membrane were common. This increase in the thickness of the filtration barrier has been reported in other salt water teleosts (Olsen, Acta Pathol. Microbiol. Scand. 212:815, 1970; Bonga, Z. Zellforsch. 137:563, 1973).

Glomeruli from fish injected with either native or cationized ferritin resulted in retention of the tracer by the GCW as evidenced by the lack of ferritin accumulation in the urinary space, tubular lumen and tubular epithelial cells (Figures 2 to 4). The cationized ferritin accumulated in clusters along endothelial cell membranes and the blood side of the glomerular basement membrane (Figure 4). No such binding of the native ferritin was observed. This binding of the positively charged ferritin to the GBM confirms the anionic nature of the GCW.

Thus we conclude that the anionic antifreeze peptides are retained in the circulation through their repulsion from the anionic GCW of the winter flounder. This research was supported by a University of Illinois Graduate College Dissertation Research Grant (to DHP) and NSF Grant PCM 77-25166 (to ALD).

27 TOPOGRAPHIC DISTRIBUTION OF STEROIDOGENIC ENZYMES IN SQUALUS TESTIS: STRUCTURAL AND FUNCTIONAL CORRELATIONS.

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The role of steroids within the testis remains a problem of great interest in the field of male reproduction. It is generally agreed that androgens derived primarily from interstitial Leydig cells are required at some stage of spermatogenesis, and androgen receptors have recently been located in both Sertoli cells and germ cells. A less well-known fact is that estrogen (formed by aromatization of androgen) is a normal product of the testis and, in certain species, comprises a large proportion of total testicular steroid secretion (e.g., stallion, boar, Necturus). Because estrogen receptors are located in Leydig cells and several enzymatic steps leading to androgen biosynthesis are inhibited by estrogen, it seems likely that it is part of a short feedback loop governing further androgen output. Androgens and estrogens are therefore interrelated biosynthetically and functionally. There is some controversy over whether Leydig cells or Sertoli cells are the primary source of intratesticular estrogen.

Most of what we know about testicular functions has been obtained from studies in laboratory mammals. In these species, all stages of germ cell development are present simultaneously from the onset of puberty. Moreover, their gross distribution throughout the testis is uniform, although on a microscopic level it can be seen that only certain stages occur together (specific cell associations) in any one segment of the semiferous tubule. Using these species it is technically difficult, without disrupting the normal relationship between the tubules and interstitium, to study how the steroidal microenvironment might change during the cycle of the seminiferous epithelium. Certain nonmammalian vertebrates would seem to be natural models for investigating this problem. In the shark, for example, germ cells in different stages of development are topographically segregated within the testis. In earlier studies we found that Squalus testis synthesized substantial amounts of estrone and estradiol-17β from ^{3}H-androgen (Callard, G.V., et al., Endocrinology 103:2283, 1978) and that aromatase activity was greatest in microsomes prepared from regions in which germ cells were undergoing meiotic divisions (Callard, G.V., and Petro, Z. The Bulletin, MDIBL 19:38, 1979). The purpose of the present investigation was to expand our study of steroidogenesis to include other key enzymes leading to androgen and estrogen bio synthesis and, using light and electron microscopy, to characterize the steroid-secreting cell types.

The testes of male sharks captured in July were sectioned transversely and each slice further separated into 3 regions based on the following criteria: opacity; color; position in relation to the epigonal tissue. Tissues were homogenized in sucrose/phosphate buffer, and microsomes prepared by centrifugation (100,000 x g x 60 min) were resuspended in phosphate buffer. Whole homogenates of free spermatozoa collected from the vas deferens

