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Localization of Cellular Retinol-binding Protein and Cellular Retinoic Acid-binding Protein in the Rat Testis and Epididymis

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The distribution of cellular retinol-binding protein (CRBP) and cellular retinoic acid-binding protein (CRABP) in rat testis and epididymis was examined by the peroxidase-antiperoxidase immunolocalization technique. In the testis, cellular retinol-binding protein was localized exclusively in the Sertoli cells. Staining varied with the stages of the seminiferous epithelium cycle and was maximal prior to the maturation divisions. Cellular retinoic acid-binding protein was localized exclusively in the germinal cells in the adluminal compartment. The results suggest that retinoic acid may be the retinoid form used by the germinal cells, and that Sertoli cells may use the cellular retinol-binding protein to transfer retinol from the basal to the adluminal compartment. In the epididymis, cellular retinol-binding protein was localized in the cytoplasm and stereocilia of the principal cells in the proximal caput epididymidis, while cellular retinoic acid-binding protein was localized in the spermatozoa and the stereocilia of the principal cells throughout the epididymis and in the epithelial cells of the distal vas deferens. Sperm staining intensity decreased from the initial segment to the cauda. The presence of high levels of cellular retinol-binding protein in the epithelial cells and high levels of cellular retinoic acid-binding protein in the spermatozoa of the caput epididymidis, known to be involved in the synthesis and secretion of factors necessary for sperm maturation, suggests that vitamin A may have a role in this process.

Key words: cellular retinol-binding protein, cellular retinoic acid-binding protein, testis, epididymis.

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The essential role of retinol (vitamin A alcohol) in mammalian spermatogenesis has been recognized for many years. In animals deprived of retinol, the germinal epithelium degenerates and spermatogenesis is interrupted (Wolbach and Howe, 1925). Spermatogenic function is restored if retinol is replaced in the diet. However, retinoic acid (vitamin A acid) given to animals maintained on retinol-deficient diets will not prevent testicular degeneration which is indistinguishable from that seen in retinol-deficient rats (Howell et al, 1963). It is important to note that retinol can give rise to retinoic acid *in vivo* (Emerick et al, 1967), but retinol cannot arise from retinoic acid *in vivo* (Dowling and Wald, 1960). Even when injected directly into the testis, retinoic acid fails to repair the lesions of retinol deficiency (Ahluwalia and Bieri, 1971). This is in contrast to the ability of retinoic acid to maintain normal morphology in most epithelial tissues in the absence of retinol (Howell et al, 1963). These results have led some investigators to conclude that retinoic acid is totally inactive in spermatogenesis (Ahluwalia and Bieri, 1971). However, a significant amount of retinoic acid has been demonstrated in rat testis (Ito et al, 1974). Further, if retinoic acid is given to retinol-deficient rats, specific changes in testicular messenger RNA can be demonstrated by *in vitro* translation as early as 1 hour after administration of the retinoic acid (Omori and Chytil, 1982). Consequently, important questions remain on the forms of vitamin A utilized by the testis.

Increased understanding of the role of retinol and retinoic acid in testicular function might come from consideration of two intracellular proteins, called cellular retinol-binding protein (CRBP) and cellular retinoic acid-binding protein (CRABP), which bind retinol and retinoic acid, respectively, with high affinity and specificity. Although a number of binding or

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carrier proteins for vitamin A-active compounds are now known, only cellular retinol-binding protein and cellular retinoic acid-binding proteins are found in all vitamin A-responsive tissues (Ong et al, 1982). There are several reasons why these proteins are believed to be important elements in a cell's ability to respond to retinol or retinoic acid (reviewed in Chytil and Ong, 1984): they have retinol and retinoic acid as endogenous ligands and their respective binding specificities for analogs of these ligands mirror the biologic activity of the analogs. In addition, cellular retinol-binding protein has been shown to transfer retinol to specific binding sites in the nucleus *in vitro*. Both proteins are present in rat testis (Ong and Chytil, 1975), which strongly suggests that both retinol and retinoic acid play some role in the testis.

In this report, we have demonstrated that cellular retinoic acid-binding protein is present in the testis, that it is localized exclusively in the germ cells, and that the cellular retinol-binding protein is localized exclusively in the Sertoli cells. We have also demonstrated the localization of both cellular retinol-binding protein and retinoic acid-binding protein in the epididymis.

Materials and Methods

Preparation of Tissues

Five normal male rats were sacrificed by decapitation and the testes were removed and immediately fixed by immersion for 3 hours in Perfix (Fisher). After dehydration and embedding in paraffin, 5-μm sections were cut for immunolocalization studies.

The epididymis was prepared by perfusion through the aorta with 50 ml of Zamboni's fixative (Stefanini et al, 1967). The epididymis was removed and immersed for an additional 3 hours in the same fixative. The tissue was then left in 70% ethanol overnight. The tissue was dehydrated and prepared for immunolocalization as described below.

TABLE 1. Distribution of Cellular Retinol-binding Protein PAP Staining Intensity in 114 Cross Sections of Rat Seminiferous Tubules

Stages Seminiferous Epithelium Cycle		Intensity of PAP Staining* Number of Tubules		
		+	++	+++
Roosen-Runge & Giesel (1950)	Clermont and Leblond (1952)			
1	IX		2	
2	X-XI		2	3
3	XII-XIII			20
4	XIV			7
5	I-III		6	16
6	IV-V		16	2
7	VI-VII	28	2	
8	VIII	4	6	

*+ Weak staining.
++ Moderate staining.
+++ Strong staining.

Preparation of Immune Reagents

Specific anti-cellular retinol-binding protein IgG was affinity-purified from immune serum as previously described (Bok et al, 1984). The same procedure was also used to affinity-purify specific anti-cellular retinoic acid-binding protein IgG, using pure rat testis cellular retinoic acid-binding protein coupled to sepharose 4B.

The specificity of the affinity-purified preparations was tested in the following manner. An extract of soluble proteins was prepared from testis as previously described (Ong et al, 1982). Aliquots of the extract, as well as pure cellular retinol-binding protein and cellular retinoic acid-binding protein, were subjected to electrophoresis on an 11% polyacrylamide gel using the method of Laemmli (1970). Proteins were then transferred from the gel to nitrocellulose paper (Towbin et al, 1979). Immunoreactive proteins were detected on the paper using the affinity-purified IgG preparations and radioiodinated protein A, as described by Soderquist and Carpenter (1984).

Immunolocalization of Cellular Retinol and Retinoic Acid-binding Proteins

The avidin-biotinylated peroxidase complex (ABC) method of Hsu et al (1981) was used (Vectastain ABC Kit, Vector Laboratories, Burlingame, California). A solution of 2.5% bovine serum albumin and 0.1% Triton X-100 (v/v) in 0.05 M Tris-HCl, *p*H 7.4, was used for dilution of all immunochemical reagents. The sections were initially incubated for 30 minutes at room temperature in 0.3% H_2O_2 in 100% methanol to quench endogenous peroxidase activity. After hydration, the sections were treated with 2% normal goat serum to reduce nonspecific binding in subsequent steps. The sections were next incubated with either 1) affinity-purified anti-cellular retinol-binding protein rabbit IgG for 1 hour at an absorbance of 0.05 at 280 nm, 2) affinity purified anti-cellular retinoic acid-binding protein rabbit IgG at an absorbance of 0.01 at 280 nm, or 3) with the same antibodies that had been preincubated with a large excess of pure cellular retinol- or retinoic acid-binding proteins for 12 hours at 4 C. The sections then were incubated with biotinylated goat anti-rabbit IgG for 30 minutes and, finally, with the ABC complex for 60 minutes. The above incubations were done in a humid chamber at 37 C and sections were washed three times with PBS between steps. Brown staining was accomplished using diaminobenzidine and H_2O_2 as substrates for the peroxidase.

Cellular retinol- and retinoic acid-binding proteins were localized on the same sections by the double staining technique of Nakane (1968). Briefly, after staining the slide with diaminobenzidine to reveal the presence of cellular retinol-binding protein, antigen-antibody complexes were dissociated by soaking overnight in three changes of glycine-HCl, *p*H 2.2. The sections were then treated to

localize the cellular retinoic acid-binding protein, as described above, ending with 4-chloro-1-napthol as substrate for the peroxidase. Consequently, staining for the cellular retinol-binding protein was brown; staining for the cellular retinoic acid-binding protein was blue.

Some testicular sections were counterstained with hematoxylin to identify the stages of the seminiferous epithelium cycle. The 14-stage classification of Leblond and Clermont (1952) could not be used since it is based on the development of the acrosome visualized by PAS staining. The cell associations of the seminiferous epithelium were classified, instead, according to the method of Roosen-Runge and Giesel (1950), based on the morphologic development of the germ cells and their spatial relationship. The correspondence between the eight-stage classification of Roosen-Runge and Giesel (Arabic numerals) and the 14-stage classification of Leblond and Clermont (Roman numerals) is given in Table 1 and throughout the text.

