The Binding Protein for Retinol from Rat Testis Cytosol

ISOLATION AND PARTIAL CHARACTERIZATION*

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This study reports the isolation and partial characterization of a soluble protein with binding specificity for retinol from rat testis cytosol. Cytosol, labeled in vitro by addition of [3H]retinol, was fractionated by ion exchange chromatography on DEAE-Sepharose, gel filtration on Sephadex G-50, and preparative polyacrylamide gel electrophoresis. The resulting cytosol binding protein for retinol had been purified at least 1,700-fold, with a yield of approximately 24%. The binding protein for retinol had a molecular weight of approximately 14,600, was homogeneous on disc gel electrophoresis, and was microheterogeneous on isoelectric focusing, showing two close bands of holoprotein with apparent isoelectric points of 4.8 and 4.9. The properties of rat testis cytosol binding protein for retinol differed from those of serum retinol binding protein in several ways. The purified testis binding protein showed no immunological reactivity when assayed in the radioimmunoassay for rat serum retinol-binding protein and did not display affinity for serum prealbumin, as determined by affinity chromatography on immobilized human prealbumin. A direct comparison of the absorption and fluorescence spectra of the rat testis cytosol binding protein for retinol with those of human serum retinol-binding protein exhibited a relatively high specificity for all-unlabeled compounds added in excess showed that the tissue binding protein had been purified at least 1,700-fold, with a yield of approximately 24%. The binding protein for retinol had a molecular weight of approximately 14,600, was homogeneous on disc gel electrophoresis, and was microheterogeneous on isoelectric focusing, showing two close bands of holoprotein with apparent isoelectric points of 4.8 and 4.9. The properties of rat testis cytosol binding protein for retinol differed from those of serum retinol binding protein in several ways. The purified testis binding protein showed no immunological reactivity when assayed in the radioimmunoassay for rat serum retinol-binding protein and did not display affinity for serum prealbumin, as determined by affinity chromatography on immobilized human prealbumin. A direct comparison of the absorption and fluorescence spectra of the rat testis cytosol binding protein for retinol with those of human serum retinol-binding protein showed significant differences between the two proteins. The purified cytosol binding protein for retinol had an ultraviolet absorption spectrum characterized by two peaks with maxima at approximately 277 and 345 nm. The fluorescence data suggest that retinol bound to serum retinol-binding protein is present in a more rigid conformation than is retinol bound to the cytosol binding protein.

Vitamin A is a nutrient that is necessary for the support of growth and life of higher animals. Vitamin A is necessary for vision (1), reproduction (2, 3), and the maintenance of differentiated epithelia and mucus secretion (4–6). Apart from the established role of retinaldehyde in vision, however, little is known of the mechanisms by which vitamin A exerts its biological effects within target tissues and cells. Vitamin A is transported from its hepatic stores to peripheral tissues in the form of retinol bound to a specific transport protein, serum retinol-binding protein (7, 8). This protein, which has been purified from human (9, 10) and rat (11, 12) plasma, and from other species (8, 13–18), has a molecular weight of approximately 20,000, and a single binding site for 1 molecule of retinol. RBP interacts strongly with plasma prealbumin, and normally circulates in plasma as a 1:1 molar RBP–prealbumin complex (7–12). Evidence has been reported suggesting that specific receptors for serum RBP are present on the surface of certain vitamin A-requiring cells (19, 20), and it has been suggested that RBP may deliver retinol to such binding sites at the cell surface and release retinol at these locations.

During the past 5 years, considerable information has been obtained demonstrating that a number of tissues in rats, humans, and other species contain soluble proteins with binding specificity for retinol or for retinoic acid. Evidence for a specific binding protein for retinol in rat tissues was first reported by Dashou et al. in 1973 (21). Subsequently, information about the species and tissue distribution, and about some of the characteristics of intracellular binding proteins for retinol, have been reported from several laboratories (22–31). These studies have mainly employed an "assay" for binding activity in which labeled retinol added to the cytosol fraction of tissue homogenates was observed to bind to a component with a sedimentation coefficient of approximately 2 S, as determined by sucrose density centrifugation. Competitive binding studies using [3H]retinol and a variety of unlabeled compounds added in excess showed that the tissue binding protein exhibited a relatively high specificity for all-trans-retinol (22–24).

