CHAPTER THREE

HISTOLOGICAL AND HISTOCHEMICAL OBSERVATIONS AND DISCUSSION ON MUCOSUBSTANCES IN OVOTESTIS, HERMAPHRODITE DUCT AND SEMINAL VESICLE OF *ONCIDIUM VERRACULATUM*

OVOTESTIS

A short review of histochemical work done on ovotestis

Ovotestis or hermaphrodite gland of the gastropods has been a target organ of several investigations for long time. The morphological details and functional role of its different cells has been studied extensively (Bayne, 1970; Ohsako, 1971 and Holman, 1972). Several other investigators have correlated the cyclic activities and changes within this gland with the neurosecretory cyclic activities of the eye stalk tentacle and the cells in thecerebral ganglions of such animals. (Gottfried and Dorfman, 1970 and Wells and Wells, 1972). Some others have contributed towards the localisation of inorganic elements (Yasuzumi, 1962 and Lusis, 1962). Besides these chemical studies the histochemical and biochemical studies in the ovotestis proper seem to have been comparatively neglected though some research workers have taken a keen interest in studies of different nutritive and useful chemical substances like mucins, proteins, lipids and enzymes of the reproductive tract of the hermaphrodite pulmonates. The following is a brief review of the work done on the histochemical and biochemical studies on the gonads of different gastropod molluscs.

Mucosubstances

In recent years some investigators have studied carbohydrates of this important organ of the reproductive system of different snails. (Kugler, 1965 and Taylor and Anderson, 1969). A brief survey of their reports shows that the neutral mucopolysaccharides (Bradbury and Meek, 1963), glycogen (Taylor and Anderson, 1969) PAS positive granules (Yamasaki, 1969) and acid mucopolysaccharides (Selwood, 1970) were found in different parts and cells of the ovotestis and its duct. Except for these studies, the details of the mucosubstances of the ovotestis remain to be fully explored.

From the critical review of the present information on the histochemical and biochemical studies on the ovotestis, it seems that although mucosubstances play important role in the hermaphrodite gastropod, this metabolite has not been extensively studied in the ovotestis. Hence the present investigation was undertaken.

OBSERVATIONS

I. Histological Observations

1) Ovotestis or Hermaphrodite Gland

The ovotestis of *Oncidium verraculatum* which is also called as hermaphrodite gland, was round and compact in form,

brownish in colour. Its size depended upon the seasonal changes and age. Externally, it appeared lobulated being composed of compact lobules, follicles or acini which were round or oval in form. The follicles being held together by connective tissue fibres. The microscopic follicular structure observed in HE, histological staining technique comprised outer germinal or follicular epithelium lining the follicular central cavity, which was filled with mature ova and sperms produced by the follicular epithelium. The oocytes were seen to be attached to the wall, while spermatozoa at the centre of the acini. There was a continuous production of ova and sperms and nutritive cells in the mature gonad. The histological picture revealed it to be formed of innumerable follicles containing ova and sperms in their various stages of development. The follicles containing spermatogonia, which give rise to sperms, are generally situated more towards the inner side of the ovotestis, the peripheral region being usually occupied by the ovarian follicles. The ova show conspicuous nuclei and the sperms are arranged in bunches with small heads and long tails.

2) Hermaphrodite Duct

The follicles of the ovotestis are connected with the small branches of hermaphrodite duct. The hermaphrodite duct is a narrow, white, thin-walled tube which carries both sperms

and eggs. Its first half portion is much convoluted but towards the albumen gland it becomes more or less straight. The histological picture visualised by the thin sectioning and histologically stained preparations, showed that the hermaphrodite duct is round and hollow. The wall of the duct consisted of two layers of cells. An outer-most layer is composed of very much flattened pavement epithelium and an inner layer is composed of conspicuously ciliated short columnar epithelium. The cilia are very long and project in the cavity of the duct. There are round and very prominent nuclei situated at the centre of the cells. As the animals were collected in the month of May, the lumen contained sperm mass in this season. Some glandular cells were found to be scattered in between the columnar epithelial cells.

