Vitamin A Transport in Rat Plasma

ISOLATION AND CHARACTERIZATION OF RETINOL-BINDING PROTEIN*

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SUMMARY

Studies were conducted to isolate and characterize rat serum retinol-binding protein (RBP), the specific transport protein for vitamin A in the rat. RBP was isolated from rat serum by a sequence of procedures which included: precipitation with ammonium sulfate between 30 and 50% saturation; chromatography on DEAE-Sephadex; gel filtration on Sephadex G-200 and G-100; and preparative polyacrylamide gel electrophoresis. These procedures resulted in RBP which had been purified approximately 2,300-fold, and which was completely pure by physical and by immunological criteria. Purified rat RBP has α₁ mobility, a sedimentation constant (s₂₀,₅₀) of 2.06 S, and a molecular weight of approximately 20,000. The properties of rat RBP resemble those of human plasma RBP in many ways. The two proteins have nearly identical ultraviolet absorption spectra (peak maxima at 280 and 330 nm) and fluorescence emission and excitation spectra. The amino acid compositions of rat and human RBP are somewhat similar, both with a fairly high content of aromatic amino acids. A specific antirat RBP antiserum was prepared in a rabbit. There was no immunological cross-reactivity between rat and human RBP, indicating that the two proteins are immunologically completely distinct.

In plasma, rat RBP circulates in the form of a protein-protein complex, with an apparent molecular weight of approximately 60,000 to 70,000. The protein (prealbumin-2) with which RBP interacts to form a complex has an electrophoretic mobility slightly greater than that of rat serum albumin. Purified prealbumin-2 has an apparent molecular weight of approximately 45,000 to 50,000. Prealbumin-2 may also represent a major transport protein for thyroid hormone in the rat.

Recent studies with human plasma have established that vitamin A circulates as retinol bound to a specific protein, retinol-binding protein (1, 2). Human RBP has a single binding site for 1 molecule of retinol, α₁ mobility on electrophoresis, and a molecular weight of approximately 21,000. In plasma, RBP circulates as a 1:1 molar protein-protein complex with plasma prealbumin (1–3). The RBP-prealbumin complex is normally highly stable, with an association constant for complex formation of the order of 10⁶ (4). The usual level of RBP in human plasma is about 40 to 50 μg per ml, and that of prealbumin 200 to 300 μg per ml (4, 5). RBP normally circulates mainly as the holoprotein, containing a molecule of bound retinol (4, 5).

The studies presented here were undertaken to determine whether a similar transport system for vitamin A exists in rat plasma. We now report the isolation and partial characterization of rat retinol-binding protein, the specific transport protein for vitamin A in rat plasma. The long range goal of this work is to develop an animal model for the detailed study of the regulation of RBP production and secretion by the liver. Studies dealing with the nutritional regulation of RBP metabolism in the rat are reported in the companion paper (6).

EXPERIMENTAL PROCEDURE

Plasma and Serum—Pilot studies were carried out on plasma, obtained from blood collected from the abdominal aorta of large (>400 g) older rats. The major, large scale isolation of RBP was carried out with 5 liters of rat serum purchased from Pel-Freeze Biochemicals, Inc., Rogers, Ark. The serum had been frozen immediately after collection and was maintained in the frozen state until used.

Column Chromatography—Gel filtration on columns of Sephadex G-200 or G-100 (Pharmacia Fine Chemicals, Inc.), and chromatography on columns of DEAE-Sephadex (Pharmacia), were conducted as described in previous publications from this laboratory (1, 7). Specific details for illustrative examples are indicated in the legends to the figures. For samples undergoing gel filtration, a small amount of blue dextran polymer (molecular weight 2 × 10⁶) was usually added before chromatography, in order to determine the void volume (V₀). All column chromatography was carried out in a cold room at 4–5°C. In most instances the effluent stream was monitored continuously for absorption of light at 280 nm with a Uvicord II absorptiometer (LKB Instruments, Inc., Rockville, Md.).

Electrophoresis—Preparative and analytical (disc) polyacrylamide gel electrophoresis were carried out as reported previously.

