Immunocytochemical Studies on the Localization of Plasma and of Cellular Retinol-binding Proteins and of Transthyretin (Prealbumin) in Rat Liver and Kidney

MICHIMASA KATO, KUNIYO KATO, and DEWITT S. GOODMAN
Department of Medicine, Columbia University College of Physicians and Surgeons, New York 10032. Dr. Michimasa’s and Kuniyo Kato’s present address is Department of Anatomy, Shinshu University, School of Medicine, Matsumoto, Nagano, 390, Japan.

ABSTRACT The immunocytochemical localization of cellular retinol-binding protein (CRBP), of plasma retinol-binding protein (RBP), and of plasma transthyretin (TTR) was studied in rat liver and kidney. The studies employed normal rats, retinol-deficient rats, and rats fed excess retinol. Antisera were prepared in rabbits against purified rat CRBP, RBP, and TTR. The primary antibodies and goat anti-rabbit IgG were purified by immunosorbent affinity chromatography, using the respective pure antigen coupled to Sepharose as the immunosorbent. This procedure effectively removed cross-reactive and heterophile antibodies, which permitted the specific staining and localization of each antigen by the unlabeled peroxidase-antiperoxidase method.

CRBP was found to be localized in two cell types in the liver, the parenchymal cells and the fat-storing cells. Diffuse cytoplasmic staining for CRBP was seen in all the parenchymal cells. Much more intense staining for CRBP was seen in the fat-storing cells. The prominence of the CRBP-positive fat-storing cells changed markedly with vitamin A status. Thus, these cells were most prominent, and appeared most numerous, in liver from rats fed excess retinol. Both RBP and TTR were localized within liver parenchymal cells. The intensity of RBP staining increased markedly in retinol-deficient rat liver, consistent with previous biochemical observations. With the methods employed, specific staining for RBP or TTR was not seen in cells other than the parenchymal cells. In the kidney, all three proteins (CRBP, RBP, and TTR) were localized in the proximal convoluted tubules of the renal cortex. Staining for RBP was much more intense in normal kidney than in kidney from retinol-deficient rats. These findings reflect the fact that RBP in the tubules represents filtered and reabsorbed RBP. The pattern of specific staining for CRBP among the various tubules was very similar to that seen for RBP on adjacent, serial sections of kidney. The function of CRBP in the kidney is not known.

It is now well established that specific binding proteins for retinol exist in plasma and in the intracellular compartment in a number of tissues. These proteins play important roles in the metabolism of vitamin A. Thus, retinol-binding protein (RBP), the specific plasma transport protein for vitamin A, transports retinol from its storage site in the liver to peripheral target tissues in the body. A different protein, cellular retinol-binding protein (CRBP) is found within cells in many tissues.

Since the initial isolation of human RBP in 1968 (1), extensive studies in many laboratories have provided considerable information about the structure, metabolism, and biological roles of RBP (see references 2–4 for recent reviews). RBP, with a molecular weight close to 21,000 and one binding site for retinol, is synthesized in (5) and secreted by the liver. RBP in plasma strongly interacts with another protein, plasma transthyretin (TTR, also more commonly referred to as plasma prealbumin2), and normally circulates as a 1:1 molar

1 Abbreviations used in this paper are: CRBP, cellular retinol-binding protein; RBP, retinol-binding protein; TTR, transthyretin.

2 The name transthyretin has been suggested by the Nomenclature Committee of IUB and the IUPAC-IUB Joint Commission on Biochemical Nomenclature for the protein commonly called prealbumin (J. Biol. Chem., 1981, 256:12-14).
RBP-TTR complex (1-4). In addition to its role in vitamin A transport, TTR plays a role in the binding and plasma transport of thyroid hormones. RBP is mainly catabolized in the kidneys (6, 7).

Vitamin A mobilization from liver is highly regulated by factors that control the rates of hepatic RBP synthesis and secretion. Retinol deficiency specifically blocks the secretion of RBP, which can then be rapidly stimulated by intravenous retinol repletion (2, 3, 8). Some information is available about the cellular and molecular mechanisms that mediate these phenomena.

A number of tissues of rats, humans, and other species contain a soluble intracellular protein with binding specificity for retinol, CRBP (see references 9 to 11 for recent reviews). CRBP from several tissue sources is a single polypeptide chain with a molecular weight of ~14,600, and a single binding site for one molecule of retinol. CRBP differs in a number of major ways from plasma RBP. It has been suggested that CRBP may play a direct role in the biological expression of retinol activity within the cell (10-12).

