CHAPTER SIX

HISTOLOGICAL AND HISTOCHEMICAL OBSERVATIONS AND DISCUSSION ON MUCOSUBSTANCES IN THE PENIS AND PENIAL GLAND COMPLEX (PENIS, PENIAL GLAND, END SACS OF PENIS AND PENIAL GLAND) OF THE *ONCIDIUM VERRACULATUM*

A short review of histochemical and biochemical work on the penis and penial gland complex

Among the pulmonates the stylomatophoran snails and slugs have attained maximum complexity of structure of the reproductive apparatus and complicated procedures of the mating. Hyman (1967). Some investigators have observed that the dart induces the penial activity at copulation and the whole penial complex (viz. dart, dart gland and penis with penis sheath) shows seasonal rhythmicity in its activity (Goddard, 1962) (Duncan, 1964). The mechanism of spermatophore insertion and sperm exchange during copulation and the copulation of many species have been observed by several investigators (Tore, 1965; Beeman, 1970).

There are very few histochemical studies on the reproductive system of the pulmonate snails (Kugler, 1965; Fantin and Vigo, 1968; Plesch et al., 1971). Nanaware and Varute (1973b) characterised the nature of acid mucopolysaccharides elaborated by the glandular structures. Depending upon the nature of the mucosubstances, the glands could be classified into sulfomucin and sialomucin secreting glands.

Among the land pulmonate gastropods, the dart gland, also known as the multifid gland, serves as a releaser of stimulus for courtship and as a stimulus to penial activity

at copulation (Goddord, 1962). Biochemical architecture of the penial complex which plays very important role during the copulation has been mostly neglected. The present investigation was, therefore, undertaken to understand the cytochemical architecture of the penial complex especially mucosubstances of the hermaphrodite marine slug, Oncidium verraculatum

OBSERVATIONS

I. Histological

1) Penis

The histological structure of the penis of Oncidium verraculatum, visualised in the HE staining technique coincides with the structure described by Awati and Karandikar (1948). Penis is a compound muscular tube containing a special type of penial armature. In T.S. three different tubes or sheaths enclose one within the other. The innermost is the Vas deferens, lined with ciliated epithelium, long cilia projecting within and almost filling the lumen of the duct and this epithelium is followed by muscles and connective tissue. The middle tube consists of a thin sheath formed of chondroid substance containing scattered fibres; it carries serially arranged curved teeth or spines on its outer side. The sections used here are from anterior region

and so these teeth are inconspicuous. The outermost tube forms a thick muscular coat inner wall of it showing thick chitinous lining.

2) Penial Gland

The histological structure of the penial gland of *Oncidium verraculatum* visualised in the HE staining technique is found to be similar with the structure prescribed by Awati and Karandikar (1948). It is a muscular tube containing needle apparatus in the anterior portion. In the present investigation the sections of penial gland are from posterior region without showing needle apparatus. Inner-most layer of penial gland is composed of columnar epithelium, bearing a chitinous lining on the inner wall. This layer is covered with muscular sheath. Some glandular cells between columnar epithelium and muscle layer are observed.

3) End Sac of Penis

The histological structure of the end sac of penis of *O.verraculatum* visualized in the HE staining technique showed Vas deferens inside and it is covered by a chitinous ring possessing spines. The vas deferens and spiny ring are present in the lumen of the end sac of penis. The end sac internally lined by tall columnar cells and inner epithelium is thrown into many folds. Just below the inner epithelium large number of glands are found to be present. This glandular part is

covered externally by muscles which are longitudinal and circular in nature.

4) End Sac of Penial Gland

Needle apparatus opens into the penial end sac like a syringe needle. The lumen of the needle apparatus is continuous with that of penial glandular tube. The histological structure of the end sac of penial gland of O.verraculatum is visualised in the HE staining technique of cellular parts of both the needle apparatus and the end sac. The histological picture of end sac of penial gland showed inner lining composed of tall columnar epithelial cells and they are continuous with needle apparatus on its outer border. At the base of this epithelium glandular region can be distinguished and which is covered by muscles. Thus, the histological picture of end sac of penial gland and needle apparatus are similar with each other.

Histologically end sacs of penis and penial gland are similar except in the end sac of penis vas deferens and chitinous ring with spines is present and in the end sac of penial gland needle apparatus is present.