1 The abbreviation used is: RBP, retinol-binding protein.
(1, 7). Sodium dodecyl sulfate-polyacrylamide disc gel electrophoresis was performed as described by Weber and Osborn (8).

Analytical Ultracentrifugation—Sedimentation velocity and equilibrium analyses were carried out by Dr. P. Feigelson in a Spinco model E ultracentrifuge equipped with a monochromator and photoelectric scanner. The absorbance of the cell contents at 280 nm was determined at intervals as a function of distance from the center of rotation. After analysis the cell contents were mixed and the analysis repeated under identical conditions but scanning for absorbance at 330 nm. The sedimentation velocity study employed a solution of 0.42 mg of rat RBP per ml in 0.1 M sodium phosphate buffer, pH 7.0. The sedimentation equilibrium analysis used a solution of RBP 0.054 mg per ml in the same buffer. The conditions for sedimentation velocity and equilibrium analyses were the same as those reported previously for the study of human plasma prealbumin and RBP (3, 7). Molecular weights were calculated as reported previously (7), with the method of Yphantis (9). These calculations employed the value of 0.74 as an estimate for the term $V_r$, and an assumed value of 1.0 for $p$. As previously discussed (7) it is felt that the assumed value for $V_p$ did not introduce any major error in the estimation of the molecular weight.

Molecular Weight Estimates—The molecular weight of rat RBP was also estimated by gel filtration on a standardized column of Sephadex G-100, and by sodium dodecyl sulfate-disc gel electrophoresis. The gel filtration method was similar to that previously used with human RBP (1), as described by Whitaker (10). In this method, a small amount of blue dextran polymer was added to each sample before chromatography to determine the void volume ($V_0$). After chromatography the effluent volume ($V_e$) corresponding to the center of the peak of eluted protein was measured, and the values of $V_e:V_0$ were plotted against the log of the molecular weight. The sodium dodecyl sulfate-disc gel electrophoresis method was similar to that described by others (8, 11). After electrophoresis the gels were stained for protein, and the values of the relative mobility of different proteins were plotted against the logs of their molecular weights. The proteins of known molecular weight used as standards for these analyses were obtained from Mann Research Labs, Inc., New York, with the following exceptions. Ribonuclease A and rat serum albumin were isolated as described previously (1, 3). Prealbumin was purified in our laboratory by preparative polyacrylamide gel electrophoresis before use. Human plasma RBP and prealbumin were isolated as described previously (1, 3, 7). Linear regression lines for each of the two plots (log molecular weight versus $V_e:V_0$ or versus relative mobility) were calculated by the method of least squares, with a Wang model 700A electronic calculator with programs prepared for that instrument.

Amino Acid Analysis—The amino acid composition of rat RBP was kindly determined by Doctors R. E. Canfield and E. J. Morgan. Because of the very limited amount of pure rat RBP available, analyses were carried out on only one sample (0.95 mg) subjected to acid hydrolysis for 24 hours. A sample of human RBP (1.2 mg) was subjected to acid hydrolysis under the same conditions at the same time. The samples were hydrolyzed in 3 N $p$-toluenesulfonic acid containing 0.2% 3-(2-aminoethyl) indole in evacuated sealed tubes at 110°, as described by Liu and Chang (12). This method of hydrolysis has been shown (12) to provide a satisfactory recovery of tryptophan in protein hydrolysates, for its direct determination on the amino acid analyzer. Amino acid analyses were performed by the method of Spackman, Stein, and Moore (13) on a Spinco model 121 amino acid analyzer. Because of the single time interval used for hydrolysis, the results may not be absolutely correct for the more slowly hydrolyzed residues (e.g. valine, isoleucine) and for the more labile amino acids (e.g. serine, threonine, tryptophan). The results do, however, provide an accurate comparison of the amino acid compositions of rat and human RBP.