Results

Specificity of the Affinity-purified IgG Preparations

Aliquots of testis extract, pure cellular retinol-binding protein, and pure cellular retinoic acid-binding protein were submitted to SDS-gel electrophoresis. The resolved proteins then were transferred to nitrocellulose paper and examined for immunoreactivity with both affinity-purified anti-cellular retinol-binding protein and affinity-purified anti-cellular retinoic acid-binding protein. As shown in Fig. 1, anti-cellular retinol-binding protein detected pure cellular retinol-binding protein, but did not react with pure cellular retinoic acid-binding protein. A single band corresponding to cellular retinol-binding protein was recognized in the testis extract. This indicated that no other proteins capable of reacting with this antibody preparation could be detected in the testis extract. Similarly, anti-cellular retinoic acid-binding protein detected pure cellular retinoic acid-binding protein, but not cellular retinol-binding protein. Again, a single band corresponding to cellular retinoic acid-binding protein was recognized in testis extract. Both lanes containing testis extract were overloaded to detect if minor proteins capable of cross-reaction were present.

From these results, we conclude that the immune reagents we employed in this study are specific for cellular retinol-binding protein and for cellular retinoic acid-binding protein, and recognize no other proteins in the testis.

Localization of Cellular Retinol-binding Protein in the Testis

Figure 2 shows the localization of cellular retinol-binding protein in an unstained section of normal rat testis. Positive staining was seen in the basal compartment of the seminiferous epithelium and in cytoplasmic processes between germ cells. In a section counterstained to identify the different cell types, positive staining was seen only in Sertoli cells (Figs. 4, 6–8). No staining was seen in the germ cells. In all cases, staining did not occur if the antibody preparation was pretreated with pure cellular retinol-binding protein (Figs. 3 and 5). Within a single tubule, all the Sertoli cells stained with approximately equal intensity. However, a substantial variation in the intensity of staining was seen between Sertoli cells of different tubules. Further, the distribution of staining within the Sertoli cell varied reproducibly in different tubules.

Figures 2 and 4 illustrate the differences in staining observed in different tubules. The basal compartment in some tubules was strongly stained while, in other tubules, it was moderately or weakly stained. To relate the variation in staining to the stages of the seminiferous epithelium cycle, the level of Sertoli cell staining was evaluated in counterstained sections of normal testis where the cell associations of the seminiferous epithelium cycle could be recognized more readily than in unstained sections. Seminiferous tubules were scanned and Sertoli cell staining was

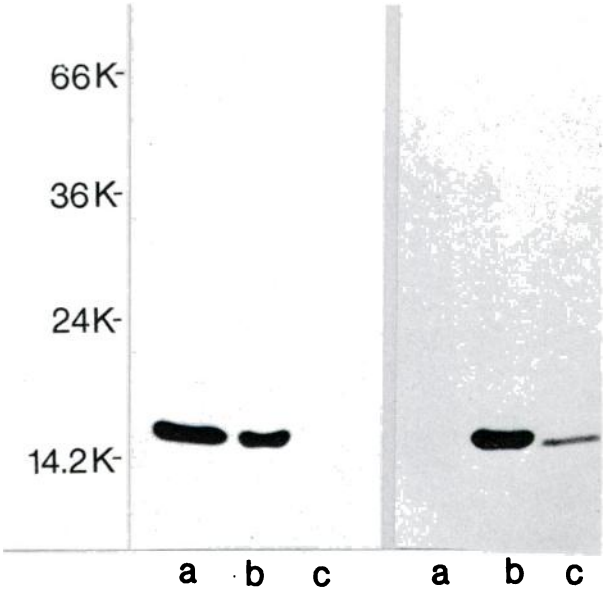
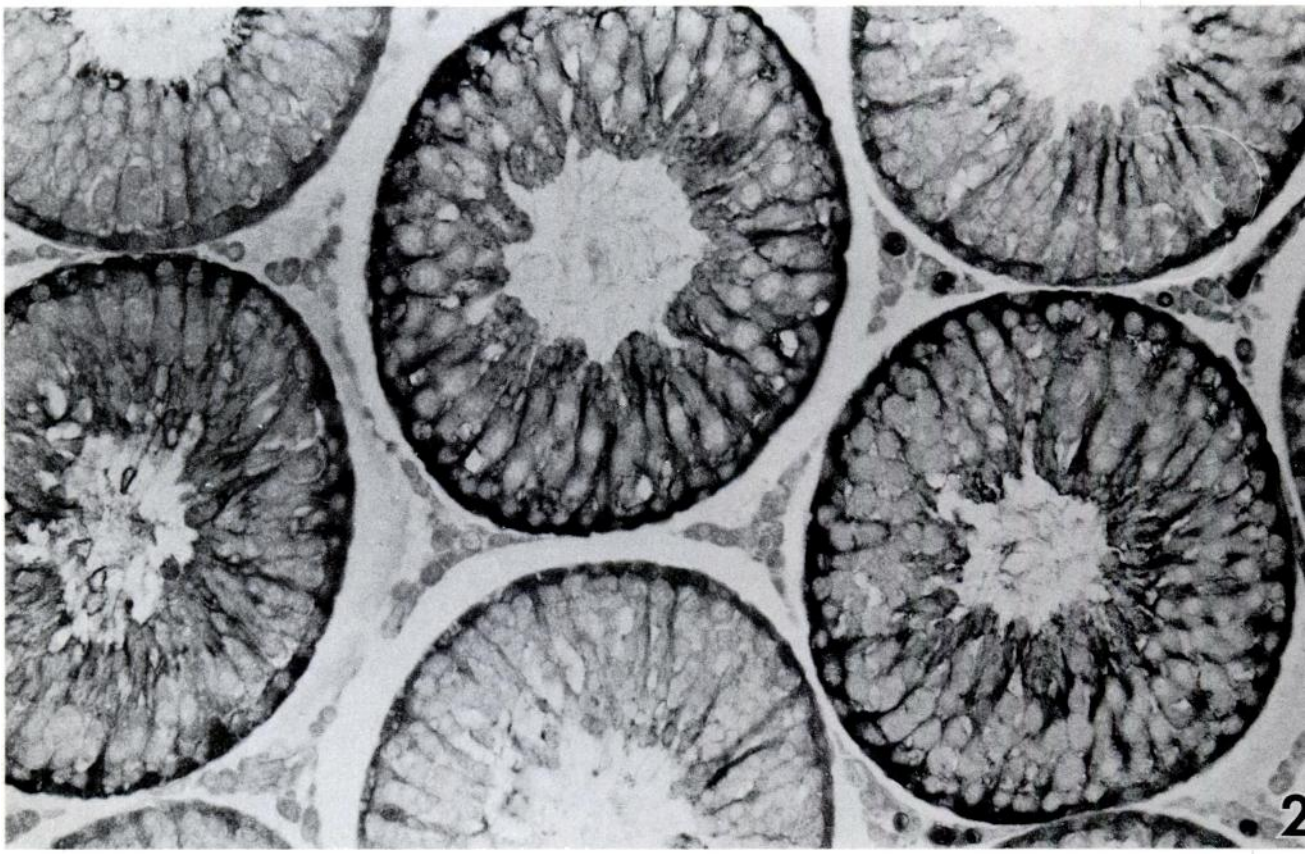


Fig. 1. Detection of immunoreactive proteins in a soluble extract of rat testis. Left panel was developed with anti-CRBP IgG. Lane a, 20 pmole pure CRBP. Lane b, testis extract containing 1.5 mg protein. Lane c, 20 pmole pure CRABP. Right panel was developed with anti-CRABP IgG; lanes identical to left panel. CRBP and CRABP have very similar molecular weights and display the same relative mobility in the gel system used.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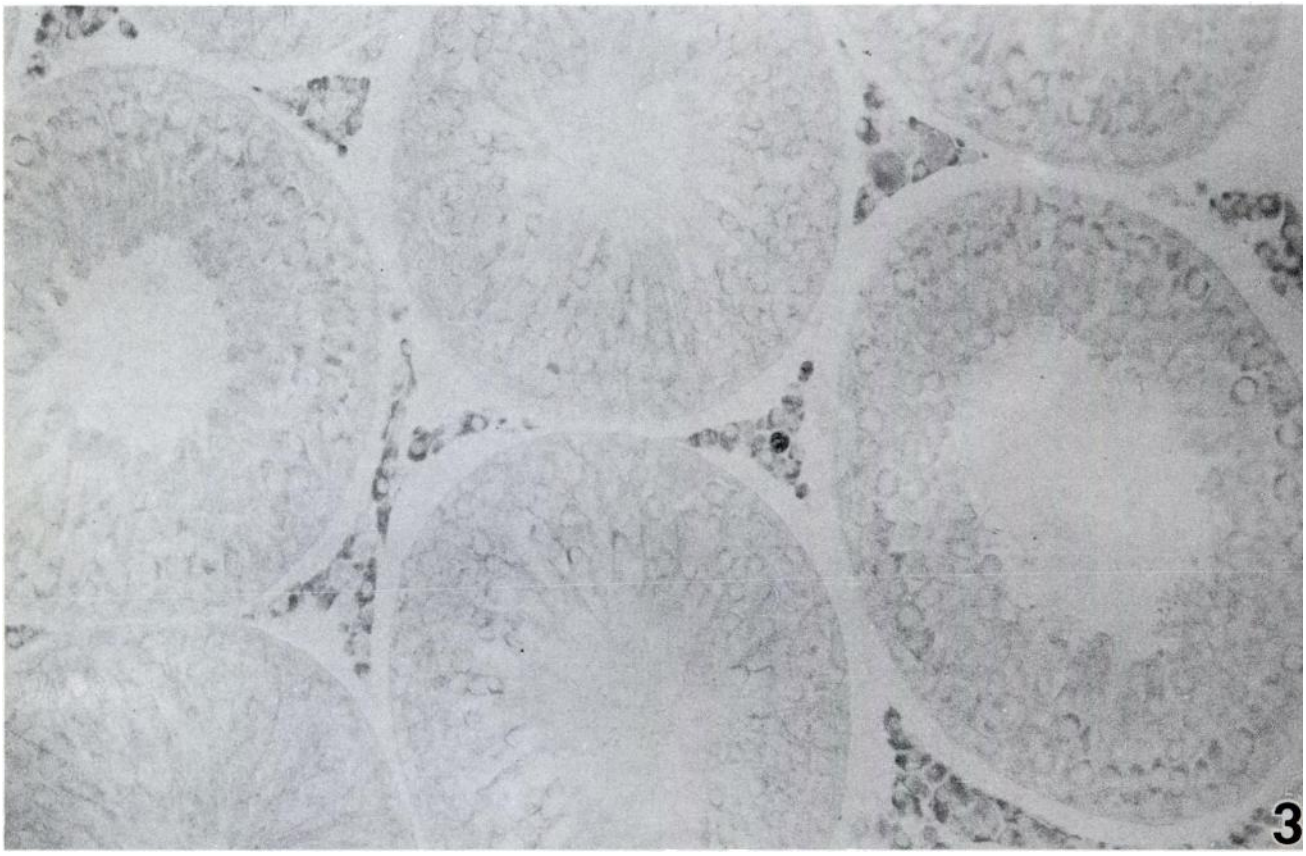
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evaluated as weak, moderate, or strong. The seminiferous epithelium cycle stage of each tubule was recorded. Seminiferous tubules in stage 7 exhibited weak staining, while those in stages 1 and 6 showed moderate staining. The strongest staining was always seen in stages 3 and 4 (Table 1, Figs. 6–8).

