

Results :

Weakly acidic sulfated mucosubstances, hyaluronic acids and sialomucins stain dark blue. Strongly acidic sulfated mucosubstances are stained weakly or not at all.

ii) Alcian blue (AB) at pH 1.0 (Lev and Spicer, 1964);

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

2) Stained for 30 minutes in 1% AB in 0.1N HCl (pH 1.0).

3) Blotted slides on Puffless filter paper.

4) Dehydrated quickly, cleared and mounted as usual.

Results :

Only sulfated mucosubstances stain intense blue.

iii) Colloidal Iron (C.I.) (Hale, 1946; Rinehart and Abul Haj, 1951; Mowry, 1961,1963) :

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

2) Rinsed in 12% acetic acid.

3) Treated with freshly prepared working colloidal

iron solution for 60 minutes at room temperature.

4) Rinsed in 12% acetic acid.

5) The sections were treated with freshly prepared mixture of equal volume of 2% HCl. and 2% potassium ferrocyanide for 20 min.

6) Washed with running water for 5 minutes.

7) Dehydrated, cleared and mounted as usual.

Results :

Sites of acidic mucosubstances are prussian blue. The results obtained with this method are very much identical to those obtained with AB pH 2.5 procedure.

C) Distinction between neutral and acidic mucosubstances :

1) AB pH. 2.5 - PAS (Mowry and Winkler, 1956; Mowry, 1963):

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

2) Rinsed briefly in 3% acetic acid.

3) Stained with 1% AB in 3% acetic acid (pH 2.5) for 30 minutes.

4) Rinsed in 3% acetic acid.

5) Washed in distilled water for 5 minutes.

6) Processed as A-1 for PAS.

Results :

Alcian blue reactive periodate unreactive acid mucosubstances stain blue, alcian blue and PAS

reactive mucosubstances stain purple-blue and PAS reactive but alcian blue unreactive mucosubstances colour magenta.

ii) AB pH 1.0 - PAS (Spicer, 1965; Spicer et al., 1967):

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

2) Stained with 1% AB in 0.1 N HCl (pH 1.0) for 30 minutes.

3) Sections were blotted on puffless filter paper.

4) Processed as in A-i for PAS.

Results :

Only sulfomucins are stained blue or blue-purple. Nonsulfated and only periodate reactive mucosubstances are stained pink-magenta.

iii) Colloidal Iron-PAS (Ritter and Oleson, 1950; Mowry, 1963) :

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

2) Rinsed briefly in 12% acetic acid.

3) Treated with freshly prepared colloidal iron working solution for 60 minutes at room temperature.

4) Rinsed with 12% acetic acid.

5) Treated with freshly prepared mixture of equal volumes of 2% HCl and 2% potassium ferrocyanide for 20 minutes.

6) Washed in running water for 5 minutes.

7) Processed as in A-i for PAS.

Results : Acidic mucosubstances colour blue or blue-purple and neutral mucosubstances colour pink-magenta. Results are mostly similar to those of AB pH 2.5-PAS.

D) Distinction between sulfomucins and carboxymucins:

i) Aldehyde fuschin (AF) (Gomori, 1950; Halmi and Davies, 1953):

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

2) Rinsed in 70% alcohol.

3) Stained with AF staining solution for 30 minutes.

4) Rinsed in 70% alcohol.

5) Dehydruted in 90% and absolute alcohol, cleared in xylene and mounted as usual.

Results:

Sulfated mucosubstances are stained dark-purple, sialomucins and hyaluronic acids stain light-purple, Some elastic fibres also stain intense purple.

ii) Aldehyde Fuschin-AB pH 2.5 (Spicer and Meyer, 1960):

1) After dewaxing and hydration, sections were

brought to distilled water.

2) Rinsed in 70% alcohol.

3) Stained in AF staining solution for 30 minutes.

4) Rinsed in 70% alcohol.

5) Washed in running water for 5 minutes.

6) Rinsed in 3% acetic acid.

7) Stained with AB (pH 2.5) for 30 minutes.

8) Rinsed in 3% acetic acid.

9) Washed in running water for 5 minutes

10) Dehydrated, cleared and mounted as usual.

Results :

Sulfated mucosubstances stain purple, nonsulfated mucosubstances like sialic acid and hyaluronic acid stain blue.

iii) Critical electrolyte concentration technique using AB at pH 5.6 with increased concentration of $MgCl_2$ (Scott et al., 1964; Scott and Dorling, 1965):

1) Eight slides after ~~dewaxing and~~ hydration were brought to distilled water.

2) Each slide was stained for 30 minutes in staining solutions: 00.0M, 0.1M, 0.2M, 0.4M, 5M, 0.6M, 0.8M and 1.0M.

3) Washed in running water for 5 minutes.

4) Dehydrated, cleared and mounted as usual.