Radioimmunoassay studies have provided information about the tissue distribution and levels in the rat of RBP (13), TTR (14), and of CRBP (15, 16). The highest levels of RBP were found (in addition to plasma) in the liver and kidneys. High levels of CRBP were observed in several organs, including the liver, kidney, testis, and epididymis. In addition, information about the subcellular distribution of RBP (13), TTR (14), and CRBP (15) among homogenate fractions separated by differential centrifugation, has been reported.

The organs that contain the highest levels of RBP and CRBP consist of several different types of cells, organized anatomically and functionally in specific ways within each organ. Accordingly, information is needed about the distribution and localization of these binding proteins for retinol within and among the different types of cells that comprise each organ. Some information is available, from immunocytochemical studies, about the localization of RBP within rat liver (17), and about the localization of human RBP and TTR within human kidney (18, 19). No information is available about the cellular localization of CRBP within these organs.

We now report studies on the immunocytochemical localization of CRBP, RBP, and TTR in rat liver and kidney. These studies employed purified primary antibodies against each protein, and examined organs from normal rats, from retinol-deficient rats, and from rats fed excess retinol. These studies aim to extend our understanding of the metabolism and of the functional roles of the plasma and the intracellular binding proteins for retinol.

MATERIALS AND METHODS

Purification of Antigens (RBP, TTR, and CRBP): A modification of the procedure described by McGuire and Cyttil (20) was employed for the isolation of RBP and TTR from rat serum. Purification involved a series of fractionation procedures as follows: (a) DEAE-cellulose chromatography in 20 mM Imidazole-acetate buffer pH 6.0 with a linear gradient of NaCl from 0 to 90 mM; (b) gel filtration on Sephadex G-100 (Pharmacia, Inc., Piscataway, NJ) in 50 mM Tris-HCl buffer pH 8.4; and (c) gel filtration on a second column of Sephadex G-100 (Pharmacia, Inc.) . CRBP and CRBP are not separated from each other during these procedures. The fractions containing CRBP and CRBP after the third chromatographic step were pooled, dialyzed against 25 mM Na acetate buffer pH 4.9, and then applied to a column of SP-Sephadex (Pharmacia, Inc.) previously equilibrated with the same buffer. CRBP and CRBP were eluted and were fully separated from each other with a 500-mM linear gradient, from 25 to 200 mM Na acetate buffer pH 4.9. Purified CRBP and CRBP were both homogeneous on disc gel electrophoresis, when analyzed as previously described (21, 22).

Preparation of Antibodies: Rabbit antisera against rat RBP and against rat TTR were obtained by immunizing rabbits with purified RBP or TTR, respectively, using the techniques described previously from this laboratory (23, 24).

To prepare a rabbit antiserum against rat CRBP, samples of purified CRBP were fractionated in different ways before injection into rabbits. The CRBP preparations were as follows: (a) unmodified CRBP (1 mg in 2 ml isotonic NaCl); (b) delipidated (apo) CRBP; (c) peroxidase oxidized CRBP, and (d) CRBP conjugated to rabbit IgG. Preparation (b) was made by extruding CRBP (1 mg in 2 ml water) with n-heptane (2 ml) for 30 min with a Vortex mixer. After centrifugation (3,000 g for 30 min) the heptane phase was removed by aspiration.

Portions of each of these four CRBP preparations equivalent to 500 mg CRBP were emulsified with equal volumes of complete Freund's adjuvant (Difco Laboratories, Detroit, MI) and were injected subcutaneously in multiple spots in the backs of male New Zealand rabbits. A different rabbit was used for each CRBP preparation. Each rabbit received booster injections intramuscularly of 250 μg of the same CRBP preparation (with which it was initially immunized) emulsified with an equal volume of incomplete Freund's adjuvant 1 and 2 mo after the initial immunization. Sera (representing potential antiserum against CRBP) were collected 1 wk after the second booster injection.

Goat antiserum against rabbit immunoglobulins was purchased from Bio-Rad Laboratories, Richmond, CA. Rabbit IgG and rat IgG were purified from normal rabbit and rat serum by affinity chromatography on Protein A-Sepharose CL-4B (Pharmacia, Inc.) . Purified monospecific antibodies against CRBP, RBP, TTR, and rabbit IgG were obtained by immunosorbent affinity chromatography of the IgG fractions prepared from the specific antiserum against each of these proteins. The IgG fractions were obtained from the respective antisera by ammonium sulfate precipitation followed by DEAE-cellulose column chromatography (17). Immunosorbent affinity chromatography, for antibody purification was carried out as described previously (18). In each case, the specific immunosorbent was prepared by coupling the antigen (CRBP, RBP, TTR, or rabbit IgG) with Sepharose 4B (Pharmacia, Inc.) . The corresponding IgG fraction was then applied to the antibody column, and proteins other than the specific antibodies were eluted with phosphate buffered saline (PBS). The purified specific antibodies were then eluted by 0.1 M glycine-HCl buffer pH 2.3 containing 0.15 M NaCl. The antibody concentration was estimated by absorbance at 280 nm. In addition, cross-reactive antibodies against rat IgG were removed from the purified goat anti-rabbit IgG by immunosorbent affinity chromatography on rat IgG coupled to Sepharose 4B. The purified goat anti-rabbit IgG antibody is subsequently referred to as the bridge antibody.