Preparation of Antiserum—A solution of purified rat RBP, 0.8 ml of concentration 2 mg per ml in 0.1 M sodium phosphate buffer, pH 7.4, was emulsified with an equal volume of complete Freund’s adjuvant (Difco Laboratories, Detroit, Mich.). One white rabbit weighing approximately 2 kg was injected intracutaneously in the back 11 times with 0.1-ml portions of the emulsified RBP. Immediately thereafter 0.5 ml of pertussis vaccine was injected subcutaneously in the back of the neck. Three weeks later a “booster” injection of emulsified RBP was made into toe pads (0.1 ml into each of three toe pads). Blood was collected from ear arteries, or veins, or both, at 2-week intervals thereafter; after clotting the blood samples were centrifuged at 2000 rpm for 30 min at 4° and the sera collected. Eight lots of sera were collected during a 20-week interval, and were stored at -20°. The sera were assayed for specificity and titer against rat RBP by immunodiffusion (double diffusion in gel) by the method of Ouchterlony (14), as described previously (1, 7).

The rabbit antihuman RBP antiserum used here had been prepared as described elsewhere (5).

Other Procedures—Absorptions and absorption spectra were usually measured with a Gilford model 2400 spectrophotometer or with a Beckman DB spectrophotometer. In some instances spectra were recorded with a Cary model 14 spectrophotometer. Fluorescence measurements were made with an Amenco-Bowman spectrophotofluorometer (American Instrument Co., Silver Spring, Md.). Fractions eluted from columns were assayed for protein-bound retinol by measuring the relative intensity of fluorescence with excitation at 334 nm and emission at 460 nm. In the figures shown here, the illustrated values for relative intensity of fluorescence all refer to the values obtained with excitation and emission at these wave lengths. More highly purified protein factions were also assayed for protein-bound retinol by measuring absorbance at 330 nm.

Protein concentrations were estimated from the absorbances at 280 nm, and by the method of Lowry et al. (15), with bovine serum albumin or human prealbumin as standards. The extinction coefficient (E$^m_{280}$) of RBP at 280 nm was estimated by measuring the absorbance at 280 nm and the protein concentration by the method of Lowry et al., on the same solution of pure RBP. Solutions of purified RBP were otherwise assayed for protein by measuring the absorbance at 280 nm.

In the major fractionation study reported here, the recovery of protein-bound retinol during the several fractionation procedures was estimated by fluorescence measurements as described above, or by direct measurement of vitamin A levels by the trifluorocetic acid method of Dugan, Frigerio, and Siebert (16) as modified by Rnels and Mahadevan (17). Small portions of the starting serum, and of the RBP-containing pools from the initial fractionation procedures, were stored at -20° and, several months later, were assayed directly for RBP content by the radioimmunoassay procedure described in the companion paper (6). The results of some of these assays are reported under “Results.”
RESULTS

Whole Plasma—When rat whole plasma was subjected to gel filtration on Sephadex G-200, protein-bound vitamin A was eluted as a single sharp peak with elution volume very similar to that of rat serum albumin (Fig. 1). The apparent molecular size of the plasma transport protein for vitamin A was estimated from this analysis to correspond to that of a protein of molecular weight approximately 60,000 to 70,000. As indicated below, this apparent size represents that of the complex between RBP, the specific transport protein, and rat plasma prealbumin-2.

Human plasma RBP and prealbumin can be separated largely from plasma albumin, and purified 80- to 100-fold, by a single chromatography of whole plasma on DEAE-Sephadex (7). A preliminary experiment was conducted to determine whether this would also prove to be an effective first step for the purification of rat RBP. In contrast to human plasma, however, chromatography of rat plasma on a column of DEAE-Sephadex, under the same conditions employed with human plasma, resulted in the elution of RBP (as monitored by protein-bound retinol) as a single peak together with rat plasma albumin. Fractionation with ammonium sulfate was therefore selected as the first procedure for RBP purification, since this appeared to result in the removal of most of the albumin with recovery of most of the RBP, sample was eluted as a single sharp peak with effluent volume very similar to that seen in whole plasma (Fig. 1). The apparent molecular weight of rat serum RBP was determined by gel filtration on Sephadex G-200, protein-bound retinol of the RBP, sample was eluted as a single peak with effluent volume similar to that of rat serum albumin (Fig. 1). The protein-bound retinol of the RBP, sample was eluted as a single peak with effluent volume similar to that seen with whole plasma (Fig. 1). In contrast, the protein-bound retinol of the RBPf pool was eluted in two overlapping peaks. The fractions comprising each of these peaks were combined to yield two pools: RBPF and RBPf, representing the second peak (see legend to Fig. 2).