Evidence is also available for the existence of a different soluble intracellular binding protein with specificity for retinoic acid (23, 25–28, 32–36). The binding protein for retinoic acid is generally similar to the intracellular binding protein for retinol with regard to sedimentation behavior, but differs as to tissue distribution (23, 27, 34).

Interest in these intracellular binding proteins has been stimulated by reports suggesting a relationship between the binding affinity of the proteins for various vitamin A-related compounds and the biological activity of the compounds. A number of retinoids with anticarcinogenic activity can associate with the tissue binding protein for retinoic acid, and the binding ability tends to correlate with biological activity for given compounds (37, 38). It has also been suggested that the affinities of cis isomers of retinol for the tissue binding protein for retinol generally parallel the activities of these isomers in vivo (22).

We now report the isolation and partial characterization of the intracellular binding protein for retinol, CRBP, from rat testis cytosol. Several properties of this protein were compared directly with those of serum RBP. Information has also been obtained about the rat testis intracellular binding protein for retinoic acid.

EXPERIMENTAL PROCEDURES

Labeled Compounds—[11,12-3H]Retinyl acetate, [15-3C]retinoic acid, CRBP, cytosol binding protein for retinol; SDS, sodium dodecyl sulfate; mS, millisiemens.

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The abbreviations used are: RBP, serum retinol-binding protein;
acid, and unlabeled retinol were generously provided by Dr. W. E. Scott of Hoffmann-La Roche, Inc., Nutley, NJ. [11,12-3H]Retinol was prepared by hydrolyzing the labeled retinyl acetate in 5% KOH, 1% pyrogallol in 95% ethanol under N₂ for 1 h at 60°C. Retinol was extracted into spectral hexanes and chromatographed on a 1-g column of aluminum oxide deactivated with 10% water. The fractions eluting with hexanes:diethyl ether (90/20, v/v) were checked for the fluorescence spectra typical of retinol, and were stored at -20°C under nitrogen or argon. Before use, [1H]retinol was diluted with unlabeled retinol to a final specific activity of 62 to 98 μCi/mg of retinol. [14C]Retinoic acid (specific activity, 59 μCi/mg) was tested for purity by thin solvent partition procedure of Borgström (39). Less than 2% of [12]C-retinoic acid activity extracted into hexanes at alkaline pH (nonacidic lipids) and more than 96% was extracted into hexanes at pH 1 (acidic lipids), indicating a purity of greater than 96%.

Preparation and Labeling of Cytosol—Cytosol from adult rat testes was prepared from batches of 500 to 516 frozen testes, purchased from West Jersey Biological Supply (Wenonah, N.J.) or from Pel-Freeze Biologicals, Inc. (Rogers, AR). Testes were thawed to ice temperature before use and all subsequent procedures were performed at 0–5°C. The tunica albuginea and the major portion of the spermatic artery were removed (the latter was done to reduce potential contamination with serum RBP). Testes were minced in 2 ml per g of tissue containing 10% glycerol in 95% ethanol under N₂ for 1 h at 60°C. Retinol was not seen. In contrast, excess nonradioactive retinoic acid did not displace the labeled retinol from its binding component in the second peak. When an excess of nonradioactive retinol (100-fold excess) was included in the cytosol incubation, the second peak of labeled retinol was not seen. In contrast, excess nonradioactive retinoic acid did not displace the labeled retinol from its binding component in the second peak. These observations indicated that the second peak contained a saturable binding component with ligand specificity for retinol, in confirmation of the results of Weber and Osborn (44), using 11% gel, pH 7.1. After analytical electrophoresis, disc gels were either fixed in 12.5% trichloroacetic acid and stained with 0.2% Coomassie brilliant blue, or were digested (45) before radioassay.