Antibody Characterization: Immunosorbent purified rabbit anti-retinol RBP, rabbit anti-rat TTR, and goat anti-rabbit IgG antibodies were examined by double immunodiffusion (25). The titers of the rabbit anti-CRBP antisera were determined by enzyme-linked immunosorbent assay (ELISA) before immunosorbent purification. These assays used 96-well microtiter plates. 200 μl of 50 mM Na barbital buffer pH 9.5 containing 50 ng CRBP was added to each well and incubated 18 h at room temperature. The plate was washed three times with PBS containing 0.05% Tween 20 (PBS-Tween). The wells were then aspirated, and re-incubated with 200 μl of one of the following solutions at room temperature, and were washed with PBS-Tween after each incubation: (a) 10% normal goat serum in PBS-Tween for 30 min; (b) one or another dilution (see below) of rabbit anti-CRBP antisera in PBS-Tween for 120 min; (c) bridge antibody (25 μg/ml in PBS-Tween) for 60 min; and (d) peroxidase-rabbit antiperoxidase (PAP) complex (prepared from Accurate Chemical and Sci-
entific Corp., Westbury, NY), diluted 100-fold in PBS-Tween, for 60 min. For each anti-CRBP antiserum a set of serial two-fold dilutions was tested, so as to examine a wide range of dilutions in a given assay. The wells were then each incubated with 200 μl of a solution of 0.05% o-dianisidine dihydrochloride in citrate-phosphate buffer (77 mM citric acid, 126 mM Na phosphate dibasic, pH 6.0) containing 0.005% H₂O₂ for 60 min. The absorbance at 488 nm of the wells was then measured with an automated vertical beam reader (Artrek System Corp., Farmingdale, NY).

The specificity of the immunoabsorbent purified rabbit anti-CRBP was also assessed by enzyme-linked immunoabsorbent assay. Samples of rat CRBP, CRABP, RBP, and liver Z-protein (kindly provided by Dr. I. Arias, Albert Einstein College of Medicine, NY) of 50 ng each were coated in a series of wells on a plate. The titers of the purified rabbit anti-CRBP antibody solution against each of these antigens were determined by the peroxidase-rabbit anti-peroxidase method as described above.

Preparation of Tissues: Male weanling rats of the Holtzman strain were divided into three groups of four rats each. The first group was fed a purified vitamin A deficient diet (14) for a period of 35 d. The second and third groups were fed the same diet supplemented with 2.4 μg of retinol equivalents in the form of retinyl ester per gram of diet for the 35-d period. From day 47 to day 52 the rats in the third group were also given 20 mg retinyl ester (in peanut oil) per kilogram body weight daily by oral administration. On day 55 all rats were anesthetized with ethyl ether. Blood was removed from the animals, and 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, 2.4 mM CaCl₂, 1.2 mM MgCl₂) for 5 min, followed by ice cold Perfus (Fisher Scientific Co., Springfield, NJ) for 15 min. Perfusion was performed at a constant flow rate of 20 ml/min. The livers and kidneys were then removed from the animal and cut into 2-mm thick slices, and fixation was continued for 2 h at 4°C. The fixed tissue slices were washed with 95% ethanol three times (8 h each time at 4°C) and embedded in paraffin. Serial sections 4–5-μm thick were cut in a tissue slicer.

The plasma vitamin A levels in the three groups of rats, as determined by the method of Thompson et al. (26) were (a) deficient rats (fed the unsupplemented deficient diet), 1.9 ± 0.7 μg/dl (mean ± SD, n = 4); (b) normal rats (fed the diet supplemented with 2.4 μg/gram of diet), 67.3 ± 6.1 μg/dl; and (c) rats fed excess vitamin A, 67.3 ± 6.1 μg/dl.