3) Seminal Vesicle

Hermaphrodite duct or common duct of *Oncidium verraculatum* is quite convoluted. On its way it gives off a finger-like whitish vesicle which is called as Seminal vesicle which serves as a store-house for mature sperms. Histological architecture of this gland shows that is is spherical gland with inner cuboidal epithelium, followed by connective and muscular tissue and outer covering. In the lumen sperm mass was found to be filled. Usual histological methods HE gave blue staining in the nuclei and the pink

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colouration in the cytoplasm.

II. Histochemical Observations

The histochemical data on some important staining reactions employed in the present investigation of the ovotestis Hermaphrodite duct and seminal vesicle are recorded in the Table Nos. 1 and 2, according to the visually estimated intensity and shade with four plus (++++) representing the strongest activity. The histochemical observations requiring further description and consideration, are presented hereafter along with the interpretations of the histochemical staining reactions.

1) Elaboration of Mucosubstances by the Ovotestis

The histochemical study on the ovotestis revealed the following significant facts about the elaboration of the mucosubstances by the female and male germ cells. (1) On the basis of their capacity to elaborate the mucosubstances the male germ cells and the female germ cells showed different histochemical reactivities. (2) Glycogen appeared to be associated with both the types of sex cells.

The localization of different mucosubstances in the different cell types in the ovotestis are photomicrographically illustrated in the Plate No. 1, Figs. 1 to 6.

Elaboration of Mucosubstances by the Follicular Epithelium

The germinal epithelial cells of the follicular wall exhibited an intense PAS reactivity which was slightly diminished by diastase or saliva digestion and which was abolished by prior phenylhydrazine treatment. These cells exhibit alcianophilia both at pH 1.0 and pH 2.5 in the combined sequential staining techniques with AB (pH 1.0, 2.5)-PAS showing pink blue colouration. The presence and characterization of acidic mucosubstances was confirmed by graded concentration of $MgCl_2$ in the Alcian blue dye technique. The alcianophilia was quite resistant upto 0.2 M $MgCl_2$, which was diminished slowly in 0.4 M $MgCl_2$ and 0.5 M $MgCl_2$. Further, in mild methylation and in active methylation alcianophilia was not diminished; neither demethylation could restore the activity indicating the acidic mucosubstances present in the follicular epithelium which are weakly and strongly sulfated type.

Thus, the follicular epithelium contains glycogen, neutral mucins and weakly as well as strongly sulfated acidic mucins.

Elaboration of Mucosubstances by the Nutritive or Nurse Cells in the Follicles

The nurse cells at the periphery of the follicle showed

intense PAS reactivity. It was slightly diminished by diastase or saliva digestion and was completely abolished by prior phenylhydrazine treatment. They did not exhibit any alcianophilia both at pH 1.0 and pH 2.5. In the combined sequential staining techniques with AB (pH 1.0, 2.5)-PAS these cells reacted only with PAS giving a pink staining, there being no trace of blue colouration. Mild and active methylation, acid hydrolysis and suppression of alcianophilia by $MgCl_2$ were showed negative reactivities towards their techniques.

The above histochemical reactions indicate that these cells contain mainly neutral mucins and the glycogen.

Elaboration of Mucosubstances by the Female Germ Cells of the Follicle or Ovum

The oocytes showed moderate PAS activity which was abolished by prior phenylhydrazine treatment and was decreased by diastase or saliva digestion indicating neutral and acidic mucosubstances. Traces of alcianophilia observed with both AB (pH 1.0 and pH 2.5) indicating trace quantity of acidic mucosubstances.

