material and methods

CHAPTER TWO

MATERIAL AND METHODS

A) MATERIAL

B) METHODS

A) MATERIAL :

I) GENERAL FEATURES -

The bird selected for present histological and histochemical investigation is Amaurornis phoenicurus phoenicurus (Trinomial nomenclature), commonly called white breasted waterhen, also named pennant (meaning triangular flag). Amauros (Greek) means dark colour. All races of A. phoenicurus phoenicurus do not possess a white breast but do exhibit white throat. Raghuvira and Dave in their book 'Indian Scientific nomenclature of birds of India, Burma and Ceylon' have described nine species under the genus Amaurornis, distributed all over Asia.

As the common name suggests, the bird has forehead, sides of the face, lower parts from chin to breast white coloured, while upper plumage, sides of the body exhibit slaty black colouration. Wings are blackish brown, bill greenish, rounded at base, while legs are olive yellow. Iris is brown in young, crimson red in breeding males. Females are slightly smaller in size than males. Habits-A. phoenicurus phoenicurus is famous for its loud cry. It wanders often in search of food and feeds in the open away from water. But it is alert enough and makes a sharp run whenever disturbed. During breeding season, it is very noisy bird and its harsh roars are audible at a great distance. The characteristic roar 'sherming' ends as a squeal or scream and may be repeated many times especially at night. During courtship display, it

does chucklings, gep-gep-gep followed by a ringing krui-krui-krui and hik-hik-hik.

Food - It feeds on young rice, water plants, seeds, grains and also on insects, worms, molluscs, larvae, thus the bird is omnivorous.

II) SYSTEMATIC POSITION :

Class - Aves
Sub-Class - Neornithes
Super-order - Neognathae
Order - Gruiformes
Family - Rallidae
Genus - Amaurornis
Species - Phoenicurus
Sub-species - Phoenicurus (Trinomial nomenclature)

III) COLLECTION, FIXATION AND SECTIONING OF THE MATERIAL :

For the present study, adult waterhens (A.phoenicurus phoenicurus) of both sexes were collected at Gandhinagar, near Kolhapur (M.S.) in the month of September 1985. The birds were injured by shooting them with the help of air-gun (precausion was taken so as not to disturb the alimentary tract). On the spot, alimentary tract of each bird was dissected out and cut into various regions such as oesophagus, proventriculus, ventriculus, duodenum, small intestine and large intestine.

These organs were then cut into small suitable pieces.

The pieces were fixed in calcium acetate formal (CAF) fixative (Leppi 1968) in separate specimen tubes (2 % calcium acetate in 10 % neutral formalin) and kept in refrigerator at 4°C (pH was adjusted at 7.2 by adding a few grains of $CaCO_3$). After prolonged fixation (24 h - 36 h), the tissues were throughly washed in running tap water followed by routine dehydration in an ethanol series of ascending concentration, clearing in xylol and paraffin embedment. As usual, sections were cut at a thickness 5-6 µm, affixed to glass slides, rehydrated in an ethanol series of descending concentration and subjected to a series of staining procedures of histology and histochemistry.

B) METHODS :

For studying nature, distribution and localisation of mucosubstances, there are series of well tested histochemical techniques employed by different investigators in the field of histochemistry. By employing a battery of recently established histochemical techniques, ins and outs of mucosubstances have been brought well in light.

Biochemical techniques though give quantitative data of mucosubstances in mathematical terms, they fail to illustrate cellular site of mucosubstances, On the contrary, histochemical techniques illustrate the identification, localisation and distribution of mucosubstances at cellular level. The contribution of various investigators like Spicer (1963), Curran (1964), Spicer and Henson (1967), Leppi (1968), Nalavade and Varute (1971, 1972a,b,c; 1973a,b; 1976a,b; 1977) is noteworthy.

The specificity of different methods can be enhanced by the use of chemical reactions such as blocking of reactive groups, their restoration, controlling pH of the basic dyes, sequential staining procedures, critical electrolytic concentration, selective removal of the moieties by acid hydrolysis, enzyme digestion techniques, methylation, saponification etc. Thus non-specific histochemical methods can be supplemented with the modified and specific ones for better understanding of chemical composition of the cellular components like mucosubstances.