Immunocytochemical Staining: The unlabeled peroxidase-antiperoxidase method of Sternberger et al. (27) was used as described previously (18). The following incubations were performed with deparaffinized sections of tissue: (a) 0.3% H₂O₂ in absolute methanol for 20 min; (b) PBS containing 10% normal goat serum for 30 min; (c) purified specific antibody (anti-RBP, anti-TTR, or anti-CRBP), 25 μg/ml in PBS, for 120 min; (d) bridge antibody (200 μg/ml in PBS) for 60 min; and (e) peroxidase-rabbit anti-peroxidase (diluted 40-fold in PBS) for 60 min. The sections were washed with PBS three times after each incubation. The sections were then rinsed in PBS for 5 min at 4°C followed by 1% 2,3-diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, MO) in 50 mM Tris-HCl buffer pH 7.6, and stained for 3–5 min with a solution of 0.02% 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, MO) and 0.003% H₂O₂. The tissues were counterstained with diluted (4 ×) hematoxylin solution, Gill’s formulation #1 (Fisher Scientific Co.) in 25% (vol/vol) ethylene glycol. Under those conditions, background peroxidase staining and endogenous peroxidase activity were not observed.

Immunocytochemical Controls: The specificity of each of the purified antibody preparations was tested immunocytochemically by absorbing small portions of each antibody solution with each of the purified antigens (CRBP, RBP, TTR) coupled to Sepharose 4B. In each instance, the immunoabsorbent (20 μl at 2–4 mg protein per ml) was added to the antibody solution (1 ml, 25 μg/ml, in PBS), followed by incubation with rotation for 18 h at 4°C. After centrifugation the absorbed antibody solutions were used for immunocytochemical staining as described above. The results obtained with the absorbed antibody solutions were compared with those obtained with the unabsorbed solutions. The specificity of the reaction was also examined by substitution of PBS and normal rabbit IgG (25 μg/ml) for the immunoabsorbent purified antibodies in the staining procedure. Additional controls that were carried out involved the omission of the bridge antibody, peroxidase-rabbit antiperoxidase, or the final substrate for the peroxidase reaction.

RESULTS
Isolation of CRBP
CRBP was purified by the sequence of procedures described in Materials and Methods. Fig. 1 shows the results of the final purification procedure, chromatography on a column of SP-Sephadex. This procedure resulted in the complete separation of CRBP from CRABP, and in the isolation of each of these proteins as purified homogeneous entities. The overall purification procedure resulted in an excellent yield of purified CRBP (8.8 mg) and CRABP (5.1 mg) from 1,000 rat testes. These yields were substantially higher than those previously obtained in our (21, 22) or in other (28) laboratories. We believe that the higher yields resulted from the use of acetone powder of whole testes as the starting material for the fractionation procedures, and from the final purification step (Fig. 1) which achieves complete separation of CRBP and CRABP with good recovery of both binding proteins.

Antibody Characterization and Specificity
Rabbits were immunized with four different preparations of CRBP (see Materials and Methods). The resulting sera were examined by the enzyme-linked immunoabsorbent assay technique to estimate their anti-CRBP antibody titers. The results are shown in Fig. 2. The highest antibody titer was obtained from the rabbit immunized with delipidated CRBP. No anti-CRBP antibody activity was detected in the serum from the rabbit immunized with unmodified CRBP.

The specific antibody against CRBP was purified by immunoabsorbent affinity chromatography from the antisera raised against delipidated CRBP. The specificity of this purified antibody preparation was then assessed by determining whether it would react against several related antigens. CRABP has been reported to show some homology with both CRBP and with Z-protein (29, 30). By the ELISA method, the purified anti-CRBP antibodies reacted only with CRBP, and not at all with CRABP, Z-protein, or RBP (data not shown).

Immunosorbent purified rabbit anti-rat RBP and anti-rat TTR antibodies each gave a single precipitin line when tested against either the purified antigen (RBP or TTR, respectively)

![Figure 1 Isolation of CRBP and of CRABP by chromatography on SP-Sephadex. The pooled fractions containing CRBP and CRABP, obtained after chromatography on DEAE-cellulose (see Materials and Methods section), were applied to a column (1 × 40 cm) of SP-Sephadex. The column was eluted with a linear gradient, from 25 to 200 mM, of Na acetate buffer pH 4.9 (fractions 1 through 125). Fractions of 4 ml each were collected at a flow rate of 12 ml/h. Beyond fraction 125, elution was carried out with 500 mM Na acetate pH 4.9. Fractions 72–85 were pooled and contained pure CRBP; fractions 96–110 were pooled to yield pure CRABP. O, conductivity (mS); □, absorbance at 280 nm A₂₈₀; Δ, relative intensity of fluorescence (of protein-bound retinol) (RIF); ▪, ³H cpm (representing CRABP-bound [³H] retinoic acid) (CPM) per 50 μl. The small peak of fluorescence seen in fractions 96–110 represents the fluorescence of protein-bound retinoic acid.](https://jcb.rupress.org/content/98/8/1698/F1)

Downloaded from jcb.rupress.org on September 29, 2017
liver (Fig. 3, A and D) diffuse cytoplasmic staining for CRBP and its role in vitamin A storage in the liver (31). In normal rat types in the liver, the parenchymal cells and the fat-storing cells have been raised (data not shown). The staining was not affected with a given antibody disappeared completely after absorption with sections of normal liver and kidney. In every instance, regarding intensity of specific immunohistochemical staining, unabsorbed antibody preparations were then compared, with tests showed a reaction-of-identity with each other.