II. Histochemical

Elaboration of Mucosubstances by Penis

The histochemical data on some important staining reactions

employed in the present investigation of the different cell types in the penis are recorded in Table No. 6 according to the visually estimated intensity and shade with four plus (++++) representing the strongest activity. The histochemical results requiring further description and consideration are presented hereafter along with the interpretations of the histochemical staining reactions. The histochemical reactivities are photomicrographically illustrated in Plate No. 5, Figs. 1, 2.

1) Elaboration of Mucosubstances by the Vas Deferens inside the Penis

Results were found to be similar with that of vas deferens described earlier in the fifth chapter.

2) Elaboration of Mucosubstances by the Chondroid Sheath of the Penis

This region shows two distinct layers. Inner sulfomucin containing layer and outer neutral mucin containing layer.

(i) Sulfomucin Containing Inner Layer of Chondroid Sheath

It exhibits intense PAS reactivity which was almost reduced by prior phenylhydrazine treatment and becomes poor after diastase digestion. With AB pH 1.0 and 2.5 it showed moderate alcianophilia. Sequential staining procedures with AB (pH 1.0, 2.5)-PAS it showed a weak alcianophilia. With aldehyde fuchsin

technique it showed weak purple reactivity. The alcianophilia was found to be resistant upto 0.5 M Mg^{++} in CEC technique. Mild and active methylation diminished the alcianophilia which could not be restored after saponification. Acid hydrolysis had no effect on it. Thus this layer contains strong sulfomucins.

(ii) Neutral Mucin Containing Outer Layer of Chondroid Sheath

It exhibits intense PAS reactivity which was completely reduced by prior phenylhydrazine treatment and was reduced slightly by diastase digestion. It is without any alcianophilia with both AB pH 1.0 and 2.5. With sequential staining techniques it showed only pink colouration. The rest of the histochemical reactivities are found to be negative. Thus, this layer contains neutral mucins.

(iii) Elaboration of Mucosubstances by the Muscles of the Penis

These muscles exhibit intense PAS reactivity which was completely abolished by prior phenylhydrazine treatment and only trace activity remained after diastase or saliva digestion. These muscles were without any alcianophilia with both AB (pH 1.0)-PAS and AB (pH 2.5)-PAS show only intense pink reactivity but there was no trace of blue reactivity. All the other histochemical techniques gave negative reactivities.

The outer muscular sheath of penis contains mainly glycogen and neutral mucins.

Elaboration of Mucosubstances by Penial Gland

The histochemical data on some important staining reactions employed in the present investigation of the different cell types in the penial gland are recorded in Table No. 7 according to the visually estimated intensity and shade with four plus (++++) representing the strongest activity. The histochemical results requiring further description and consideration are presented hereafter along with the interpretations of the histochemical staining reactions. The histochemical reactivities are photomicrographically illustrated in Plate No. 5, Figs. 5 and 6.

1. Elaboration of Mucosubstances by the Columnar Epithelium of the Penial Gland

Columnar epithelial cells of the inner lining of penial gland exhibit moderate PAS reactivity and it was almost diminished after diastase or saliva digestion and prior phenylhydrazine treatment. These cells show no alcianophilia with both the AB (pH 1.0) and AB (pH 2.5). Sequential staining procedures with AB (pH 1.0, 2.5)-PAS in these cells show only pink reactivity and there is no trace of blue reactivity. All other histochemical reaction gave negative reactivities.

Thus, from the above observations it is clear that the columnar epithelial cells of the inner lining of penial gland contain only glycogen and neutral mucins.

2. Elaboration of Mucosubstances in the Glands of the Penial Gland

These cells show moderate PAS activity which was reduced by diastase or saliva digestion and prior phenyl hydrazine treatment. These cells show weak alcianophilia with both AB (pH 1.0) and AB (pH 2.5). In the sequential staining procedures with AB (pH 1.0)-PAS and AB (pH 2.5)-PAS weak blue and pink reactivity was observed. Aldehyde fuchsin technique also shows weak purple reactivity in this region indicating the presence of sulfomucins. Mild methylation was without any effect on alcianophilia, but active methylation effected a complete and irreversible loss of alcianophilia at pH 2.5. Acid hydrolysis failed to abolish alcianophilia at pH 2.5. Further studies on the extinction of the alcianophilia by addition of graded concentration of Mg^{++} to the staining solution. The alcianophilic reaction remains unaffected by the addition of 0.1 M Mg^{++} and decreases with increasing concentration but it is not completely abolished at 0.5 M Mg^{++}. All these histochemical reactivities confirm the presence of strong acidic sulfomucins just beneath the epithelial layer.

