THE MALE RABBIT

II. HISTOCHEMISTRY OF THE EPIDIDYMIS AND AMPULLA AS INFLUENCED BY SPERM OUTPUT

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Summary. Fifteen rabbits were allotted equally to three groups: (I) two successive ejaculations every 48 hr, (II) sexual rest, and (III) unilateral vasectomy followed by sexual rest. One month later they were killed. Acid phosphatase was found in the Golgi area of epididymal columnar cells. Scattered columnar cells with intense acid phosphatase activity were also found in epididymal regions 1 and 6 and they were most numerous after vasectomy. Alkaline phosphatase was found apically in epididymal columnar cells and in their stereocilia. The weaker reaction in the proximal ductus deferens and epididymal regions 7 and 8 of the ejaculated rabbits supports the concept that regular ejaculation diminishes the rate of sperm resorption within the epididymis. The ampullae of ejaculated rabbits contained more alkaline phosphatase than those of non-ejaculated animals. Cytoplasmic PAS-positive droplets were most abundant in regions 1 and 7 of the epididymis. Glycogen was found only in region 6. Little evidence was found for a holocrine cell cycle in the epididymis. Large, PAS-positive cells, considered to be phagocytes, were most common in regions 1 and 6 of the epididymis and were present in increased numbers after vasectomy. Phagocytes may resorb materials emanating from the testis.

INTRODUCTION

A holocrine secretory cycle for certain non-ciliated epididymal cells has been described for several species (Martan & Allen, 1964; Martan & Risley, 1963a, b; Martan, Risley & Hruban, 1964). With rats, daily mating affected the cycle length and changed the secretory characteristics of these holocrine cells. The caput epididymidis apparently has an important role in absorption of fluids and substances emanating from the testis (Burgos, 1964; Ladman & Young, 1958; Mason & Shaver, 1952; Nicander, 1965). Other reports have indicated that the cauda epididymidis is involved in the resorption of non-ejaculated spermatozoa (Orgebin-Crist, 1961; Gaddum & Glover, 1965; Lambiase, 1967) and that the number of spermatozoa resorbed may vary inversely with sperm output (Amann & Almquist, 1962).

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If spermatozoa are resorbed at a variable rate within the epididymides of sexually active and sexually rested males, the epididymal epithelium might reflect this in its histochemical characteristics. In previous studies where the sexual status of the animals was controlled, species unadaptable to semen collection by artificial vagina were used. Thus, histochemical differences in the epididymal epithelium could be related only to mating frequency. Mating frequency may not accurately portray sperm output. Quantitative data on sperm output can be obtained with rabbits. Therefore, the histochemistry of the epididymis and ampulla was investigated using rabbits known to have widely different sperm outputs.

MATERIALS AND METHODS

Fifteen male New Zealand White rabbits, accustomed to semen collection by artificial vagina, were divided equally among three treatment groups. Group I rabbits were ejaculated twice successively every 48 hr for 29 days. Two false mounts preceded each ejaculation (Amann, 1966). The gel plug, if present, was removed from the ejaculate which was then transferred into a graduated cylinder. The collection tube and the liner of the artificial vagina were flushed with distilled water. The combined ejaculates, including flushings, were diluted with distilled water to a total of 50 ml. Sperm concentration of the diluted semen was determined from haemocytometer counts and sperm output was calculated. Group II rabbits were sexually rested for 30 days. Group III rabbits were sexually rested for 30 days after unilateral vasectomy about 3 to 5 cm distal to the cauda epididymidis. Vasectomy was through a midventral abdominal incision.

At the end of the experimental period, animals were killed with Nembutal and their epididymides and ampullae removed. Rabbits in Group I were killed 2 hr after the last ejaculation. Each epididymis was cut into two parts: (a) the caput and proximal corpus epididymidis and (b) the distal corpus and cauda epididymidis. For rabbits in Groups I and II, tissues from one side were prepared for examination of periodic acid-Schiff (PAS) positive materials while the contralateral tissues were used for enzyme studies. For Group III animals, both vasectomized and control sides of a rabbit were treated in the same way, three rabbits being used for enzyme studies and two for PAS staining. Each Group III rabbit served as its own control.