Isoelectric Focusing—Isoelectric focusing in 6% polyacrylamide gel electrophoresis was performed as previously described (39) with ligand specificity for retinol, in confirmation of the results of Weber and Osborn (44), using 11% gel, pH 7.1. After analytical electrophoresis, disc gels were either fixed in 12.5% trichloroacetic acid and stained with 0.2% Coomassie brilliant blue, or were digested (45) before radioassay.

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specificity for retinoic acid, and not for retinol (23, 24).

In other preliminary experiments, cytosol was labeled with 1 nmol/ml of [3H]retinol and filtered on Sephadex G-50. The fractions comprising the second peak (i.e. the specific and saturable binding component) were pooled and applied to a small column of DEAE-Sepharose. Elution with a linear gradient of NaCl resulted in a single peak of [3H]retinol that eluted at a conductivity near 3.7 mS. Subsequently, in the large scale purification reported below, whole cytosol was applied first to a larger column of DEAE-Sepharose and the sharp peak containing [3H]retinol that eluted at a conductivity near 3.7 mS was collected for further purification of CRBP.

Purification of CRBP—The purification sequence reported here (Table I) has been repeated three times with virtually identical results each time. These procedures generally resulted in the isolation of a sample of CRBP that had been purified at least 1,700-fold. Concurrently, these procedures resulted in the partial purification (approximately 370-fold) of a separate binding protein for retinoic acid that was separated from CRBP by electrophoresis in the final step for purification of CRBP.

DEAE-Sepharose Chromatography—Cytosol prepared from 665 g of rat testes was incubated with [3H]retinol and labeled with [14C]retinoic acid and was chromatographed on DEAE-Sepharose (Fig. 1). A linear gradient of NaCl was applied to elute coincident peaks of protein-bound [3H]retinol and [14C]retinoic acid, at a specific conductivity of 3.8 to 4.2 mS. The pooled fractions comprising this peak were estimated to contain approximately 73% of the specifically bound [3H]retinol, which overlapped the major peak of eluted protein.

After DEAE-Sepharose chromatography was subjected to gel electrophoresis (Fig. 1, Gel B), a single, coincident peak of protein-bound [3H]retinol was observed. This value was determined in four experiments where an average of 13% of added [3H]retinol was eluted in the second (specifically bound) peak from a Sephadex G-50 column (see "Preliminary Studies" under "Results").

Table I

| Procedure            | Total A280 | Recovery of total A280 (%) | Recovery of specifically bound [3H]retinol (%) | Apparent specific radioactivity (dpm/mg) | Apparent purification |
|----------------------|------------|---------------------------|---------------------------------------------|----------------------------------------|-----------------------|
| Labeled cytosol      | 3.22 x 10⁵ | 100                       | 100                                         | 3.61 x 10⁵                             | 1.0                   |
| DEAE-Sepharose       | 1.32 x 10⁵ | 4.10                      | 72.6                                        | 6.40 x 10⁵                             | 1.8 x 10⁴             |
| 1st Sephadex G-50    | 1.83 x 10⁵ | 0.508                     | 54.5                                        | 3.47 x 10⁵                             | 9.6 x 10⁴             |
| 2nd Sephadex G-50    | 4.31 x 10⁵ | 0.134                     | 45.5                                        | 1.23 x 10⁵                             | 3.4 x 10⁴             |
| Preparative gel electrophoresis | 4.45 | 0.014                   | 24.0                                        | 6.27 x 10⁴                             | 1.7 x 10⁴             |

Total A280 = volume (in milliliters) x absorbance at 280 nm (A280).

Specifically bound [3H]retinol was estimated as 13% of the total [3H]retinol added to the whole cytosol. This value was determined in four experiments where an average of 13% of added [3H]retinol was eluted in the second (specifically bound) peak from a Sephadex G-50 column (see "Preliminary Studies" under "Results").

Calculated as total disintegrations per min of specifically bound [3H] divided by total absorbance at 280 nm (i.e. disintegrations per min per unit of A280).

Expressed in cumulative terms.