Thus, the glandular layer below the epithelium contains

glycogen, neutral mucins and strong acidic sulfomucins.

3. Elaboration of Mucosubstances by the Muscles of the Penial Gland

These muscles exhibit weak PAS reactivity which was completely abolished by prior phenylhydrazine treatment and was completely diminished after diastase or saliva digestion. The muscles were without alcianophilia with both AB (pH 1.0) and AB (pH 2.5) sequential staining procedures with AB (pH 1.0)-PAS and AB (pH 2.5)-PAS show only weak pink reactivity. All the other histochemical techniques gave negative reactivities.

Thus, the muscle sheath of penial gland contains glycogen and neutral mucins only.

Elaboration of Mucosubstances by End Sacs of Penis and Penial Gland

The histochemical data on some important staining reactions employed in the present investigation of the different cell types in the end sac of penis are recorded in Tables No. 6 and 7 according to the visually estimated intensity and shade with four plus (++++) representing the strongest activity. The histochemical results requiring further description and consideration are presented hereafter along with the interpretations of the histochemical staining reactions. The histochemical reactivities are photomicrographically illustrated

in Plate No. 5, Figs. 3,4, 7 and 8.

1) Elaboration of Mucosubstances by the Vas deferens in the End Sac of Penis

Results were found to be similar with that of vas deferens described earlier in the fifth chapter.

2) Elaboration of Mucosubstances by the Chitinous Ring With Spines

This outermost part of vas deferens in the end sac exhibits weak PAS reactivity which was diminished by diastase or saliva digestion and was completely abolished by prior phenylhydrazine treatment. This region shows moderate alcianophilia with AB (pH 1.0) and weak alcianophilia with AB (pH 2.5). Sequential staining procedures with AB (pH 1.0)-PAS and AB (pH 2.5)-PAS show weak pink blue activity. With aldehyde fuchsin a weak purple activity was noticed indicating the presence of weak sulfated mucins. Mild methylation was without any effect on alcianophilia but active methylation effected a complete and irreversible loss of alcianophilia at pH 2.5. Acid hydrolysis failed to abolish alcianophilia at pH 2.5. Further studies on the extinction of the alcianophilia by addition of graded concentration of Mg^{++} to the staining solution. The alcianophilia reaction remains unaffected by the addition of 0.1 M Mg^{++} but it decreases with increasing

concentration and was completely abolished at 0.4 M Mg^{++} concentration. All these histochemical reactivities confirm the presence of weak sulfomucins.

3) Elaboration of Mucosubstances by the Inner Epithelium of End Sacs of Penis and Penial Gland

Epithelial lining of the end sac of penis and penial gland exhibits moderate PAS reactivity which was without any effect after diastase or saliva digestion and was completely abolished after prior phenylhydrazine treatment. These cells exhibited moderate alcianophilia with AB (pH 1.0) and weak with AB (pH 2.5). This alcianophilia was found tobe located on the inner border of the cells. With sequential staining procedures like AB (pH 1.0)-PAS and AB (pH 2.5)-PAS they exhibit weak and moderate pink blue reactivity respectively. With aldehyde fuchsin technique it exhibited weak purple activity towards the inner border. Mild methylation was without any effect on alcianophilia but active methylation effected a complete and irreversible loss of alcianophilia at pH 2.5. Acid hydrolysis failed to abolish alcianophilia at pH 2.5. Further studies on the extinction of the alcianophilia by addition of graded concentration of Mg^{++} to the staining solution. The alcianophilic reaction remains unaffected by the addition of 0.1 M Mg^{++} but it decreases with increasing concentration of Mg^{++}. It was found that in some cells of this layer

the alcianophilia is resistant upto 0.5 M Mg^{++} concentration but in other cells alcianophilia is completely abolished at 0.5 M Mg^{++} concentration. Thus it is an indication of some cells containing strong acidic sulfomucins and others with weak sulfomucins.

Thus, the epithelial lining of end sacs of penis and penial gland contain two types of cells in one weak sulfomucins are present while in other strong sulfomucins are present in addition to neutral mucins and glycogen.