The terminology suggested by Spicer et al. (1965) for carbohydrate rich tissue components is followed in the present investigation. A series of recent and well established histochemical methods were employed for the characterisation of the mucosubstances in the alimentary tract of A.phoenicurus phoenicurus. The summary of the histochemical techniques employed in the present investigation, procedures, chemical reactions involved in the staining and their interpretation is presented in Table No. 1.

HISTOCHEMICAL TECHNIQUES -

1) NEUTRAL MUCOSUBSTANCES -

1-A) Periodic acid Schiff reaction (PAS).
[McManus, 1946; and Hotchkiss, 1948]

Procedure - 1) After dewaxing and hydration, the sections were brought to distilled water. 2) Oxidised 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 with 0.5 % Sodium-meta-bisulphite for 6 min. 6) Washed in distilled water, followed by ethanol dehydration, clearing and mounting in DPX.

Result - Periodate reactive, hexose containing mucosubstances stain pink-magenta.

1-B. Phenylhydrazine - PAS

[Spicer, 1965; Spicer et al., 1967]

Procedure - 1) After dewaxing and hydration, sections were brought to distilled water. 2) Oxidized with 0.5 % periodic acid for 10 min. 3) Followed by treatment with 5 % phenylhydrazine for 30 min. 4) Washed with distilled water. 5) Immersed in Schiff's reagent for 10 min. 6) Rinsed three times (total 6 min.) with 0.5 % sodium meta-bisulphite. 7) Washed, dehydrated, cleared routinely and mounted in Canada balsam.

Result - Periodate reactive acid mucosubstances are selectively stained periodate engendered dialdehydes are blocked.

1-C. Diastase digestion - PAS technique for identification of glycogen

[Lillie, 1954; Lison, 1960]

Procedure - 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 1-A for PAS.

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

2. Acid Mucosubstances

2-A. Alcian Blue (AB) at pH 1.0

[Lev and Spicer, 1964]

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

2) Stained for 30 min. in 1 % AB in 0.1 N HCl (pH 1.0). 3) Blotted on puffless filter paper. 4) Dehydrated quickly, cleared and mounted as usual.

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

3. Distinction Between Neutral and Acidic Mucosubstances

3-A. AB pH 1.0 - PAS Sequential Staining Technque

[Spicer, 1965; Spicer et al., 1967]

Procedure - 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 min. 3) Sections were blotted on puffless filter paper.
4) Processed as in 1-A for PAS.

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

3-B. AB pH 2.5 -PAS Sequential Staining Technique

[Mowry and Winkler, 1956; Mowry, 1963]

Procedure - 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 min. 4) Rinsed in 3 % acetic acid. 5) Washed in distilled water for 5 min.
6) Processed as 1-A for PAS.

Result - 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.

4. Distinction between Sulfomucins and Carboxymucins

4-A. Aldehyde Fuchsin (AF)

[Gomori, 1950; Halmi and Davies, 1953]

Preparation of AF Crystals -

The crystals of AF were prepared according to the method suggested by Cameron and Steal (1959). : To 200 ml boiling distilled water, 1 gm of basic fuchsin was added and the solution was let to boil for one min. then cooled and filtered. To the filtrate, 2 ml of conc. HCl and 2 ml of paraldehyde were added. The solution was left stoppered at room temperature. When the solution had lost its reddish colour, usually after 3-4 days, it was filtered and the filtrate was discarded. The precipitate was dried on the filter paper at 60°C.

Staining Solution - The staining solution was prepared by dissolving 0.5 gm of dry crystals in 70 % alcohol.

Procedure - 1) After dewaxing and hydration, sections were brought to distilled water. 2) Rinsed in 70 % alcohol. 3) Stain with AF staining solution for 30 min. 4) Rinsed in 70 % alcohol. 5) Dehydrated in 90 % and absolute alcohol, cleared in xylene and mounted as usual.

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

4-B. Aldehyde Fuchsin - AB (AF - AB pH 2.5) Sequential Staining Technique

[Spicer and Meyer, 1960]

Procedure - 1) After dewaxing and hydration, sections were brought to distilled water. 2) Rinsed in 70 % alcohol. 3) Stained in Af staining solution for 30 min. 4) Rinsed in 70 % alcohol. 5) Washed in running water for 5 min. 6) Rinsed in 3 % acetic acid. 7) Stained with AB (pH 2.5) for 30 min. 8) Rinsed in 3 % acetic acid. 9) Washed in running water for 5 min. 10) Dehydrated, cleared and mounted as usual.