The pink staining was observed in the sequential staining techniques AB (pH 1.0, 2.5)-PAS indicating mainly

neutral mucosubstances in them. The mucosubstance contents in the cytoplasm in matured oocyte did not exhibit any alcianophilia at pH 1.0 but they did exhibit metachromasia at very high pH levels (pH 4.5 to 5.5). The reactions indicated absence of sulfomucins in them but presence of carboxy mucins. With AB (pH 2.5), they exhibited an intense alcianophilia which was completely abolished by mild and active methylation and subsequent saponification could restore the original degree of alcianophilic staining. Acid hydrolysis was without any effect on alcianophilia of these oocytes. Similar results were obtained with neuraminidase treatment. The hyaluronidase digestion could practically abolish the alcianophilia at pH 2.5 and high pH metachromasia. The above alcianophilic and metachromatic reactions were enhanced in these oocytes after the pepsin digestion. These histochemical reactions, thus, indicated presence of PAS-positive protein-bound hyaluronic acid in cocytes.

Elaboration of Mucosubstances by the Sperms

The mature sperms found in bundles only showed positive reactions towards the histochemical techniques employed in this investigation.

Sperms show intense PAS reactivity which was slightly reduced after prior treatment of phenylhydrazine and also

diastase or saliva digestion AB (pH 1.0) and AB (pH 2.5) alcianophilia was observed which was abolished by mild methylation whereas saponification could restore little of the original. The histochemical reactions, thus, indicate the presence of carboxy mucins. Acid hydrolysis effected a complete loss of alcianophilia. Sialidase digestion could practically abolish the alcianophilia and low pH azurophilia from these sites. The histochemical reactions, thus, indicated presence of sialic acid in carboxymucins. Such sialomucins seem to be bound with protein, since proteolysis is necessary for the visualisation of their basophilia.

The PAS reactivity in the sperm tails was completely lost after prior saliva or diastase digestion and phenylhydrazine treatment. Towards other histochemical techniques, these mucosubstances showed negative reactivities indicating the presence of only glycogen in these sites. Thus, the sperm tails contained only glycogen in them.

2) Elaboration of Mucosubstances by the Hermaphrodite Duct

The histochemical study on the hermaphrodite duct revealed the significant fact about the elaboration of the mucosubstances by the epithelial cells of this duct that some glandular cells elaborating mucosubstances were distributed in between the epithelial cells. Since this

observation is made on the animals collected in the summer (month of May) and hence there are only sperms passing through this duct and not a single site was observed carrying the eggs through it. Therefore, our observations are confirmed to the elaboration of mucosubstances by the hermaphrodite duct during the passing of male germ cells (sperms) through it. Although there may be either similar or dissimilar mucosubstance elaboration by this duct during passing of female germ cells (ova) through it. This study is confined to the localization of mucins and their seasonal variations form a big and separate topic of research and will be published in due course of time.

Elaboration of Mucosubstances by the Hermaphrodite Duct During the Passing of Male Germ Cells (Sperms) Through It

The Columnar Epithelium

The columnar epithelial cells of the harmaphrodite duct exhibited an intense PAS reactivity which was slightly diminished due to diastase or saliva digestion and which was also abolished by prior phenylhydrazine treatment. The cytoplasmic content of these cells reacted opposite to the reactions of the cells of spermatheca of *Semperula maculata*. They did show alcianophilia both at pH 1.0 and pH 2.5 but their intensity is low. In combined sequential staining

procedures, with AB (pH 1.0, 2.5)-PAS, these cells reacted only with PAS giving a pink staining, there being no trace of blue colouration. In AB pH 2.5 graded with $MgCl_2$ the cells showed alcianophilia upto 0.4 M $MgCl_2$, suggesting the presence of sulfated acidic mucins in them. Metachromasia was exhibited by these cells at low pH levels.

The above histochemical reactions indicate that these cells contain mainly glycogen, neutral mucosubstances and weakly sulfated acidic mucins in them.

