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Immunohistochemical Studies on the Localization of Cellular Retinol-Binding Protein in Rat Testis and Epididymis

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ABSTRACT

The immunohistochemical localization of cellular retinol-binding protein (CRBP) was studied in rat testis and epididymis. Parallel studies were also carried out on the localization of plasma retinol-binding protein (RBP) and transthyretin (TTR) in testis. The studies employed antibodies purified by immunosorbent affinity chromatography, permitting the specific staining and localization of each antigen by the unlabeled peroxidase-antiperoxidase method. For RBP and TTR, specific immune staining was found in the interstitial spaces between the seminiferous tubules, and not in the tubules themselves. In contrast, strong specific immune staining for CRBP was found in the seminiferous tubules, with a striking localization within Sertoli cells. Moreover, a distinct cyclic variation of specific staining for CRBP within Sertoli cells was observed during the spermatogenic cycle. This cyclic variation was seen with regard to both the intensity of staining and to the anatomic distribution of CRBP within the Sertoli cells. Within the epididymis CRBP was selectively localized to the proximal portion of the caput epididymidis, with variations in intensity of the staining of the epithelium of the ducts in different histological zones. Specific immune staining for CRBP was very weak or absent in the other portions of the epididymis. These results were confirmed by radioimmunoassay. Vitamin A-deficient rats showed markedly reduced specific immune staining for CRBP in both testes and epididymides, and greatly reduced levels of CRBP in these tissues on radioimmunoassay. These studies on the localization of CRBP provide information concerning the specific cells and anatomic loci within the testis and epididymis where retinol may be playing an important role in sperm formation and maturation.

INTRODUCTION

Retinoids are known to be required for reproduction, and in the male for the maintenance of normal testicular structure and function (Coward et al., 1969; Howell et al., 1963; Thompson et al., 1964). Moreover, a specific requirement for retinol (vitamin A alcohol) for the maintenance of normal spermatogenesis is well established (Coward et al., 1969; Howell et al., 1963; Huang and Hembree, 1979; Thompson et al., 1964). Thus, in retinol deficiency, spermatogenesis does not proceed beyond early meiosis, and extensive loss of germinal epithelium is seen (Huang and Hembree, 1979; Krueger et al., 1974). The germ cell loss is reversible with retinol repletion (Huang and Hembree, 1979; Huang et al., 1983).

Retinol circulates in blood bound to a specific plasma transport protein, retinol-binding protein (RBP) (Goodman, 1984; Kanai et al., 1968). Delivery of retinol to extrahepatic target tissues, may involve cell surface receptors that recognize RBP (Chen and Heller, 1977; Rask and Peterson, 1976). Within many tissues, retinol is present bound to a soluble intracellular protein, cellular retinol-binding protein (CRBP) (Chytil and Ong, 1978, 1979, 1984). Although the biological role that CRBP plays within cells has not been established, it has been suggested

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that CRBP may be involved in the biological expression of retinol activity within the cell (Chytil and Ong, 1979, 1984; Liau et al., 1981).

Information is available from radioimmunoassay (RIA) studies about the tissue distribution and levels of CRBP in the rat (Adachi et al., 1981; Ong et al., 1982). Although CRBP is widely distributed in the body, CRBP levels vary considerably from tissue to tissue. High levels of CRBP were observed in liver, kidney, testis and epididymis.

We have recently reported studies on the immunocytochemical localization of CRBP in rat liver and kidney (Kato et al., 1984). The studies employed purified primary antibodies against CRBP, and examined tissues from normal rats, from retinol-deficient rats, and from rats fed excess retinol. CRBP was found to be localized in two cell types in the liver, the parenchymal cells and the fat-storing cells; specific staining for CRBP was more intense in the fat-storing cells. In the kidney, CRBP was localized in the proximal convoluted tubules of the renal cortex. The immunocytochemical localization of CRBP in liver and kidney was compared with that of RBP and of transthyretin (TTR).

We now report studies on the immunohistochemical localization of CRBP in rat testis and epididymis. Parallel studies on the localization of RBP and TTR in testis are reported as well. In the testis, CRBP appears to be strikingly localized within Sertoli cells, and to undergo cyclic variation in relation to the spermatogenic cycle. CRBP is also selectively localized in the initial segments of the caput epididymidis. The highly selective cellular localization of CRBP within both the testis and epididymis suggests that the CRBP-rich cells may be the major cellular sites of action of retinol within the male reproductive system.

MATERIALS AND METHODS

Preparation of Tissues

Male weanling rats of the Holtzman strain were divided into two groups of 8 rats each. One group was fed a purified vitamin A-deficient diet (Muto et al., 1972), and the second group was fed the same diet supplemented with 2.4 μg of retinol equivalents in the form of retinyl esters per gram of diet. The studies reported were carried out at the end of a 7-wk period on these diets. For the immunohistochemistry study, 4 normal and 4 deficient rats were anesthetized with ethyl ether, and blood was drawn from the animal via the right atrium. The organs were fixed by perfusion of the whole animal through the left ventricle (with exit through the right atrium) with an ice-cold solution of Hepes buffered saline (10 mM Hepes buffer, pH 7.4, 122 mM NaCl, 6.6 mM KCl, 1.2 mM $CaCl_2$) for 5 min, followed by ice-cold Perfix (Fisher Scientific Co., Springfield, NH) for 15 min. Perfusion was performed at a constant flow rate of 20 ml/min. The testes and epididymides were then removed from the animal, and fixation was continued for 2 h in Perfix with rotation at 4°C. The hardened testes were cut into 3- to 4-mm thick slices, and these slices, together with the intact epididymides, were fixed for 3 more h in the same manner. The fixed tissues were washed with 95% ethanol three times (8 h each time at 4°C) and embedded in paraffin. Serial sections of 4- to 5-μm thickness were mounted on glass slides. Adjacent sections of testis were subsequently stained for CRBP (immunohistochemically, see below), or with hematoxylin and eosin (H-E).

For the CRBP radioimmunoassay study, the remaining 4 normal and 4 deficient rats were also anesthetized with ethyl ether, followed by removal of blood. Each rat was then subjected to whole body perfusion through the left ventricle with 0.9% NaCl for 5 min at a constant flow rate of 20 ml/min. Testes and epididymides were removed from the animals, and the epididymides were divided into two parts: the proximal portion of the caput and the remainder of the epididymis. In this paper we refer to the proximal portion of the head (caput) region of the epididymis as the *p-cap*, and to the remainder of the epididymis (including the distal caput, the corpus, and the cauda) as the *other* portion. The *p-cap* is easily distinguished from the *other* portion by the shape and color of the tissue, and by an obvious cleft. It corresponds approximately to the initial segments of the rat epididymis as defined by Fawcett and Hoffer (1979), and to histological Zones 1 and 2 (Reid and Cleland, 1957; see below). The tissues were each minced, weighed and homogenized with a Polytron Homogenizer (Brinkmann Instruments, Westbury, NY) in 4 vol (v/w) of 50 mM imidazole buffer, pH 7.4, containing 0.79% NaCl, 0.03% bovine serum albumin, 0.1% thimerosal, 0.01% leupeptin (Peninsula Labs., San Carlos, CA), and 1% Triton X-100 (referred to as *RIA buffer*). The homogenates were centrifuged at 18,000 rpm for 2 h in a Sorvall RC-5B centrifuge. The resulting clear supernatant solutions were frozen immediately in dry ice-acetone and stored at −80°C until used.

The serum vitamin A levels in the two groups of rats, determined by the method of Thompson et al. (1971) were: normal rats, 62.0 ± 7.2 μg/dl (mean ± SD); deficient rats, 1.9 ± 0.7 μg/dl.

Immunohistochemical Staining for CRBP, RBP and TTR

The unlabeled peroxidase-antiperoxidase (PAP) method of Sternberger et al. (1970) was used as described previously (Katoh et al., 1982; Kato et al., 1984). Purified antigens (rat CRBP, RBP and TTR) were isolated, and rabbit antisera against these purified rat proteins (antigens) were prepared, as described elsewhere (Kato et al., 1984). Purified monospecific antibodies against each antigen (CRBP, RBP or TTR) were obtained as described in detail (Kato et al., 1984) by immunosorbent affinity chromatography of

the immunoglobulin G (IgG) fractions prepared from the specific antisera against each of these proteins. In each case, the affinity chromatography procedure used the respective pure antigen coupled to Sepharose 4B as the immunosorbent. Similarly, specific goat antibodies against rabbit IgG were obtained by immunosorbent affinity chromatography on rabbit IgG linked to Sepharose; cross-reactive antibodies against rat IgG were removed from the purified goat antirabbit IgG by a further immunosorbent affinity chromatography step on rat IgG coupled to Sepharose (Kato et al., 1984).

