CHAPTER TWO

MATERIAL AND METHODS

A) MATERIAL

For the present investigation adult frogs, Euperodon systoma were used. Both, male and female (ten each) frogs were collected during their active breeding period (July to September). The frogs were anesthetized by ether and esophagus, stomach (cardiac and pyloric), duodenum, small intestine (ileum), and large intestine were dissected out. These organs were cut into small pieces, not measuring more than 5 mm^3 and were immediately fixed in 2% calcium acetate in 10% formalin (CAF) at $4^{o}C$. After prolonged fixation (24 hrs.) the tissues were well washed in chilled distilled water, followed by prolonged washing in running tap water. After dehydration in ethanol grades, clearing in xylene and paraffin embedment, the sections were cut at 6 μm. some of the sections of each tissue were routinely stained by Haematoxylin - Eosin (H-E) for histological observations, while the adjacent sections were subjected for various histochemical techniques described hereafter for the identification and characterization of mucosubstances.

B) METHODS

For the visualization of mucosubstances there are series of histochemical techniques evolved by different investigators in this field. Such histochemical techniques have an advantage over biochemical techniques in the fact that, though the latter techniques give reliable data on quantities of mucosubstances in exact mathematical terms, they are not of much use in illustrating the cellular site in the given organ or tissue where they are elaborated and occur. The specificity of different methods can be enhanced by restoring the use of chemical reactions such as control of pH of basic dyes, sequential staining techniques, methylation, saponification, critical electrolyte concentration, acid hydrolysis, and enzyme

digestion tests. Thus the non-specific histological methods can be supplemented with the histochemical and ancillary ones for the better understanding of the chemical composition of the cellular components. The various histochemical techniques with their merits and demerits for mucosubstances localization have been critically analyzed and reviewed by Spicer(1963),Spicer and Henson(1967), Spicer et al.(1967 b), Curran(1964), Barka and Anderson(1965),Lillie (1965),Thompson(1966),Leppi(1968),Nalavade(1975),and Nalavade and Varute(1971,1972 a,b,c,1973 a,b,1976 a,b,1977). Nomenclature applied to the mucosubstances is taken from the discussion of a proposed general terminology of histochemically recognizable materials(Spicer et al.,1965).

In the present investigation the following series of histochemical techniques for the visualization of mucosubstances in Amphibian alimentary tract were employed.

I - Neutral Mucosubstances

A. 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 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,hexose containing mucosubstances stain pink-magenta.

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

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

2) Oxidised 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-bi-sulphite.

7) Washed,dehydrated, cleared routinely and mounted in Canada balsam.

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

C. 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 I-A for PAS.

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

II - Acid Mucosubstances

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

B. 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 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: Only sulfated mucosubstances stain intense blue.

C. Colloidal Iron (CI) (Hale, 1946; Rinehart and Abul-Haj, 1951; Mowry, 1961, 1963)

Stock Solution: To the boiling 250 ml. of distilled water, 4.4 ml. 29 % ferric chloride solution was added with constant stirring. When the solution turned dark red it was allowed to cool and then dialysed against distilled water,using dialysing membrane.

Working Solution

Glacial acetic acid	-	05 ml.
Distilled water	-	15 ml.
Stock colloidal iron solution	-	20 ml.

Procedure

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 volumes of 2 % HCl and 2 % potassium ferrocyanide for 20 min.
6) Washed with running water for 5 min.
7) Dehydrated, cleared and mounted as usual.

Result: 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.

III - Distinction Between Neutral and Acidic Mucosubstances

A. AB pH 2.5 -PAS Sequential Staining Technique (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 min.
4) Rinsed in 3 % acetic acid.
5) Washed in distilled water for 5 min.
6) Processed as I-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.

B. AB pH 1.0 - PAS Sequential Staining Technique

(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 min.

3) Sections were blotted on puffless filter paper.

4) Processed as in I-A for PAS.

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

C. Colloidal Iron - PAS Sequential Staining Technique

(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 min.at room temperature.

4) Rinsed with 12 % acetic acid.

5) 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 min.

7) Processed as in I-A for PAS.

Result: 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.

IV - Distinction Between Sulfomucin and Carboxymucins

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.

B. Aldehyde Fuchsin - AB (AF - AB pH 2,5) Sequential Staining Technique.

(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 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.

C. Critical Electrolyte Concentration Technique 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.

D. Azure A Metachromatic Staining Technique at Controlled pH Levels (Wislocki et al.,1947; 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.

E - Mild Methylation - AB pH 2.5

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 II-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.

G - Mild methylation - saponification - AB pH 2.5

H - 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 min. After washing briefly with distilled water, they were stained with AB pH 2.5 as in II-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.

I. 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 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.

V. Enzyme Digestion Tests

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.

B. Hyaluronidase Digestion (Barka 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_2PO_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.

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 thoroughly in running water.

4) Stained with AB pH 2.5, C.I.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, CI and azure A negative)stained with basophilic dyes after removal of protein masking.

A bird's eye view of the various histochemical techniques employed in the present investigation along with the chemical reactions involved in the staining and the histochemical interpretation of staining reactions is given in Table No.1.

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 phenyl-hydrazine 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 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).
5	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).

Table No.1 contd.

1	2	3	4	5
6	Colloidal Iron (CI)	Probably formation of complexes between cationic colloidal ferric aggregates and carboxyls.Sulfate and phosphate esters.	Non-sulfated acidic muco substances and some sulfated mucosubstances colour blue.	Hale (1946), Rinehart and Abdul-Haj (1951),Mowry (1961,1963).
7	AB pH 2.5 - PAS	Addition of results by single methods.	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.	AB pH 1.0- PAS	-do-	Sulfomucins stain blue or blue-purple.Neutral and nonsulfated periodate reactive mucosubstances stain pink-magenta.	Spicer (1965), Spicer et al. (1967).
9	Colloidal Iron -PAS	-do-	Colloidal iron-reactive, periodate unreactive acid mucosubstances stain blue.Colloidal Iron and PAS reactive mucosubstances colour purple-blue.Neutral mucosubstances colour pink-magenta.	Ritter and Oleson (1950);Mowry (1963).

Table No.1 contd.

1	2	3	4	5
10	Aldehyde Fuchsin (AF)	Formation of salt compexes 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).
11	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 Mayer (1960).
12	Alcian blue at pH 5.6 with graded concentration of $MgCl_2$	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).
13	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).
14	Mild-methylation AB pH 2.5.	Esterification of carboxyl groups.	Generally mild methylation abolishes the alcianophilia of carboxymucins.	Fisher and Lillie (1954); Spicer (1960).

Table No.1 contd.

1	2	3	4	5
15	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).
16	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).
17	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 (1959); Spicer (1960).
18	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).
19	Salidase (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).

Table No.1 contd.

1	2	3	4	5
20	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).
21	Pepsin digestion-AB pH 1.0, 2.5 or Azure A pH 1.5, 3.0 and 4.5.	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).