Elaboration of Mucosubstances by the Glandular Cells in the Epithelium

The glandular cells located in the epithelium showed similar reactivities with the histochemical procedures; in addition to that they exhibited alcianophilia reduced in mild methylation and its restoration after saponification. Similarly, acid hydrolysis completely abolished alcianophilia. These histochemical reactivities indicated that these cells contained glycogen, neutral mucosubstances, weakly sulfated acidic mucins and in addition carboxyl group containing acidic mucins, mainly sialic acid (Plate No.1 Figs. 7 & 8).

Elaboration of Mucosubstances by the Muscles

The muscles of the hermaphrodite duct exhibited intense

PAS reactivity which was slightly diminished due to diastase or saliva digestion and which was also abolished by prior phenylhydrazine treatment. These cells moderately reacted with the rest of the histochemical techniques employed, such as AB pH 1.0 and at pH 2.5. In combined sequential staining procedures, with AB (pH 1.0, 2.5)-PAS these cells reacted only with PAS giving a pink staining, there being no trace of blue colouration. No metachromasia at higher pH level.

The above histochemical reactions indicate that these cells contain mainly glycogen, neutral mucosubstances and slightly (weakly) sulfated acidic mucosubstances in them.

Elaboration of Mucosubstances by Luminal Contents

The lumen of the hermaphrodite duct contained material showing intense PAS reactivity which was completely abolished by prior phenylhydrazine and malt diastase digestion. In combined AB (pH 1.0, 2.5)-PAS sequential staining, stained only pink indicating the presence of glycogen and neutral mucosubstances in them. Alcianophilic staining procedures involving suppression by $MgCl_2$ grades, mild and active methylation and the saponification, acid hydrolysis showed and confirmed the presence of strongly sulfated acidic mucosubstances also in them.

3) Elaboration of Mucosubstances by Seminal Vesicle

Elaboration of Mucosubstances by Epithelial Layer

The cells of this layer showed moderate PAS reactivity which was completely abolished by prior phenylhydrazine treatment and was reduced by diastase or saliva digestion indicating the presence of neutral mucins and glycogen. These cells show poor alcianophilia to both AB (pH 1.0, 2.5). With sequential staining procedures like AB (pH 1.0, 2.5)-PAS these cells showed weak pink blue colouration. Mild methylation was without any effect on the alcianophilia at pH 2.5 but active methylation effected a complete and irreversible loss of the alcianophilia. Acid hydrolysis failed to abolish alcianophilia at pH 2.5. These staining reactions indicated the presence of weak sulfomucins. This was further confirmed by the studies on the extinction of the alcianophilia by the addition of the graded concentration of Mg^{++} to the staining solution. The alcianophilic reaction remained unaffected by the addition of 0.1 M and .2 M Mg^{++} but any further addition led to an extinction of the alcianophilia.

Thus epithelial cells contain glycogen, neutral mucins and weak sulfomucins.

Elaboration of the Mucosubstances by the Luminal Contents

The luminal contents contain spermatophores or large

bodies of sperm masses. These get intensely stained with PAS (Plate No. 7, Fig. 9) and the reactivity was completely abolished with prior diastase or saliva digestion and phenylhydrazine treatment. Luminal content was without alcianophilia with both AB (pH 1.0, pH 2.5). With sequential staining procedures AB (pH 1.0, 2.5)-PAS it showed only pink reactivity and the sperm masses stained intense pink. All the other histochemical reactions were negative. Thus, presence of only glycogen in the sperm masses is suggested.

DISCUSSION

1) Ovotestis

The investigation of the mucosubstances in the ovotestis of the marine gastropod was undertaken with a view to augmenting the histochemical nature of the mucosubstances of the ovotestis and their probable role in physiology of reproduction. Until recently the mucosubstances studied in the ovotestis showed neutral mucopolysaccharides in spermatid (Bradbury and Meek, 1963), PAS-positive granules in sperms (Yamasaki, 1969) and in testicular nutritive cells (Yasuzumi, 1962), acid mucopolysaccharide in developing oocytes (Selwood, 1970), glycogen in sperms (Anderson and Personne, 1970) and in the leydig cells (Taylor and Anderson, 1969), glycoproteins (Taylor and Anderson, 1969), protein-carbohydrate yolk

precursors in previtellogenesis and embryogenesis (Guraya, 1969). From a critical study of these histochemical investigations it seems that hardly a few investigators have fully characterised the mucosubstances in the cells of the ovotestis of Oncidium. The present investigation deals with the complete cytochemical characterisation of the different cells of the ovotestis from the mucosubstances elaboration point of view.