Immunohistochemical staining was carried out by performing the following incubations with deparaffinized sections of tissue: *1*) 0.3% H_2O_2 in absolute methanol for 20 min; *2*) phosphate-buffered saline (PBS), pH 7.4, containing 10% normal goat serum for 30 min; *3*) purified primary antibody (anti-CRBP, anti-RBP or anti-TTR), 25 μg/ml in PBS, for 120 min; *4*) purified goat antirabbit IgG antibody (referred to as the *bridge antibody*), 200 μg/ml in PBS, 60 min; and *5*) PAP (Accurate Chemical & Scientific Corp., Westbury, NY), diluted 40-fold in PBS, 60 min. The sections were washed with PBS three times after each incubation. They were then rinsed in 50 mM Tris-HCl buffer, pH 7.6, and reacted with a solution of 0.02% 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, MO) in 50 mM Tris-HCl buffer, pH 7.6, containing 0.003% H_2O_2 for 5 min. Some tissues were counterstained with diluted (4-fold) hematoxylin (Gill formulation #1, Fisher) in 25% (v/v) ethylene glycol. Under these conditions, background peroxidase staining or endogenous peroxidase activity were not observed.

As described elsewhere (Kato et al., 1984) for these same antibody preparations and procedures, a variety of control experiments were conducted to examine the specificity of the immunocytochemical procedures used. The specificities of the purified primary antibodies were demonstrated by double immunodiffusion (for anti-RBP and anti-TTR) and by enzyme-linked immunoabsorption assay (ELISA) (for anti-CRBP), and were tested immunocytochemically by absorbing small portions of each antibody solution with each of the purified antigens coupled to Sepharose (Kato et al., 1984). When tested with sections of testis and epididymis, the specific staining associated with each antibody disappeared after absorption with the particular antigen against which the antiserum had been raised. The staining was not affected by absorption with the other antibodies. Some of the results of this absorption study are presented below in the *Results* section (Figs. 3a and 8b). No specific immune staining was observed after omission of the primary antibody from the staining procedure, or when one or another of the following components of the staining procedure was omitted: the bridge antibody; PAP; or the final substrate for the peroxidase reaction.

Variations Found During the Spermatogenic Cycle

A study was conducted to determine whether the intensity of specific staining for CRBP varied during the cycle of the seminiferous epithelium. The stages of the cycle of the seminiferous epithelium were identified according to the classification of Roosen-Runge and Giesel (1950). Sections of testis with H-E staining were examined by a single investigator, and each tubule seen in complete cross-section was classified as being in one of eight stages, defined according to the morphological criteria described (Roosen-Runge and Giesel, 1950). It was felt that this classification was preferable to the more complex one of Leblond and Clermont (1952) for the exploration of the question of whether or not a cyclic variation in CRBP staining was present. It is possible, if one desires, to correlate the eight stages of the cycle in the classification used here (Roosen-Runge and Giesel, 1950) with the fourteen stages of the cycle as defined by Leblond and Clermont (1952), or with the six stages as defined by Huckins (1978). In the present study, the relative intensity of specific staining for CRBP was estimated on sections of testis immediately adjacent to the ones used for H-E staining and cycle staging. Each tubule seen in complete cross-section was assigned a value of 1 (weak), 2 (moderate), 3 (strong) or 4 (very strong) to describe the relative intensity of staining of the Sertoli cells (see *Results*) for CRBP. Tubules that did not stain for CRBP were designated as 0. The intensity of CRBP staining was estimated by a single investigator who was different from the one who carried out the classification of the tubules on the H-E sections, and without knowledge of the results of the staging of the tubules.

RIA of CRBP

CRBP was quantitatively measured by RIA, using a modification of the method previously reported (Adachi et al., 1981). The assay employed ^{125}I-labeled CRBP, and freshly prepared specific turkey antibodies against rat CRBP. CRBP was acylated with ^{125}I-labeled Bolton-Hunter reagent (New England Nuclear, Boston, MA; 4000 Ci/mmol) according to the original method (Bolton and Hunter, 1973). The specific radioactivities of the acylated CRBP preparations ranged from 22 to 32 mCi/mg in several acylations. Turkey antiplasma against CRBP and rabbit antiserum against turkey IgG were freshly prepared as described previously by Adachi et al. (1981). Purified monospecific IgG against CRBP was obtained by immunosorbent affinity chromatography of the whole turkey IgG fraction with CRBP linked to Sepharose, as described above. One mg of purified turkey antibody against rat CRBP was mixed with 99 mg preimmune turkey IgG in 5 ml 0.9% NaCl and stored at −80°C until used.

The RIA for CRBP was carried out as follows. All reagents were dissolved in or diluted by RIA buffer (defined above). The concentration of pure CRBP in the standard solution was estimated from 280 nm absorbance, using an $E^{1\%}_{1cm}$ of 14 (Ross et al., 1978). All procedures were carried out at 4°C. Reagents were added to the polystyrene tubes (12 × 75 mm) and incubated in the following sequence: *a*) 100 μl of the sample to be assayed (standard CRBP solution or diluted tissue cytosol); *b*) 50 μl of the turkey anti-CRBP IgG solution (0.05 μg of purified specific IgG and 4.95 μg of nonimmune IgG); *c*) 300 μl RIA buffer, incubated 2 h; *d*) 50 μl of ^{125}I-CRBP (approx. 20,000 cpm), incubated 24 h with rotating by a roller drum (Bellco Glass Inc., Vineland, NJ); *e*) 25 μl of rabbit antiserum against turkey IgG, incubated 3 h with rotating by roller drum; and *f*) 500 μl of RIA buffer. After mixing, the samples were centrifuged at 5000 rpm for 30 min in an HS-4 rotor in a Sorvall RC-5B centrifuge. The supernatants were removed by

aspiration, and the precipitates were assayed for ^{125}I in a LKB 1274 RIAGAMMA counter.

The RIA as used here was much more sensitive (by almost an order of magnitude) than the assay previously reported (Adachi et al., 1981), and effectively measured CRBP levels in the range of 2 to 20 ng per assay tube. A pooled sample of testis cytosol from three normal rats was assayed for CRBP in 16 tubes (8 different dilutions, each in duplicate) in 4 different immunoassay runs. The values for the within-assay and between-assay variabilities were estimated as 2.7% and 4.8%, respectively.

The assay was highly specific for CRBP. Neither cellular retinoic acid-binding protein (CRABP), purified as described by Kato et al. (1984), RBP, nor liver Z-protein [which has been reported to show some sequence homology to CRBP and CRABP (Takahasi et al., 1982)] displaced ^{125}I-CRBP from the turkey antirat CRBP antibodies. Identical displacement curves were obtained with increasing concentrations of pure CRBP, and of the cytosol from rat testis or from the proximal caput of the epididymis.

RESULTS

Localization of RBP and TTR in the Normal Rat Testis

Specific immunohistochemical staining for both RBP and TTR was observed in the interstitial spaces between the seminiferous tubules (Fig. 1). No staining for either RBP or TTR was found in the seminiferous tubules. Within the interstitial space, immunoreactive RBP and TTR were both localized in the lymphatic sinusoids (Figs. 1b and d) and not within the blood vessels that were seen on cross-section (*arrows* in Figs. 1a and c). No evidence of localization of RBP or TTR within interstitial cells was observed. Lines of positive staining were sometimes, but not always, observed along the outer edges of seminiferous tubules (*arrowheads* in Figs. 1a and c) and of some interstitial cells (*arrowheads* in Fig. 1b).