Analytical Gels—Before the final electrophoretic step of purification, the preparation showed a number of different protein bands on disc gel electrophoresis (Fig. 4, Gel A). The final CRBP preparation showed a single dark band of protein (Fig. 4, Gel B), and a single, coincident peak of retinol radioactivity (Fig. 5A). SDS-disc gel electrophoresis showed a single sharp band of protein (Fig. 4, Gel C) with a very faint trace of a higher molecular weight component of undetermined nature.
FIG. 2. Gel filtration on Sephadex G-50. The sample obtained after DEAE-Sepharose chromatography was applied to a column (8 x 110 cm) equilibrated with 40 mM Tris-HCl, 250 mM NaCl, 2 mM EDTA, 12 mM thiglycerol, 0.02% NaN₃, pH 7.5. Elution with this buffer was carried out at a flow rate of about 210 ml/h; fractions of 20 ml each were collected. The void volume of the column was at Fraction 100. The pooled eluate (bar, i) included Fractions 140 to 163 with $V_s/V_o$ ratios from 1.40 to 1.62.

FIG. 3. Preparative polyacrylamide gel electrophoresis of one-half of the pooled eluate obtained after gel filtration. The sample, concentrated to 6 ml and containing 5% sucrose and bromphenol blue tracking dye, was applied to a 9% polyacrylamide gel column (7 cm high, 80-ml gel) at pH 8.91. The cathodic reservoir contained pH 8.3 buffer (according to Davis (43)), and the anodic and elution buffers were the same buffer at a higher concentration (0.016 M Tris-HCl, 0.128 M glycine, pH 8.3). The major portion of the run was carried out at 28 mA current (approximately 300 mV). Fractions of 2.75 ml each were collected at a flow rate of 15 ml/h. The elution position of dye is marked with an arrow (1). Absorbance at 280 nm was monitored continuously with an Uvicord II recording spectrophotometer. The electrophoresis was conducted in two runs to process all of the G-50 eluate. The main portion of the peak of $[^{3}H]$retinol (Fractions 45 to 51) was combined from each of the two runs and characterized by the analytical procedures described in the text. Fractions 54 to 59, containing the descending limb of the $[^{14}C]$retinoic acid peak, were pooled and designated "partially purified retinoic acid-binding protein."

After isoelectric focusing, two bands could be seen that had apparent isoelectric points of 4.8 and 4.9 (Fig. 4, Gel D). Virtually all of the applied $[^{3}H]$retinol was found to migrate in a peak of radioactivity (pH 4.75 to 4.9) that overlapped the two bands (Fig. 5 B). In order to determine whether one or both bands represented purified holo-CRBP containing bound $[^{3}H]$retinol, the isoelectric focusing was repeated with two identical gels which were stained very briefly in order to visualize the two bands. Subsequently, each band was cut out, digested, and assayed for tritium. Half of the recovered tritium...
was found associated with the band focused at pH 4.9, and half was contained in the band focused at pH 4.8. Moreover, when an unstained gel similar to Gel D in Fig. 4 was visualized in the dark under ultraviolet light, two yellow fluorescent bands corresponding to the two close bands of protein were observed. Thus, each of the two close bands is a form of CRBP capable of binding exogenous retinol.

The partially purified retinoic acid-binding protein was also analyzed by isoelectric focusing. The protein-bound [14C]retinoic acid focused at a pH of 4.7.

**Molecular Weight**—The molecular weight of the purified CRBP was estimated by SDS-disc gel electrophoresis to be 14,600 ± 720 (mean ± S.D. for seven determinations). An independent molecular weight estimate of 15,900 was obtained from gel filtration, using a portion of the partially purified CRBP obtained after the second gel filtration step. By this same procedure, partially purified retinoic acid-binding protein was estimated to have a molecular weight very slightly larger (approximately 16,600) than CRBP. Because of the much greater precision of the value obtained by SDS-disc gel electrophoresis (purified protein used for analysis, multiple replicate analyses), 14,600 was selected as the molecular weight estimate for CRBP.