The Follicular Epithelium and the Nurse Cells

The nurse or nutritive cells of the ovotestis of pond snails were shown to contain PAS-positive granules. The nature of those granules was not fully characterised (Yasuzumi, 1962). Nanaware and Varute (1975) have characterised the mucosubstances of a land snail Semperula maculata, in the follicular epithelium and nurse cells of ovotestis and has shown that these cells contain neutral mucosubstances and glycogen in them. The present investigation of marine slug Oncidium, the follicular epithelium and nurse cells seem to contain neutral mucosubstances and glycogen in them. But in addition the epithelium contains weakly sulfated acidic mucins. From the existing literature it appears that there are some differences in mucin contents in these cells of the ovotestis in fresh water, marine and land molluscs. But

such differences cannot be interpreted on the basis of habitat with the scanty information or studies available. The study of mucin contents of these cells of the different snails and slugs from different habitats, will be very interesting, as they may give some clue to the relation between the capacity of elaboration of the mucosubstances by the follicular epithelium and the nutritive cells and their habitat.

The presence of neutral mucins and glycogen both in the follicular epithelium and nutritive cells is understandable, since during proliferation of epithelial cells, the nutritive cells supply their nutritive elements to the germinal cells (Barth and Jansen, 1959). A protective role of the nutritive cells as stated by Barth and Jansen (1959) is not clearly understood. The role of weakly sulfated mucins in the epithelium cannot be interpreted.

Oocytes

The neutral polysaccharides (Bayne, 1968), glycogen (Moncuso, 1956), acid mucopolysaccharide (Anderson, 1969), protein-carbohydrate complexes have been shown to be present in maturing oocytes or eggs. But except for Mariano (1967) and Varute and Nanaware (1974) classification of mucosubstances in oocytes of molluscs, no body has yet completely analysed mucins from the oocytes. In the present

investigation are found only hyaluronic acid bound with the proteins. No sialic acid could be detected in the oocytes of these slugs.

Protein-bound hyaluronic acid found at the periphery of the ovum can have a protective role in the oocytes from sperm attack (Hubner, 1955). According to Bayne (1968) mucins in the molluscan eggs have importance in reducing the dessication. The hyaluronic acid containing protein-polysaccharide complexes at the periphery of eggs form tiny granules which appear similar to the cortical granules described in the vertebrate oocytes. Hence in all probability mucins towards the egg-periphery are cortical granules, which play an important role in the process of fertilization.

Sperms

Very few attempts have been made to understand the nature of mucins in the different parts of the sperm from the non-stylommatophoran and stylommatophoran gastropods. Bradburi and Meek (1963) observed glycogen in mitochondria of sperm. PAS-positive granules were observed in atypical sperms and in sperm tails (Bulnheim, 1968). Nanaware and Varute (1975) have found sialic acid in sperm head and glycogen in tails of sperms. In the present investigation, we also found sialic acid and glycogen in sperm head and

tails respectively. Such differences in the content of mucosubstances in the oocytes and sperms of different gastropods, indicate a distinct heterogeneity, but the probable reason and significance of such heterogeneity remain to be elucidated.

The presence of sialic acid in the sperm heads helps in the smooth entry of the sperm strands towards the hermaphrodite duct and thereby in the copulating partner's spermatheca, during sperm exchange at the time of copulation. It should be noted that the sialic acid containing mucosubstances are localised at the tip of the head of the sperms, thus indicating their probable presence in the acrosome of the sperm. Such a presence of sialic acid in the acrosome indicates its probable importance in acrosome reaction in the process of fertilization. The glycogen from the tails might serve as a reserve food for the matured sperms.