Localization of CRBP in the Normal Rat Testis

Figures 2–4 illustrate the localization of CRBP in the normal testis. Strong specific immune staining for CRBP was found in the seminiferous tubules, with very weak staining observed in the interstitial tissue (Fig. 2a). The specific staining was completely eliminated by the absorption of anti-CRBP by pure CRBP (Fig. 3a). The staining of the cells of the seminiferous tubules was selective (discussed below), and showed variation in intensity from tubule to tubule. As shown in Fig. 3b, the cells that were strongly positive for CRBP staining could be identified as Sertoli cells (*SC*) by their distinctive columnar shape extending from the base of the seminiferous epithelium to the tubule lumen. Only weak staining for CRBP was seen in spermatogonia (*Sg*), spermatocytes (*P*, pachytene spermatocyte), spermatids (*Spt*), and the limiting membrane comprising myoid cells and lymphatic endothelium (*arrowheads*, Fig. 3b). We could not determine whether or not CRBP was localized in the spermatids undergoing elongation, since we could not distinguish these from cytoplasm of Sertoli cells. Weak staining was also observed in some cells of the interstitial tissue (*arrowheads* in Fig. 3c). These cells may have been Leydig cells, but definite identification of these cells was not possible with the resolving power of our immunohistochemical technique.

Cyclic Variation of CRBP in Sertoli Cells

A study was carried out to explore the possible cyclic variation of intensity of immune staining for CRBP during the cycle of the seminiferous epithelium. As described in the *Materials and Methods* section and shown in Fig. 2b, seminiferous tubules were classified as being in one or another of 8 stages of the spermatogenic cycle. The relative intensity of specific staining for CRBP of the Sertoli cells of each tubule was then estimated on adjacent sections by a different investigator, using a semiquantitative scale from 0 to 4. The mean values of intensity of CRBP staining for tubules at each stage of the cycle were then calculated.

Two experiments were carried out, with very similar results. Figure 5 shows the combined results of the two experiments, in which a total of 427 tubules were evaluated. A distinct cyclic variation in the intensity of staining for CRBP during the spermatogenic cycle was observed. This variation is illustrated, for a single section, in Fig. 2a. Sertoli cell CRBP content (i.e., intensity of immune staining) increased during Stages 1 and 2, to reach very high levels at Stages 3 through 5. During Stages 6 and 7 the CRBP content decreased, becoming quite low at Stage 8.

Figure 4 illustrates, at higher magnification than Fig. 2a, the variation in intensity and distribution of specific staining for CRBP that was observed in tubules at different stages of the spermatogenic cycle. CRBP staining of Sertoli cells was moderately intense in tubules at Stage 2 (Fig. 4a), and was very strong in tubules at Stages 3 and 4 (Fig. 4b). It has been reported that lipids occur as large droplets in the basal part of the Sertoli cells during

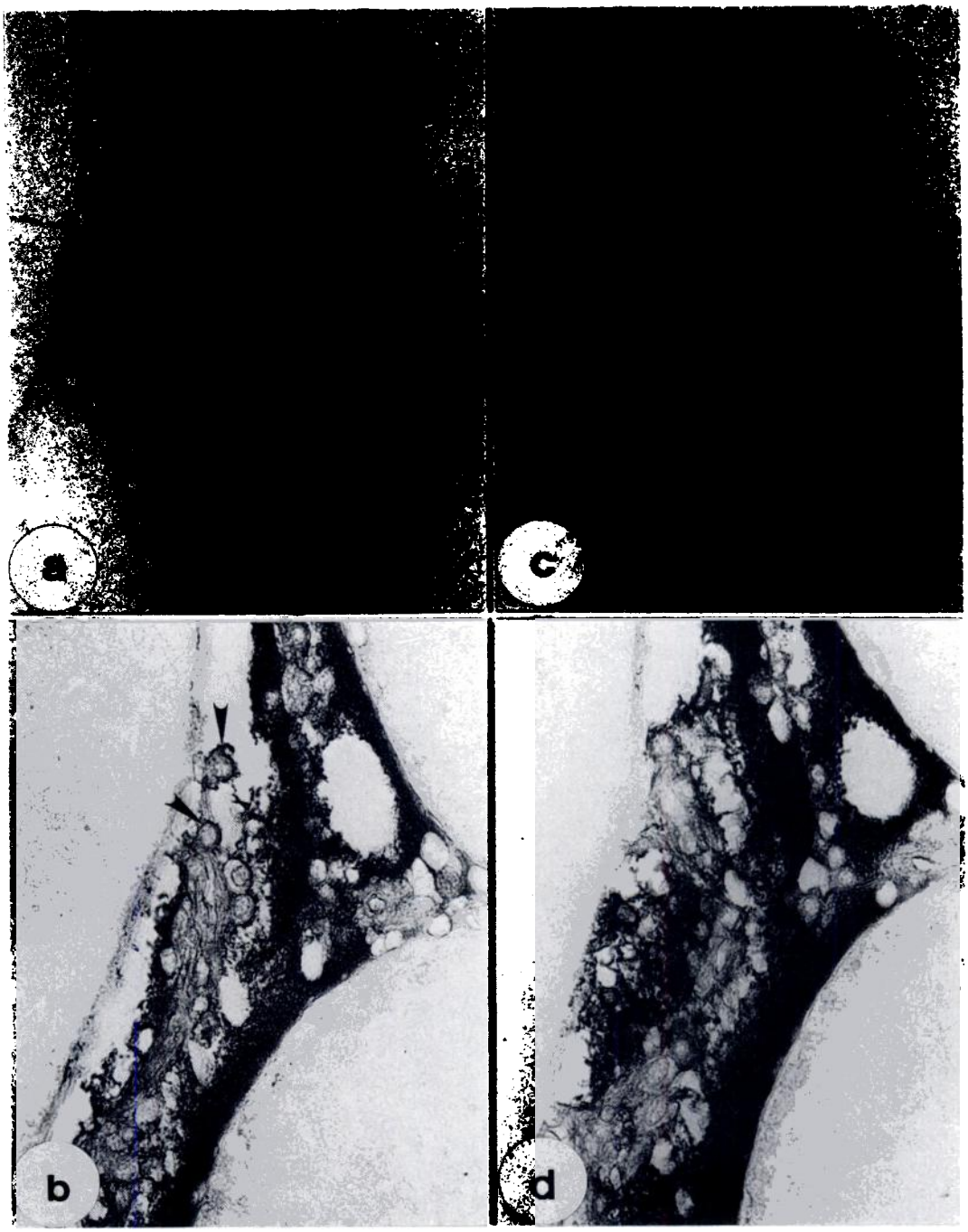

FIG. 1. Localization of RBP (*a* and *b*) and TTR (*c* and *d*) in the normal rat testis. Figs. *b* and *d* are higher magnification (X210) micrographs of portions of Figs. *a* and *c* (X70) respectively. Blood was washed out from the blood vessels (*arrows* in *a* and *c*) by perfusion, and both immunoreactive RBP and TTR were found to be localized in the interstitial space. No reaction product (immune staining) was found in the seminiferous tubules nor within the interstitial cells. The lines of staining along the edges of some seminiferous tubules (*large arrowheads* in *a* and *c*) and of some interstitial cells (*small arrowheads* in *b*) were often observed.