**Immunological Distinction from Serum RBP**—Although the molecular weight of testis CRBP was clearly less than that of rat serum RBP (19,650, see Ref. 11), the possibility existed that the two proteins might be related antigenically. An experiment was conducted to determine whether CRBP could displace [125I]-RBP from antibodies to rat serum RBP in the radioimmunoassay procedure for rat serum RBP. Fig. 6 demonstrates that testis CRBP was totally ineffective in displacing labeled RBP, when tested over a range of 1 to 500 ng of protein. By this immunoassay, therefore, CRBP showed no immunological relationship whatsoever to rat serum RBP.

**Lack of Affinity for Prealbumin**—Rat serum CRBP is known to interact strongly and form a tight protein-protein complex with both rat and human serum prealbumin. In order to determine whether rat testis CRBP or retinoic acid-binding protein also displayed affinity for serum prealbumin, partially purified CRBP and retinoic acid-binding protein (obtained after the second G-50 chromatography) were analyzed by affinity chromatography on a prealbumin-Sepharose column. Under the conditions used (50), rat serum RBP bound tightly to the affinity gel and eluted only when the conductivity of the buffer was greatly decreased. In contrast to serum RBP, both protein-bound [3H]retinol and [14C]retinoic acid from the rat testis preparation were recovered quantitatively in the void volume, indicating that neither of these intracellular binding proteins has affinity for serum prealbumin.

**Amino Acid Composition**—The amino acid composition of purified rat testis CRBP was determined by microanalytical procedures and was compared to the amino acid composition of serum CRBP. Rat testis CRBP is characterized by a relatively high content of acidic amino acids (or their amides) (Table II).

**Absorption Spectroscopy**—The extinction coefficient (Ε cooling) at 280 nm was measured with a solution of CRBP showing absorbance at 280 nm of 0.176. With this solution, Ε cooling was estimated to be 14. Because of some uncertainty as to the absolute amount of CRBP measured by the method of Lowry et al. (54) when standardized against other known proteins, this value should be considered as only an approximation.

The absorption spectrum of purified CRBP was compared directly with that of pure human serum RBP (55, 56). Serum RBP displays two absorption peaks, with maxima at approximately 280 and 330 nm, respectively (Fig. 7). The second peak of absorption, representing protein-bound retinol, has been shown to have a position and shape virtually the same as that displayed by retinol itself in solution in benzene (55). The absorption spectrum of the purified CRBP had two peaks, of nearly equal height and with maxima at approximately 277 nm and at 349 to 348 nm, respectively (Fig. 7). The second (retinoid) peak of the absorption spectrum of rat testis CRBP differed from that of serum RBP (and hence of retinol alone in solution) in several ways. First of all, the second peak maximum of CRBP was located at a higher (15 to 20 nm) wavelength; secondly, the second peak was broader for CRBP than for serum RBP; and finally, the second peak of CRBP was more irregular in shape than that of serum RBP, with a prominent shoulder apparent at approximately 365 to 370 nm.

The interpretation of these differences is not clear (see "Discussion").

It is possible that the final, purified CRBP preparation contains both exogenous ligand ([3H]retinol) as well as endog-
enous retinoid ligand. Calculations were made in order to try to estimate the amount of each type of ligand. The molar ratio of exogenous retinol associated with purified CRBP was calculated from the measured radioactivity in the final preparation and the specific radioactivity of the \[^{3}H\]retinol added to the cytosol, together with the molecular weight estimate of 14,600 for CRBP. For two preparations of purified CRBP, an average of 0.25 molecule of exogenous \[^{3}H\]retinol was estimated to be present per molecule of CRBP.

In order to estimate endogenous ligand content, the assumption was made that all of the ligand bound to CRBP had the same molecular weight as retinol, and the same extinction coefficient at its absorbance maximum as that of all-trans-retinol bound to serum RBP (\(E_{1cm}^{1%} 1625\) (9)). With these assumptions, it was estimated that 0.49 molecule of retinoid ligand (both exogenous and endogenous) were present per molecule of CRBP. Thus, only approximately one-half of the absorbance found in the second peak could be accounted for by the bound, exogenous \[^{3}H\]retinol.