The staining intensity also varied within the Sertoli cell, depending on the stage of the seminiferous epithelium cycle. Figure 8 shows that staining in Sertoli cells from stage 7 was weak at the base of the cell and in the processes between germ cells, and strong in the apical processes. In contrast, in the neighboring tubule in stage 3 of the seminiferous cycle, staining was strong at the base of the Sertoli cell and in the processes between germ cells. Thus, it appears that cellular retinol-binding protein was localized exclusively in Sertoli cells, and that the immunoreactive retinol-binding protein location and content varied with the stage of the seminiferous epithelium cycle.

Localization of Cellular Retinoic Acid-binding Protein in the Testis

Cellular retinoic acid-binding protein was localized exclusively in the adluminal compartment. No staining was seen in the basal compartment (Fig. 15). Staining was not seen if the antibody preparation was pretreated with pure cellular retinoic acid-binding protein (Fig. 16). In counterstained sections, positive staining for cellular retinoic acid-binding protein was confined to the late spermatocytes and spermatids; the Sertoli cells showed no staining (Figs. 9–11). This result was consistent from one tubule to the next and did not vary with the stage of the spermatogenic cycle.

All of the germ cells, however, did not stain positively for cellular retinoic acid-binding protein. The spermatogonia and early spermatocytes showed no staining (Figs. 9–12). Weak staining was first seen in the pachytene spermatocytes in late stage 7 (Fig. 12). This increased somewhat as the cells matured in stage 8 (Fig. 9), but the first strong staining was seen in the pachytene spermatocytes of stages 1 and 2 (Figs. 9 and 10) and the subsequent diplotene spermatocytes of stage 3. Round and elongated spermatids showed strong staining for cellular retinoic acid-binding protein (Figs. 9–14).

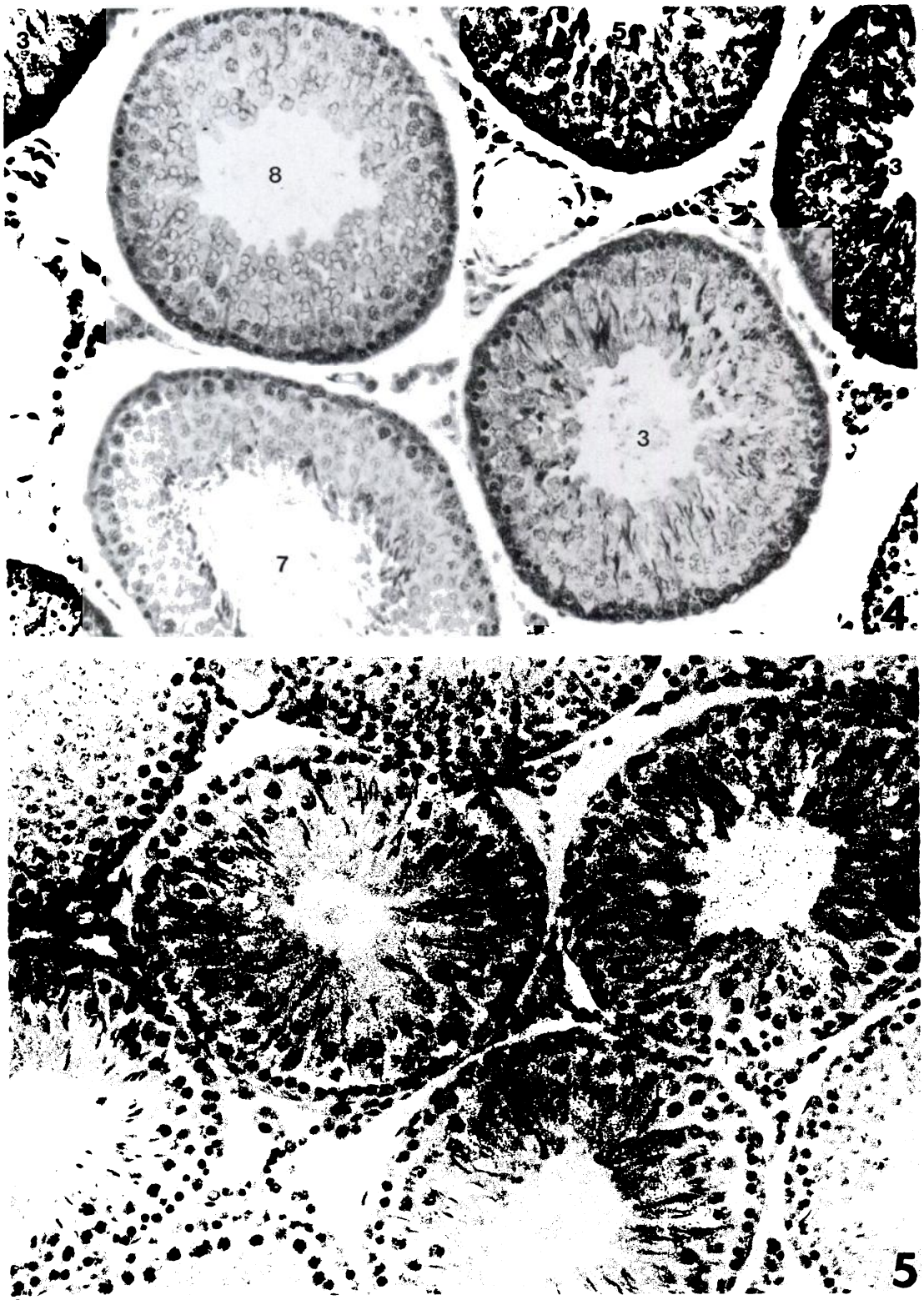
Double Localization of Cellular Retinol- and Retinoic Acid-binding Proteins in the Testis

Figures 13 and 14 show the localization of both retinol- and retinoic acid-binding proteins in an unstained section of normal rat testis. During stage 8 (Fig. 13), the brown cellular retinol-binding protein staining in the Sertoli cells varied in intensity within the cell itself. The basal cytoplasm was stained lightly, but the apical processes were strongly stained. The blue cellular retinoic acid-binding protein staining was restricted to the germ cells in the adluminal compartment and the elongated spermatids that border the lumen beyond the Sertoli cell apical processes. In a slightly more advanced tubule (Fig. 14), the same localization was observed. However, in the tubule area where spermiation had occurred, the Sertoli cell apical processes were less visible and the basal Sertoli cell cytoplasm was stained strongly while, in the area where the apical processes were still visible, the basal cytoplasm was lightly stained.

Localization of Cellular Retinoic Acid-binding Protein in the Epididymis

As already reported (Porter et al, 1983), high levels of cellular retinol-binding protein in the epididymis were confined to the epithelial cells and stereocilia in the initial segment and proximal caput regions of the organ, and staining was not observed if the antibody preparation was pretreated with pure cellular retinol-binding protein (Figs. 17 and 18). Staining was present throughout the body of the cells. It was of interest to examine the distribution of cellular retinoic acid-binding protein in this tissue as well. As shown in Figs. 19 to 21, strong staining for cellular retinoic acid-binding protein was also seen in the initial segment and proximal caput epididymidis. In contrast to cellular retinol-binding protein, however, the staining was associated predominantly with the spermatozoa in the lumen of the tubule, as well as the stereocilia of the epithelial cells. Staining intensity decreased abruptly in segment 4 of the caput (Fig. 23)

Fig. 2. Immunocytochemical localization of cellular retinol-binding protein in normal rat testis. Staining was localized in the basal compartment of the seminiferous tubules. Note the difference in staining intensity among the different tubules (× 340). **Fig. 3.** Immunocytochemical localization of cellular retinol-binding protein in normal rat testis. A section similar to that in Fig. 1 with affinity-purified anti-CRBP IgG absorbed with pure CRBP prior to incubation with peroxidase-conjugated second antibody (× 340).