Tissues for enzyme studies were frozen on cryostat tissue holders at -20°C , sectioned at $8\text{ }\mu$ on a cryostat microtome, thawed on clean glass slides and allowed to air dry. Histochemical procedures using α -naphthyl phosphate as the substrate were: (a) azo dye method for acid phosphatase (Barka & Anderson, 1962) using staining times of 0, 2.5, 5.0, 10.0 and 20.0 min and (b) a modification of the azo dye method for alkaline phosphatase (Pearse, 1960) using staining times of 0, 2.5, 5.0, 7.5 and 10.0 min. Control sections for acid phosphatase were simultaneously incubated in 0.01 M NaF. Alkaline phosphatase controls were prepared by omitting the α -naphthyl phosphate from the staining solution. Phosphatase-stained sections were lightly counterstained with Harris haematoxylin and mounted in glycerin jelly.

Tissues used for PAS staining were quenched and then freeze-substituted for 15 days in Lison's Gendre fluid (Pearse, 1960). They were then cleared, embedded in Tissuemat and sectioned at 10 μ . The PAS-stained (Pearse, 1960) slides were counterstained with Harris haematoxylin. Control slides were incubated in malt diastase for 1 hr at 37° C to remove glycogen or extracted 16 hr at 58° C with a 1:1 mixture of methanol and chloroform to remove glycolipids.

The localization and intensity of the staining reactions were evaluated in each of the eight histologically distinct regions of the rabbit epididymis. These regions were described by Nicander (1957).

RESULTS

The experimental treatments allowed comparisons among rabbits with: (a) essentially a maximum daily sperm output, Group I; (b) a relatively low sperm output with sperm loss possible only through masturbation and micturition, Group II and non-operated side in Group III; and (c) no external loss of spermatozoa, vasectomized side in Group III.

Daily sperm outputs for the five Group I rabbits averaged 44, 56, 103, 117 and 122×10^6 spermatozoa per day. Mean body weight for the fifteen rabbits was 3.64 kg.

Post-mortem observations revealed that the cauda epididymidis and the ductus deferens on the vasectomized side of all Group III rabbits were enlarged and turgid. Because of this distension by spermatozoa, the volume of the cauda epididymidis on the vasectomized side exceeded that on the control side by 50 to 300%. However, this distension did not appear to affect the histochemistry of these tissues.

Histochemistry of the epididymis

Acid phosphatase activity was located immediately above the nucleus of the columnar cells in all regions of the epididymis and also extended apically in regions 4, 6, 7 and 8. The columnar cells of region 3 had the highest acid phosphatase activity and a strong perinuclear reaction was obtained after 5 min of staining. After 20 min, regions 4, 6 and 7 had moderate staining reactions while those of the efferent ducts, regions 1, 2, 5 and 8 of the epididymis and the ductus deferens were only weak to moderate. Some weak activity also was detected basally in regions 5 and 6. The stereocilia were practically devoid of acid phosphatase. Basal cells generally were negative for acid phosphatase. Certain columnar cells with a very intense cytoplasmic acid phosphatase reaction were scattered in the epithelium of the proximal portion of region 1, in distal region 5 and in region 6 (Pl. 1, Fig. 1). They were particularly numerous in the vasectomized side of Group III rabbits. The staining of these cells was considerably reduced by simultaneous incubation with 0.01 M NaF.

Accumulations of moderately to strongly tinted cells were found, usually basally, in regions 1 and 6 (Pl. 1, Fig. 2). Their cytoplasm retained its atypical colour even after simultaneous incubation with NaF. In the remainder of this report, such a cell will be referred to as a phagocyte.

Compared with all other epididymides, those from the vasectomized side of Group III rabbits contained (a) a greater number of phagocytes and (b) more of the scattered cells which stained intensely for acid phosphatase. There were no differences in acid phosphatase activity or localization between Group I and Group II animals.