**Fluorescence Spectra** — The uncorrected fluorescence spectra of testis CRBP were compared directly with those of pure human serum RBP in the same experiment. When CRBP was excited at 340 nm, a peak of emission was recorded with its maximum at approximately 475 to 480 nm (Fig. 8A). The shape and position of this emission spectrum were almost the same as those observed for retinol alone in solution in ethanol (Fig. 8A). In contrast, serum RBP showed an emission peak with a lower peak maximum, at approximately 460 nm (Fig. 8A).

The excitation spectrum for CRBP, with emission measured at 465 nm showed a peak with maximum at 355 nm, and with peak shoulders near 290 and 370 nm (Fig. 8B). In contrast, serum RBP showed an excitation spectrum with peak maximum at approximately 336 nm with only one shoulder at approximately 290 cm (Fig. 8B).

The emission spectrum of CRBP with excitation at 280 nm showed a major peak at approximately 340 nm, and a considerably smaller peak at approximately 470 to 480 nm (Fig. 8C). The first peak (340 nm) presumably represents the emission spectrum of the protein itself (particularly its tryptophanyl and tyrosyl residues) whereas the peak at 470 to 480 nm represents the emission spectrum of the retinoid ligand bound to the protein (comparable to the spectrum shown in Fig. 8A).

**DISCUSSION**

This report describes the isolation and partial characterization of the binding protein for retinol from rat testis cytosol. The CRBP had been purified at least 1,700-fold, with a yield of approximately 34%. Purified CRBP had an ultraviolet absorption spectrum characterized by two peaks with maxima at approximately 277 and at 345 nm, and was also fluorescent due to its content of bound retinoid acting as a fluorescent chromophore. The molecular weight of purified CRBP, 14,600, was similar to estimates made in preliminary experiments with whole testis cytosol. In addition, previous studies of others (23, 24) have estimated the sedimentation constant of CRBP in whole cytosol to be approximately 2 S. Taken together, these data suggest that CRBP exists in monomeric form in the tissue cytosol.

Our preparation of purified CRBP was judged to be homogeneous by the criteria of disc gel electrophoresis, and microheterogeneous as evaluated by isoelectric focusing in polyacrylamide gel. In the latter procedure, two bands of holotestis CRBP having apparent isoelectric points of 4.8 and 4.9 were observed. Microheterogeneity has been reported previously for preparations of human (57) and rat (11) serum RBP which were homogeneous by immunological criteria but which

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**Fig. 7.** Absorption spectra of purified rat testis CRBP (solid curve) and of human serum RBP (dotted curve). The two spectra were recorded in the same experiment, on the same instrument.

**Fig. 8.** Fluorescence spectra (uncorrected) of purified testis CRBP and of pure human serum RBP. Solutions of the two proteins were prepared and their spectra recorded. Various dilutions were then prepared just prior to recording the fluorescence spectra shown. The absorbances at 280 and 340 nm of the solutions used for fluorescence analyses were calculated from the measured dilutions of the original solutions. Panel A, emission spectra with excitation at 340 nm. Absorbances at 280 and 340 nm, respectively: CRBP, 3.55 \(\times 10^{-3}\) and 2.67 \(\times 10^{-3}\); serum RBP, 1.53 \(\times 10^{-3}\) and 8.73 \(\times 10^{-4}\); retinol in ethanol, \(A_{280}^{1cm} = 5.28 \times 10^{-5}\). Panel B, excitation spectra with emission at 465 nm. Absorbances at 280 and 340 nm, respectively: CRBP, 3.55 \(\times 10^{-3}\) and 3.19 \(\times 10^{-3}\); serum RBP, 2.23 \(\times 10^{-3}\) and 1.61 \(\times 10^{-3}\); Panel C, emission spectra with excitation at 280 nm. Absorbances at 280 and 340 nm, respectively: CRBP, 5.32 \(\times 10^{-3}\) and 3.95 \(\times 10^{-3}\); serum RBP, 4.60 \(\times 10^{-3}\) and 2.62 \(\times 10^{-3}\). In contrast, excitation of serum RBP at 280 nm produced two major peaks of emission, with maxima at approximately 340 and 400 nm (Fig. 8C).