Results :

Generally carboxymucins like sialic acid and hyalurinic acid are not stained at or above 0.1 M Mg^{++} concentrations. Sulfomucins are selectively stained at and above 0.2 M Mg concentnation. Vdrious sulfomucins loose their alcianophilia at different levels of Mg^{++} concentration.

iv) Azure A metachromatic staining technique at controlled pH levels (Wislocki et al., 1947; Spicer, 1960; Spicer et al., 1967 and Pearse, 1968):

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

2) Stained with azure A at desired pH for 30 minutes.

3) Quickly washed in distilled water.

4) Wet sections were observed under microscope.

5) Dehydrated in alcohol and observed under microscope.

6) Cleared in xylene and mounted as usual.

Results :

Strangly sulfated mucosubstances exhibited metachromatia below pH 1.5, Sialomucins generally stain metachromatically between pH 2.5 to 3.5. Some protein masked sulfomucins and hyaluronic acid exhibited metachromasia at and above pH 4.5. Generally the metachromasia of sulfomucins resist alcohol dehydration.

v) Mild methylation - AB pH 2.5

vi) Active methylation - AB pH 2.5 (Fisher and Lillie, 1954; Spicer, 1960) :

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

2) Rinsed in absolute methanol.

3) Sections were placed in coupling jars containg 0.1 N HCL in absolute methanol (preheated) for 4 hours at 37°C (mild methylation) and at 60°C (active methylation). Correspondingly the control sections were kept at 37°C and 60°C in methanol only (without HCl).

4) Rinsed in absolute methanol.

5) Followed by 5 minutes washing in running water.

6) Stained with AB pH 2.5 as in B-i.

7) After washing, dehydration and clearing sections were mounted as usual.

Results :

Generally mild methylation abolishes the basophilia of carboxymucins by esterification,while active methylation hydrolyses most of sulfate esters.

vii) Mild methylation - saponification-AB PH 2.5 :

viii) Active methylation-saponification-AB PH 2.5 :

(Spicer and Lillie, 1959; Spicer, 1960) :

Sections were methylated separately at 37°C and 60°C as above. After brief washing with distilled water, they were treated with 1% KOH in 70% alcohol for 20 minutes.

After washing briefly with distilled water, they were stained with AB pH 2.5 as in B-i after washing, dehydration and clearing, the sections were mounted as Usual.

Results :

Restoration of the basophilia after saponification indicates the presence of carboxymucins but failure of restoration of basophilia indicates the presence of the sulfate esters.

ix) Acid hydrolysis (Quintarelli et al., 1961) :

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

2) They were treated with 0.1 N HCl at 60°C for 4 hrs.

3) Washed in running water for 5 minutes.

4) Stained with AB pH 2.5 or azure A pH 3.0

5) Dehydrated, cleared and mounted as usual.

Results :

Complete or partial loss of alcinophilia or metachromasia indicates the probable presence of sialomucins.

E) Enzyme digestion tests :

i) Sialidase (Neuraminidase) digestion

(Spicer and Warren, 1960) :

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

2) The slides were placed on glass rods, close to surface of water in petridish kept at 37°C. Sections were covered with enough sialidase (Vibrio cholerae, type V, Sigma)

in 0.1 M sodium acetate at pH 5.3 containing 0.04 M $CaCl_2$. Control sections were covered with buffer only (0.1 M Sodium acetate at pH 5.3 cantaining 0.04 M $caCl_2$). Sections were incubated for 16 to 24 hours.

3) Rinsed with distilled water.

4) Stained with AB pH 2.5

5) Dehydrated, cleared and mounted as usual.

Results :

Complete or partial loss of alcinophilia indicated the presence of sialic acid.

ii) Hyaluronidase digestion (Barka and Anderson, 1965; Spicer et al., 1967) :

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

2) Sections were incubated at 37°C for 6 hours in 0.05% hyaluronidase (Testicular, Sigma) in freshly prepared buffer at pH 5.5 (94 ml. 0.1 M KH_2PO4+ 6 ml 0.1 M $Na2HPO4$). Control sections were incubated only in buffer.

3) Washed in running water for 5 minutes.

4) Stained with AB pH 2.5

5) Dehydrated, cleared and mounted as usual.

Results :

Complete or partial loss of alcinophilia indicates the presence of hyaluronic acid, chondrotin sulphate A and C.

iii) Pepsin digestion (Pearse, 1960; Spicer, 1960; Quintarelli, 1963; Thompson, 1966) :

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

2) Digested in 0.1% pepsin in 0.1 N HCl at 37°C for 4 hrs..

3) Washed thoroughly in running water.

4) Stained with AB pH 2.5.

5) Dehydrated, cleared and mounted as usual.

Results :

Protein masked mucosubstonces (PAS Positive but AB, CI and azure A negative) stained with basophilic dyes after removal of protein masking.