A point of some academic interest is that, though the fully mature ova and sperms show a distinct difference in their mucosubstance contents, the former containing hyaluronic acid and the latter sialomucins in their heads and glycogen in their tails, the germinal cells from which these two types of sex cells arise, have the same type of mucosubstance

content, i.e., glycogen and glycosaminoglycans. When a germ cell differentiates into ovum, hyaluronic acid elaboration takes place, whereas, when the sperm differentiates, elaboration of sialomucin occurs. The intrinsic control which directs the differentiation of the germ cells, either towards the formation of an egg or sperm, is not known. This intrinsic control, whatever it may be, must also be responsible for the differential elaboration of the mucosubstances in these two types of cells.

2) Hermaphrodite Duct

The hermaphrodite duct showed capacity of elaboration of both the types of mucosubstances i.e., neutral mucosubstances, weakly sulfated acidic mucosubstances and glycogen by its columnar epithelial cells. The glandular cells scattered in the epithelium were specific in their histochemical reactivities and contained glycogen, neutral mucosubstances, weakly sulfated and carboxyl group containing sialic acid acidic mucosubstances in them. The muscles contain glycogen, neutral mucins and weakly sulfated mucins. These mucins are present in the above mentioned cells, when the male germ cells are passing through the hermaphrodite duct. Rangrao (1963) has found AB-positive mucins and glycogen in the hermaphrodite duct of *Ariophanta ligulata* whereas Nanaware and Varute (1975) have

found only neutral mucosubstances and glycogen in this duct of Semperula maculata, when male germ cells were passing through it. The findings of the present investigation are quite different. Ariophanta ligulata and Semperula maculata are land snails whereas oncidium is a marine slug. Whether this difference is associated with the phylogenic characteristic of different habitat, cannot be said from the limited literature at hand on the acid mucosubstances secreted by the columnar epithelial cells, glandular cells and the muscles when sperms are passing through this passage. This could be well understandable since same biological lubricating role has been attributed to the molluscan mucins (Nanaware and Varute, 1975).

Until recently, mucins in the molluscan hermaphrodite duct were not fully characterised. According to Kugler (1965) these were thionine metachromatic, alcianophilic, PAS-positive and amylase fast. Now why the glycogen is found with the association of sperms ? Or does this glycogen come from the tail of the sperms in the duct cells ? It seems that the glycogen from these cells might supply nutrition to the stored sperms for a long time during their passage through this duct.

In other investigations seasonal differentiation in the elaboration of mucosubstances in this duct has been

observed (Nanaware and Varute, 1975). Whether similar seasonal changes are present in this animal also is not known. Such seasonal changes in the mucosubstances are under neurosecretory control. It will, hence, be very interesting as well as speculative to study the rhythmic activities and the correlations, between the synthesis and elaboration of the mucosubstances in different cell types of this duct and neurosecretory substances of the eye-stalk and brain during the reproductive events in the seasonal breeding cycle.

3) Seminal Vesicle

The histological picture of the seminal vesicle of *Oncidium verraculatum* is different when compared to seminal vesicle of *Ariolimax caruanal* (Sirgel, 1973). In former species as opposite to the latter, there is no cellular differentiation in the epithelial cells. The present histochemical observation showed that the epithelium contained glycogen, neutral mucins and weakly sulfated acidic mucins. The luminal contents which contained spermatozeugmata showed strong PAS reactivity and presence of glycogen was predominant. It might be due to the presence of sperms which contain glycogen in their tails. Although phagocytotic role has been attributed to the cell buds (large vesicles given off from the epithelial cells) in seminal vesicle (Buckland *et al.*, 1976), such a role cannot be interpreted from the present investigation.