Four chromatographic runs on DEAE-Sephadex were carried out in order to fractionate the entire sample obtained after (NH₄)₂SO₄ precipitation. The total recovery of RBP after chromatography was 91%.

In order to determine why RBP appeared to be eluted from DEAE-Sephadex in two partly separated peaks, small portions of the RBPF and of the RBPf pools were analyzed by gel filtration on columns of Sephadex G-200. The protein-bound retinol of the RBPF sample was eluted as a single peak with effluent volume (Vₑ/Vₒ = 1.75) similar to that seen with whole plasma (Fig. 1). In contrast, the protein-bound retinol of the RBPf sample was eluted in two peaks: a small peak with Vₑ/Vₒ approximately 1.7 and a larger peak with Vₑ/Vₒ of 2.1 (see Fig. 3). These findings indicated that the RBPF pool contained RBP present mainly as a moiety of smaller molecular size than that seen in whole plasma or in the RBPf pool. The data suggested that RBP was present
A gel filtration on Sephadex G-200 of a portion of the RBP pool obtained after DEAE-Sephadex chromatography (see Fig. 2). The sample (70 ml containing 2.24 g of protein) was applied to a column (5 x 116 cm) equilibrated with 0.02 M potassium phosphate buffer, pH 7.6, 0.2 M NaCl. Elution with this buffer was carried out at a flow rate of about 45 ml per hour; fractions 15 ml each were collected. The void volume of the column ($V_v$) was at Fraction 50. The RBP-containing (fluorescent) fractions were combined into two pools: (a) R-f1, Fractions 77 to 90, and (b) R-f2, Fractions 91 to 118.

**Fig. 3.** Gel filtration on Sephadex G-200 of a portion of the RBP pool obtained after DEAE-Sephadex chromatography (see Fig. 2). The sample (70 ml containing 2.24 g of protein) was applied to a column (5 x 116 cm) equilibrated with 0.02 M potassium phosphate buffer, pH 7.6, 0.2 M NaCl. Elution with this buffer was carried out at a flow rate of about 45 ml per hour; fractions 15 ml each were collected. The void volume of the column ($V_v$) was at Fraction 50. The RBP-containing (fluorescent) fractions were combined into two pools: (a) R-f1, Fractions 77 to 90, and (b) R-f2, Fractions 91 to 118.

The protein present in the R-f2 pool was concentrated by ultrafiltration, and then subjected to gel filtration on a column of Sephadex G-100. RBP was eluted almost entirely as a single peak, with a value of $V_e:V_0$ of approximately 1.75 (corresponding to a $V_e:V_0$ ratio of about 2.1 on Sephadex G-200). A small amount of nonfluorescent, higher molecular weight protein was removed by this procedure. The RBP-containing fractions were pooled, concentrated by ultrafiltration, and again subjected to gel filtration on Sephadex G-100. As shown in Fig. 4, RBP was now eluted as a single peak of highly purified protein.

**Fig. 4.** Gel filtration on Sephadex G-100 of RBP obtained after purification by repeated chromatography on Sephadex G-200 and G-100. The sample (5 ml containing approximately 14 mg of RBP) was applied to a column, 2.5 x 60 cm in size. Elution with 0.02 M potassium phosphate buffer, pH 7.6, 0.2 M NaCl was carried out at a flow rate of about 15 ml per hour; fractions of 4.5 ml each were collected.

**Fig. 5.** Preparative polyacrylamide gel electrophoresis of purified RBP obtained after gel filtration as shown in Fig. 4. Approximately 12 mg of RBP were applied to a gel column 7 cm high (about 80 ml volume). The major portion of the electrophoretic run was carried out at 300 volts with 14 ma current. Fractions of 2.5 ml each were collected at a flow rate of about 15 ml per hour Apo, apo-RBP; H1 and H2, forms of holo-RBP.

