CHAPTER TWO

MATERIAL AND METHODS

1. Material

For the present investigation adult Oncidium verraculatum were used. The animals were collected from their natural habitate, from the rocky shore near Ratnagiri, the Western coast of Maharashtra, India, in the month of May, 1981. Some Oncidium were also maintained in glass vessels in the laboratory for observation and histological work. The freshly collected animals were used for histochemical study. The animals were dissected and different organs of the alimentary tract were removed separately. 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 tissue were well washed in chilled distilled water, followed by prolonged washing in running tap water. After dehydration in alcohol 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.

2. 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 occured. The specificity of different methods can be enhanced by resorting 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 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 various workers.

In the present investigation the following series of histochemical techniques for the visualization of muco-substances in O. verraculatum alimentary tract were employed.

I) Neutral mucosubstances :

A) 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 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 metabisulphite.
6) Washed in distilled water, followed by alcoholic dehydration, cleared in xylene and mounted in canadabalsm.

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

B) Phenylhydrazin - 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 % phenyl hydrazine for 30 minutes.

4) Washed with distilled water.

5) Immersed in Schiffs reagent for 10 minutes.

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

7) Washed, dehydrated, cleared routinely and mounted in canadabalsm.

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, staining procedure.

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

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

B) Alcian blue (AB)

1) After dewaxing and hydration sections were brought to distilled water.
2) Stained for 30 minutes 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 sulfomucins stain intense blue.

III) Distinction between neutral and acidic mucosubstances :

A) AB pH 2.5 - PAS sequential staining technique

(Mowry and Winkler, 1966, 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 in I - A for PAS staining technique.

Result : Alcian blue reactive periodate unreactive acid mucosubstances stain blue, alcian blue and PAS reactive mucosubstances stain blue-purple 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.) for 30 minutes.
3) Sections were blotted on puffless filter paper.
4) Processed as in I - A for PAS staining technique.

Result : Only sulfomucins are stained blue or blue-purple Nonsulfated and only periodate reactive muco-substances are stained pink magenta.

IV) Distinction between sulfomucins and carboxymucins

A) Aldehyde fuchian (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 fuschin was added and the solution was allowed to boil for 1 minute, 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^{o}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) Stained with AF staining solution for 30 minutes.

4) Rinsed with 70% alcohol.

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

Result : Sulfated mucosubstances are stained dark-purple, sialomucin and hyaluronic acids stain light purple, some elastic fibers also stain intense purple.

B) Aldehyde fuchsin - AB (AF - AB 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 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.

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

C) Azure A metachromatic staining technique at controlled pH levels (Wislocki et al. 1947; Spicer, 1960; Spicer et al. 1967 and 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 NHCl + 20 ml. 0.1 M KH_2PO_4)

PH 2.0 - 0.02% azure A in 50 ml. 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_2HPO_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_2HPO_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_2HPO_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_2HPO_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_2HPO_4.

Procedure :

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 and the the observations were recorded.
5) Dehydrated in alcohol and observed under microscope.
6) Cleared and mounted as usual.

Result : Strongly sulfated mucosubstances exhibit metachromasia below pH 1.5, sialomucins generally stain metachromatically between pH 2.5 and 3.5 ; some protein masked sulfomucins and hyaluronic acid exhibit metochromasia at and above pH 4.5 . Generally the metachromasia of sulfomucins resists alcoholic dehydration.

D) 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 to 0.05 M sodium acetate/acetic acid buffer at pH 5.6 . Then more $MgCl_2$ was added and a series of increasing concentration of Mg^{++} was 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) 8 dewaxed slides after hydration were brought to distilled water.
2) Each slide was stained for 30 minutes in staining solutions 0.0, 0.1, 0.2 etc. respectively.
3) Washed in running water for 5 minutes.
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^{++} concentrations. Sulfomucins are selectively stained at and above 0.2 M Mg^{++} concentrations. Various sulfomucin lose their alcianophilia at different levels of Mg^{++} concentration.

E) Mild methylation - AB pH 2.5

F) Active methylation - AB pH 2.5 :

Procedure :

1) After dewaxing and hydration, sections were brought to distilled water.
2) Rinsed in absolute methanol.
3) Sections were placed in couplin jars containing 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 II A.
7) After washing, dehydration and clearing, sections were mounted in candabalsam.

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 minutes. 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 canadabalsam.