The specificity of the purified antibodies was also demonstrated immunocytochemically by absorbing each antibody preparation with each of the following antigens: CRBP, RBP, and TTR (see Materials and Methods). The absorbed and unabsorbed antibody preparations were then compared, with regard to intensity of specific immunohistochemical staining, with sections of normal liver and kidney. In every instance, for each of the three antibodies, the specific staining associated with a given antibody disappeared completely after absorption with the particular antigen against which the antisera had been raised (data not shown). The staining was not affected by absorption with the other antigens.

No specific peroxidase staining was observed after omission of the primary antibody from the staining procedure, after its replacement with PBS, or when one or another major component (see Materials and Methods) was omitted from the staining procedure. Weak and diffuse staining was observed when the primary antibodies were replaced with normal rabbit IgG (25 μg/ml), as reported previously (18). This observation suggests that our method may be sensitive enough to detect rabbit heterophile antibodies, and illustrates that the purification of the primary antibodies is important for removing nonspecific antibodies, which was necessary for the successful progress of this work.

Localization of Immunoreactive CRBP in Liver

Specific immune staining for CRBP was seen in two cell types in the liver, the parenchymal cells and the fat-storing cells. Fat-storing cells have been considered to play a significant role in vitamin A storage in the liver (31). In normal rat liver (Fig. 3, A and D) diffuse cytoplasmic staining for CRBP of low intensity was seen in all the parenchymal cells. Much more intense staining for CRBP was, however, seen in the fat-storing cells. These strongly positive cells were identified as fat-storing cells for the following reasons: (a) perisinusoidal location; (b) anatomical size and irregular shape (hence, referred to as perisinusoidal stellate cells [31]); (c) the presence of small vacuoles that presumably represent the lipid droplets or granules characteristically seen in fat-storing cells (Fig. 3 D), arrows. These strongly positive cells were less dense in the central zone of the lobule than in the peripheral area (Fig. 3 A).

The immunocytochemistry of CRBP was examined in livers from vitamin A deficient rats (Fig. 3, B and E) and from rats fed excess vitamin A (Fig. 3, C and F), and compared with the results obtained with normal liver. Similar staining, of weak intensity, was seen in parenchymal cells of livers from vitamin A deficient, or vitamin A excess-fed rats. In contrast, distinct differences were seen in the fat-storing cells. Thus, fat-storing cells strongly positive for CRBP appeared most prominent in liver from rats fed excess vitamin A (Fig. 3 C). In contrast, these strongly positive cells were less prominent, and appeared less numerous, than normal in livers from vitamin A deficient rats (Fig. 3 B). The vacuoles within the cytoplasm of the strongly positive fat-storing cells were more numerous in rats fed excess vitamin A (Fig. 3 F, arrow). The number of vacuoles (and the volume of the cytoplasm) was markedly decreased in the strongly positive cells from vitamin A deficient rats (Fig. 3 E). These observations help establish the identity of these cells as fat-storing cells, since it is well known that the number and size of the lipid droplets in the fat-storing cells increase considerably after large doses of vitamin A (31, 32).

Localization of Immunoreactive RBP and TTR in Liver

Specific cytoplasmic staining for RBP was observed in all parenchymal cells. The intensity of staining was mild in normal liver (Fig. 4 A) and was much more strong in liver from vitamin A deficient rats (Fig. 4 B). These observations are consistent with the known fact that vitamin A deficiency leads to a marked increase in the level of RBP in liver as measured by radioimmunoassay (3, 8, 13). No differences were observed between livers from normal rats or from rats fed excess vitamin A with regard to the intensity or distribution of RBP positive staining (data not shown).

Positive staining for TTR was also seen in parenchymal cells (Fig. 4 C). No significant differences were observed in the staining for TTR in the liver sections from normal, vitamin A deficient, or excess vitamin A-fed rats (data not shown). With the staining methods used for RBP and TTR in this study, only parenchymal cells could be distinguished (from other cell types in the lobule) as showing clearly positive specific immune staining for each protein. The possibility that some nonparenchymal cells might have been weakly stained for RBP and/or TTR could not be excluded; with the methods employed, however, such cells could not be clearly identified as showing positive staining.