4) Elaboration of Mucosubstances by the Glands in End Sac of Penis and Penial Gland

According to the histochemical reactivities glands can be grouped into two distinct types. They are:

Type 1: These glands exhibit intense PAS reactivity which was slightly reduced by prior phenylhydrazine treatment and still more diminished by diastase or saliva digestion. These glands show intense alcianophilia with AB (pH 1.0) and weak with AB (pH 2.5). With sequential staining AB (pH 1.0, 2.5)-PAS show intense blue pink reactivity and with aldehyde fuchsin they stained intense purple. In CEC technique alcianophilia was found to be resistant upto 0.5 M Mg^{++}. Mild methylation was without any effect and active methylation diminished the reactivity which could not be

restored after saponification. This confirms the presence of mainly strong sulfomucins.

Type 2: These glands showed same reactivities with PAS, prior phenylhydrazine treatment - PAS and diastase digestion - PAS to those of Type 1. These glands showed intense alcianophilia with AB (pH 2.5) and weak with AB (pH 2.5). With sequential staining AB (pH 1.0, 2.5)-PAS they showed intense blue pink reactivity. With aldehyde fuchsin they remain unstained. Mild methylation reduced the reactivity which was restored after saponification. In CEC technique the alcianophilia reduced with increasing concentration of Mg^{++} and was completely lost at 0.5 M Mg^{++}. Acid hydrolysis effected a complete loss of alcianophilia. Presence of sialic acid was confirmed by sialidase digestion technique. Thus these glands contain mainly carboxy mucins containing sialic acid and neutral mucins.

5) Elaboration of Mucosubstances by the Muscle of End Sac of Penis and Penial Gland

The longitudinal and circular muscle bundles from penis end sac showed moderate PAS reactivity, which was not resistant to the prior treatment of phenylhydrazine. They showed no alcianophilia with AB both at pH 1.0 and 2.5. The PAS reactivity was abolished by prior treatment with saliva or diastase digestion. These results indicated that these muscles contained

only glycogen.

DISCUSSION

Penis and End Sac of Penis

The investigation of the mucosubstances elaborated by penis and the end sac of penis was undertaken with a view to augmenting the general understanding of the biochemical nature of these structures and the secretion of the mucosubstances in the penis and its end sac and their probable role in physiology of reproduction of the slugs in particular. Till recent times the secretion product from the penial complex in marine slugs was classified as acid mucopolysaccharides (Plesch et al., 1971), its histochemical characterization in different acid mucopolysaccharides had remained to be studied. It was also not known in which glands in the penis these mucosubstances are elaborated. The present detailed histochemical investigation of the mucopolysaccharides and in addition deals with the characterisation of different mucin elaborating glands in penis and in the penis end sac.

The chondroidal sheath inner layer contained strongly sulfated mucins whereas outer sheath layer contained only neutral mucins. Muscles of the penis contained glycogen and neutral mucins in them. The end sac of penis has many multicellular glands which could be differentiated into two types: (1) Sulfomucin secreting glands and (2) neutral mucin

and sialic acid secreting glands the reactivities and biochemical nature are very much identical with those of the penial gland.

A point of some academic interest, which needs to be emphasised here is the presence of sialic acid in the secretion of the penis end sac. Warren (1963) had failed to detect any sialic acid in the molluscs and hence stated that the molluscs lack capacity to elaborate endogenous sialic acid. But it is important to emphasise that he had employed biochemical techniques in which he could measure quantitatively sialic acid to a particular critical minimum quantity, beyond which biochemical assay of sialic acid was not possible. As against such biochemical techniques, the histochemical techniques such as those employed in the present investigation, afford a better understanding of the sialic acid content in very small quantities. The histochemical techniques, thus, afford more advantages over the bioassay techniques. By employing such histochemical techniques, sialic acid has been reported in dart gland and penis of Semperula maculata (Nanaware and Varute, 1973a, 1973b). The present investigation affords evidence in support of presence of sialic acid in the glands of penis and sac. It has to be now consided that the invertebrates do have a capacity to elaborate sialic acid endogenously. The problem of phylogenetic importance of sialic acid, which has already been studied biochemically, now needs to be approached histochemically.

During the copulation the penis is protruded and it helps

in the release of sperms (Beeman, 1970) and the acid mucopolysaccharides probably play a role during copulation (Plesch et al., 1971). Thus, the acid mucosubstances, sulfomucins and sialomucins found in the present investigation might be functioning as a biological lubricant during the copulation. This lubrication might be useful to avoid the friction at the sides of copulatory organs and smooth transfer of the sperm strands into the female gonopore of copulating partner. Goudsmit (1972) and Nanaware (1974) have also attributed such a role of lubrication to the molluscan mucosubstances. A similar functional significance has also been attributed to the acidic mucosubstances (Odin, 1958; Longley et al., 1963). During copulation the endogenous sperms are tightly bound to one another by extracellular material (Beeman, 1970); this strand formation function might be taken up by the acidic mucosubstances secreted by the penis and penis end sac.