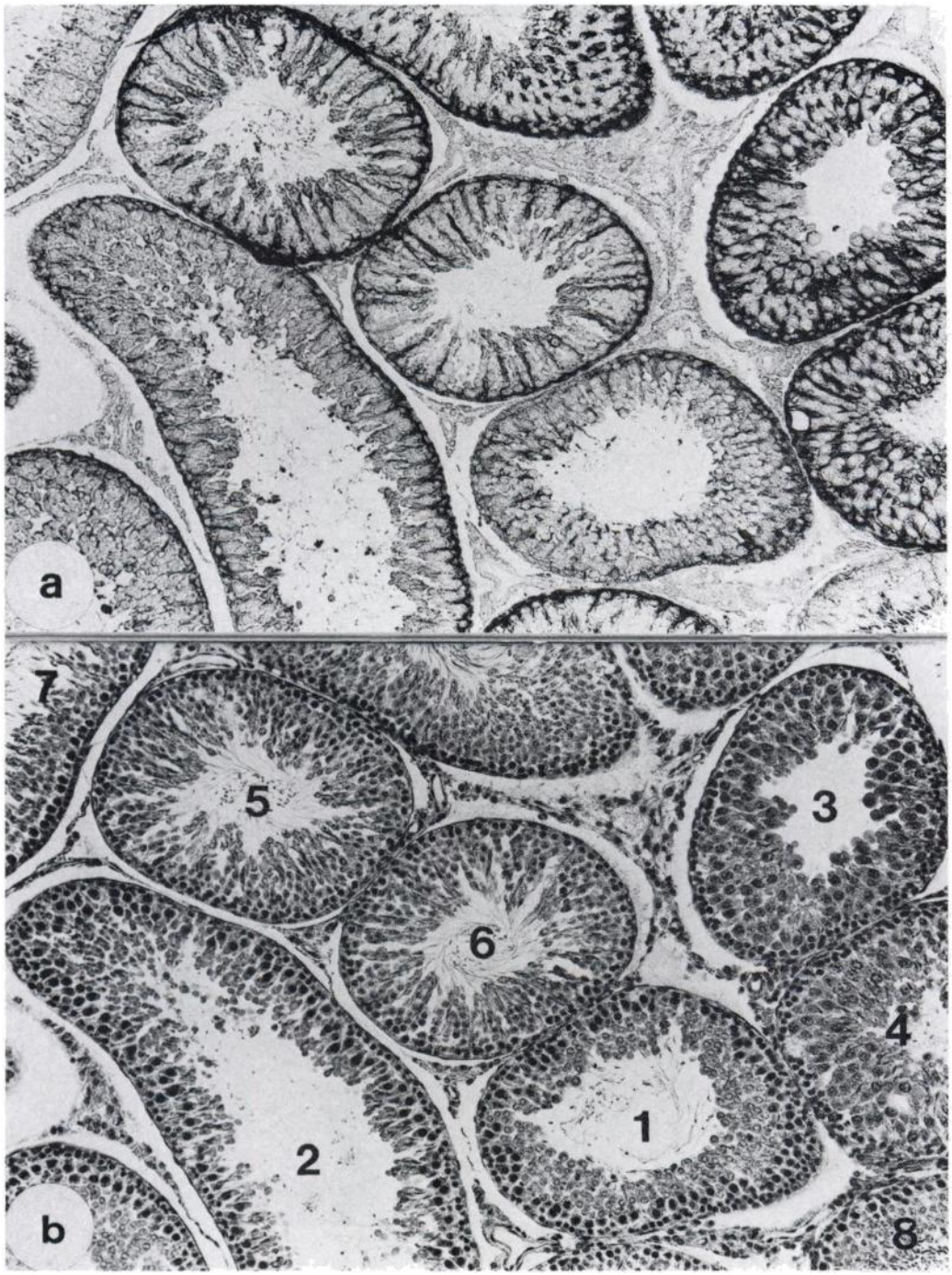

FIG. 2. Localization of CRBP in the normal rat testis. Adjacent serial sections of testis were stained immunohistochemically for CRBP (*a*), or with hematoxylin-eosin (H-E). In the H-E section (*b*), the tubules were classified into the 8 stages of the spermatogenic cycle (numbers *1* through *8*, see *Materials and Methods* section). Note the cyclic variation of intensity of CRBP staining among the seminiferous tubules, and the absence of strong staining in the interstitial tissues (*a*). ×55.

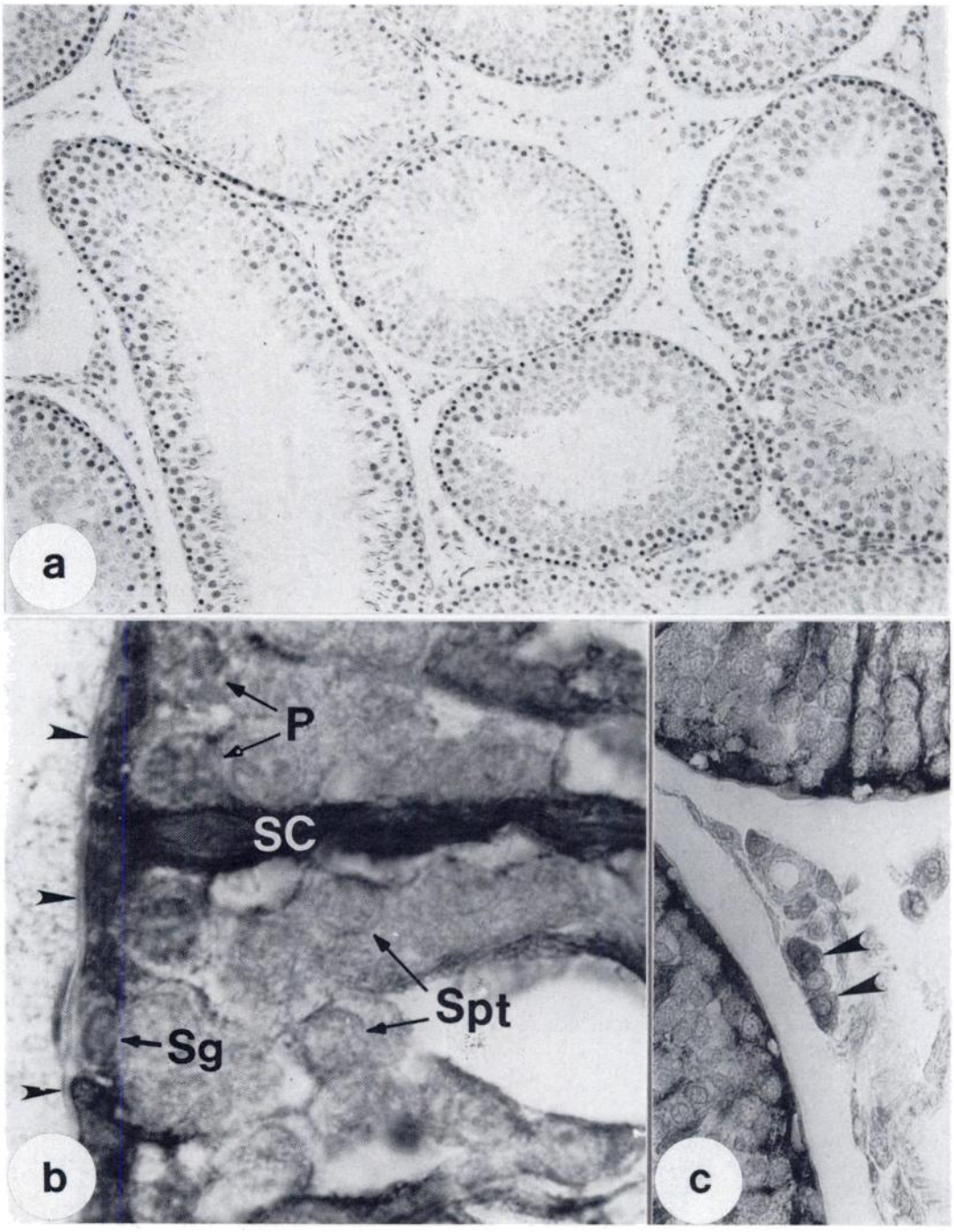

FIG. 3. Localization of CRBP in the normal rat testis. *a*) Control section, stained with the solution of anti-CRBP antibodies that had been absorbed with CRBP (×55). This section of testis was the section immediately adjacent to one shown in Fig. 2a. No specific immunohistochemical staining was seen with the absorbed antibody solution. The stained nuclei visible in this section (Fig. 3a) were stained with the hematoxylin counterstain, and not by the peroxidase reaction. *b*) Localization of CRBP in the Sertoli cell (*SC*) (×480). Note the absence of strong staining in the following: spermatogonia (*Sg*), spermatocyte (*P*, pachytene spermatocyte), spermatid (*Spt*), or the limiting membrane of the seminiferous tubule (*arrowheads*). *c*) Interstitial cells with moderate staining for CRBP (*arrowheads*) (×160).