Figs. 4 and 5. Immunocytochemical localization of cellular retinol-binding protein in normal rat testis. Counterstained with hematoxylin. **Fig. 4.** Note the strong staining in the basal compartment of stage 3 (XII–XIII) seminiferous tubules, the moderate staining in stage 5–6 (II–V), and the weak staining in stage 7 (VII) (× 272). **Fig. 5.** Similar to that of Fig. 4 with affinity purified anti-CRBP IgG absorbed with pure CRBP prior to incubation with peroxidase-conjugated second antibody (× 272).

and gradually thereafter from the distal caput to the cauda epididymidis (Figs. 25 and 26, Table 2).

A weak cellular retinoic acid-binding protein staining was observed in the cytoplasm of epithelial cells and it varied along the length of the epididymis. In the initial segment, staining was weak (Fig. 19). In segments 2 to 3 of the caput (Figs. 20–22), cytoplasmic staining increased and was confined to the supranuclear region (Fig. 22). Maximum staining was seen in segments 3 and 4 of the caput (Fig. 23). Cytoplasmic staining even in segments 3 and 4 was weak compared with luminal staining in regions 1 to 3. Cytoplasmic staining decreased further down the epididymis and was not detected either in the principal or the clear cells of the cauda epididymidis or of the proximal vas deferens (Figs. 25 and 26). In the distal vas deferens, however, the cytoplasm of the principal cells stained strongly for cellular retinoic acid-binding protein (Figs. 27–30). Staining was consistently more intense at the base of the epithelial infoldings (Figs. 19 and 20).

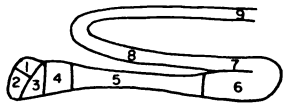
Throughout the epididymis, immunoreactive cellular retinoic acid-binding protein was seen in the stereocilia. The intensity of staining correlated with the staining intensity in the lumen, except in the cauda, where stereocilia staining appeared stronger than luminal staining (Fig. 25).

Cellular retinoic acid-binding protein staining was also observed in the layers of peritubular cells surrounding the epididymal tubule and in the lamina propria and the muscle layers surrounding the vas deferens. In all cases, stereocilia and luminal staining was not seen after pretreatment of the antibody preparation with excess amounts of pure cellular retinoic acid-binding protein, but peritubular staining was blocked less effectively.

TABLE 2. Immunolocalization of Cellular Retinol-binding Protein and Retinoic Acid-binding Protein in Rat Epididymis

	Principal Cells	Stereocilia	Lumen
Segment 1*			
CRBP	+++	++	0
CRABP	±	+++	++++
Segments 2-3			
CRBP	+++	++	0
CRABP	+	+++	++++
Segment 4			
CRBP	+	+	0
CRABP	+	++	+++
Segment 5			
CRBP	0	0	0
CRABP	±	++	++
Segment 6			
CRBP	0	0	0
CRABP	0	++	++
Segment 7			
CRBP	0	0	0
CRABP	0	+++	+
Segment 8			
CRABP	0	++	+
Segment 9			
CRABP	+++	+++	

*Diagram shows the location of the epididymal segments.

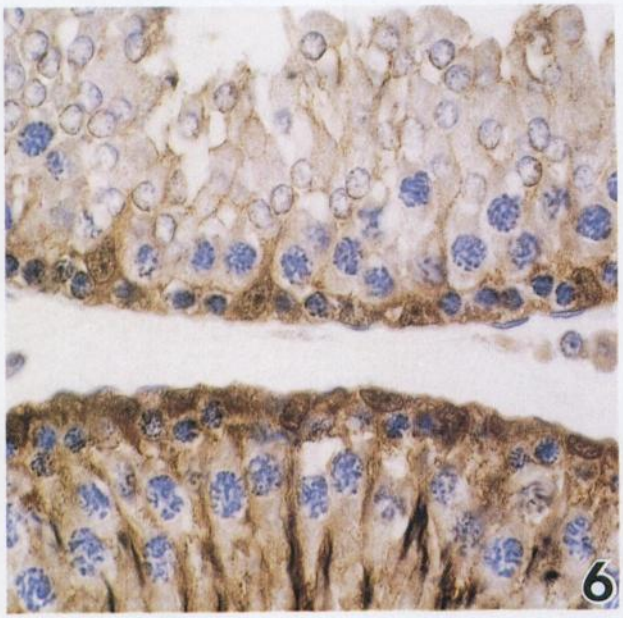


Discussion

The results presented here show that, in the testis, the germ cells contained immunoreactive cellular retinoic acid-binding protein but no detectable cellular retinol-binding protein. In contrast, cellular retinol-binding protein was confined to the Sertoli cells. This finding contradicts the hypothesis that retinoic acid, the natural ligand for cellular retinoic acid-binding protein (Saari et al, 1982), has no role in spermatogenesis, and suggests that it may be necessary to reevaluate the action of vitamin A in the testis.

Cellular retinoic acid-binding protein was not uniformly present in all of the germ cells. No detectable cellular retinoic acid-binding protein was observed in spermatogonia or preleptotene spermatocytes. These cells are present in the basal compartment of the seminiferous tubule and thus are not sequestered by the blood-testis barrier. Although it has been reported that retinoic acid increases spermatogonial proliferation in the cryptorchid mouse testis cultured *in vitro* (Haneji et al, 1982), such an effect is not observed *in vivo* (Howell et al, 1963; Ahluwalia and Bieri, 1971). Furthermore, the number of spermatogonia is less affected than the number of spermatocytes and spermatids during vitamin A deficiency (Mitranond et al, 1979). Thus, the spermatogonia may be the least vitamin A-dependent cells in the seminiferous tubule, an idea consistent with the finding that these cells do not contain detectable levels of cellular retinol or retinoic acid-binding protein.

The early primary spermatocytes are also present in the basal compartment. As these cells mature, they move upward into the adluminal compartment, where they become dependent upon the Sertoli cells for their supply of nutrients, including vitamin A. It is during this period of maturation that the spermatocytes also began to stain strongly for cellular retinoic acid-binding protein. It is noteworthy that spermatocytes did not stain for cellular retinoic acid-binding protein immediately after migrating into the adluminal compartment; they become reactive in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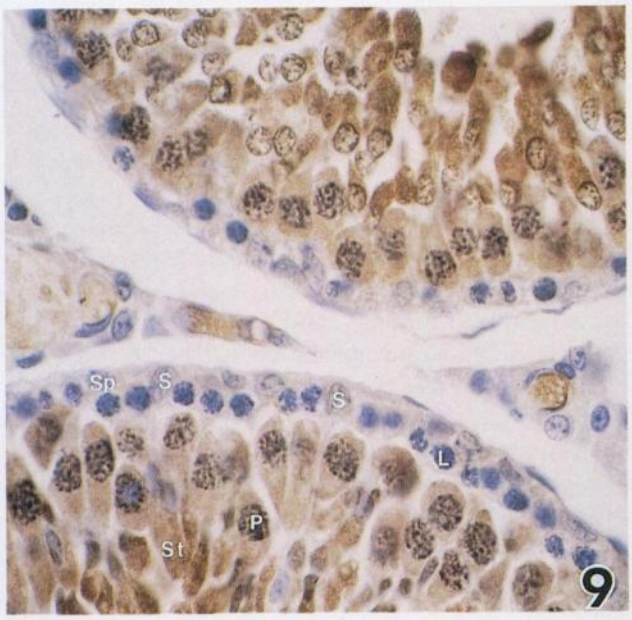
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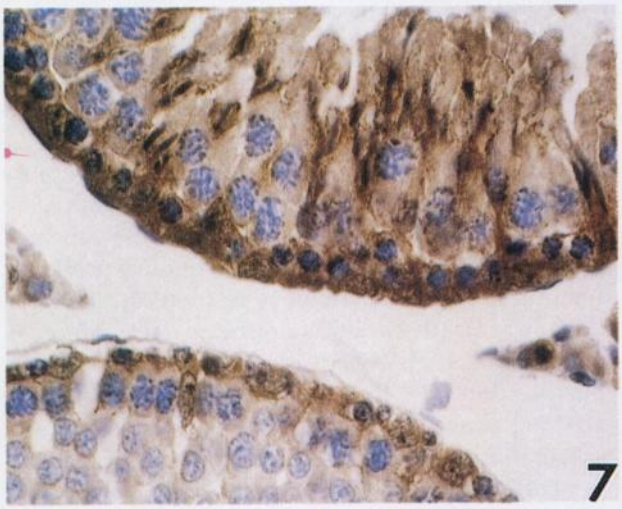
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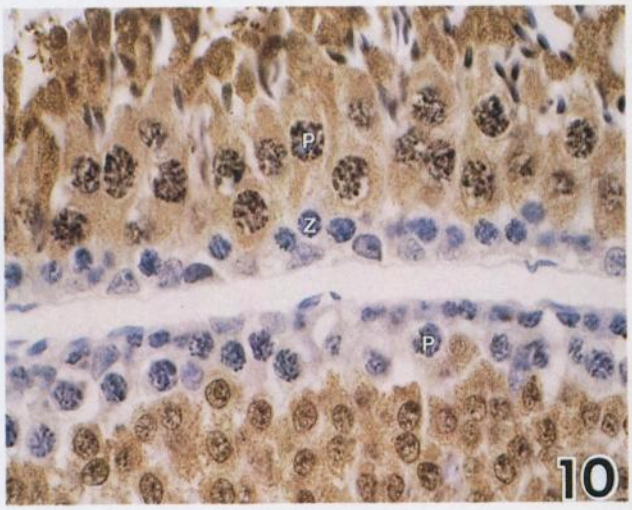
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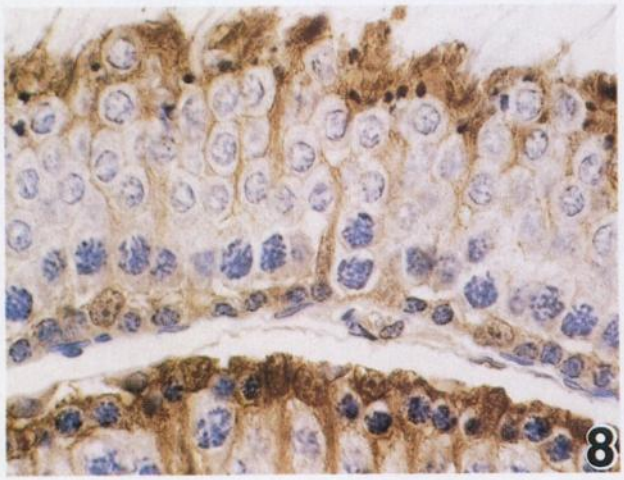
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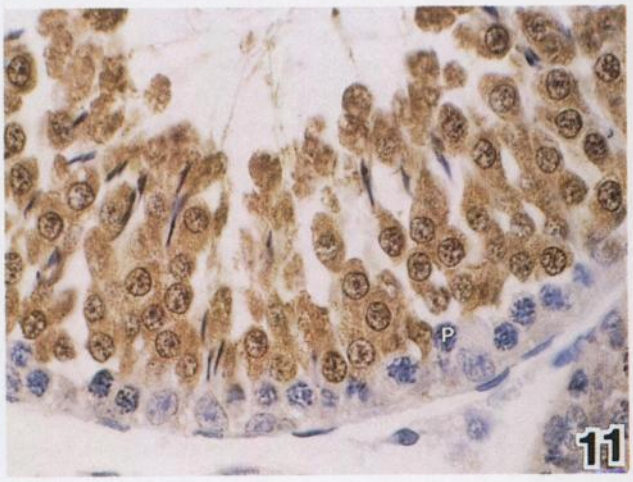
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mid-pachytene only (stages 8–4), so they are negative for a large part of meiosis. Strong staining for cellular retinoic acid-binding protein continued throughout the remainder of spermatogenesis and was still associated with spermatozoa in the proximal regions of the epididymis. At no time during this process were the germ cells observed to stain positively for cellular retinol-binding protein.