served as germ cell controls. [^{3}H]-Progresterone and [^{3}H]-17α-hydroxyprogesterone (both 100 mCi/mmol; final concentration = 10 μM) were used to assay 17α-hydroxylase and C-17, 20-lyase, respectively. [^{3}H]-Androstenedione (11 Ci/mmol; final concentration = 266 nM) was the substrate for aromatization. To start the reaction, 50 μl of phosphate buffer containing the radiolabeled substrate and an NADH/NADPH generating system were added to 450 μl of tissue protein. The incubations were carried out at 22°C for 10 and 20 min (17α-hydroxylase, C-17, 20-lyase) or 30 and 60 min (aromatase). Zero-time incubates served as incubation controls. Reaction products were measured after extraction, thin layer chromatography, and phenolic partition (estrogens only). In selected samples, authenticity of the steroid products was verified by repeated crystallization to constant specific activity. Details of the assay procedures have been described (Callard, G.V., et al., Biol. Reprod. 23:461, 1980). Dissected testicular fragments or whole testes were fixed for 24 hr in Sorensen's phosphate buffer (pH 7.4) or seawater containing 5% glutaraldehyde. Following post-fixation and dehydration, the tissues were embedded in glycol butyl methacrylate for light microscopy or Epon for light and electron microscopy by previously published procedures (Callard, G.V., et al., 1980).

Micrographs in Figure 1 (A-D) show the stages of germ cell development in Squalus testis in July. The composition of the dissected samples used for the steroidogenic assays was as follows: zone I - A, spermatogonia; zone II - B and C, mainly primary and secondary spermatocytes with some round spermatidfilled labules; zone III - C and D, some round spermatids, mainly spermatozoa. Electron microscopic analyses are still in progress. The topographic distribution of steroidogenic enzyme activity is summarized in Table 1. Following incubation with

TABLE 1.--Topographic distribution of steroidogenic activity in Squalus testis and in free sperm

		STEROIDOGENIC ACTIVITIES[a]			
Zone	Components	17α-hydroxylase (pmol/min/mg)	20β-oxidoreductase (pmol/min/mg)	C-17, 20-lyase (pmol/min/mg)	aromatase (fmol/min/mg)
I	spermatogonia	5.84 ± 0.81	2.78 ± 0.43	2.90 ± 1.33	2.93 ± 0.50
II	spermatocytes some round spermatids	4.26 ± 0.61	4.44 ± 1.04	6.89 ± 1.10	7.10 ± 0.40
III	some round spermatids spermatozoa	10.76 ± 2.05	25.82 ± 0.62	24.18 ± 5.61	2.83 ± 0.40
	free sperm	2.35[b]	165.14[b]	-0-[b]	-0-[b]

[a]Product yield per mig microsomal protein (mean ± sem) for separate microsomal preparations from 3 animals.

[b]Product yield per mg homogenate protein for a single pool of free sperm.

labeled progesterone, both 17α-hydroxylated and 20β-hydroxylated products were measured. Within the testes, activities for both enzymes were somewhat higher in the most advanced testicular zone; however, 20β-hydroxysteroid oxidoreductase (dehydrogenase) predominated in free sperm. As a consequence of lyase activity, small amounts of androstenedione and testosterone were found in testicular microsomes from all zones but product yields were greatest in samples that were most mature. Although no lyase was detectable in free sperm, high yields of a polar metabolite with the characteristics of 17α, 20β-dihydroxy-pregn-4-ene-3, 20-dione was tentatively identified. Estradiol-17β and smaller amounts of estrone were produced from radiolabeled androstenedione in all 3 testicular regions, although yields were highest in zone III. No aromatization was detectable in sperm collected from the vas.