In all regions, diffuse staining representing alkaline phosphatase was uniformly distributed at the extreme apical end of the columnar epithelial cells and in their stereocilia. Fine particles also were present near both poles of the columnar cells. Positive basal cells were not observed. The phagocytes had a yellow, granular appearance which was not a true positive staining reaction. They were particularly numerous in tissue from the vasectomized side of Group III rabbits.

The epithelium of the efferent ducts was negative for alkaline phosphatase and in region 1 of the epididymis only a weak reaction was obtained after incubation for 10 min. Regions 2 (Pl. 1, Fig. 3) and 4 had the strongest alkaline phosphatase activity; the reaction was intense after 2.5 min incubation. Regions 3 (Pl. 1, Fig. 4) and 5 were less reactive than regions 2 and 4 since only moderate to strong reactions were obtained after incubation for 5 min. In region 6, the reaction was strong to intense after 2.5 min incubation. Treatment differences for alkaline phosphatase were found only in regions 7 and 8 of the epididymis and in the ductus deferens. Tissues from the sexually rested rabbits of Groups II and III had strong to intense reactions after 10 min incubation (Pl. 2, Fig. 5). However, in ejaculated rabbits only weak to moderate alkaline phosphatase reactions were obtained (Pl. 2, Fig. 6). In Group III rabbits, no differences were found between the vasectomized and control sides.

Diffuse PAS-positive material of weak to moderate intensity was most prevalent between the nucleus and the tubule lumen, but it also was found below the nucleus in regions 3, 5, 6 and 8. This PAS-positive material and that described below was not glycogen. The stereocilia were strongly stained. In region 1, numerous, fine PAS-positive droplets were found below the nucleus and large, strongly stained droplets characterized the apical area (Pl. 2, Fig. 7). Similar large droplets were occasionally seen apically in region 2. In region 3, the columnar cells sometimes contained PAS-positive basal granules. Small droplets of moderate staining intensity were occasionally found both above and below the nucleus in region 5. Region 7 differed markedly from adjacent

EXPLANATION OF PLATE 1

FIG. 1. Acid phosphatase in region 6 of the epididymis. A diffuse moderate reaction was localized in the apical half of the cells after 10.0 min incubation. Note the intensely stained columnar cells. Basal cells (b) occasionally were stained. $\times 350$.

FIG. 2. Acid phosphatase in region 1 of the epididymis. An inconsistent reaction, varying from negative to weak, was found in the cytoplasm above the nucleus after 5.0 min incubation. Phagocytes (arrows), stained an atypical brownish colour even after incubation with NaF, usually were located near the basement membrane (bm). $\times 550$. The insert shows phagocytes at higher magnification. $\times 1275$.

FIG. 3. Alkaline phosphatase in region 2 of the epididymis. The stereocilia and apical ends of the columnar cells were intensely stained after 2.5 min incubation. $\times 350$.

FIG. 4. Alkaline phosphatase in region 3 of the epididymis. The stereocilia and apical ends of the columnar cells were moderately stained after 2.5 min incubation. Fine basal particles were more prominent. $\times 350$. Compare with Fig. 3 from the same tissue section.

PLATE 1

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(Facing p. 347)

regions. Moderately to strongly stained fine droplets were located above the nucleus and large intensely stained droplets were found below the nucleus (Pl. 2, Fig. 8). Moderately to strongly PAS-staining basal cells were most numerous in regions 1, 6, 7 and 8.

Large, strongly to intensely stained phagocytes were found in sections from almost all animals (Pl. 2, Fig. 7), but were particularly numerous in tissue from the vasectomized side of Group III rabbits. These cells usually possessed irregular, pyknotic, darkly stained nuclei.

Glycogen was present only in the cytoplasm of basal and columnar cells in region 6. No differences associated with experimental treatments were detected.

Histochemistry of the ampulla

With each staining system, the strongest reaction was found in those epithelial cells contiguous with the muscle surrounding the ampulla. A diffuse acid phosphatase reaction was found throughout the cytoplasm after 20 min incubation. The staining intensity for Group I rabbits may have been slightly greater than that for animals in Groups II and III.