Gambhir and Ahluwalia (1975) have reported that vitamin A, in the form of either retinol or retinyl ester, is present in the acrosome of bovine spermatozoa. Since we found no cellular retinol-binding protein in the acrosome, it would suggest that the form was probably retinyl ester. Their assay does not detect retinoic acid. The high level of cellular retinoic acid-binding protein in germinal cells seen in our study suggests that a physiologically significant form of vitamin A in these cells may well be retinoic acid. At any rate, it would appear that retinoic acid may be the form of vitamin A required or utilized by the developing germ cells.

Cellular retinol-binding protein was localized exclusively in the Sertoli cells. Huggenvik and Griswold (1981) have identified cellular retinol-binding protein in the cytosols of cultured rat Sertoli cells, but have not detected the binding protein in spermatids or spermatocytes. In addition, Rich and de Kretser (1977) have reported that the amount of androgen binding protein (ABP) secreted by the Sertoli cells is greatly decreased in testes of retinol-deficient rats fed retinoic acid. These observations, along with the results presented here, indicate that the Sertoli cell utilizes retinol in the testis.

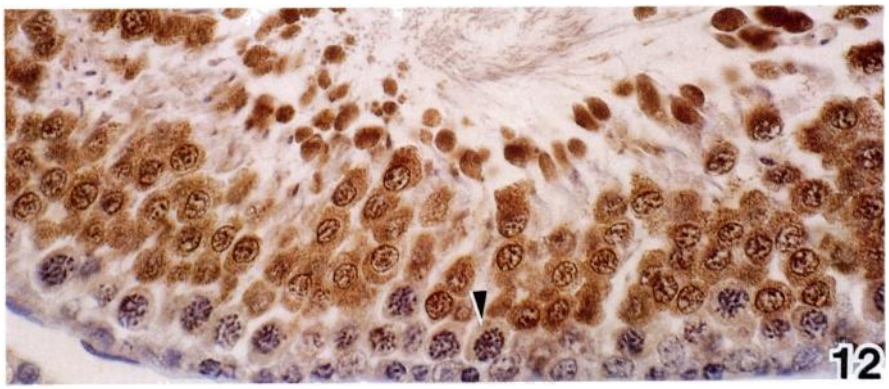
The Sertoli cells showed a wide fluctuation in the content of immunoreactive cellular retinol-binding protein, which varied in a strikingly reproducible fashion with the cycle of the seminiferous epithelium. The cyclic nature of the Sertoli cell metabolism is well known. It was first demonstrated by Von Ebner (1888). Since then, the lipid content (Niemi and Kormano, 1965; Kerr and de Kretser, 1975) and hormone responsiveness (Gordeladze et al, 1982) have been shown to also vary as a function of the cycle of the seminiferous epithelium. The present study provides additional evidence for the cyclical nature of the functional activities of the Sertoli cell.

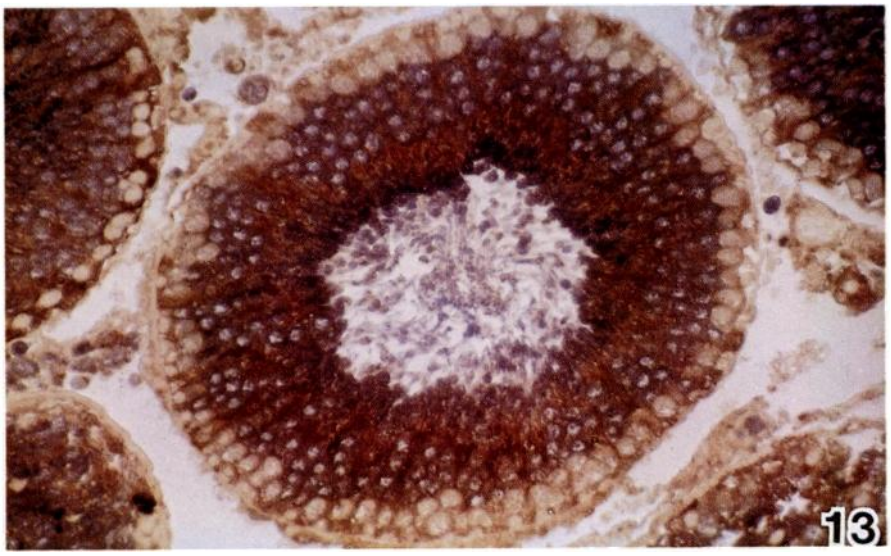
It is of interest to note that the strongest staining for cellular retinol-binding protein in the Sertoli cells was seen in stages 3 and 4, which correlated with the stage at which the developing spermatocytes are moving through the blood-testis barrier. They became reactive for cellular retinoic acid-binding protein only after being sequestered for a while in the adluminal compartment. Vitamin A is present in the blood primarily in the form of retinol bound to a specific transport protein (Smith and Goodman, 1979). To supply the germ cells, the retinol must pass from the interstitium into the Sertoli cells, which may utilize the cellular retinol-binding protein in this process. It is tempting to speculate that this transfer may be maximal during stages 3 and 4 of the spermatogenic cycle when the late spermatocytes and spermatids stain strongly for cellular retinoic acid-binding protein. This would also require the conversion of retinol to retinoic acid. The site of the presumed conversion remains to be demonstrated and could be the Sertoli cells, the spermatocytes, or the spermatids.

It is interesting to note that a similar blood-organ barrier exists for the retina which, in retinol-deficient animals, is not maintained by retinoic acid. The barrier is formed by the tight junctions of the pigment epithelial cells which, like the Sertoli cells, contain high levels of cellular retinol-binding protein (Bok et al, 1984), suggesting that a similar system for the delivery of vitamin A may exist in this tissue.

In birds, unlike mammals, spermatogenesis can be maintained in retinol-deficient animals by retinoic acid (Thompson et al, 1969). The typical Sertoli-Sertoli cell junctions observed for mammals are

Figs. 6–8. Immunolocalization of cellular retinol-binding protein in normal rat testis. Counterstained with hematoxylin. **Fig. 6.** Stage 8 (VIII) (top) and stage 3 (XII–XIII) (bottom) of the seminiferous epithelium cycle. Note the staining in the basal cytoplasm of the Sertoli cell and the processes between germ cells (× 576). **Fig. 7.** Stages 3 (XII–XIII) and 7 (VII) of the seminiferous epithelium cycle. Note the difference in staining in Sertoli cells in stage 7 (bottom) and 3 (top) of the seminiferous epithelium cycle (× 576). **Fig. 8.** Stages 7 (VII) and 3 (XII–XIII) of the seminiferous epithelium cycle. Note the strong staining in Sertoli cell apical processes in stage 7 (top) and the basal cytoplasm in stage 3 (bottom), and the weak staining in the lateral and basal cytoplasm in stage 7 (× 576). **Figs. 9–11.** Immunocytochemical localization of cellular retinoic acid-binding protein in normal rat testis. Counterstained with hematoxylin. **Fig. 9.** Stage 8 (VIII) (top) and 2 (X) (bottom) of the seminiferous epithelium cycle. Note the lack of staining in the spermatogonia (Sp), Sertoli cells (S), and leptotene spermatocytes (L), the light staining in the pachytene spermatocytes (P), and the strong staining in the elongating spermatids (St) (× 576). **Fig. 10.** Stage 2 (XI) (top) and 7 (VII) (bottom) of the seminiferous epithelium cycle. Note the lack of staining in the zygotene spermatocyte (Z) of stage 2 and the early pachytene spermatocytes (P) of stage 7 (× 576). **Fig. 11.** Stage 5–6 (II–V) of the seminiferous epithelium cycle. Note the lack of staining in the early pachytene spermatocytes (P) (× 576).