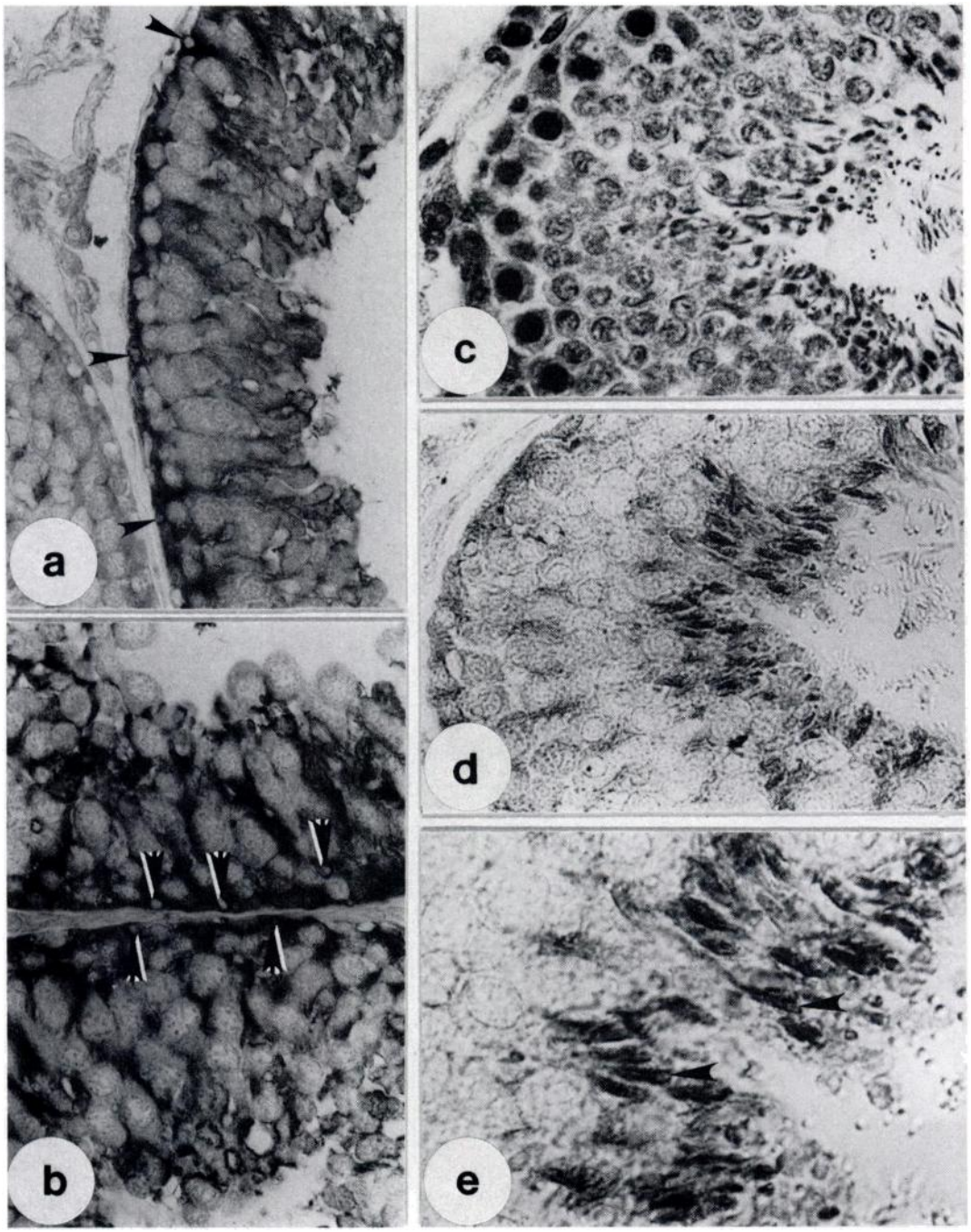

FIG. 4. Localization of CRBP in seminiferous tubules at different stages of the spermatogenic cycle in the normal rat testis. *a*) Seminiferous tubule at Stage 2 of the cycle; note the moderate staining for CRBP of the Sertoli cells and the large vacuoles in some of these cells (*arrowheads*). *b*) Seminiferous tubule at Stage 3 (*upper portion*) and at Stage 4 (*lower portion*) of the cycle; note the very strong staining for CRBP in the Sertoli cells and the many vacuoles present in their basal portions (*arrows*). *c-e*) Seminiferous tubules at Stage 8 of the spermatogenic cycle. *c*) H-E staining on the section adjacent to the section of *d*; note the spermatozoa which almost completely line up at the apical end of the seminiferous epithelium. *d*) Note the positive immunoreactive CRBP stain at the apical portion of seminiferous epithelium where the heads of spermatozoa are lining up. *e*) The high magnification of the apical portion of the seminiferous tubule which is shown in *d*; note the local accumulation of immunoreactive CRBP in the end of Sertoli cell processes which hold the heads of spermatozoa (*arrowheads*). a-d X160; e X360.

Stages 2–4 [X-XIV by the classification of Leblond and Clermont (1952)] of the spermatogenic cycle (Kerr and De Kretser, 1975; Posalaki et al., 1968). In the present work large vacuoles were observed in the basal part of the Sertoli cells at Stages 2 (Fig. 4a), 3 and 4 (Fig. 4b). Thus, the peak of CRBP content in Sertoli cells appeared to overlap and follow the occurrence of large lipid inclusions in these cells.

During Stages 7 and 8, specific immune staining for CRBP was mainly localized in the apical (luminal) portion of the tubules (Figs. 4d and e), in a distribution pattern very different from that seen at Stages 1 through 6. At Stage 8, spermatozoa are lined up at and embedded within the apical end of the seminiferous tubule wall. The distribution and localization of CRBP staining at this stage was similar to the distribution of spermatozoa in the tubule wall (Figs. 4c and d). However, in most sections the sperm heads (*arrowheads* in Fig. 4e) and the residual bodies of the rat spermatozoa did not appear to stain for CRBP, and the structures stained for CRBP appeared different with regard to both shape and size from the heads of the spermatozoa or the residual bodies. These CRBP-containing structures could not be identified with certainty. We believe, however, that they represent the apical portions of the processes of Sertoli cells, in close apposition to the newly formed spermatozoa.

Effects of Vitamin A Deficiency on the Testis

Figure 6a shows a section of testis from a vitamin A-deficient rat, stained immunohistochemically for CRBP. The intensity of staining for CRBP in the Sertoli cells was markedly decreased in the vitamin A-deficient testes, as compared to the normal ones. This striking decrease in specific staining for CRBP was seen in all seminiferous tubules examined, in sections of testis from all 4 vitamin A-deficient rats.

In 3 of the 4 deficient rats, the seminiferous tubules could not be classified into stages of the spermatogenic cycle because of severe germ cell loss that was present throughout the testes. In 1 of the 4 deficient rats, however, the testes exhibited a transitional period of degeneration or loss of germ cells, but retained enough normal morphology to permit classification of tubules into the 8 stages of the spermatogenic cycle. Figure 6b shows a section of testis from

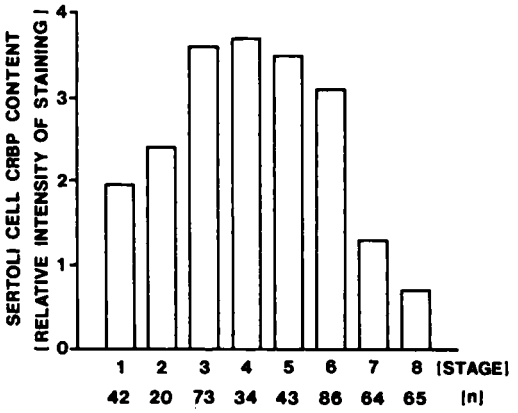

FIG. 5. Variation in the relative intensity of staining for CRBP in the Sertoli cells during the cycle of the seminiferous epithelium. The relative intensity of staining for CRBP was estimated semiquantitatively in arbitrary units as: *0*=absent; *1*=weak; *2*=moderate; *3*=strong; and *4*=very strong. These estimations, of relative intensity of CRBP staining, were mainly based upon examination of the basal portions of the Sertoli cells. Each bar shows the mean value for the relative intensity of tubules at a given stage of the spermatogenic cycle. Tubules were classified into one or another of 8 stages of the cycle by morphological criteria (see *Materials and Methods* and Roosen-Runge and Giesel, 1950); the stage is indicated below each bar by the *numbers 1* through *8*. The number of tubules (*n*) identified in each stage (and estimated with regard to CRBP staining) is indicated below each bar for each stage.

