CHAPTER I

INTRODUCTION

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Mucopolysacharides

The first classification based on the chemical composition of the carbohydrate moiety of mucoproteins was made by Levene (1925). Then number of classifications were given (Meyer 1938; Meyer 1945, 1953; Jeanloz 1960; Dorfman, 1963; Curran, 1964; Gottschalk, 1960; Stacey, Barker, 1962; Hunt, 1970).

Some times confusion arises because a particular substance is known by many names e.g. "Chondroitin sulfate" is also known as "β-heparine", as "dermatan sulfate", and as "dermatoidin sulfate". Besides the confusion in terminology the structure of many carbohydrates is yet to be fully elucidated which poses a problem for biochemists to classify carbohydrates. Biochemically it is possible to discriminate glycoproteins from mucoproteins but histochemically it is not (Casselman 1959). Hence, at present two types of classifications are in existence. i) Biochemical classification. ii) Histochemical classification. For the detailed information on the chemical nature and structure of polysaccharides or mucopolysaccharides, the reviews by Kent and Whitehouse (1955), Hale (1957), Meyer et al., (1957), Wolstenholme and O'connor (1958), Muir (1961), Hirst (1962), Hoffman and Meyer (1962), Dorfman (1963) and Gottschalk (1966) are very helpful.

The histochemical classification has been given by Hale (1957), Casselman (1959), Pearse (1960), Spicer (1963), Curran (1964), Spicer et al., (1965), Spicer and Henson (1967) and Spicer et al., (1965). The classification is mainly based on the same lines as initially suggested by Meyer (1938, 1945, 1955). Since then the carbohydrate histochemistry has advanced significantly because of new tools, techniques and stains. As a result of this more information is made available to the histochemists. Spicer et al., (1965) classified carbohydrate-rich tissue components and suggested the word "mucosubstance" a general term for histochemical reference to any carbohydrate-rich component.

The histochemical classification of mucosubstances as suggested by Spicer et al., (1965) in which the mucosubstances are named by 1) Staining site where they are found, and 2) Subgrouping them as well possible as neutral mucosubstances, sulfomucins, sialomucins etc. It is suggested that further subdivisions of these subtyped mucosubstances can be achieved by means of the following histochemical reactions.

i) Affinity for basic dyes such as Azure A.

ii) Affinity for alcian blue.

iii) Lability to testicular hyaluronidase.

iv) Lability with respect to Vibrio cholerae neuraminidase.

Below is setforth a working histochemical classification of mucosubstances which is substantially that of Spicer et al., (1965).

Histochemical classification of mucosubstances.

I. Neutral mucosubstances.

Neutral glycoproteins, immunologically reactive glycoproteins, fucomucins, mannose-rich mucosubstances in epithelial and connective tissue. All react towards PAS and some towards periodic acid-p-diamine procedures.

II. Acidic mucosubstances.

A. Sulfated.

1. Connective tissue mucopolysaccarides (Periodate reactive).

(a) Resistant to testicular hyaluronidase.

i. Alcianophilic at or below 1.0 M $MgCl_2$ Keratan Sulfate Heparin.

ii. Alcianophilic in presence of 0.7 M (or less) $MgCl_2$-Dermatan Sulfate.

(b) Susceptible to testicular hyaluronidase.

i. Alcohol resistant, affinity for 0.02% Azure A at or below pH 2.0 Chondroitin sulfate in cartilage.

ii. Alcohol resistant, affinity for 0.02% Azure A at or above pH 4.5.

B. Non-sulfated.

1. Hexuronic acid-rich mucopolysaccharides-hyaluromic acid, chandroitin.

2. Sialic acid-rich mucosubstances.

(a) Connective tissue mucopolysaccharides containing sialic acid.

(b) Epithelial sialomucins - Acid glycoproteins.

i. Highly susceptible to Vibrio cholerae sialidase periodate reactive and metachromatic.

ii. Slowly digestible with Vibrio Cholerae sialidase.

i) Periodate reactive

ii) Periodate unreactive

iii. Resistant to Vibrio cholerae sialidase.

1) Rendered metachromatic and susceptible to enzyme by prior saponification.