Studies with human RBP have revealed that all three peaks correspond to high mobility minor bands, which are not present in the RBP pool of rat plasma. The results presented here indicate that rat plasma RBP can be purified to homogeneity by gel filtration and ultrafiltration, and that it retains its biological activity. The possibility that the very faint bands represent minor impurities in the sample was subjected to preparative polyacrylamide gel electrophoresis, as shown in Fig. 5. Protein was eluted in three peaks (labeled Apo, H1, and H2 in Fig. 5) from the gel column. Similar elution patterns, with varying ratios of the three peaks, have been observed with human RBP (1, 3).
tain pure RBP, with the first peak representing apo-RBP (RBP not containing bound retinol) and with the HI and HS peaks representing two forms of holo-RBP (RBP with bound retinol) (1, 3, 5). It was assumed that rat RBP was similar to human RBP in this regard, and that the three peaks shown in Fig. 5 represented apo-RBP and two forms of holo-RBP. Evidence that the peaks all represent pure RBP is presented in the companion paper, where it is shown that apo- and holo-RBP separated by gel electrophoresis as in Fig. 5, are immunologically identical as assessed in the radioimmunoassay for rat RBP.

The collected fractions comprising the apo-RBP peak (Fig. 5) and those comprising the two holo-RBP peaks (HI and HS) were separately pooled, and the protein in each pool subjected to gel filtration on a small column of Sephadex G-100. The RBP-containing fractions from the Sephadex G-100 columns were dialyzed exhaustively against distilled water, and the solutions then lyophilized to dryness. The two freeze-dried preparations of purified RBP (apo-RBP and holo-RBP) were stored at -20° and used for further studies. Except for the amino acid analysis, all of the further studies of the properties of rat RBP reported here employed this preparation of purified holo-RBP.

Analytical Ultracentrifuge Studies—Sedimentation velocity studies showed that purified holo-RBP migrated as a single homogeneous protein with a sedimentation constant (s_20,w) of 2.06 S. Fig. 6 shows the results of sedimentation equilibrium analysis of RBP. When scanned at either 280 or 330 nm, RBP appeared to be a single homogeneous component, as can be judged from the linearity of the data. The molecular weight of RBP was estimated to be 20,380 from the 280-nm data, and 19,700 from the 330-nm data, giving a mean value for the estimate of molecular weight of 20,040.

Immunological Studies—The antiserum obtained from the rabbit injected with purified rat RBP gave a single precipitin line when tested against purified RBP by immunodiffusion (see Fig. 7). A single precipitin line was also obtained by testing this antiserum against rat whole serum, and this line showed a reaction of identity with the line obtained by reacting the antiserum against purified RBP. The finding that a monospecific antiserum was produced when the purified rat RBP was used as antigen provides strong additional evidence for the purity of the rat RBP preparation.

An immunodiffusion study was carried out to determine whether there was immunological cross-reactivity between human and rat RBP. As shown in Fig. 7, the antiserum against rat RBP showed no reaction with purified human RBP; similarly the antiserum against human RBP showed no reaction with purified rat RBP. These results indicate that human and rat RBP are immunologically distinct and different from each other.

Spectral Studies—The absorption spectrum of the purified holo-RBP is shown in Fig. 8. The spectrum had two peaks, of nearly equal height, with maxima at 280 and 330 nm, respectively. As with human RBP (1-3, 18), the peak at 280 nm presumably represents the absorption spectrum of the protein, and the peak at 330 nm that of the protein-bound retinol. Isolated apo-RBP did not show an absorption peak at 330 nm. (Fig. 5 illustrates the relative 330-nm absorbances of apo- and holo-RBP.)