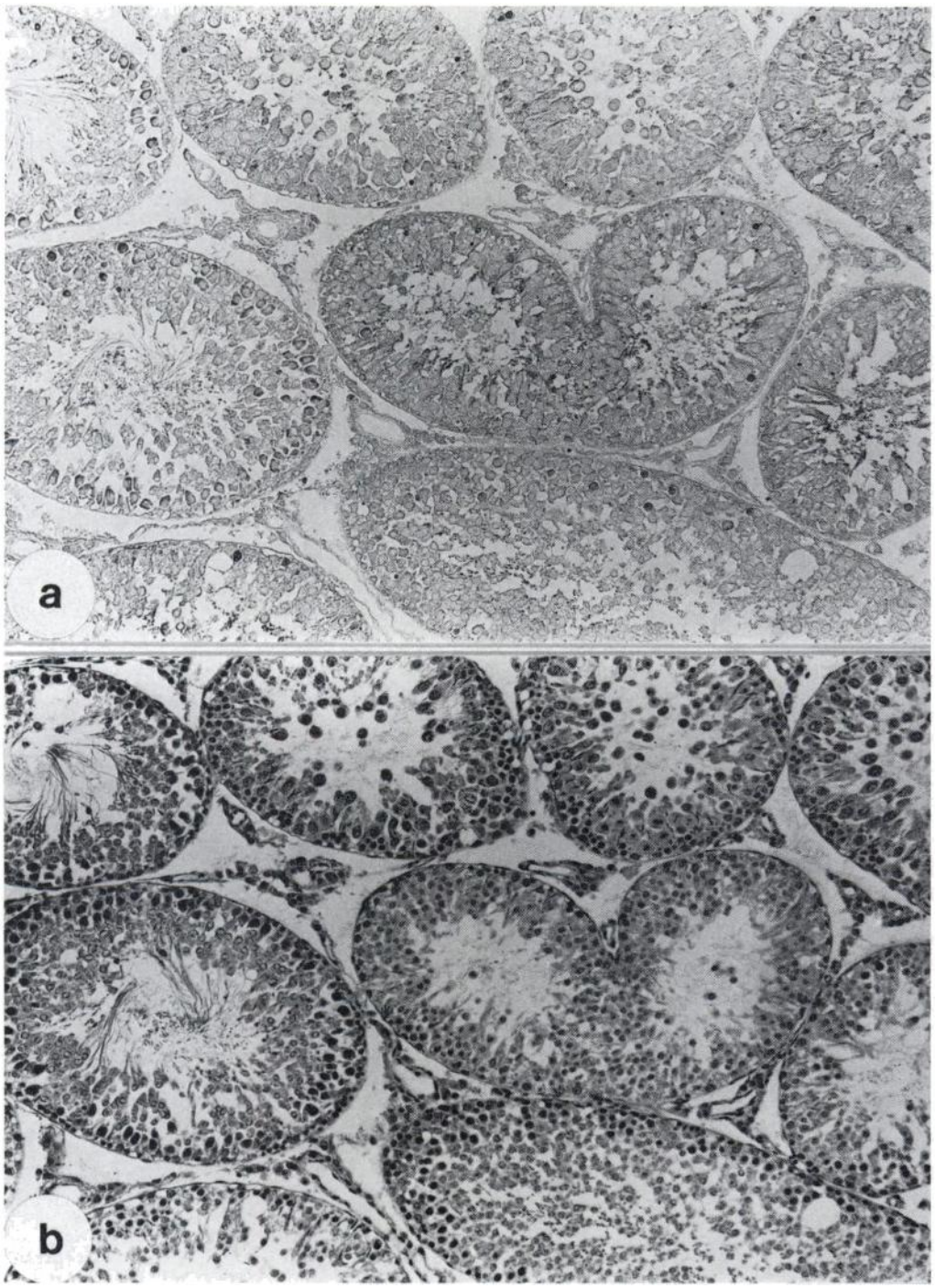

FIG. 6. Localization of CRBP in the testis from a vitamin A-deficient rat. Adjacent serial sections of testis were stained immunohistochemically for CRBP (*a*) or with H-E (*b*). The testis shown in this figure displayed relatively mild damage from vitamin A deficiency. Only weak staining for CRBP was observed in both the seminiferous tubules and the interstitial space (*a*). ×55.

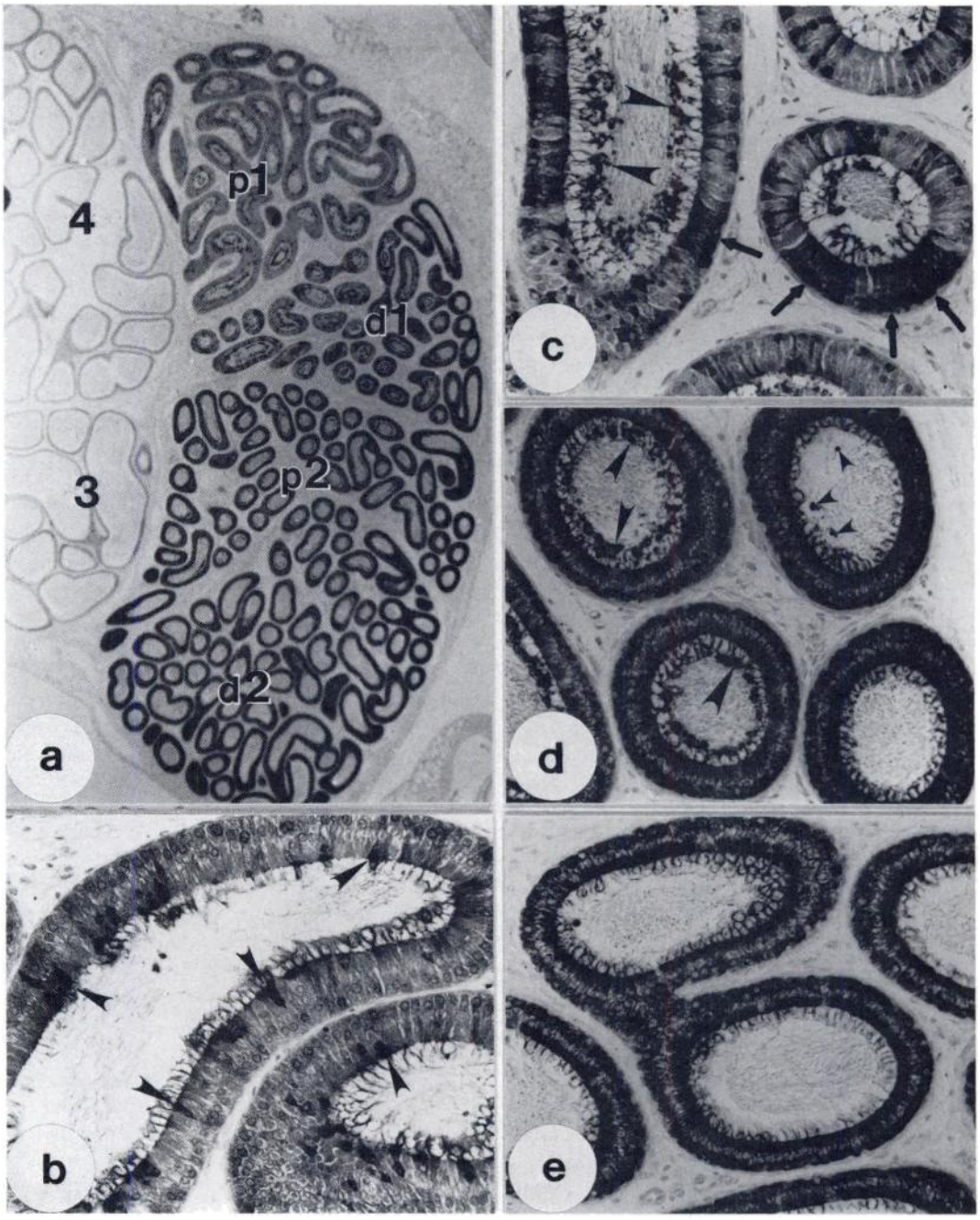

FIG. 7. Localization of CRBP in the head region of the normal rat epididymis. *a*) Highly selective localization of CRBP in the proximal caput of the epididymis. The numbers (*1* through *4*) identify the different histologic zones (Reid and Cleland, 1957; Reid, 1958; Reid, 1959) of the epididymis. Note the variation of staining in the different regions of the *p-cap*, which comprises Zones 1 and 2 (*p*=proximal; *d*=distal), and the virtual absence of staining in Zones 3 and 4. ×25. *b-e*). Higher magnification (×200) micrographs of Zones p1, d1, p2 and d2, respectively. The proximal portion of Zone 1 (*b*) showed some small cells with strongly positive CRBP staining, most of which were located at the lumen side of the duct (*arrowheads*). The distal portion of Zone 1 (*c*) showed a variable intensity of staining, with some intensely stained cells (*arrows*) that resembled the staining seen in the proximal portion of Zone 2 (*d*). In some portions of the ducts, granules with strongly positive CRBP staining were observed at the apical edge of the stereocilia (*large arrowheads* in *c* and *d*); some of these were seen in the lumen apart from stereocilia (*small arrowheads* in *d*). The section was counterstained with hematoxylin.