Localization of Immunoreactive CRBP, RBP, and TTR in the Kidney

Fig. 5 shows sequential sections of normal rat kidney that were stained by anti-CRBP (Fig. 5 A), anti-RBP (Fig. 5 B), and
FIGURE 3 Localization of immunoreactive CRBP in rat liver. A and D, liver from a normal animal; B and E, liver from a vitamin A deficient rat; C and F, liver from a rat fed excess vitamin A. Note the light staining of parenchymal cells and the intense staining of the fat-storing cells in all micrographs. Fat-storing cells strongly positive for CRBP were most prominent, and appeared most numerous, in livers from rats fed excess vitamin A. The short-stemmed arrows in D and the long-stemmed arrow in F point to vacuoles in the rat storing cells that presumably represent the lipid droplets characteristically seen in these cells. The short-stemmed arrows in F point to vacuoles that are surrounded by a ring strongly stained for CRBP. These vacuoles are presumed to represent vacuoles within processes of fat-storing cells surrounded by a ring of cytoplasm. Bar, 200 μm. × 60 (A–C). Bar, 10 μm. × 1,300 (D–F)

anti-TTR (Fig. 5 C), respectively. Fig. 5, D–F are the higher magnification micrographs of portions of the cortex of Fig. 5, A–C, respectively. CRBP was mainly localized in the renal cortex; very weak staining was also detected in the proximal straight tubules of the upper portion of the medulla (see Fig. 5 A). In the cortex, staining for CRBP was found in the cells of the proximal convoluted tubules (Fig. 5 D). Extremely strong staining for RBP was also observed in the proximal convoluted tubular cells of the cortex (Fig. 5, B and E); RBP was not found in the medulla (lower portion of Fig. 5 B). TTR was also only localized in the proximal convoluted tubular cells of the renal cortex (Fig. 5, C and F).

When Fig. 5, D–F (which represent adjacent serial sections) were compared with each other, it was noted that the relative intensity and distribution of positive staining for CRBP (Fig. 5 D) and for RBP (Fig. 5 E) were very similar to each other among the different tubules that comprised the two adjacent sections. Thus, although the staining in Fig. 5 E is stronger than that in Fig. 5 D, the pattern of staining among the various tubules is similar in the two sections. In contrast, some tubular cells were negative for TTR staining (arrows in Fig. 5 F), whereas the corresponding tubules were positive for RBP (Fig. 5 E) and for CRBP (Fig. 5 D). Specifically, all of the early, initial portions of tubules (adjacent to the corresponding glomeruli) were negative for TTR staining (larger arrows in Fig. 5 F), but were positive for RBP (Fig. 5 E) and for CRBP (Fig. 5 D). In addition, the intensity of staining for TTR was less than that for RBP.

Sequential sections of kidney from a vitamin A deficient rat were also stained with antibodies against CRBP (Fig. 6, A),
FIGURE 4  Localization of immunoreactive RBP and TTR in rat liver. A and B, localization of RBP in normal liver (A) and in liver from a vitamin A deficient rat (B). C, localization of TTR in normal liver. Cytoplasmic staining of parenchymal cells was seen in all sections. Note the extremely strong staining for RBP in liver from a vitamin A deficient rat (B), and the much less intense staining seen in the normal liver (A). The apparent nuclear staining seen here entirely represents nuclei stained with the hematoxylin counterstain. Bar, 100 μm. × 150.

RBP (Fig. 6 B), and TTR (Fig. 6 C). Somewhat weaker staining for CRBP was observed in the proximal convoluted tubules of the vitamin A deficient rat (Fig. 6 A) as compared with the normal animal (Fig. 5 D). Specific staining for RBP was markedly decreased in the vitamin A deficient rat kidney (Fig. 6 B), compared with the normal kidney (Fig. 5 E). Moreover, staining for RBP was particularly localized in the apical portions of the tubular cells (Fig. 6 B). The intensity of staining for TTR in vitamin A deficiency (Fig. 6 C) was similar to that seen in the normal kidney (Fig. 5 F). As observed with the normal kidney, staining for TTR was not observed in the early (initial) portion of the proximal convoluted tubes (arrow in Fig. 6 C). Both RBP (Fig. 6 B) and CRBP (Fig. 6 A) were identified in the corresponding early portions of the tubules on adjacent sections.