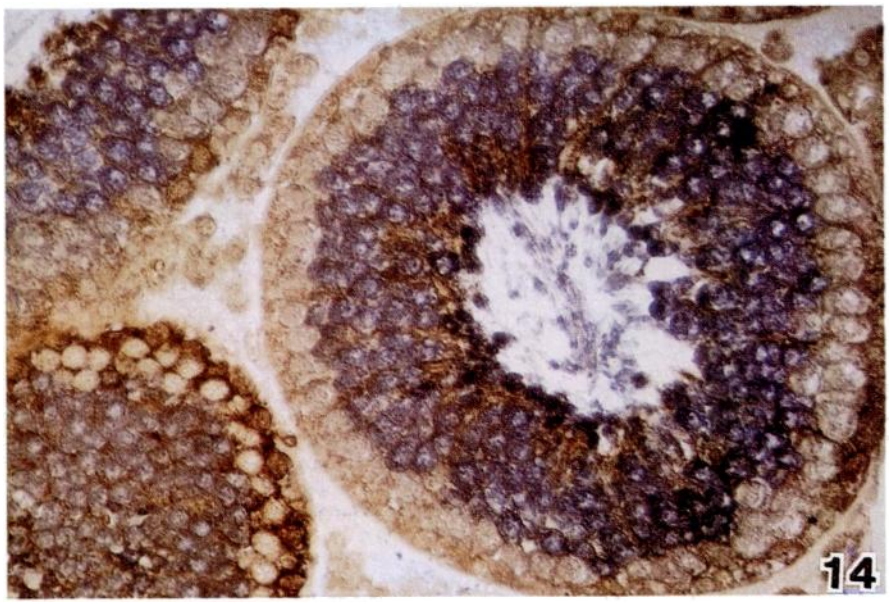
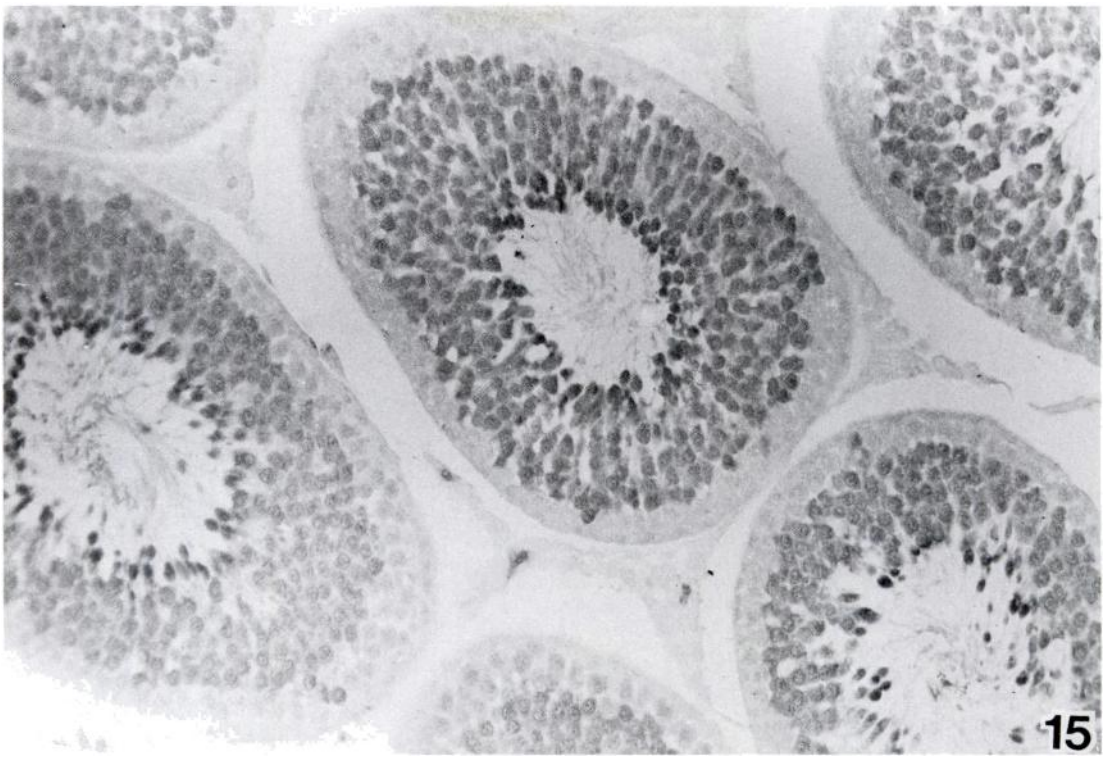
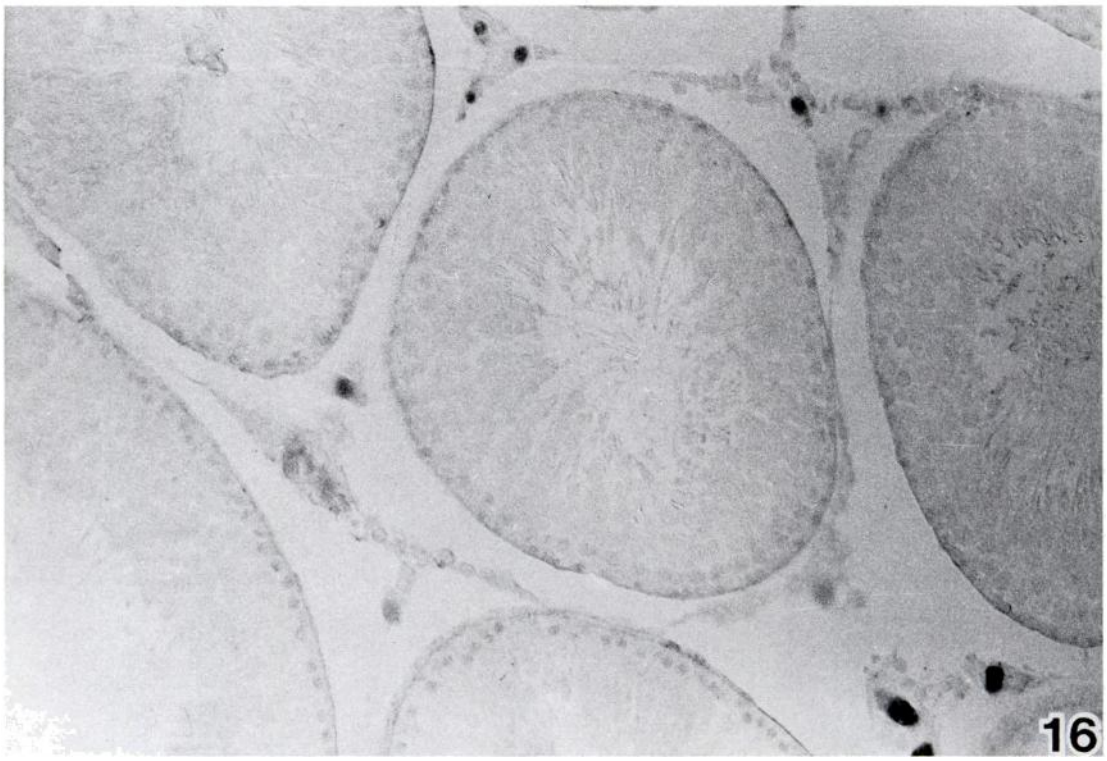
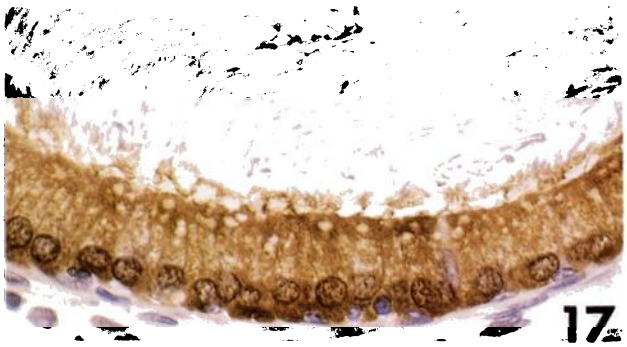


Fig. 12. Immunocytochemical localization of cellular retinoic acid-binding protein in normal rat testis. Counterstained with hematoxylin. Stage 7 (VII) of the seminiferous epithelium cycle. Note the light staining in the pachytene spermatocytes (arrow) (× 576). **Figs. 13 and 14.** Distribution of cellular retinol-binding protein and cellular retinoic acid-binding protein in stage 7 (VII) of the seminiferous epithelium cycle as revealed by the technique of immunohistochemical localization. Cellular retinol-binding protein is shown by the brown stain to be present in Sertoli cells, while cellular retinoic acid-binding protein is shown by the blue stain to be present in late spermatocytes and spermatids. The cellular retinol-binding protein brown staining is seen in the Sertoli cell apical processes, the processes between germ cells, and the basal cytoplasm. The blue cellular retinoic acid-binding protein staining is restricted to the germ cells and the luminal spermatozoa. Note in Fig. 13 the relationship of the spermatids near spermiation and the apical Sertoli cell processes. In a more advanced stage (Fig. 14), the Sertoli cell apical processes are strongly stained and the basal cytoplasm lightly stained on the left side of the tubule where elongated spermatids are present while, on the right, where spermiation has occurred, the apical processes are not visible but the basal cytoplasm is strongly stained. It suggests that a retraction of the Sertoli cell cytoplasm occurs after spermiation (× 350).