this rat, with H-E staining. A preliminary study was carried out with the sections of testis from this rat, in order to examine relationships between loss of germ cells and the stage of the cycle. Examination of 697 tubules showed that the most severe loss was shown for late spermatocytes undergoing meiotic divisions at Stages 3 and 4. In these stages, many diplotene spermatocytes and dividing meiotic cells were found sloughed into the lumen. The late spermatids were also lost severely in the seminiferous tubules at Stages 5, 6 and 7. Changes (loss) of other types of cells, in other stages, were much less marked. These findings are consistent with the observations of others (Huang and Hembree, 1979; Huang et al., 1983) that retinol deficiency particularly arrests spermatogenesis in early meiosis.

Localization of CRBP in the Normal Rat Epididymis

Specific immune staining for CRBP was strikingly localized to the proximal caput of the epididymis, and was very weak or absent from the other portions of the epididymis (Figs. 7a and 8a). In the proximal caput the epithelium of the ducts showed strongly positive staining for CRBP, with variations in intensity among the different kinds of epithelial cells and in different histological zones. In the rat epididymis there are regional histological variations, which have been classified into six zones (Reid and Cleland, 1957; Reid, 1958, 1959). The intensity of staining for CRBP appeared to increase from the proximal portion of Zone 1 (*p*1 in Fig. 7a) (into which the efferent ducts empty) to Zone 2, and then to abruptly decrease (and virtually disappear) in the ducts of Zone 3. The proximal portion of Zone 1 showed strong staining in small cells (*arrowheads* in Fig. 7b), and weak staining in principal cells (tall columnar cells). The distal portion of Zone 1 (*d*1 in Fig. 7a) showed an increasing number of columnar cells with strongly intense staining (*arrows* in Fig. 7c). In Zone 2, the majority of the columnar cells showed strong staining for CRBP (Figs. 7d and e). In the lumina of some of the ducts of Zones 1 and 2, granules showing strongly positive staining for CRBP were observed (*arrowheads* in Figs. 7c and d). Some of these granules, intensely stained for CRBP, were located in the lumen apart from stereocilia (*small arrowheads* in Fig. 7d). As shown in Fig. 8c, the basal cells (*B*), the peritubular cells (*PT*) and some epithelial cells interposed between principal cells (*arrow*) showed no positive staining for CRBP. All positive staining for CRBP was eliminated by absorption of the anti-CRBP solution with pure CRBP (Fig. 8b).

Effects of Vitamin A Deficiency on the Epididymis

The intensity of specific immune staining for CRBP was markedly decreased in the epididymides of all of the 4 vitamin A-deficient rats, as compared to the normal rats. Figures 8d and e show the decrease in intensity of CRBP staining in the distal region of Zone 1 and the proximal region of Zone 2, respectively (compare with Figs. 7c and d). Luminal granules strongly positive for CRBP were not observed in any region of the vitamin A-deficient epididymis.

CRBP Levels in Testes and Epididymides

The concentrations of CRBP were measured by RIA in the testis, the proximal caput of the epididymis, and the *other* portion of the epididymis in each of 4 normal and 4 vitamin A-deficient rats. The results are shown in Table 1. The highest concentration of CRBP was found in the proximal caput of the epididymis of normal rats. The *other* portion of the epididymis contained only a low level of CRBP. The CRBP content of all three tissues was markedly decreased in vitamin A deficiency. Thus, these quantitative data agree with the qualitative/semiquantitative results obtained from the immunohistochemical studies.

DISCUSSION

These studies provide detailed information on the immunohistochemical localization of CRBP within different types of cells in the rat testis and epididymis. As discussed previously (Kato et al., 1984), an important methodological aspect of this work was the use of primary and bridge antibodies purified by immunosorbent affinity chromatography to avoid background and nonspecific staining. Highly selective localization of CRBP within certain, specific cells was observed in both organs. Within the testis, CRBP was particularly localized within the cytoplasm of the Sertoli cells of the seminiferous tubules. Only weak staining for CRBP was seen in the germinal cells and in