A quantitative comparison was made between serum RBP and testis CRBP to compare the fluorescence intensity emission at peak maxima (460 or 475 nm) of solutions having equal absorbance at 340 nm and excited at 340 nm. This study indicated that serum RBP was 4.3 times more fluorescent than CRBP under these conditions. Thus, if retinol is solely responsible for the 340 nm absorbance of CRBP, then the fluorescence energy yield of retinol bound to CRBP is only one fourth that of the fluorescence yield of retinol bound to serum RBP.
showed three closely spaced bands after disc gel electrophoresis. It is possible that CRBP may also display microheterogeneity on polycrylamide disc gel electrophoretic analysis under some circumstances. For example, one recent preparation of CRBP displayed a faint, fluorescent band of protein which migrated just anodic to the major CRBP band. Microheterogeneity could result from genetic polymorphism and the use of a mixed genetic population of tissue donors. Alternatively, it could result from alterations (e.g. deamidation) occurring during aging in vivo or during processing of the cytosol or subsequent protein fractions.

A major objective of the present investigation was to compare the properties of purified CRBP with those of the plasma vitamin A transport protein, RBP. Serum RBP has been characterized extensively since its original isolation (9), with regard to a wide range of physical and chemical characteristics, and with regard to its physiological roles and its metabolic regulation (7, 8). A clear distinction between these two proteins is particularly necessary because immunoreactive serum RBP has been measured and localized within several organs as well as in the blood plasma, using both quantitative immunnoasssay (49) and immunofluorescence (50) methods. Thus, rat liver and kidney (which are probable sites of synthesis and degradation, respectively, of serum RBP) were shown to contain an average of 30 and 151 ng of RBP per g wet weight of tissue, while rat serum contained an average of 44 ng of RBP per ml (49).

Comparison of CRBP with serum RBP demonstrated that the two proteins differed from each other with regard to all characteristics examined. First, CRBP is clearly smaller than serum RBP. Second, when compared in the radioimmunoassay for rat serum RBP, rat testis CRBP showed no immunological cross-reactivity whatsoever with rat serum RBP. Third, rat testis CRBP failed to show affinity for serum prealbumin, as determined by affinity chromatography. Fourth, the spectral properties (both absorption and fluorescence) of testis CRBP and serum RBP were delineated together and found to be significantly different from each other (see discussion below). Fifth and finally, the amino acid compositions of CRBP and human serum RBP were determined together and compared. Although the compositional differences in amino acids were not strikingly large, they were clearly more extensive than the minor differences previously observed between rat and human serum RBP (11). Throughout its purifications, CRBP was assayed by its content of bound labeled retinol, which had been added to the cytosol. We have calculated that the final purified preparation of CRBP contained an average of 0.25 mol of [3H]retinol per mol of protein. From the absorbance at 340 nm, however, we estimated that purified CRBP contained about 0.6 mol of total retinoid ligand per mol of protein (see “Results”). Thus, if the assumptions involved in the latter estimate are correct, approximately half of the bound retinoid ligand in the purified CRBP was of endogenous origin. It would be of interest to know if the endogenous ligand is also all-trans retinol, or whether it is some other retinoid. A detailed investigation into the nature of the bound ligand will require extraction and identification of the bound retinoid.