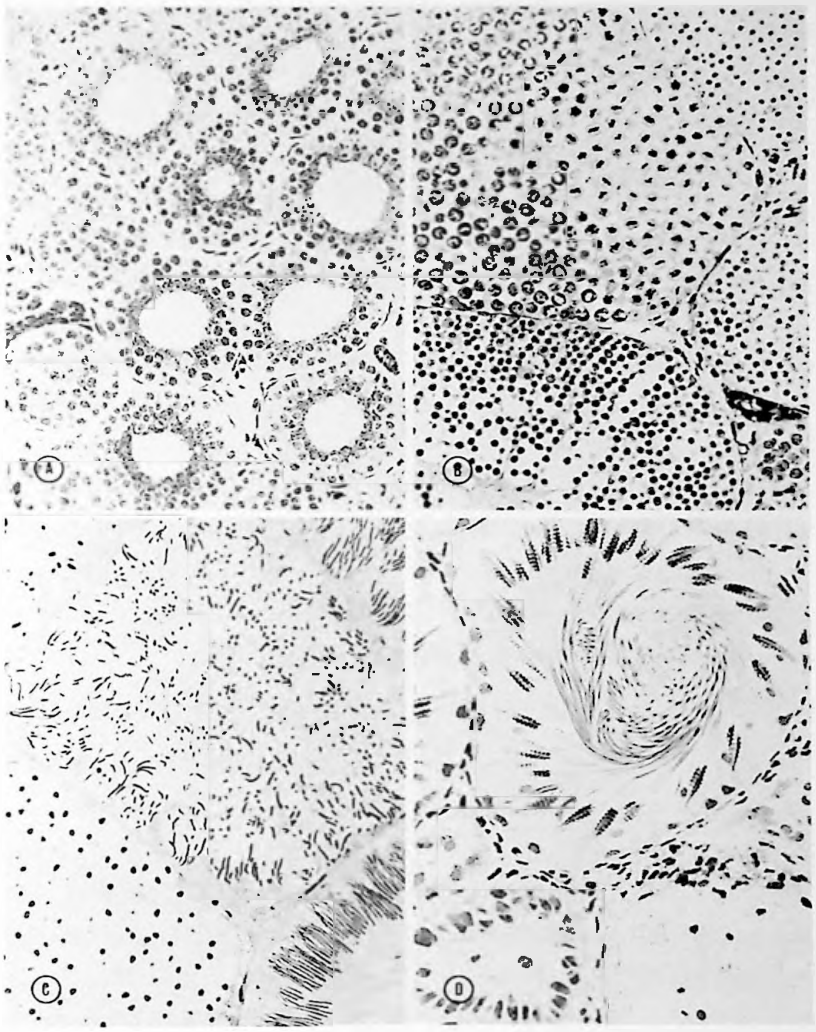
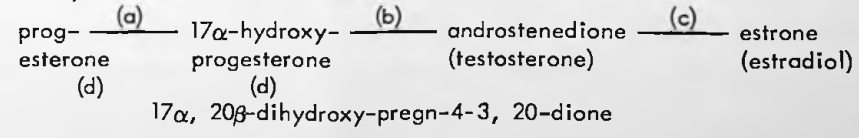


Figure 1.--Transverse sections through different regions of the shark testis (Squalus acanthias) (x180). (A) Immature lobules containing spermatogonia. Sertoli cell nuclei form a ring adjacent to the lumen while germ cells are located peripherally. Cyst organization is not apparent at this stage. (B) Lobules containing primary spermatocytes in the pachytene stage (lower right corner) or undergoing meiotic divisions (upper left) to produce secondary spermatocytes (lower left). Organization of clones of germ cells into cysts is now apparent within each lobule. Note that meiosis is synchronized in all cells of any one cyst, although several stages of meiosis are represented in the same lobule. Also, adjacent cysts tend to be synchronized. (C) Lobule containing spermatids at varying stages of differentiation, ranging from round spermatids (lower left) to elongate spermatids (center) to eventually form distinct bundles of spermatozoa (upper and lower right corners). The heads of maturing spermatozoa are all oriented toward the periphery of the lobule. (D) In upper part of micrograph, mature lobule containing tight bundles of spermatozoa embedded in Sertoli cytoplasm. Sperm tails form whorls in the lumen. Sertoli nuclei are now obvious adjacent to the basement membrane. Each bundle of spermatozoa is seen to be associated with one Sertoli nucleus, together forming a single cyst. Following spermiation lobule diameter is much reduced and the remaining Sertoli cells undergo degeneration (lower half of micrograph).