2) Sialidase resistant after saponification.

a) Periodate reactive

b) Periodate unreactive

A brief survey of literature

OVARY

The oogonial proliferation, oogenesis and folliculogenesis in nonmammalian vertebrates have been reviewed by Tokarz (1978). Oogonia are found in the ovaries of possibly all adult reptiles (Munson, 1904; Loyez, 1906; Boyd, 1940;

Miller, 1948; Franchi et al., 1962; Varma, 1970). These oogonia are located in one or more discrete regions known as germinal beds (Loyez 1906). Besides oogonia, oocytes in varying stages of development are found in the germinal beds of reptiles (Boyd, 1940; Miller 1948; Varma 1970; Jones et al., 1976). The entrance of oogonia into meiotic prophase and differentiation to primary oocytes continues in the ovaries of adult reptiles (Franchi et al., 1962). Following differentiation, the primary oocytes of reptiles complete a series of changes typical of meiotic prophase (Loyez, 1906; Boyd, 1940; Arronet, 1973). By early diplotene, however, the intercellular bridges are disrupted and primary oocytes are isolated from one another by investing follicular cells (Filosa and Taddei 1976). During the prolonged diplotene stage, the oocyte greatly enlarges (Franchi et al., 1962). Oocytes contained a yolk nucleus which develops in the juxtra nuclear cytoplasm (Guraya 1963a). The Oogenesis varies with the stage of the seasonal reproductive cycle. (Miller, 1948; Goldberg, 1970; Cieslak, 1945; Bragdon, 1952).

Folliculogenesis begins when prefollicular cells are derived from the germinal epithelium (Loyez, 1906; Boyd, 1940; Miller, 1948; Betz, 1963; Varma, 1970). They attach to oocyte and surround it; usually before or at the onset of diplotene stage. (Loyez, 1906; Boyd, 1940; Hubert, 1971 Arronet, 1973) forming the "primordial follicle."

The development and structure of the follicular epithelium vary greatly in different groups of reptiles, the literature on which is reviewed by Guraya (1978). This layer exhibits greater development and complexity in saurians and ophidians than in chelonians. The 'primordial follicle' has single layer of flattened follicle cells having similar morphology. After the oocyte has left the germinal bed, the follicular epithelium becomes first bilayered and then multilayered in the ovaries of lizards and snakes (Miller, 1948; Brambell, 1956; Guraya 1958, 1959 a, b, 1965 b, 1968 e, Betz 1963; Wilhoft, 1963; Ghiara et al., 1968, 1970; Varma, 1970 b; Hubert, 1971 a, 1973 a, 1976; Blanc, 1971 a, b; Gerrard et al., 1973; Olmo and Taddei, 1974; Jones et al., 1975 a) but in Chelonia it remains single layered (Guraya, 1959 c; Rahil and Narbaitz, 1973). There are three types of cells in the follicular wall of lizards and snakes; small, intermediate and pyriform cells. The small follicle cells can be further distinguished into basal and apical cells, depending on their localization in the follicular epithelium (Jones et al., 1975 a). The various cell types can be distinguished from their size, shape and other morphological features (Guraya, 1978). Only small follicle cells in the follicular wall divide (Hubert and Andrivon, 1971; Olmo and Taddei, 1974). These small cells are transformed into the pyriform follicle cells (Hubert and Andrivon, 1971; Jones et al., 1975 a;

Tokarz, 1977). Their proliferation occurs under the influence of FSH (Tokarz 1977). Each flask-shaped pyriform cell has a narrow protoplasmic prolongation which appears to traverse the zona pellucida. Thus the cyloplasm of the oocyte and pyriform cells becomes confluent through these cytoplasmic prolongations. (Varma, 1970 b; Gerrard et al., 1973; Guraya and Varma, 1976) or inter cellular bridges (Porte and Zahnd, 1961; Ghiara et al., 1968, 1970; Hubert, 1971 b, 1973 a, 1976; Blanc, 1971 a, b; Neaves, 1971; Taddei, 1972 a; Jones et al., 1975 a).

In vitellogenic follicles, the pyriform cells become smaller, and the follicular epithelium becomes single layered and monomorphic (Betz, 1963; Varma, 1970 b; Blanc, 1971 a, b; Gerrard et al., 1973; Guraya and Varma, 1976). All that remains are small follicle cells. Pyriform follicle cells degenerate and disappear and their degeneration is one of the reasons for the reduction of the follicular epithelium (Ghiara et al., 1968, 1970; Hubert, 1971 a, 1973 a, 1976; Gerrard et al., 1973; Jones et al., 1975 a). In the fully mature eggs, the cells of the follicular epithelium are stretched, reduced in size, and separated from each other.