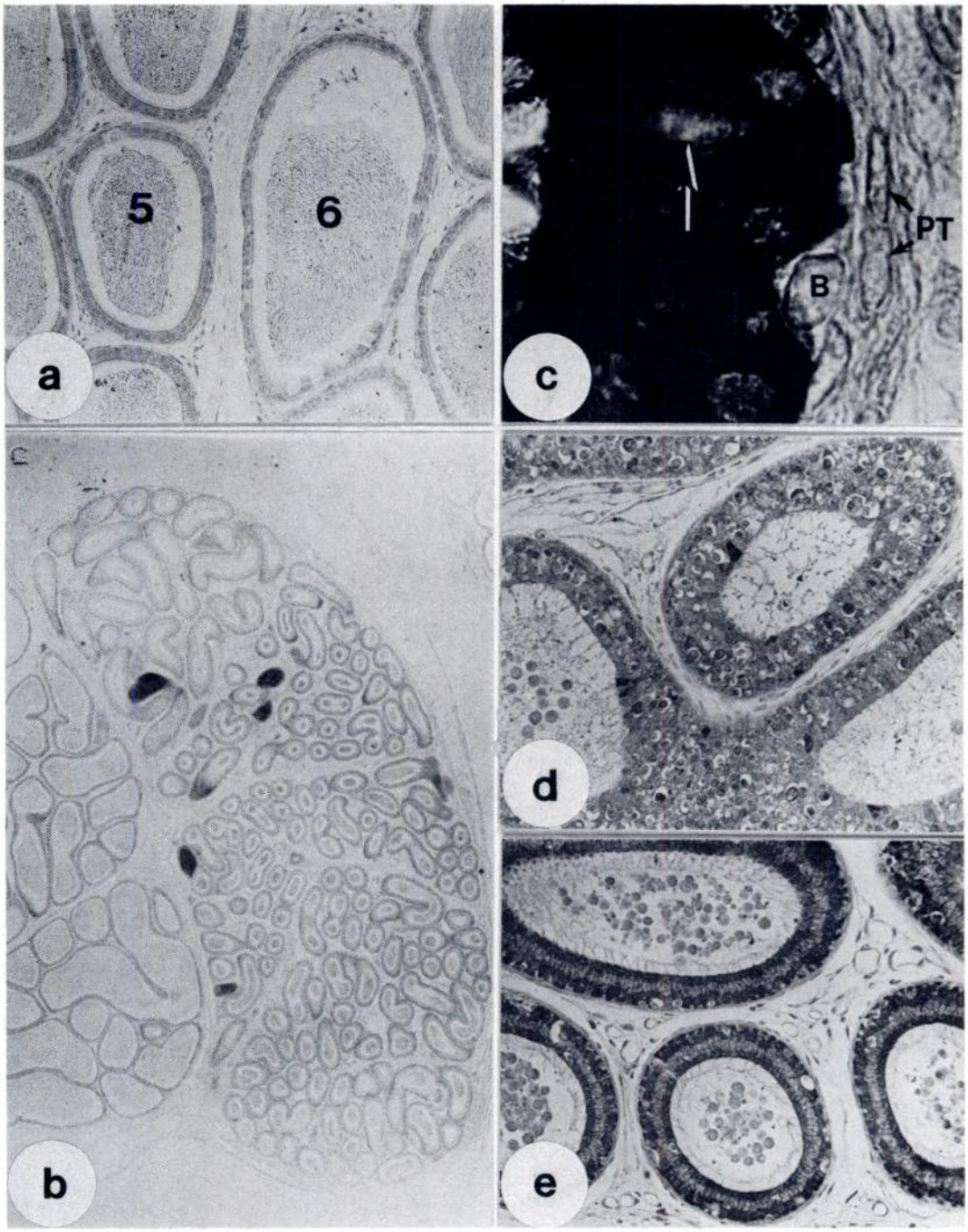

FIG. 8. Localization of CRBP in the normal (*a* and *c*) and vitamin A-deficient (*d* and *e*) rat epididymis and control section of the head region of the normal rat epididymis stained with the solution of anti-CRBP antibodies that had been absorbed with pure CRBP (*b*). *a*) Zone 5 (*left portion*) and Zone 6 (*right portion*) of cauda epididymidis. X25; note the virtual absence of staining in both zones. *b*) The section adjacent to the section shown in Fig. 7a; note the absence of any immunostaining for CRBP after absorption of anti-CRBP by pure CRBP (*see Materials and Methods*). X25. *c*) Proximal portion of Zone 2; note the absence of immunoreactive CRBP in an epithelial cell (*arrow*), and in basal cells (*B*) and peritubular cells (*PT*). X1360. *d* and *e*) Distal portion of Zone 1 (*d*) and proximal portion of Zone 2 (*e*) of the epididymis from a vitamin A-deficient rat; note the marked decrease in intensity of staining for CRBP in both zones as compared with the respective zones of the normal epididymis (Figs. 7c and d). X200. Nuclei were counterstained in all sections except the section for Fig. 8a.

TABLE 1. Concentrations of CRBP in the testis, the proximal caput of the epididymis (*p-cap*), and the remainder of the epididymis (*other*), in normal and in vitamin A-deficient rats.[a]

	Normal	Vitamin A deficient
Testis	22.6 ± 1.2[b]	6.2 ± 1.4
Epididymis, *p-cap*	117.3 ± 10.7	17.1 ± 4.6
Epididymis, *other*	7.0 ± 2.2	2.9 ± 0.4

[a]CRBP levels are in μg per g wet weight of tissue.

[b]Mean ± SD (n=4) values are shown.

the interstitial tissue. Within the epididymis, CRBP was strikingly localized in the cytoplasm of the ductular epithelium of the proximal portion of the caput. In contrast, weak to no staining for CRBP was seen in the ductular epithelium of the distal caput or of the remainder of the epididymis.

Unlike CRBP, both plasma RBP and TTR were found to be localized in the interstitial space of the testis, but not within the seminiferous tubules. These immunohistochemical findings are in agreement with the autoradiographic studies of McGuire et al. (1981), who injected radioactively labeled RBP into rats and found radioactivity in the testis localized to the interstitium. Since RBP delivers retinol from hepatic stores to the testis (and other extrahepatic tissues), and since retinol has been found in seminiferous tubule cells (Ahluwalia et al., 1975; Rajguru et al., 1982) and even in spermatozoa (Gambhir and Ahluwalia, 1975), these observations raise the question of the detailed mechanism whereby retinol reaches the seminiferous tubule cells. One possibility is that RBP interacts with the basal membrane of Sertoli cells (or with cells in the basal compartment of the tubule), transfers its retinol ligand to the cell, and then leaves the cell membrane without significant accumulation of RBP at that site. This possibility is consistent with a report of apparently specific binding of RBP to a membrane fraction from chicken testis homogenates (Bhat and Cama, 1979). A second possibility is that retinol delivery from interstitial to seminiferous tubule cells involves an interstitial binding protein for retinol, distinct from plasma (or cellular) RBP. Such an interstitial binding protein for retinol has been reported in the interphotoreceptor matrix of the retina (Bunt-Milam and Saari, 1983; Liou et al., 1982). In this regard, it has recently been reported that a cell line derived from murine Sertoli cells secretes a relatively high molecular weight binding protein for retinol, distinct from RBP and CRBP, into the medium (Carson et al., 1984).

Sertoli cells play an essential role in spermatogenesis, providing the germ cells both with physical support and with metabolically needed "nutrients" (Ritzen et al., 1981; Steinberger and Steinberger, 1977). Morphologically, Sertoli cells provide a structural framework that organizes the migration of developing germ cells into and through the adluminal compartment. Because of the blood-testis barrier, all nutritional or regulatory substances have to pass through the Sertoli cell cytoplasm to reach germ cells within the adluminal compartment (Ritzen et al., 1981; Steinberger and Steinberger, 1977). Studies on the distribution of vitamin A in the testis have suggested that the Sertoli cell may be a significant site of localization of retinol (Ahluwalia et al., 1975; Rajguru et al., 1982). In addition, CRBP activity, assayed as binding activity for retinol, has been identified in the cytosols of cultured Sertoli cells and peritubular cells from rat testis (Huggenvike and Griswold, 1981).

In vitamin A deficiency with germ cell depletion, Sertoli cells morphologically remain relatively intact (Huang and Hembree, 1979; Huang et al., 1983). Despite this, studies with vitamin A-deficient rats have suggested that vitamin A does affect Sertoli cell function (Huang and Hembree, 1979; Rich and de Kretser, 1977). Moreover, studies with cultured Sertoli cells have demonstrated direct effects of retinol on these cells, including effects on the production of androgen binding protein (Karl and Griswold, 1980), and effects on uridine nucleotide metabolism (Carson and Lennarz, 1983).

Sertoli cells are known to undergo cyclic variation in morphology and function during the spermatogenic cycle (Ritzen et al., 1981). In the present studies, a distinct cyclic variation of specific staining for CRBP was observed, both with regard to the intensity of staining for CRBP and with regard to the anatomic distribution of CRBP within the Sertoli cell.

The finding that CRBP is strongly localized and demonstrates a cyclic variation in Sertoli cells suggests that CRBP may play an important role in the metabolism and/or function of

retinol in these cells. The observations also support the concept that the Sertoli cell plays a critical role in the mechanisms whereby vitamin A influences and maintains spermatogenesis. The nature of these mechanisms, and of the specific roles played by CRBP, remain to be established. CRBP in the Sertoli cell may be involved in the uptake of retinol into the cell, in the direct actions of retinol on the Sertoli cell, in the possible secretion of retinol into seminiferous fluid (see below), or in the transfer of retinol to developing germ cells. Data are needed about these and other possibilities.

The highly selective localization of CRBP in Zones 1 and 2 of the epididymis suggests that retinol plays a particular functional role at these sites. After the completion of these studies, moreover, and while this manuscript was in preparation, Porter et al. (1983) reported similar findings concerning the immunolocalization of CRBP in the rat epididymis. In both the present work and that of Porter et al. (1983), the immunohistochemical findings were confirmed by RIA. It is well established that during transport of spermatozoa in the epididymis they undergo a number of changes (maturation) which result in the ability to fertilize (Burgos, 1974). Some of these changes occur in the caput epididymidis, including the development of sperm motility (Hinton et al., 1979) and morphologic changes in the acrosome (Bedford, 1965). If we assume that the presence of CRBP reflects a role for retinol at a particular site, then the present findings suggest that retinol may play a particular role in sperm maturation in the proximal caput. The nature of this selective role remains to be explored.

Fawcett and Hoffer have reported that the initial segments of the rat epididymis are dependent upon substances in the testicular fluid (Fawcett and Hoffer, 1979). Following castration and androgen administration a selective regression of the initial segments, but not of more distal segments, was observed. It was concluded that the initial segments require high intraluminal concentrations of androgen bound to androgen binding protein; the possibility that they are dependent upon some other, unidentified, constituent of testicular fluid was also raised. It is of interest that the initial segments correspond to the zones (1 and 2) of CRBP localization as observed here. It is known, moreover, that androgen binding protein, which is concentrated in the proximal regions of the epididymis, originates in the testis (Hansson et al., 1974). Thus, the possibility that CRBP in the proximal caput also originates in the testis, and represents the unidentified constituent of testicular fluid suggested by Fawcett and Hoffer, can be considered. Studies directed at the site of biosynthesis of CRBP in these tissues would help address this question.

The vitamin A (retinoid)-deficient rats studied here showed markedly decreased levels of CRBP in both testis and epididymis. This was demonstrated by immunohistochemistry and confirmed by RIA. More information is needed about the role of retinoids in regulating the metabolism and levels of CRBP in various cells and tissues.

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