CHAPTER TWO

MATERIAL AND METHODS

MATERIAL

The gastropod selected for the present studies, *Oncidium verraculatum*, is available on the rocky shore at Ratnagiri. It was previously classified as stylommatophoran pulmonate. But according to the present classification it is included under opisthobranchs (Hyman 1967). Its reproductive system is quite complex and described in detail in a later part of the present chapter.

Site of the Collection of the Animals

Animals were collected from the rocky shore at Ratnagiri, Maharashtra, India, near the fort located on seashore from their natural habitat in the month of May. While collecting animals at every time, the care was taken to obtain only the mature *Oncidium*. They were externally recognised by the large size.

Some animals were also maintained in glass vessels in the laboratory for observations on their behaviour during copulation. But for the histochemical work, freshly collected *Oncidium* only were employed. The animals were sacrificed, dissected and different organs in the reproductive tract were removed separately and treated for the histochemical studies described in the later part of this chapter.

General Plan of the Reproductive System of *Oncidium verraculatum* (After Awati and Karandikar, 1948)

The reproductive system of *Oncidium verraculatum* is complicated with a number of accessory glands. Though the animal is hermaphrodite, the male and female genital apertures are quite distinct and separately located. A brief morphological description will point out the structural complexity of the system and interesting positions of the glands at its various regions.

The reproductive system of *Oncidium verraculatum* consists of an unpaired ovotestis or hermaphrodite gland, a common or hermaphrodite duct, male and female reproductive tracts, accessory glands and a well developed male copulatory apparatus. The ovotestis lies in the posterior region of the body cavity just in front of the diaphragm, slightly on the left side. The hermaphrodite duct starts from the ovotestis as a narrow and greatly convoluted tube. On its way it gives off a finger-like whitish vesicle called the vesicula seminalis, then the common duct becomes somewhat thicker in form and continues into a greatly coiled glandular tube, the sperm-oviduct. Associated with the sperm-oviduct there are two glandular masses which constitute the albumen gland. The secretion of this gland is poured into the proximal portion of the sperm-oviduct through

a small common duct. There is another gland, the prostate gland, which is associated with the sperm-oviduct. It opens into the sperm-oviduct at its distal end by a somewhat longitudinal slit without the interposition of a regular duct.

The sperm-oviduct divides distally into two unequal tubes of which the thinner one is the vas deferens and the thicker one, the oviduct. The oviduct runs posteriorly below the pulmonary chamber, and is met on the way by a small duct from a round vesicle the receptaculum seminis or spermatheca. Posteriorly the oviduct enters the floor of the body cavity and opens to the exterior on the ventral side, just near the anus.

The vas deferens runs to a short distance posteriorly in company with the oviduct, it penetrates the floor of the body cavity turn cephalad, proceeds anteriorly through the floor tissue, and emerges again into the body cavity near the oral end on the right side. It then forms a long coil in the body cavity and finally enters a strongly developed elongated muscular tube called the penis. The penis leads anteriorly in a swollen muscular sac called the penis end sac which opens on the ventral side at the base of the right tentacle. There is one more accessory structure associated with the male genital duct known as the penial gland. It

consists of a very long greatly coiled glandular tube forming an intricate network in the right anterior region of the body cavity. Posteriorly the gland ends blindly in the body cavity. Anteriorly it becomes continuous with a strongly developed muscular tube which runs cephalad and enters a swollen sac called as end sac of penial gland.

The details of each organ regarding its histological structure, function will be dealt with separately in each chapter of the respective organ.

METHODS

I. Histological Techniques

The small pieces of the reproductive organs were fixed in the ice cold calcium acetate formalin (CAF - 2% in 10% formalin) fixative for 24 hrs. The fixation of the tissues was followed by washing in chilled distilled water, in running tap water, dehydration in alchohol, clearing in xylene and paraffin embedment. The sections were cut at 5 to 6 μ. Some sections were routinely stained by haematoxylene-eosin technique for histological observations.

II. Histochemical Techniques

For visualization of mucosubstances there are series of histochemical methods evolved by different workers in this

field. The specificity of various methods can be enhanced by restoring the use of chemical reactions such as control of pH of basic dye, sequential staining techniques, methylation, saponification, acid hydrolysis and enzyme digestion tests. Thus, the non-specific histochemical methods can be supplemented with the histological 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 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 different reproductive organs of O.verraculatum were employed.

Fixation and Post-fixation Procedures

The different tissues of the reproductive organs were quickly cut into smaller pieces and immediately immersed in ice-cold solution ($4^{o}C$) of 2% of calcium acetate in 10% formalin (CAF). After prolonged fixation (24 hrs.), the tissues were well-washed in chilled distilled water, followed by washing in running tap water. After dehydration in alcohol, clearing in xylene and paraffin embedment, the sections were

cut at 5 to 6 μ. The sections were subjected to various histochemical techniques hereafter described for the detection of mucosubstances.

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 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) 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 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, staining procedure.

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

D. - Metachromasia Technique for Galactogen

(Varute and Nanaware, 1972a-d, 1973)

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

2) Azure A at pH 0.5 to 2.5 - no metachromasia.

3) Azure A at pH 3.5 to 4.5 - intense alcohol-labile metachromasia.

4) Pepsin or papain digestion - Azure A pH 0.5 to 2.5 - no metachromasia.

5) Neuraminidase - Azure A pH 3.5 to 5.5 - metachromasia unaffected.

6) Hyaluronidase - Azure A pH 4 to 5.5 - metachromasia unaffected.

7) Azure A pH 3.5 to 5.5 + 1% NaCl - intense metachromasia.

8) Azure A pH 3.5 to 5.5 + 1% albumen - intense metachromasia.

9) Acid hydrolysis Azure A pH 3.5 to 5.5 - metachromasia unaffected.

10) Pectinase digestion - Azure A pH 3.5 to 5.5 - no metachromasia.

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 sialomucins stain dark blue. Strongly acidic sulfated mucins 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 minutes in 1% AB in 0.1 N HCl (pH 1.0).

3) Blotted on a 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

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 1.0) for 30 minutes.

3) Sections were blotted on a 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 mucosubstances are stained pink-magenta.

IV - Distinction Between Sulfomucins 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 Camerson and Steal (1959). To 200 ml. boiling distilled water, 1 gm of basic fuchsin was added and the solution was allowed to boil for one minute, then cooled and filtered. To the filtrate, 2 ml of concentrated 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 filterate 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) 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, sialomucins and hyaluronic acids stain light-purple. Some elastic fibers 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 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. - Critical Electrolyte Concentration Technique Using AB at pH 5.6 With Increased Concentrations 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 concentrations 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) 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 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 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 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 metachromasia at and above pH 4.5. Generally the metachromasia of sulfomucins resists alcoholic 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 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 washed in running water.

6) Stained with AB pH 2.5 as in II - A.

7) After washing, dehydration and clearing, sections were mounted in Canada balsam.

Result: Generally mild methylation abolished the basophilia of carboxymucins by esterification while active methylation hydrolyses most of the 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 Canada balsam.

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 hydration, sections were brought to distilled water.

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

3) Stained either with AB pH 2.5 or azure A pH 3.0.

4) Washed in running water for 5 minutes.

5) Dehydrated, cleared and mounted as usual.

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

V. - Enzyme Digestion Tests

A. - Sialidase (Neuraminidase Vibrio Cholerae, Type V) 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 hours.

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 indicates the presence of sialic acid.

B. - 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_2PO_4 + 6 ml 0.1 M Na_2HPO_4). Control sections were incubated only in buffer.

3) Washed in running water for 5 minutes.

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 hours.

3) Washed thoroughly 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.