Result : Restoration of the basophilia after saponification indicates the presence of carboxyl groups but failure of restoration of the basophilia indicates the presence of the sulfate esters.

I) Acid hydrolysis (Quintarelli et al., 1961)

1) After dewaxing and hysration, sections were brought to distilled water.
2) They were treated with 0.1 N HCl at 60°C for 4 hours.
3) Washed in running water for 5 minutes.
4) Stained either 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 probable of sialomucins.

A birds eye view of various histochemical technique employed in the present investigation along with the chemical reaction involved in the staining and histochemical interpretations of the staining reaction with the literature is given in Table No. 1.

Table - No.1

Histochemical methods employed for visualizing mucosubstances

Sr. No.	Histochemical method	Chemical reaction involved	Histochemical results	References
1	2	3	4	5
1	Periodic acid - Schiffs reaction (PAS)	Oxidation of vicinal hydroxyls to dialdehydes by periodate and formation of coloured complexes with Schiff's reagent.	All polysaccharides and mucosubstances colour magnenta to pink.	McManus (1946)
2	Periodic acid Phenylhydrazine-Schiff.	Phenylhydrazin selectively blocks periodates engendered dialdehydes in mucosubstances, leaving unblocked dialdehydes in periodate reactive muco-substances available to subsequent Schiff staining.	Periodate reactive acid mucosubstances stained red presumably are those in which acid groups are proximal to vicinal glycols.	Spicer(1965), Spicer et al. (1967)
3	Diastase digestion-PAS	Hydrolyzes 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 some sulfate groups.	Sialomucins and weakly acidic sulfomucins stain blue; the most strongly acidic sulfomucins stain weakly or not at all.	Lev and Spicer(1964)
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)

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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 non-sulfated periodate reactive mucosubstances stain pink magenta.	Spicer(1965) Spicer et al. (1967)
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)
8	Aldehyde-Fuschin(AF)	Formation of salt complexes between cationic staining entity and sulfated and carboxyl group.	Sulfated mucosubstances stain dark purple, sialomucins and hyaluronic acid stain 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 group.	Sulfomucins stain purple or blue purple, sialomucins and other nonsulfated acidic mucosubstances stain blue.	Spicer and Meyer(1960)
10	Azure A or Toluidine blue at controlled pH level.	Formation of blue arthochromatic or purple to red metachromatic salt complexes with extinction values indicating degree of acidity of polymer.	Strongly sulfated mucosubstances, stain purple to red at pH 0.5 to 1.5, sialomucins stain purple red at pH 2.5-3.5, hyaluronic acid and weakly acidic mucosubstances stain purple at pH 4.5-5.0	Spicer(1960) Denopsey et al.(1947) Wislocki et al. (1947) Pearse(1968)

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1	2	3	4	5
11	Alcian blue at pH 5.6 with increasing concentration of $MgCl_2$ (CEC method)	Alcian blue 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^{++} conc.	Scott and Dorling(1965) Scott et al. (1964)
12	Mild methylation AB pH 2.5	Esterification of carboxyl groups.	Generally mild methylation abolishes the alcianophilia of carboxyl mucins.	Fischer and Lillie(1954) Spicer(1960)
13	Mild methylation - saponification- AB pH 2.5	Restoration of carboxyl group.	Restoration of the alcianophilia after saponification of methylated sections, indicates the presence of carbosyl groups.	Fischer 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 sulfate groups.	Fischer and Lillie(1954) Spicer(1960)
15	Active methylation-saponification AB pH 2.5	Restoration of carboxyl groups. Sulfomucins are hydrolytically removed during active methylation are not stored following subsequent saponification.	Restoration of the alcianophilia after subsequent saponification indicates the presence of carboxyl groups and loss of alcianophilia indicates the presence of sulfated groups.	Spicer and Lillie(1959) Spicer(1960)

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1	2	3	4	5
16	Acid hydrolysis-AB pH 2.5 or azure A pH 3.0	Removes sialic acids from mucosubstance.	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 acids from mucosubstances.	Complete or partial loss of alcianophilia or metachromasia confirms the presence of sialomucin.	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, chondroitin sulfate A and C.	Barka and Anderson (1965) Spicer et al. (1967)
19	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 amino acids of proteins.	Protein masked mucosubstances stain with basophilic dyes after removal of protein masking.	Pearse(1960) Spincer(1960) Quintarelli (1963) Thompson(1966)