Uncorrected fluorescence spectra were recorded under several conditions. When RBP was excited at 334 nm, a peak of emission was recorded with maximum at approximately 464 nm. The excitation spectrum for RBP, with emission measured at 464 nm, showed a peak with maximum at 334 nm and with a shoulder at about 285 to 290 nm. Fig. 9 shows the emission
FIG. 8. Ultraviolet absorption spectrum of purified holo-RBP (RBP-H1, see Fig. 5).

FIG. 9. Fluorescence emission spectrum (uncorrected) of purified holo-RBP (RBP-H1) with excitation at 280 nm.

The spectrum with excitation at 280 nm. The spectrum has two major peaks, with maxima at about 340 to 345 nm and at about 462 to 464 nm. The first peak (340 to 345 nm) presumably represents the emission spectrum of the protein itself (particularly its tryptophanyl and tyrosyl residues), whereas the peak at 464 nm represents the emission spectrum of retinol bound to RBP. This emission spectrum (Fig. 9) indicates that there is efficient transfer of energy within the holoprotein, probably mainly from excited tryptophanyl residues to the bound retinol.

The extinction coefficient (E) at 280 nm was estimated to be approximately 19 to 20. Because of some uncertainty as to the absolute amount of RBP measured by the method of Lowry et al. (15) when standardized against other known proteins this value should be considered as only an approximation.

Molecular Weight—In addition to the sedimentation equilibrium study described above, the molecular weight of purified RBP was also estimated by gel filtration on a standardized column of Sephadex G-100 (Fig. 10), and by sodium dodecyl sulfate-disc gel electrophoresis (Fig. 11). These studies provided, respectively, molecular weight estimates for rat RBP of approximately 18,900 and 19,600. By considering these values together with the two values obtained from the sedimentation equilibrium analysis (at 280 and at 330 nm), an average value of 19,650 was obtained as an estimate for the molecular weight. As shown in Figs. 10 and 11, rat RBP appeared to be slightly smaller in size than human RBP in both of the analytical systems illustrated.

Amino Acid Composition—The results of the amino acid analysis of rat RBP are presented in Table I (left-hand column).
The largest peak (labeled RSA) consisted of rat serum albumin. The major peaks of protein were eluted from the gel column. The complex form after DEAE-Sephadex and Sephadex G-200 chromatography (see Fig. 3) was subjected to further study. Fig. 12 shows the results of preparative polyacrylamide gel electrophoresis of a portion of the proteins of the R-f1 pool. Three major peaks of protein were eluted from the gel column. The largest peak (labeled RSA) consisted of rat serum albumin.

The other two, smaller peaks had anodic mobilities greater than that of serum albumin, and were called, respectively, prealbumin-1 and prealbumin-2. The other two, smaller peaks had anodic mobilities greater than that of serum albumin, and were called, respectively, prealbumin-1 and prealbumin-2.

Studies of Protein-Protein Complex—As indicated above, RBP circulates in rat plasma as a complex of apparent molecular weight 3 to 36 times that of RBP itself. In order to explore the nature of the complex, the R-f1 pool, containing RBP in complex form after DEAE-Sephadex and Sephadex G-200 chromatography (see Fig. 3) was subjected to further study. Fig. 12 shows the results of preparative polyacrylamide gel electrophoresis of a portion of the proteins of the R-f1 pool. Three major peaks of protein were eluted from the gel column. The largest peak (labeled RSA) consisted of rat serum albumin.

The results should be compared with those obtained with a sample of human RBP which was hydrolyzed at the same time and under the same conditions (Table I, middle column). The amino acid analysis employed a sample of purified RBP which was obtained from a portion of the R-f1 pool. For rat RBP, methionine and histidine appeared to be the amino acid residues present at least abundance. Rat RBP was fairly rich in aromatic amino acid residues, although its relative content of tryptophan and tyrosine appeared to be slightly smaller than that of human RBP. The amino acid composition of rat RBP was generally similar to that of human RBP. The major quantitative differences in the two proteins were the higher levels of histidine and isoleucine found in rat RBP. Smaller differences were also observed with regard to several other amino acid residues (Table I).