Result - Sulfated mucosubstances stain purple, non-sulfated mucosubstances like sialic acid and hyaluronic acid stain blue.

4-C. Critical Electrolyte Concentration Technque Using AB at pH 5.6 with Increased Concentration of $MgCl_2$

[Scott et al., 1964; Scott and Dorling, 1965]

Staining Solution - 0.1 % AB was added in 0.05 M sodium acetate/acetic acid buffer at pH 5.6. Then $MgCl_2$ was added and a series of increasing concentration of Mg^{++} were prepared such as 0.0 M, 0.1 M, 0.2 M, 0.4 M, 0.5 M, 0.6 M, 0.8 M and 1.0 M.

Procedure - 1) Eight dewaxed slides after hydration were brought to distilled water. 2) Each slide

stained for 30 min. in staining solutions 0.0 M, 0.1 M, 0.2 M etc. respectively.

3) Washed in running water for 5 mins.

4) Dehydrated, cleared and mounted as usual.

Result - Generally carboxymucins - like sialic acid and hyaluronic acid are not stained at or above 0.1 M Mg^{++} concentration. Sulfomucins are selectively stained at and above 0.2 M Mg^{++} concentration. Various sulfomucins lose their alcianophilia at different levels of Mg^{++} concentration.

4-D. Azure A Metachromatic Staining Technique at Controlled pH levels

[Wislocki et al., 1967; Spicer, 1960; Spicer et al., 1967; Pearse, 1968]

Staining Solutions :

pH 0.5 - 0.02 % azure A in 0.5 N HCl.

pH 1.0 - 0.02 % azure A in 0.1 N HCl.

pH 1.5 - 0.02 % azure A in 50 ml buffer (30 ml 0.1 N HCl + 20 ml 0.1 M KH_2PO_4)

pH 2.0 - 0.02 % azure A in 50 ml of buffer (20 ml 0.1 N HCl + 30 ml 0.1 M KH_2PO_4)

pH 2.5 - 0.02 % azure A in 48 ml distilled water + 2 ml 0.1 M citric acid.

pH 3.0 - 0.02 % azure A in 48 ml distilled water + 1.65 ml 0.1 M citric acid + 0.35 ml 0.2 M $Na_2H\ PO_4$.

pH 3.5 - 0.02 % azure A in 48 ml distilled water + 1.4 ml 0.1 M citric acid + 0.6 ml 0.2 M $Na_2H\ PO_4$.

pH 4.0 - 0.02 % azure A in 48 ml distilled water + 1.25 ml 0.1 M citric acid +0.75 ml 0.2 M $Na_2H\ PO_4$.

pH 4.5 - 0.02 % azure A in 48 ml distilled water + 1.1 ml 0.1 M citric acid + 0.9 ml 0.2 M $Na_2H\ PO_4$.

pH 5.0 - 0.02 % azure A in 48 ml distilled water + 1.0 ml 0.1 M citric acid + 1.0 ml 0.2 M $Na_2H\ PO_4$.

Procedure - 1) After dewaxing and hydration, sections were brought to distilled water. 2) Stained with azure A at desired pH for 30 min. 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.

Result - Strongly sulfated mucosubstances exhibited metachromasia below pH 1.5, sialomucins generally stain metachromatically between pH 2.5 and 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.

4-E. Mild Methylation - AB pH 2.5

4-F. Active Methylation - AB pH 2.5

(Fisher and Lillie, 1954; Spicer, 1960]

Procedure - 1) After dewaxing and hydration, sections were brought to distilled water. 2) Rinsed in absolute methanol. 3) Sections were placed in coplin jars containing 0.1 N HCl in absolute methanol (pre-heated) for 4 hrs. 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 min. washing in running water. 6) Stain with AB pH 2.5 as 2-A. 7) After washing, dehydration and clearing, sections were mounted in Canada balsam.