DISCUSSION

These studies provide detailed information on the immunocytochemical localization of CRBP, of RBP, and of TTR within different types of cells in the liver and the kidney of the rat. A critical methodological feature of this work was the use of primary and bridge antibodies purified by immunosorbent affinity chromatography to avoid background and nonspecific staining. As discussed previously (18), this purification procedure effectively removed cross-reactive and heterophile antibodies, permitting the specific staining and localization of each antigen by the unlabeled PAP method.

The liver plays a central role in the uptake, storage, and mobilization of vitamin A in the body. Vitamin A is mobilized from the liver as retinol bound to RBP. Secretion of RBP from the liver is strongly influenced by the nutritional vitamin A status of the animal. In addition to studies in vivo, the synthesis and secretion of RBP, and the regulation of RBP secretion by retinol, have also been observed with isolated differentiated rat hepatoma cells in culture in vitro (33).

TTR, like RBP, is also synthesized in and secreted into the plasma by the liver. The hepatic secretion of TTR and of RBP appear to be independently regulated processes (14).

The results reported here on RBP and TTR confirm and extend previous studies (17) on the localization of RBP in rat liver by immunofluorescence microscopy. In both the present and previous (17) studies, specific localization of RBP was observed within liver parenchymal cells. A marked increase in the intensity of specific staining for RBP was observed here in vitamin A deficient liver. TTR was also found to be localized in the parenchymal cells, and the intensity of staining for TTR did not change with vitamin A deficiency. These immunocytochemical findings are consistent with previous biochemical observations (2, 8, 13, 14). With the methods used, only parenchymal cells could be clearly identified as showing specific immune staining for both RBP and TTR. It is likely that both RBP and TTR are synthesized in the parenchymal cells, and secreted by these cells into the plasma.

Very different results were obtained with CRBP. CRBP was found to be localized in two cell types in the liver, the parenchymal cells and the fat-storing cells. Moreover, whereas relatively weak staining for CRBP was seen in the parenchymal cells, the fat-storing cells displayed strongly intense staining for CRBP. The prominence of the CRBP-positive fat-storing cells changed markedly with vitamin A status. Thus, these cells were most prominent, and appeared most numerous, in liver from rats fed excess vitamin A. Conversely, these cells were least prominent, and appeared least numerous, in liver from vitamin A deficient rats. The intensity of the specific staining for CRBP in the parenchymal cells did not change with vitamin A status.

There is much evidence that the hepatic fat-storing cells play a major role in vitamin A storage under hypervitaminotic conditions. These cells comprise a small proportion of the nonparenchymal cells of the liver, and have been referred to in the literature by various names, including perisinusoidal stellate cells (31), lipocytes (34), vitamin A-storing cells (35), and Ito cells (36) (see reference 32 for an extensive review). Fat-storing cells are located in the perisinusoidal space and
FIGURE 5  Localization of immunoreactive CRBP, RBP, and TTR in rat kidney. A and D, CRBP; B and E, RBP; C and F, TTR. A, B, and C represent adjacent sequential sections (A to B to C) of normal kidney; D, E, and F are higher magnification micrographs of cortex from A, B, and C, respectively. In A, B, and C, the upper stained portion of each section consisted of renal cortex, whereas the lower, unstained portions of these sections represented renal medulla. CRBP, RBP, and TTR all localize in the renal cortex (upper portions of A, B, and C), and specifically in the cells of the proximal convoluted tubules (D, E, and F). A very light staining for CRBP was also observed in the proximal straight tubules (st in A). With this one exception, the renal medulla did not stain for any of these antigens. Some proximal convoluted tubules were negative for TTR (arrows in F) but were strongly positive for RBP as seen on the adjacent section (E). Bar, 1,000 µm. × 16 (A–C). Bar, 100 µm. × 130 (D–F).

contain numerous small lipid droplets. When large doses of vitamin A are given to experimental animals, the number and size of these lipid droplets increase considerably, and the fat-storing cells become larger and more prominent histologically (31, 32). Vitamin A, mainly in the form of retinyl esters (37–38), has been localized in the lipid droplets of the fat-storing cells by fluorescence microscopy (31, 32), by electron microscope radioautography (39), and directly by isolation and analysis of the fat-storing cells (37) or their lipid granules (38). Recently, evidence was reported in normal rats for the transfer of retinol from parenchymal to nonparenchymal cells, after initial hepatic uptake (40). It was suggested (40) that the nonparenchymal cells involved in this transfer and storage process might be the fat-storing cells. In addition to their role in hypervitaminosis A, these cells may play a significant role in vitamin A storage under normal conditions (31, 37, 40).