In reptiles zona pellucida is formed between the oocyte surface and follicular epithelium. In the early stages of oocyte growth, the zona pellucida forms a homogeneous layer. But, with further growth, it is differentiated into outer homogeneous layer and inner striated layer (Gabaeva,

1970; Varma, 1970 b; Jones et al., 1975 a; Guraya and Varma, 1976).

Ultrastructural studies have revealed that the basic processes involved in the formation of the zona pellucida in the reptilian follicle are same as those in fishes and amphibians (Blanc, 1971 a, b; Hubert, 1971 b, Neaves, 1971; Rahil and Narbaitz, 1973). The oocyte surface forms many microvilli; and simultaneously the follicle cell processes are formed (Ghiara and Filosa 1966; Ghiara et al., 1968, 1970). Electron dense zona material is deposited between the microvilli and follicle cell processes. In vitellogenic oocytes the microvilli are greatly increased in number and size and form zona radiata. As the follicle grows in size, the surrounding stromal tissue becomes organized in the form of fibrous theca externa (Varma 1970 b, Blanc 1971 a, b; Guraya and Varma 1976; Jones et al., 1975 a; Tokarz 1977). The mast cells are found in the theca of maturing follicles in the ovaries of the lizard Anolis carolinensis (Jones et al., 1975 b). They increase as the follicles grow. Oocyte growth in reptilians has been reviewed by Wallace (1978). The changes in serum chemicals are the function of season or estrogen stimulation. (Dessauer, 1970). Vitellogenin like macro-molecules have been observed in the serum of the ribbon snake, during the period of ovarian enlargment (Dessauer and Fox, 1959), in estrogen treated turtles and lizards (Urist and Schjeide,

1961, Hahn, 1967). They also found 43% lipid and 1.7% protein-phosphorus in vitellogenin preparation. The tracer studies have shown in lizard Anolis carolinensis that the tracer substances pass through the extracellular spaces of the follicular epithelium and are incorporated into the developing oocyte by micropinocytosis (Neaves, 1972; Rahil and Narbaitz, 1973). Simkiss (1967), reviewed the work on yolk proteins and concluded that the yolk proteins originate in the liver, but they may be modified in different ways in different animals by the other influences like phosphorylation. The extent of the rise in the level of serum calcium caused by transporting the yolk proteins as Ca^{++} complexes, is greatest in the reptiles (Dessauer et al., 1956). The calcium metabolism and yolk production has been studied in reptiles (Urist and Schjeide, 1961; Dessauer et al., (1956) and that is estrogen dependent (Dessauer and Fox, 1959; Urist and Schjeide, 1961).

The presence of cortical granules is a specific feature of the lecithal eggs in number of non-mammalian vertebrates (Arndt, 1960; Kille, 1960; Motomura, 1952, Osanai, 1960, Wartenberg and Schmidt, 1961; Wartenberg, 1962). Katagiri, (1959) and, Kemp and Istock (1963, 1967,) reported that the cortical granules erupt after fertilization and exude their contents into the perivitelline space.

The follicular atresia has been reviewed by Byskov (1978) and Saidapur (1978) in reptiles. The follicular

atresia are studied in Indian lizards by Varma, (1970); Varma and Guraya, (1973); Gouder and Nadkarni, (1976). Follicular atresia may occur in the follicles of any stage of development and is more frequently seen in follicles with polymorphic granulosa or in mature follicles that have failed to ovulate. Betz (1963) has given a detailed description of process which can be generalized in other reptiles, (Guraya, 1965; Goldberg, 1970; Varma, 1970; Gouder and Nadkarni, 1976).

Hormonal control

Tokarz (1978) has briefly discussed the Hormonal control of oogonial proliferation, oogenesis and folliculogenesis. Callard et al., (1972 a, b), Callard and Lance (1977), Licht et al., (1977); Crews (1978); and Lance and Callard (1978) also reviewed the hormonal control over reptilian ovary. The sheep pituitary extract and estrone treatment increased the number of oogonia and mitotic figures in immature alligator (Forbes, 1937, 1938). Jones et al., (1976) have also shown the mamalian FSH stimulation of oogenesis either directly or indirectly through stimulation of ovarian steroid. Mamalian FSH treatment to hypophysectomized A. carolinensis increases the oogonial number significantly (Tokarz, 1977; Jones et al., 1975, 1976).