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Fig. 13. Formation of a complex between rat RBP and prealbumin-2 (PA-2), as shown by gel filtration on Sephadex G-100. Four solutions of identical volume (0.5 ml), and each containing approximately 0.25 mg of RBP (with or without prealbumin-1 or PA-2) were chromatographed serially on a single column of Sephadex G-100, 1 × 60 cm in size. The first solution contained only RBP (Panel A). The second solution contained RBP + PA-1, with an absorbance ratio (at 280 nm) of 1:5 (Panel B). The third and fourth solutions contained RBP + PA-2, with absorbance ratios (at 280 nm) of 1:1 (Panel C) or 1:3 (Panel D). Elution was carried out with 0.02 M potassium phosphate buffer, pH 7.0, 0.2 M NaCl, at a flow rate of 5 ml per hour. Fractions of 1.5 ml each were collected. Each solution contained a small amount of blue dextran in order to determine the void volume for each chromatographic run (indicated by the small arrows labeled V₀). (The absorbance at 280 nm due to the blue dextran polymer is not plotted in the figure.)

of RBP and an even greater excess of rat serum albumin. In contrast, when RBP was mixed with a small molar excess of prealbumin-2, the RBP was eluted much earlier from the column and together with the prealbumin-2, at an elution volume corresponding to a molecular weight of approximately 60,000 to 70,000 (Fig. 13, Panel D). When RBP was mixed with a lesser amount of prealbumin-2, so that RBP was present in molar excess, the RBP was eluted in two peaks, corresponding to the RBP-prealbumin-2 complex and to free RBP, respectively (Fig. 13, Panel C). In addition to showing the formation of a complex between RBP and prealbumin-2, this study thus indicates that the capacity of prealbumin-2 for complex formation with RBP is limited, and suggests that the proteins interact in a molar ratio of 1:1.

Prealbumin-2—The molecular weight of prealbumin-2 was estimated by gel filtration on a standardized column of Sephadex G-100 (Fig. 10), and by sodium dodecyl sulfate-disc gel electrophoresis (Fig. 11). These studies provided molecular weight estimates for prealbumin-2 of 32,000 and of 45,000, respectively. In both studies (see Figs. 10 and 11) rat prealbumin-2 appeared to be slightly smaller in size than human prealbumin.

Discussion

This report describes the isolation and partial characterization of rat serum retinol-binding protein, the specific transport protein for vitamin A in rat blood. Rat RBP has α₁ mobility, a sedimentation constant (s₂₀,₅₀) of 2.06 S, and a molecular weight of approximately 20,000. The molecular weight was assessed by sedimentation equilibrium analysis, and also by gel filtration on a standardized column of Sephadex G-100 and by sodium dodecyl sulfate-disc gel electrophoresis. The interaction of retinol with RBP is of considerable physiological importance since, as previously pointed out (1), this interaction serves to solubilize the water-insoluble retinol molecule, and to protect the unstable retinol molecule against chemical degradation. In plasma, rat RBP circulates in the form of a protein-protein complex, with an apparent molecular weight of approximately 60,000 to 70,000. The protein with which RBP interacts to form a complex has an electrophoretic mobility slightly greater than that of rat serum albumin, and has been called by us prealbumin-2. Purified prealbumin-2 has an apparent molecular weight of approximately 45,000 to 50,000.

The isolation of rat RBP proved to be extremely difficult technically, much more so than the isolation of human RBP from human plasma. This was particularly so because of the fact that rat RBP did not separate from rat serum albumin on columns of DEAE-Sephadex. The preparation of isolated rat RBP described here was purified approximately 2300-fold from whole serum, and was completely pure, as assessed in the analytical ultracentrifuge, and also as indicated by the production of a monospecific antiserum when the purified RBP was used as antigen.