Penial Gland and End Sac of Penial Gland

The investigation of the mucosubstances elaborated by the penial gland of the marine slug, O.verraculatum was undertaken with a view to augmenting the understanding of the histochemical nature of the penial gland. The present detailed histochemical investigation confirms the presence of sulfomucins as a major constituent elaborated by penial gland; especially by the glandular part beneath the epithelium. End sac of penial gland

is the major site of elaboration of mucosubstances. In addition to sulfomucins end sac of penial gland also elaborates neutral mucosubstances and sialomucins. The sulfomucin elaborating and carboxy mucin elaborating glands can be easily distinguished.

Besides making the above significant addition to our existing knowledge of the histochemical nature of the penial gland secretion the present investigation also reveals another interesting aspect concerning the cellular sites of elaboration of these mucosubstances. The glands in the end sac of penial glands can be distinguished into two types. Some are secreting only carboxy mucins while others only sulfomucins. For its secretory function the penial gland end sac seems to have evolved a specialised division of labour at glandular level, certain glands being specialised to elaborate sulfomucins others only carboxymucins, sialomucins, and neutral mucins. The penial gland end sac of the marine slugs thus, affords an excellent example of biochemical division of labour at glandular level for its secretory function.

The penial gland like dart gland in other pulmonate seems to be involved in stylet formation which plays an important role during the courtship behaviour in evoking heightened sexual excitement and serving as a releaser of stimulus for courtship (Hyman, 1967). The secretion of the gland is ejected into partner's body, where it acts as a lubricant. The mucosubstances in the secretion of the penial gland might be responsible for

such a function as lubricant. In his recent review on the carbohydrates and carbohydrate metabolism of molluscs, Goudsmit (1972) has also attributed this role as a lubricant to the molluscan mucosubstances. Similar lubrication function of dart gland mucins in land snail, Semperula maculata has been shown by Nanaware (1974). In the gastropods, the fertilised eggs are coated with egg-jellies which also contain several sulfated and non-sulfated mucopolysaccharides (Fantin and Vigo, 1968). Though it has been shown that the glandular structures associated with the female reproductive tract are responsible for the formation of the egg-jellies, it will be interesting to investigate whether the secretion of the penial gland which is ejected into the copulant also contributes towards the formation of the egg-jellies.

CONCLUDING REMARKS

Thus, the aims and objectives with which the present investigation was undertaken have been successfully fulfilled. The main aim of the present investigation was to study the histochemical characterization of mucosubstances, their distribution in various histological sites of these organs, and to project idea about the functional significance of mucosubstances in various organs based on circumstantial evidences. It is hoped that these aims and objectives have satisfactorily been achieved.

While concluding the present M.Phil. dissertation on the reproductive organ mucosubstances in the marine gastropod slug, O.verraculatum, the author would like to humbly state that the present work is by no means complete. He is fully aware of his shortcomings. For the purpose of studying the concentration or content of mucosubstance/s in a given histological site in an organ, the author had to entirely depend on the visually estimated intensity of staining.

This, therefore, forms an excellent subject matter for further studies. Further studies should be carried out in the following directions:

1) The mucosubstances identified in the present investigation should further be studied by employing the bioassay studies to find out the exact quantities of mucosubstances in the given organ of the reproductive tract in mathematical terms.

2) The mucosubstances in the various organs investigated should further be isolated by chromatography and further be characterized.

3) The mucosubstances in the various organs should further be confirmed by employing autoradiographic studies.

4) Some of the mucosubstances such as neutral mucosubstances, sulfomucins etc. should be further identified.

5) Whether any other atypical mucosubstance like galactogen is present in the albumen gland should be studied.

6) To find out the diversity or similarity in the distribution of mucosubstance with respect to habitat in different animals, i.e., marine or terrestrial.

7) The species diversity of reproductive tract mucosubstances should further be confirmed by studying large number of animals including some closely related species.

8) To find out changes in the quantity and distribution of mucosubstances with respect to annual breeding aestivation cycle and their role in the reproductive physiology of gastropods.

Thus, the present investigation shows that there are several avenues open for further researches.

"To make an end is to make a beginning
The end is where we start from"

- T.S. Eliot