Alkaline phosphatase activity in the ampulla differed among treatment groups. After 10 min incubation, the epithelium of Group I animals was moderately stained while that for Groups II and III was weakly stained or negative. Alkaline phosphatase activity was restricted to the apical cytoplasm and the stereocilia.

A moderate, diffuse PAS reaction characterized the epithelium of the ampulla. Strongly to intensely stained PAS-positive material was usually found in the lumen. This extracellular material appeared to be similar in amount and staining intensity regardless of treatment.

DISCUSSION

Amann (1966) reported that the collection of two successive ejaculates every 48 hr was sufficient to maximize daily sperm output in rabbits. The daily sperm output of Group I rabbits, ejaculated at this frequency, averaged 88×10^6 . Thus, the difference in mean daily sperm output between the rabbits in Group I and those in Group II probably approached the physiological maximum.

Acid phosphatase activity generally was localized above the nucleus in the

EXPLANATION OF PLATE 2

FIG. 5. Alkaline phosphatase in region 7 of the distal corpus epididymidis from a sexually rested rabbit. Moderate reactions characterized the apical ends and stereocilia of the columnar cells after 2.5 min incubation. $\times 350$.

FIG. 6. Alkaline phosphatase in region 7 of the distal corpus epididymidis from an ejaculated (twice/48 hr) rabbit. The apical ends and stereocilia of the columnar cells had only a weak reaction after 5.0 min incubation. $\times 350$. Compare with Fig. 5 representative of a sexually rested rabbit.

FIG. 7. PAS-positive materials in region 1 of the epididymis. In addition to a diffuse cytoplasmic reaction, small droplets were below the nucleus and large droplets generally were between the nucleus and tubule lumen. Basal cells and stereocilia also were stained. Phagocytes (p) were strongly stained. $\times 550$. The insert shows a phagocyte at higher magnification. $\times 1275$.

FIG. 8. PAS-positive materials in proximal portion of region 7 of the epididymis. Fine droplets were above the nucleus and large intensely stained droplets (arrows) were below the nucleus. Stereocilia (s) and some basal cells (b) also were PAS-positive. $\times 1275$.

columnar cells of the epididymis. This corresponds to the Golgi region of rabbit (Nicander, 1957) and mouse (Allen & Slater, 1958) epididymal columnar cells. The Golgi complex and associated lysosomes contain large amounts of acid phosphatase (Novikoff, Essner & Quintana, 1964). Nicander (1965) suggested that dense bodies found in the rabbit epididymis probably were lysosomes which digested protein of testicular origin after its absorption into the epididymal epithelium by pinocytosis. The present data suggest that the resorption rate of this protein is not drastically influenced by ejaculation frequency. There were no differences in acid phosphatase activity between Group I and II rabbits.

An epididymal, holocrine cell cycle has been described for rats, mice, guinea-pigs and humans (Martan & Allen, 1964; Martan & Risley, 1963a, b; Martan *et al.*, 1964). However, recent studies of rat epididymides, using both light and electron microscopy, revealed no evidence of a holocrine cell secretory cycle (Fährmann & Schuchardt, 1965; Kreth, 1965). In the present study, columnar epididymal cells with intense acid phosphatase activity were observed. They were most prevalent in regions 1 and 6, but also were seen in regions 2 and 5. Their incidence and morphology appeared to be similar in sexually rested and ejaculated rabbits. In fact, the highest incidence of these acid phosphatase containing cells was in the epididymides from the vasectomized sides of Group III rabbits. These findings are contradictory to the results for rats where the number of holocrine cells was increased by mating (Martan & Risley, 1963a, b). However, the Group I rabbits were killed 2 hr after ejaculation while the greatest increase in holocrine cells in rats was not apparent until 7 to 13 hr after mating.