Figs. 15 and 16. Immunocytochemical localization of cellular retinoic acid-binding protein in normal rat testis. **Fig. 15.** Staining is localized in the germ cells in the adluminal compartment and in the interstitium (× 272). **Fig. 16.** Section similar to that in Fig. 15 with affinity-purified anti-CRABP IgG absorbed with pure CRABP prior to incubation with peroxidase-conjugated second antibody. Nonspecific staining is present in the interstitium. Staining in the germ cells is abolished (× 272).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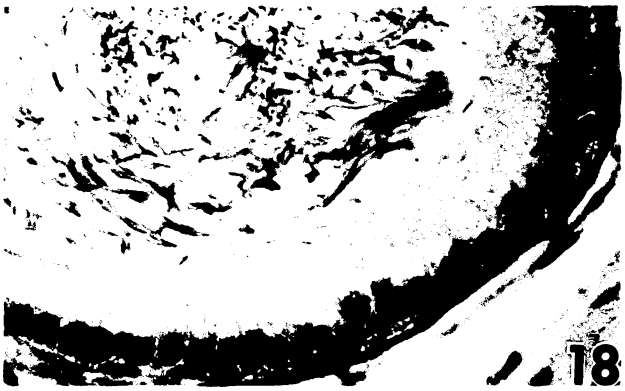
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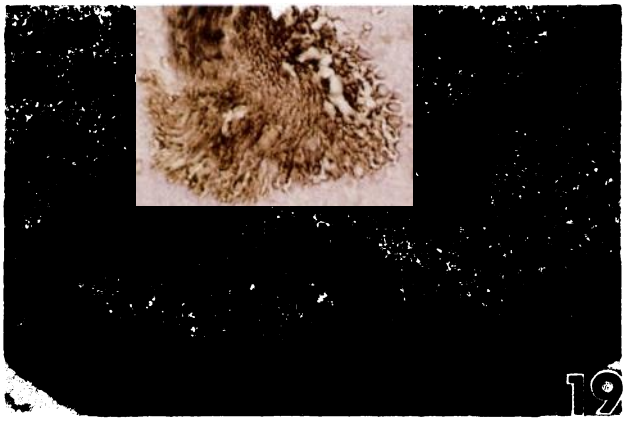
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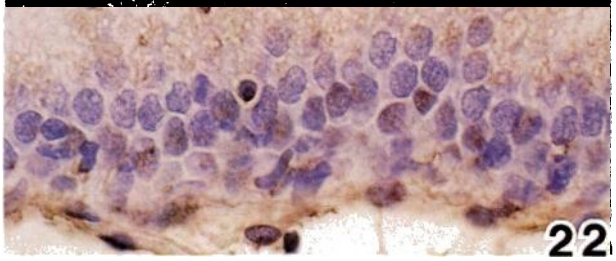
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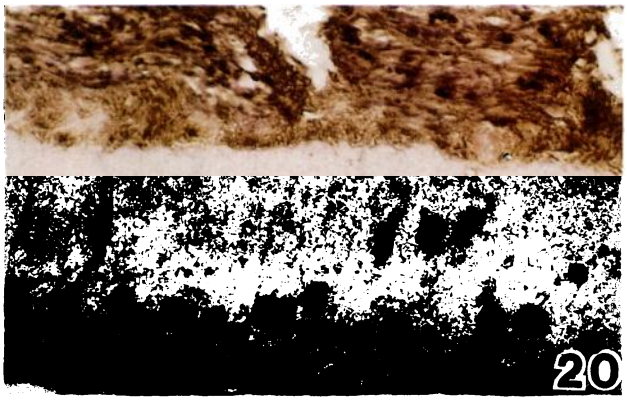
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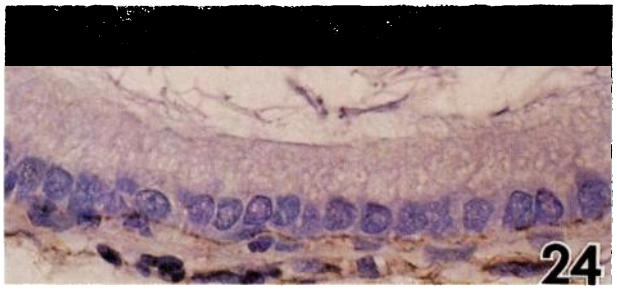
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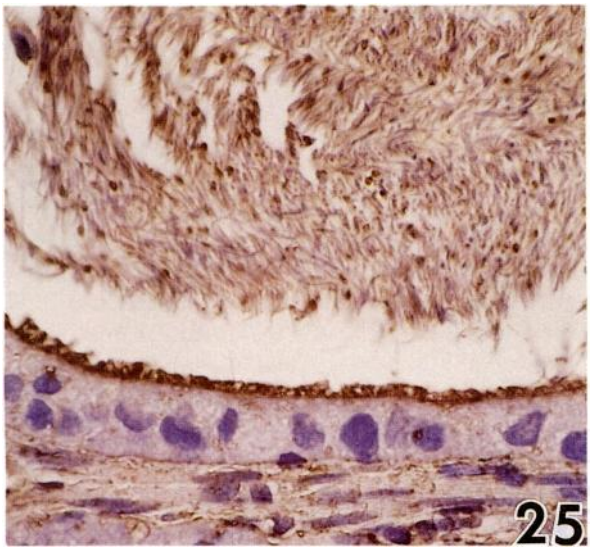
absent; they are replaced by subsurface cisternal junctions (Cooksey and Rothwell, 1973; Osman et al, 1980). Although these junctions restrict tracer diffusion into the adluminal compartment (Osman et al, 1980), it remains to be shown whether the morphologic barrier demonstrated by tracers is also a physiologic barrier. One can speculate that, in birds, the blood-testis barrier is not as restrictive as in mammals, and that administered retinoic acid may have better access to the germinal elements of the seminiferous epithelium.

The shape and size of the Sertoli cells change during the seminiferous epithelium cycle, particularly at the time of spermiation (Leblond and Clermont, 1952; Roosen-Runge, 1955; Ulvik and Dahl, 1981). Following the migration of late spermatids toward the surface of the seminiferous epithelium, these cells are retained by tubulobulbar complexes anchoring spermatids to Sertoli apical processes (Russell and Clermont, 1976). As spermatids are released from the seminiferous epithelium, the tubulobulbar processes disintegrate and the apical cytoplasmic processes of the Sertoli cells disappear. It has been suggested that this change is a result of the retraction of the cytoplasm toward the base of the cell (Russell, 1980). The localization of cellular retinol-binding protein in Sertoli cells in stages 7 (Fig. 8) and 8 (Figs. 6 and 14) observed in this study is consistent with the notion that the cytoplasm in apical processes surrounding the head of late spermatids flow down toward the base of Sertoli cells following the departure of spermatozoa. The strong cellular retinol-binding protein staining in the Sertoli apical processes coincides with the dissolution and phagocytosis of the tubulobulbar complexes anchoring spermatids to Sertoli cells. In the eye, the pigment cells of the retina are also involved in the phagocytosis of portions of neighboring cells (rod cells) (Young, 1976). This is another similarity between the Sertoli cells in the testis and the pigment cells in the retina.

We have previously reported that high levels of cellular retinol-binding protein in the epididymis are confined to the epithelial cells of the initial segment and proximal caput regions (Porter et al, 1983). In this study, we confirmed that immunoreactive cellular retinol-binding protein is restricted to the epithelium of the proximal epididymis and is not detected in the lumen. It does not appear, therefore, that the binding protein is exported to the lumen. We have also shown that strong staining for cellular retinoic acid-binding protein, predominantly associated with the spermatozoa, also occurred in these regions of the epididymis. Since cellular retinoic acid-binding protein is localized in the testicular spermatozoa, it is likely that it is also localized in epididymal spermatozoa. In the distal caput, sperm staining for cellular retinoic acid-binding protein was substantially decreased. Strong staining was seen on the stereocilia of the epithelial cells. When the distal cauda is flushed with saline, cellular retinoic acid-binding protein, as detected by the elution position of the cellular retinoic acid-binding protein: retinoic acid complex on an ion exchange column, was found associated only with homogenates of the flushed cauda and not in the soluble proteins recovered in the saline wash (Ong, unpublished data). This suggests that cellular retinoic acid-binding protein is firmly bound to the stereocilia in the distal cauda epididymidis. The presence of strong cellular retinoic acid-binding protein staining on the stereocilia and weak staining in the supranuclear regions of the principal cells in the caput epididymidis suggest that some cellular retinoic acid-binding protein may be released from spermatozoa and taken up by epithelial cells in this region. This could explain the decrease in luminal immunoreactive cellular retinoic acid-binding protein more distally. Epithelial cells throughout the rat epididymis have the capacity to endocytose substances from the lumen, but the apical cells in the caput and the clear cells in the cauda show significantly greater endocytotic activity than principal cells (Moore and Bedford, 1979). A selective cellular retinoic acid-binding protein absorption by the apical cells in the caput or the clear cells in the cauda was not observed in our study.