The present finding that CRBP is strongly localized in hepatic fat-storing cells suggests that CRBP may play an important role in the metabolism of vitamin A (retinol) in these cells. The biological role that CRBP plays within cells is not known. It has been suggested that CRBP may be involved in the biological expression of retinol activity (e.g., analogous to steroid hormone receptors) within the cell (10–12). Another possibility is that CRBP serves in the cell as an intracellular transport protein, acting to transport retinol from one locus to another between metabolic events and processes. CRBP in the fat-storing cell may be involved in the uptake of retinol into the cell, in facilitating its storage, or in the mobilization of retinol (back to the parenchymal cell, for secretion as the retinol-RBP complex) from the cell. Data are needed about these and other possibilities.

The kidneys play an important role in the normal catabo-
lism of RBP. RBP itself (not as a complex with TTR) is small enough to be filtered readily by the renal glomeruli, whereas TTR and the TTR-RBP complex are not. Formation of the RBP-TTR complex serves to protect RBP by preventing its glomerular filtration and subsequent renal catabolism (1, 6).

Although very little RBP in plasma is normally present in the free state, its glomerular filtration and renal metabolism are sufficiently large to constitute the major catabolic route for RBP. Normally, very little RBP appears in the urine, as nearly all of the filtered RBP is reabsorbed and degraded by the renal tubules (6, 41).

The results reported here, showing localization of RBP in the proximal convoluted tubules of the renal cortex, are consistent with this formulation. The data confirm and extend those recently reported in studies of the localization of RBP in human kidney tissue (18). An earlier report (19) that both RBP and TTR were found in glomerular capillaries of human kidneys, probably reflected the use of postmortem kidney slices and unpurified antibodies in that study. RBP in the proximal tubular cells presumably represents RBP that had undergone glomerular filtration and tubular reabsorption. This conclusion is supported by the finding (Fig. 6) that markedly reduced specific staining for RBP was seen in kidneys from vitamin A-deficient, compared with normal, rats. Because retinol-deficient rats have low plasma levels of RBP, the amounts of RBP filtered and reabsorbed in the kidneys should be reduced. Previous radioimmunoassay studies have shown that renal levels of RBP are greatly decreased in vitamin A-deficient rats, and that most of the RBP in kidney homogenates is recovered in the soluble, supernatant fraction (13).

TTR was also found localized in the proximal convoluted tubules of the renal cortex. This finding is difficult to interpret in physiological terms. The kidneys do not appear to play a significant role in TTR catabolism (6, 7), and the tissue sites of TTR degradation in the body are not known. TTR, unlike RBP, was not found localized in the earliest, initial portion of the tubule, adjacent to the glomerulus. This observation suggests that at least part of the TTR in the tubular cells may not have arrived there via glomerular filtration and tubular reabsorption. TTR levels in the kidney are not affected by retinol deficiency (14), and the intensity and distribution of TTR staining was not affected in the present study by vitamin A deficiency.

Radioimmunoassay studies have shown that kidneys contain relatively high levels of CRBP (15, 16). No information has, however, been available as to where, within the kidney, this CRBP might reside. The present studies demonstrate that CRBP is localized mainly in the proximal convoluted tubules of the renal cortex. Moreover, the pattern of specific staining for CRBP among the various tubules was very similar to that seen for RBP on adjacent, serial sections of kidney.

The function that CRBP plays within the kidney is not known. The present observation, of similar patterns of tubular localization for CRBP and RBP, suggests that there may have been some relationship between CRBP in the tubular cell and the tubular reabsorption and catabolism of RBP. One possibility is that the CRBP may serve to bind and conserve some of the retinol that is filtered and reabsorbed as the retinol-RBP complex. It has been suggested that quantitatively significant recycling and reutilization of retinol may occur in the animal body (42, 43). Turnover rate considerations support this conclusion. Thus, the total body turnover rate of RBP in humans has been estimated at ~5 mg/kg/d, equivalent to ~16–17 μmol per day for a 70 kg man (44). This value is at least double the estimated average intake (in molar terms) of vitamin A in well-nourished adults in Western countries. If we assume that well-nourished adults are in a steady-state with regard to vitamin A nutriture, these estimates suggest that a significant portion of the retinol that is transported by RBP may be reutilized in the body. It is possible that CRBP in the tubular cell plays a role in this reutilization process. No data are available concerning this or other possible functions of CRBP within the kidney.

We thank Dr. W. E. Scott, Hoffmann-LaRoche, Inc., Nutley, NJ for the gift of [15-14C]retinoic acid, and Dr. I. M. Arias, Albert Einstein College of Medicine, Bronx, NY, for the gift of rat liver Z-protein.

This work was supported by National Institutes of Health grants AM 05968 and HL 21006 (SCOR).
REFERENCES