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

4-G. Mild methylation - Saponification - AB pH 2.5

4-H. Active methylation - saponification - AB pH 2.5

[Spicer and Lillie, 1959; Spicer, 1960]

Procedure - 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 min. After washing briefly with distilled water, they were stained with AB pH 2.5 as in 2-A. After washing dehydration and clearing, the sections were mounted in Canada balsam.

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

4-I. Acid Hydrolysis

[Quinterelli et al., 1961]

Procedure - 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 min. 4) Stained with AB pH 2.5 or azure A pH 3.0. 5) Dehydrated, cleared and mounted as usual.

Result - Complete or partial loss of alcianophilia or metachromasia indicates the probable presence of sialomucins.

5. Enzyme Digestion Tests

5-A. Sialidase (Neuraminidase) Digestion

[Spicer and Warren, 1960]

Procedure - 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 containing 0.04 M $CaCl_2$). Sections were incubated for 16 to 24 hrs. 3) Rinsed with distilled water. 4) Stained with AB pH 2.5 or azure A pH 3.0. 5) Dehydrated, cleared and mounted as usual.

Result - Complete or partial loss of alcianophilia or metachromasia indicated the presence of sialic acid.

5-B. Hyaluronidase Digestion

[Berke and Anderson, 1965; Spicer et al., 1967]

Procedure - 1) After dewaxing and hydration, sections were brought to distilled water. 2) Sections were incubated at 37°C for 6 hrs. in 0.05 % hyaluronidase (Testicular, Sigma) in freshly prepared buffer at pH 5.5 (94 ml 0.1 M $KH_2 PO_4$ + 6 ml 0.1 M $Na_2H PO_4$). Control sections were incubated only in buffer. 3) Washed in running water for 5 min. 4) Stained with AB pH 2.5 or azure A pH 4.5. 5) Dehydrated, cleared and mounted as usual.

Result - Complete or partial loss of alcianophilia or

metachromasia indicates the presence of hyaluronic acid, chondroitin sulfate A and C.

3-C. Pepsin Digestion

[Pearse, 1960; Spicer, 1960; Quintarelli, 1963; Thompson, 1966].

Procedure - 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 throughly in running water. 4) Stained with AB (pH 2.5) or Azure A (pH 1.5, 3.0 and 4.5). 5) Dehydrated, cleared and mounted as usual.

Result - Protein masked mucosubstances (PAS-positive but AB and Azure A negative) stain with basophilic dyes after removal of protein masking.

Table No. 1

Histochemical methods employed for visualizing mucosubstances

No.	Histochemical Method	Chemical reactions involved	Histochemical result	References
1	2	3	4	5
1	Periodic acid–Schiff's reaction (PAS)	Oxidation of vicinal hydroxyls to dialdehydes by periodate and formation of coloured complexes with Schiff's reagent.	All polysaccharides and mucosubstances colour pink to magenta.	McManus (1946); Hotchkiss (1948).
2	Periodic acid phenylhydrazine Schiff	Phenylhydrazine selectively blocks periodate engendered dialdehydes in mucosubstances, leaving unblocked dialdehydes in periodates reactive mucosubstances available to subsequent Schiff staining.	Periodate reactive acidic mucosubstances stained red presumably are proximal to vicinal glycols.	Spicer (1965); Spicer et al., (1967).
3	Diastase digestion–PAS	Hydrolyses and removes glycogen.	Loss of PAS reactivity in sites containing glycogen.	Lillie (1954), Lison (1960).
4	Alcian blue pH 1.0	Probably formation of alcian blue complexes with sulfate groups.	Weakly and strongly acidic sulfomucins are selectively stained.	Lev and Spicer (1964).
5	Alcian blue pH 2.5	Probably formation of alcian blue complexes with carboxyls and sulfate groups.	Sialomucins and weakly acidic sulfomucins stain blue, the most strongly acidic sulfomucin stains weakly or not at all.	Mowry (1956).

Table No. 1 (Contd...)