Figs. 17–19. Immunocytochemical localization of cellular retinol-binding protein in normal rat caput epididymidis counterstained with hematoxylin. **Fig. 17.** Note the lack of staining in the lumen and the peritubular cells (× 576). **Fig. 18.** Section similar to that in Fig. 17 with affinity-purified anti-CRBP IgG absorbed with pure CRBP prior to incubation with peroxidase-conjugated second antibody (× 576). **Figs. 19–24.** Immunocytochemical localization of cellular retinoic acid-binding protein in the normal rat caput epididymidis counterstained with hematoxylin. Initial segment (Fig. 19), segment 2 (Fig. 20), segment 3 (Figs. 21 and 22), and segment 4 (Fig. 23). Note the strong staining in the lumen and the stereocilia of the initial segment and the proximal caput epididymidis (Figs. 19–21), the decreased reactivity in the lumen of the distal caput epididymidis (Fig. 23), and the light staining in the supra-nuclear region (Figs. 21–23) (× 576). **Fig. 24.** Section similar to that of Fig. 23 with affinity-purified anti-CRABP IgG absorbed with pure CRABP prior to incubation with peroxidase-conjugated second antibody (× 576).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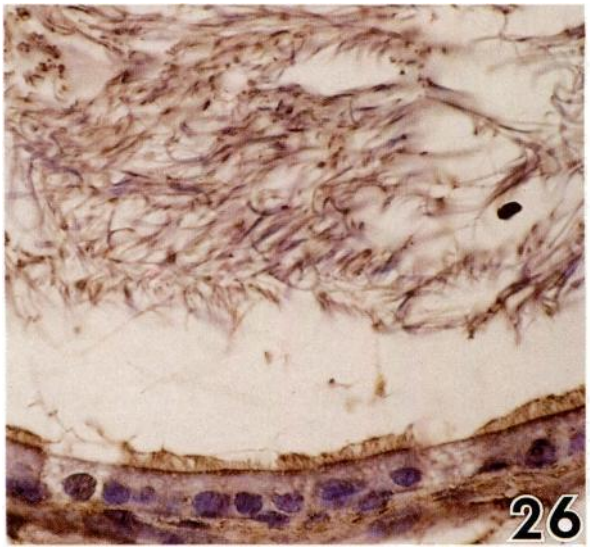
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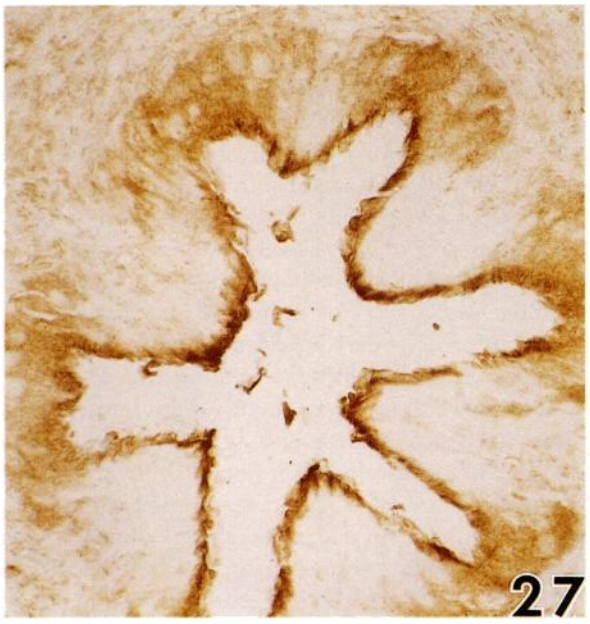
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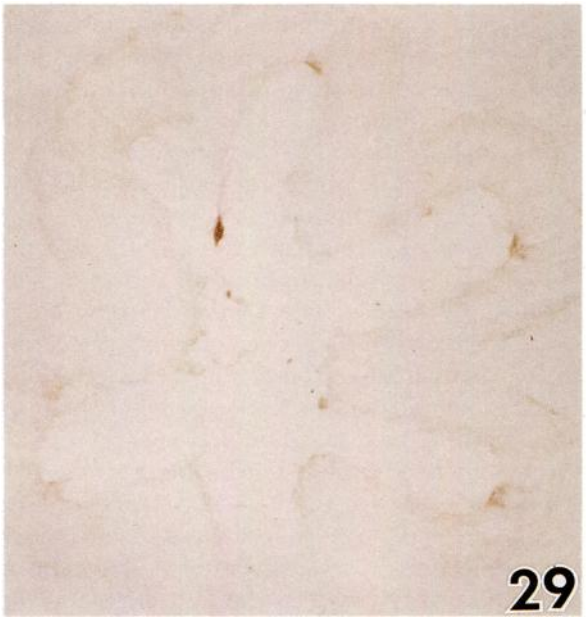
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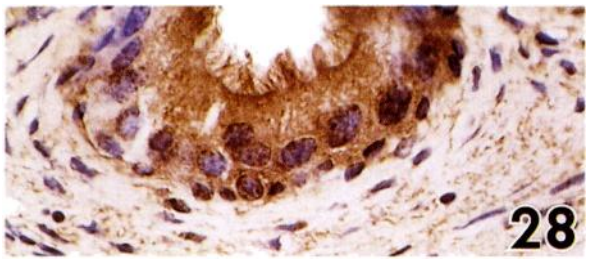
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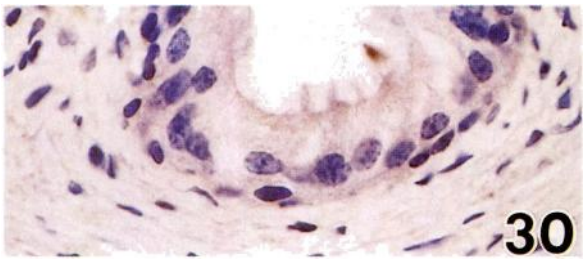
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Figs. 25 and 26. Immunocytochemical localization of cellular retinoic acid-binding protein in the normal proximal (Fig. 25) and distal (Fig. 26) rat cauda epididymidis. Note the staining on the stereocilia border, its absence on clear cells, and the progressive decline in staining of the luminal spermatozoa from the initial segment to the distal cauda (compare with Figs. 19–23) (× 576). **Figs. 27–30.** Immunolocalization of cellular retinoic acid-binding protein in normal rat distal vas deferens. Figures 28 and 30 counterstained with hematoxylin. Note the staining on the stereocilia border, the cytoplasmic staining at the base of the epithelial infoldings, and the light staining in the muscular layers of the vas. **Figs. 29–30.** Sections similar to those in Figs. 27 and 28 with affinity-purified anti-CRABP IgG absorbed with pure CRABP prior to incubation with peroxidase-conjugated second antibody. Figures 27 and 29 (× 238); Figs. 28 and 30 (× 576).

Further studies will be required to determine the fate and possible transfer of cellular retinoic acid-binding protein in the epididymis.

In the vas deferens, the absence of immunoreactive cellular retinoic acid-binding protein in the epithelial cells of the proximal vas and its presence in the epithelium of the distal vas indicate that these two regions express different levels of cellular retinoic acid-binding protein. Our observation confirms other reports that have shown ultrastructural, histochemical, and biochemical differences between the proximal and distal rat vas deferens (Hamilton and Cooper, 1978; Kennedy and Heidger, 1979; Chinoy and Chinoy, 1983).

The functional significance of high levels of cellular retinoic acid-binding protein in the lumen of the proximal epididymis and high levels of cellular retinol-binding protein in the epithelial cells of the same epididymal region is unclear. Their presence, however, invites speculation that a continuous transfer of vitamin A may be occurring in this epididymal segment, which is known to be involved in the synthesis and secretion of factors necessary for sperm maturation (Orgebin-Crist et al, 1981), and that vitamin A may have a role in this process.

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Eighteenth Annual Meeting of the Society for the Study of Reproduction

The Eighteenth Annual Meeting of the Society for the Study of Reproduction will be held at McGill University in Montreal, Canada from July 23 through July 25, 1985. The program will include a Symposium entitled "Gonadal and Fetal Function under Stress," an awards ceremony with a lecture by the recipient of the research award, as well as sessions devoted to research papers. To obtain additional information about the Symposium or submission of contributed papers, please contact:

Executive Secretary
The Society for the Study of Reproduction
309 West Clark Street
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Acta Endocrinologica Satellite Symposium

A satellite symposium, "Novel Aspects in the Hormonal Regulation of Testicular Function," will be arranged before the XV Acta Endocrinologica Congress in Helsinki, Finland, on August 2 through 4, 1985. The program will include invited lectures and free communications on the following topics: regulation of testicular function by GnRH and its analogs, basic and clinical aspects, paracrine interactions between testicular cell compartments, and role of non-gonadotropic hormones in testicular regulation. For further information, please write to:

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