Pathways of steroid metabolism in shark testis can be summarized as follows:



Rate limiting steps in the synthesis of androgen and estrogen are (a) 17α-hydroxylation; (b) cleavage of the side-chain at C-17 (lyase action); and (c) aromatization. Testicular microsomes have all three enzymes. Whereas 17α-hydroxylase and lyase are greatest in zone III, the present experiment confirms our earlier results showing that aromatization is highest in regions with primary or secondary spermatocytes. It is unlikely that germ cells within the tissue samples account for these activities since free sperm are incapable of forming C-19 steroids or of aromatizing added androgen. Instead, free spermatozoa divert substrate via (d) a 20β-reducing pathway considered to be primarily inactivating since it leads to products which are not substrates for lyase action and which, in fact, inhibit this important step in androgen biosynthesis. The acquisition of 20β-oxidoreductase activity may occur in maturing germ cells while they are still within the testis since 20β-hydroxylated products in zone III comprised mainly of spermatozoa are much higher than in other zones containing less mature germ cells. Examination of the testis with the electron microscope is still incomplete; however, it can be seen from the light micrographs in Figure 1 that the interstitium is undeveloped. Although undifferentiated Leydig cells, if present, might synthesize small but important quantities of steroids, it is more likely that Sertoli cells which are well-developed are responsible for the majority of the androgen and estrogen synthesizing activity in Squalus testis. These in vitro assays suggest that the actual steroidal milieu of the different regions of Squalus testis may be quite specific, depending on the stage of germ cell development, and demonstrate the usefulness of unconventional animal models in obtaining new information (NSF-PCM 78-23214 and NICHD 15595).

PREPARATION OF FLOUNDER RENAL TUBULES FOR THE COLLECTION OF SECRETED FLUID

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While several investigators have obtained evidence for fluid secretion in the kidneys of fish (Hickman, C.P., Can. J. Zool., 46:427, 1968; Forster, R.P., J. Cell. Comp. Physiol., 42:487, 1953; Schmidt-Nielsen, B. and J.L. Renfro, Am. J. Physiol., 228:420, 1975; Renfro, J.L., Am. J. Physiol., 238:F92, 1980), the measurement of tubular secretion rates and the collection of secreted fluid for analysis has not been possible. Using the methods of working with isolated renal tubules (Burg, M., et al., Am. J. Physiol., 210:1293, 1966), I have measured the secretion rates and the concentrations of Na, Cl, Mg, and S* in fluid secreted by flounder renal tubules.

Direct demonstration of tubular fluid secretion. Renal tubules from the winter flounder Pseudopleuronectes americanus were isolated by microdissection and bathed in flounder Ringer's (in mmoles/ℓ NaCl 145, $NaHCO_3$ 20, KCl 5, Na_2HPO_4 1.65, Na_2HPO_4 0.3 $MgSO_4$ 1, glucose 10). The lumen was perfused with light mineral oil. When perfusion of the lumen was stopped, fluid droplets were observed to split the column of oil in the lumen at several places (Fig. 1). As the original fluid droplets expanded laterally and newly formed droplets appeared, the continued fluid secretion forced the flow of fluid and oil towards the open end of the tubule, eventually clearing the lumen of oil. This was consistently observed in all tubules filled with oil (n = 6).

Collection of secreted fluid. When secreted fluid (SF) was collected, the tubule lumen was not initially filled with oil. Instead, one end of the tubule was closed by pulling it into a narrow glass pipet and the other end (open) was aspirated into a snug fitting holding pipet and sealed from the bath with Sylgard (Figure 2). Bathed in Ringer solution the tubules generated their own luminal fluid, which flowed out of the open end of the tubule where it accumulated under oil. Every tubule studied secreted fluid (n = 36). Fluid secretion averaged 27.1 ± 3.2 pl/min-mm tubule length in those tubules in which control fluid secretion rates were measured (19

*Electron probe analysis measures concentrations of elements (S) and not the concentrations of chemical species such as SO_4^{-2}.