Information about some of the characteristics of the ligand bound to CRBP was obtained by spectroscopy studies of purified testis CRBP in comparison with pure human serum RBP. Detailed absorption (55) and fluorescence (56) spectral studies of human serum RBP have been reported previously from our laboratory, and it has been shown, moreover, that human and rat serum RBP have essentially identical absorption and fluorescence spectra (11, 55, 56). We hypothesized that if the endogenous ligand bound to CRBP is all-trans retinol, and if the manner in which retinol is bound to the two proteins is similar, then the spectral properties of CRBP should resemble closely those of serum RBP. On the other hand, substantially different spectra would suggest either 1) that CRBP is complexed to an endogenous retinoid ligand that is different from retinol, or 2) that the characteristics of the binding of retinol to CRBP differ from those of the binding of retinol to RBP. The absorption spectra reported here demonstrate that the second (retinoid) peak of CRBP differed from that of serum RBP in several ways. In particular, the second peak of absorbance of CRBP was located at a 15 to 20 nm higher wavelength, and it was more broad and irregular in shape than that of RBP. In addition, the fluorescence spectra indicated clear differences between retinol bound to serum RBP and the ligand bound to CRBP. Retinol bound to RBP has an emission maximum (approximately 460 nm) that is located approximately 15 nm below that of retinol in solution in organic solvents. Furthermore, the relative intensity of fluorescence of retinol bound to RBP is 5- to 15-fold greater than that of retinol alone in solution (Ref. 56 and present studies). These data suggest that retinol is bound tightly to serum RBP in a relatively fixed state (56). In contrast, the present studies demonstrated that CRBP displayed an emission maximum at 475 to 480 nm, nearly identical to that observed for retinol in solution in ethanol. If the ligand bound to CRBP was retinol (and at least half of it was the labeled retinol added in vitro), then the absence of a blue shift suggests that the retinol is not so rigidly immobilized when associated with CRBP. The quantitative data showing a lower fluorescence intensity for retinol bound to CRBP than for retinol bound to serum RBP are also consistent with this conclusion.

Very recently, we have purified cytosol CRBP without any addition of retinol in vitro.2 The spectral characteristics of the endogenous ligand bound to this purified protein were essentially identical to those reported here for preparations of CRBP which we have estimated to contain nearly equal amounts of exogenous [3H]retinol and of endogenous native ligand. Taken together, these data suggest that the endogenous ligand may indeed be retinol.

In the course of purifying rat testis cytosol CRBP we have also partially purified a cytosolic protein with binding specificity for exogenous retinoic acid. This protein has a very slightly larger molecular weight than that of CRBP, a slightly slower electrophoretic mobility in gels at pH 8.9, and an apparent isoelectric point near 4.7. Like CRBP, the binding protein for retinoic acid showed no affinity for serum prealbumin. This binding protein resembles and is presumably identical with the retinoic acid-binding protein which has been partially purified by Ong and Chytil (23).

Evidence has been reported for the presence of binding proteins for retinol in the whole cytosol fractions of a number of organs of the rat (24, 27). In future studies we hope to explore whether the same or different binding proteins are present in the various tissues that contain such binding protein activity. Very recently, Ong and Chytil (58) have purified from rat liver an intracellular binding protein for retinol which appears to be very similar in its major properties to testis CRBP. It will be of interest to explore possible immunological and other relationships between these proteins in future studies.

At the present time, the physiological functions of CRBP and the cytosol binding protein for retinoic acid are not known. Retinol is known to be a surface active compound that is “membrane seeking” and potentially membranolytic (50).

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2 A. C. Ross and D. W. S. Goodman, unpublished results.
When retinol is bound to serum RBP, however, the hypervitaminosis A effects are not observed (60). It has therefore been suggested that RBP may deliver retinol to specific sites of action, while protecting biological membranes against the effects of nonspecific and excessive uptake of the vitamin (8, 61). It is possible that the cytosol binding proteins serve similar functions within the cell. It is also possible that the cytosol binding proteins play a more direct role in the biological expression of vitamin A activity, analogous to the direct role played by steroid hormone receptors within target cells (62, 63). With regard to the testis, it is known that retinol depletion results in degenerative changes in the germinal epithelium, and it has been suggested that RBP may serve to deliver retinol to these specific sites of action, while protecting biological membranes from nonspecific losses (64). It has therefore been proposed that CRBP plays a role in these regenerative processes, and warrants future investigation.

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