Evidence from PAS-stained sections also does not support the presence of a holocrine cell cycle in rabbits. Throughout the epididymis, cells were found in the process of being sloughed into the tubule lumen. These cells, however, contained only small amounts of weak-staining PAS-positive material. In mated rats, the intact holocrine cells which were released into the tubule lumen retained their PAS-positive material. Furthermore, the majority of the PAS-positive cells in the rabbit epididymis were ciliated, whereas holocrine cells are non-ciliated (Martan & Risley, 1963b). For other species, a close relationship between acid phosphatase and PAS staining is characteristic of immature epididymal holocrine cells (Martan & Allen, 1964; Martan & Risley, 1963a, b; Martan *et al.*, 1964). In the present study, the intensely acid phosphatase positive cells were predominantly in regions 1 and 6. Columnar cells containing many PAS-positive droplets were most conspicuous in regions 1 and 7. Thus, in rabbits the acid phosphatase containing cells are not necessarily rich in PAS-positive material. Unless the function(s) and staining characteristics of epididymal holocrine cells in rabbits differ from those of other species, the intensely stained acid phosphatase containing cells found in this study probably are not holocrine cells.

The role of alkaline phosphatase in tissues remains uncertain. However, Moog & Wenger (1952) have suggested that alkaline phosphatase is involved in the transfer of organic molecules across cell membranes. Epididymal alkaline phosphatase may function in the membrane transport of sugars and related substances (Allen & Slater, 1958; Bern, 1949; Maneely, 1955). Since alkaline

phosphatase was concentrated in the apical rather than the basal pole of the columnar cells of regions, 2, 4 and 6, these cells might function in the absorption of carbohydrates.

Lambiase (1967) reported that routine collection of two successive ejaculates each 48 hr from rabbits reduced the sperm content of the cauda epididymidis and ductus deferens. Just before two successive ejaculations, these structures contained only 58% as many spermatozoa as did those of sexually rested rabbits. Lambiase (1967) also found that 39% of the spermatozoa produced by the rabbit testes were not obtained by ejaculation at this high frequency and presumably were resorbed within the excurrent ducts.

In the present study, alkaline phosphatase activity in epididymal regions 7 and 8 (corpus and cauda) and the ductus deferens differed among treatments. Rabbits in Groups II and III had stronger reactions in these regions than those in Group I. It is presumed that more spermatozoa were present within these regions, especially region 8, in the sexually rested and vasectomized rabbits than in those rabbits regularly ejaculated. As discussed above, the increased alkaline phosphatase activity in sexually rested rabbits may reflect increased membrane transport. If this is true, more organic material probably was passing through the cell membrane in epididymides from Group II and Group III rabbits than in those from ejaculated rabbits. It seems unlikely that secretion by the epithelium of the cauda epididymidis would be increased during sexual rest or following vasectomy. Under these conditions, absorption by the cell would be more logical. Possibly the material postulated to be entering the cell from the tubule lumen represents breakdown products of spermatozoa. Thus, alkaline rather than acid phosphatase may be more indicative of sperm resorption.

In many species the ampulla secretes fructose and lactic acid (Mann, 1964). Presumably, in frequently ejaculated rabbits the epithelium of the ampulla elaborates and secretes more seminal components than in sexually rested animals. Thus, if alkaline phosphatase functions in membrane transport of sugars, Group I rabbits might be expected to have the strongest alkaline phosphatase reaction. The ampullae of Group I rabbits were more intensely stained for alkaline phosphatase than those of sexually rested rabbits in Groups II and III.

Large, intensely PAS-positive, atypical cells were found which have been referred to as phagocytes. The cytoplasm of these cells apparently was devoid of phosphatase activity. Special staining revealed that these cells did not contain iron or calcium. The function and origin of these phagocytes is unknown. They were found most often in regions 1 and 6. Resorption by the proximal caput epididymidis of fluids and substances emanating from the testis is well established for many species (Burgos, 1964; Ladman & Young, 1958; Mason & Shaver, 1952; Shaver, 1954). However, little evidence has been reported indicating that region 6 has a resorptive function. In the present study, the epithelium of region 6 was infiltrated with phagocytes and also had a high alkaline phosphatase activity. Thus, the phagocytes in regions 1 and 6 of normal rabbits may have a role in absorption of material which enters the columnar cells from the tubule lumen.

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