1	2	3	4	5
6	AB pH 1.0-PAS	Addition of results by single method.	Sulfomucins stain blue or blue-purple. Neutral and nonsulfated periodate reactive mucosubstances stain pink-magenta.	Spicer (1965); Spicer *et al.* (1967).
7	AB pH 2.5 - PAS	Addition of results by single method.	Alcian blue reactive periodate unreactive acid mucosubstances stain blue. Alcian blue and PAS-reactive mucosubstances colour purple-blue. Neutral mucosubstances colour pink-magenta.	Mowry and Winkler (1956); Mowry, 1963.
8	Aldehyde Fuchsin (AF)	Formation of salt complexes between cationic staining entity and sulfated and carboxyl groups.	Sulfated mucosubstances stain dark purple. Sialomucins and hyaluronic acid colour light purple.	Gomori (1950); Halmi and Davies (1953).
9	AF-AB pH 2.5	Formation of salt complexes between cationic staining entity and sulfate and carboxyl groups.	Sulfomucins stain purple or blue-purple. Sialomucins and other non-sulfated acidic mucosubstances stain blue.	Spicer and Meyer (1960).
10	Alcian blue at pH 5.6 with	Alcian blue forms complexes with sulfate groups. Different sulfomucins vary in the critical electrolyte concentration at which alcianophilia is lost.	Non-sulfated acidic mucosubstances are not stained at and above 0.1 M Mg^{++} concentration. Sulfomucins stain selectively at and above 0.2 M Mg^{++} concentration.	Scott *et al.* (1964); Scott and Dorling (1965).

Table No. 1 (Contd....)

1	2	3	4	5
11	Azure A or toludine blue at controlled pH levels.	Formation of blue orthochromatic or purple to red metachromatic salt complexes with the extinction values indicating degree of acidity of the polymer.	Strongly sulfated mucosubstances stain purple-red at pH 0.5 to 1.5. Sialomucins stain purple-red at pH 2.5 to 3.5. Hyaluronic acid and weakly acidic mucosubstances stain purple at pH 4.5 to 5.0.	Wislocki et al. (1947); Spicer (1960); Spicer et al. (1967) Pearse (1968).
12	Mild-methylation AB pH 2.5.	Esterification of carboxyl groups.	Generally mild methylation abolishes the alcianophilia of carboxymucins.	Fisher and Lillie (1954); Spicer (1960).
13	Mild-methylation-saponification AB pH 2.5.	Restoration of carboxyl groups.	Restoration of the alcianophilia after saponification of methylated sections indicates the presence of carboxyl groups.	Spicer and Lillie (1959); Spicer (1960).
14	Active methylation-AB pH 2.5.	Carboxyl groups are esterified. Sulfomucins are desulfated.	Active methylation abolishes alcianophilia of carboxymucins through esterification and of sulfomucins through hydrolytic removal of the sulfate groups.	Fisher and Lillie (1954); Spicer (1960).

Table No.1 (Cont.....)

1	2	3	4	5
15	Active methylation-Saponification-AB pH 2.5.	Restoration of carboxyl groups. Sulfomucins are hydrolytically removed during active methylation are not restored following subsequent saponification.	Restoration of the alcianophilia after subsequent saponification, indicates the presence of carboxyl groups and loss of alcianophilia indicates the presence of sulfate groups.	Spicer and Lillie (1969); Spicer (1960).
16	Acid hydrolysis AB pH 2.5 or Azure A.	Removes sialic acids from mucosubstances.	Complete or partial loss of alcianophilia or metachromasia indicates the probable presence of sialomucins.	Quintarelli et al. (1961).
17	Sialidase (Neuraminidase)-AB pH 2.5 or Azure A pH 3.0.	Removes sialic acid from mucosubstances.	Complete or partial loss of alcianophilia or metachromasia confirms the presence of sialomucins.	Spicer and Warren (1960).
18	Hyaluronidase AB pH 2.5 or Azure A pH 4.5.	Depolymerization of hyaluronic acid chondroitin sulfate A and C.	Complete or partial loss of alcianophilia or metachromasia indicates the probable presence of hyaluronic acid and chondroitin sulfate A and C.	Barka and Anderson (1965); Spicer et al. (1967).
19	Pepsin digestion AB pH 1.0, 2.5 or	Hydrolysis of internal peptide bonds as well as those of the terminal aminoacids of proteins.	Protein masked mucosubstances stain with basophilic dyes after removal of protein masking.	Pearse (1960); Spicer (1960); Quintarelli (1963); Thompson (1966).