The granulosa cells of all reptiles are probably the major site of steroid synthesis during vitellogenesis(Morat

1969; Jones et al.; 1974), but Guraya (1976) believes that the interstitial gland derived from atretic follicles is the source of ovarian steroids. Lupo di prisco et al., (1968) have identified eight of the known mammalian sex steroids. Lance and Callard (1978) have reviewed the harmonal experimental induction in number of reptiles and have concluded that in female reptiles, estrogen secretion by the granulosa and/or theca of vitellogenic follicle and progesterone secretion by the preovulatory follicle and corpus luteum are probably of universal occurrence. It is also likely that ovulation is preceded by a surge of gonadotropin secretion in all reptiles; but still it is not clear how reptilian LH and FSH regulate the various stages of the cycle.

OVIDUCT

The oviducts in sub-mammalian vertebrates are derived from the Mullerian ducts and develop under the influence of estrogens. The oviducts are surrounded by a sheath of smooth muscle fibres which with rhythmical contractions, can propel objects like eggs towards the exterior.

Hormonal Control

Generally, the ovariectomy results in regression of the oviducts, whereas administration of estrogens stimulated the growth and development of these structures. Panigel

(1956) showed that progestrone was more effective than estrogen; he further suggested that estrogens might act most effectively on the glandular portion of the oviduct and progesterone on the muscle wall. The effects of female sex hormones in reptiles have been reported by Turner, (1935); Mellish and Meyer, (1937); Forbes (1938); Gorbman, (1939); Evans and Clapp, (1940); Panigel, (1956).

The inner layer of the oviduct contains numerous glandular cells and may be modified in various ways so, that it contributes to the well being of the egg. The oviparous species, segmental differences in function may occur along the length of the oviduct, such as one associated with the formation of albumen glands and the secretion of hard outer shell, (Van Tienhoven, 1968). The oviducts of lizards and snakes lack well defined albuminous glands in the upper portions (Goin and Goin, 1962).

These glands are involved in the development of egg. The egg yolk is the only source of nutriment to the young. In most species the eggs increase in size considerably due to water uptake from a network of maternal blood vessels in the area of oviduct adjacent to the eggs. The water intake takes place through the porous shell (Goin and Goin, 1962).

The eggs of geckos are soft when laid, with lower content of calcium carbonate as compared to the bird egg shells but harden after exposure to air (Sadleir 1969).

Histochemical reports on the ovaries and oviducts of lizards.

Ovary

Guraya (1963 a) reported the yolk nucleus containing homogenous spherical mass of protein and RNA in developing oocytes; he also noted the association of mitochondria with yolk nucleus. Synthesis of RNA and proteins has been reported in the primary oocytes (Hubert and Andrivon, 1971).

The ultrastructure of follicular wall cells has shown the presence of mitochondria, rough endoplasmic reticulum, (ER), smooth ER, free ribosomes, well developed Golgi complex, glycogen like particles and annulate lamellae. The maximum development of all these organelle is found in pyriform cells. (Porte and Zahnd, 1961; Ghiara and Taddei, 1966, 1970; Ghiara et al., 1968; Hubert, 1971 b, 1973 a, 1976, Blanc, 1971 a, b; Neaves, 1971; Taddei, 1972 a).

Histochemical techniques have demonstrated the presence of abundant RNA and lipid bodies (phospholipids) in the follicular epithelium of lizards; snakes and turtle (Guraya, 1958; 1959 a, b, c; 1961; 1965 b; 1968 e; 1976 a; Blanc, 1971 a, b; Guraya and Varma, 1978). The histochemical, ultrastructural and autoradiographic techniques have indicated that the follicle cells are very active in the synthesis of RNA proteins, glycogen and phospholipids which are apparently contributed to the growing oocyte (Guraya, 1958; 1959 a, b, c; 1961; 1963; 1965 b; 1968 e; 1976 a; 1977 a; Blanc 1971 a;

Ghiara and Taddei, 1966; Guraya and Varma, 1978; Ghiara et al., 1968, 1970; b, c; Hubert, 1970; 1971 a, b; 1973 a, b; 1975 a, b; 1976; Hubert and Andrivon, 1971; Taddei, 1972 a).