The properties of rat RBP are similar in many ways to those of human RBP. The two proteins have virtually identical ultraviolet absorption spectra, and fluorescence emission and excitation spectra. Studies with human RBP have shown that the absorption peak at 330 nm represents the absorption of the protein-bound retinol, that the molar extinction of retinol bound to RBP is identical with that of retinol in solution in benzene, and that RBP appears to have a single binding site for 1 molecule of retinol (1, 18). It is likely that these statements also apply to rat RBP. Both human and rat RBP are small proteins with a fairly high content of aromatic amino acids. Rat RBP is slightly smaller than human RBP, as indicated by comparison of the present results with those of analytical ultracentrifuge studies previously reported for human RBP (3) (sedimentation coefficient 2.13 S, molecular weight 21,300), and also by direct comparison by gel filtration on Sephadex G-100 (Fig. 10) and by sodium dodecyl sulfate-disc gel electrophoresis (Fig. 11). The amino acid composition of rat and human RBP are fairly similar, with rat RBP having higher levels of histidine and isoleucine than human RBP, and with smaller differences being seen for several other amino acid residues (Table I). Both proteins, when isolated from plasma or serum, are microhetero-
geneous on disc gel electrophoresis, and consist of two forms of the holoprotein (H1 and H2), together with a smaller amount of retinol-free apo-RBP.

Despite these similarities, rat RBP is immunologically completely distinct from human RBP. No immunological cross-reactivity was evident when the two proteins were both tested against each of their respective antisera by immunodiffusion (Fig. 7). Subsequent studies, with radioimmunoassay procedures for each of the two proteins, have confirmed the complete lack of immunological cross-reactivity between rat and human RBP. It is thus evident that certain important structural differences must exist between the two proteins, particularly with regard to those aspects of structure involved in antigenic determination. The nature of these differences remains to be explored.

Rat RBP also resembles human RBP in that both proteins interact with a larger protein and circulate in plasma in the form of a protein-protein complex. Considerable information is now available about the structure of human plasma prealbumin, and about the interaction of human prealbumin and RBP (3, 7, 19-23). Human prealbumin appears to consist of a tetramer of four very similar and possibly identical subunits (20-23). In addition to its binding site for RBP, the human prealbumin molecule contains an independent binding site with high affinity for thyroxine (7, 24). The interaction of human RBP and prealbumin significantly stabilizes the retinol-RBP complex (18, 19, 25, 26). Furthermore, as previously pointed out (1), the formation of the protein-protein complex protects the relatively small RBP molecule by preventing its glomerular filtration, and hence the loss of RBP via urinary excretion, or renal catabolism, or both. The formation of a complex between rat serum RBP and prealbumin-2 clearly serves a similar physiological function of protecting rat RBP against glomerular filtration and renal loss. No information is available regarding other possible effects of this protein-protein interaction. The gel filtration studies reported in Fig. 13 suggest that rat RBP and prealbumin-2 interact in a molar ratio of 1:1.

Prealbumin-2 is a rat serum protein which has not been isolated previously, and which warrants more detailed examination; studies to this end are planned in this laboratory. It has recently been reported that the major transport protein for thyroid hormone in rat serum may be a protein with electrophoretic mobility slightly greater than that of rat serum albumin ("slowmigrating prealbumin" (27)). Preliminary studies have indicated that prealbumin-2 can bind thyroxine, with an affinity which appears to be greater than that of rat serum albumin for thyroxine. It is thus possible that prealbumin-2 isolated here may represent the major transport protein for thyroid hormone in the rat. Further detailed study of the interaction of prealbumin-2 and thyroxine will be required in order to explore this possibility. If prealbumin-2 does play a role in thyroxine transport in the rat, this would indicate that in both species studied in detail (man and rat) a molecular relationship exists between the transport of vitamin A and thyroxine in plasma. The possibility that such a relationship exists widely, in other mammalian orders and vertebrate classes, warrants exploration. The functional significance, if any, of this relationship is at the present time obscure.

As indicated in the introduction, this work was undertaken in part in order to try to develop an animal model for the detailed study of the regulation of RBP production and secretion by the liver. The isolation of rat RBP, and the preparation of an antirat RBP antiserum, were necessary for the development of a radioimmunoassay for rat RBP, to be used in studies on regulation and control. Detailed studies dealing with the regulation of immunoreactive RBP levels in rat serum and liver by means of changes in nutritional vitamin A status are reported in the companion paper (6).

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