The activity of 3β-HSDH has been reported in the follicular epithelium of developing oocytes (Botte and Delrio 1965; Callard, 1966; Morat, 1969; Callard et al., 1972 and Jones et al., 1974), indicating the development of follicular wall in the steroid synthesis.

The zona pellucida which is formed between the oocyte surface and follicular epithelium contains carbohydrates and proteins (Guraya, 1958; 1959, a, b, c). Faratade and Nalawade (1980) have demonstrated the carbohydrates in cortical granules.

The presence of alkaline phosphatase, acid phosphatase, adenosine triphosphatase and 5'-nucleotidase in the theca interna of developing, mature and postovulatory follicles (or corporalutea) and distribution of Δ5-3β hydroxy steroid dehydrogenase, 17β-hydroxysteroid dehydrogenase, 11β-hydroxy steroid dehydrogenase, glucose-6-phosphate dehydrogenase, isocitrate dehydrogenase, lactate dehydrogenase and NADH diphosphatase enzyme activities in ovaries of H. flaviviridis has been studied by Gouder and Nadkarni (1976). They found the enzyme activities in theca interna, granulosa cells of large preovulatory, post-ovulatory and atretic follicles,

interstitial cells of the ovarian stroma and ooplasm of the growing oocytes indicating their steroid-synthesizing capacity.

Saxena (1979) studied the cortical zone of oocytes of H. flaviviridis. The cortical zone consists of proteins, lipoproteins, carbohydrates, fatty yolk, RNA and DNA. He has only mentioned the PAS reactivity of cortical zone and zona pellucida.

Oviduct

Jahangeer et al., (1976) studied seasonal alterations in oviducal glycogen in U. hardwickii. They noted the two peaks of milligram pencent glycogen content, one during breeding season and the other during hibernation. Botte (1973) distinguished four regions in oviducts of L. sicula viz. infundibulum, tuba, uterus and vagina. The mucosa contained two types of cells i) Ciliated ii) Secreting in tuba, uterus and vagina, the latter type elaborated only acidic mucins in tuba but acidic and neutral mucins in uterus and vagina. The oviducal glands in the middle part of the tuba elaborated albumen, whereas the glands in the terminal part of the tuba and uterus are involved in shell organization. Callard and Leathem (1970) studied glycogen content and two enzymes in oviducts of snake, Natrix sipendon pictiventris. They noted ~~a~~ increase in glycogen content in preovulatory animals ~~than~~ winter or spring animals and remained higher during pregnancy.

Ian and Leathem (1966) reported β-glucuronidase activity in oviducts of ovoviviparous snakes during pregnancy and oviducts of Natrix sipendon pictiventris respectively. Botte (1973) reported acid phosphatase in the oviduct of L. sicula.

Reasons to undertake the Present Problem

The reptilia class shows variations in the reproductive patterns. Mainly oviparity, ovoviviparity and viviparity. So, the reproductive processes in these animals appear to be puzzling. The review of literature shows the frequent morphological anatomical data but histochemical study is scanty. And the detail investigation on the mucosubstances from reproductive organs and accessories during the reproductive cycles appears to be neglected. As proteins, lipids and carbohydrates form the building blocks of animal life and they play an important role in every metabolism of animal and as the mucosubstances form a part of the carbohydrates and protein complexes, their study in a complete metabolic process like reproduction may help to explain the possible process involved in reproduction.

As the scope of the present project is limited one, and the animals had to be studied throughout the reproductive phases only, female of the H. flaviviridis has been selected for study.

The recently developed histochemical techniques are used to study the reproductive organs and their accessories, wherever needed; few other well settled recent techniques have been utilized to study the organs in detail.

Presentation of Work :

This small project is divided into five chapters. First chapter introduction, includes a survey of morphological, anatomical and histochemical data available on the reptiles, and the general ideas about mucosubstances and their recent classification. Second Chapter includes material and methods, where the details of methods used for the study and the material i.e. H.flaviviridis, its collection, dissection, processing of the material to be investigated etc. and the approach of the study is given. The Third Chapter contains the studies on ovaries, detailed observations and possible discussions. The Fourth Chapter consists of studies on oviducts, detailed observations and possible discussion. The Fifth, last Chapter covers summary and concluding remarks. Which is followed by bibliography, which is used throughout the project.