Extraction and recombination studies of the interaction of retinol with human plasma retinol-binding protein

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Abstract Methods have been developed for the removal of retinol from human plasma retinol-binding protein (RBP), so as to form the retinol-free apoprotein, and for the recombination of apo-RBP with retinol to again form the holoprotein. Retinol is removed from RBP by gently shaking a solution of RBP with heptane under controlled conditions. During the shaking, retinol is gradually extracted from the RBP and into the heptane phase. The reassociation of apo-RBP with retinol is achieved by exposing a solution of apo-RBP to Celite coated with a thin film of retinol, followed by isolation of the RBP by gel filtration on Sephadex G-100. This procedure results in the recombination of apo-RBP with an amount of retinol almost identical with that previously removed by extraction.

The two-phase extraction procedure was used to explore some of the factors which affect the interaction of retinol with RBP. The retinol–RBP complex was most stable in the lower portion of the pH range 5.6 to 10. The rate of removal of retinol from the RBP–prealbumin complex (the form in which RBP normally circulates in plasma) was markedly less than the rate of its removal from RBP alone. The interaction of retinol with RBP appears to be stabilized by the formation of the RBP–prealbumin complex.

The recombination procedure was employed to examine the specificity of the binding of retinol to RBP, by determining whether compounds other than all-*trans*-retinol would effectively bind to apo-RBP. Apo-RBP did not bind cholesterol, but displayed a slight affinity for phytol. The affinity of RBP for β-carotene was minimal, whereas both retinyl acetate and retinal were bound about one-third as effectively as all-*trans*-retinol. In contrast, retinoic acid bound to apo-RBP almost as effectively as did retinol. Each of two isomers of retinol, 13-*cis* and 11,13-di-*cis*-retinol, bound to apo-RBP to some extent. The 13-*cis* isomer appeared to bind somewhat less effectively than did the 11,13-di-*cis* isomer. The binding of retinol to RBP is highly but not absolutely specific.

Supplementary key words vitamin A · prealbumin · holoprotein · apoprotein · lipid–protein interaction · protein–protein interaction

The PLASMA TRANSPORT SYSTEM for vitamin A comprises two proteins: retinol-binding protein (RBP), the specific transport protein, and prealbumin (PA) (1, 2). RBP interacts strongly with PA and circulates in plasma as a 1:1 molar RBP–PA complex (1–3). RBP has a molecular weight of approximately 21,000 and has a single binding site for one molecule of retinol. The usual level of RBP in plasma is about 40–50 μg/ml, and that of PA, 200–300 μg/ml (4, 5). RBP normally circulates mainly as the holoprotein, containing a molecule of bound retinol (4, 5).

RBP provides an interesting and physiologically important model for the study of the interaction of a specific lipid (retinol) with a protein. It was previously pointed out that the interaction of retinol with RBP serves to solubilize the water-insoluble retinol molecule and to protect the unstable retinol molecule against chemical degradation (1). We now report a method for the removal of retinol from RBP without apparent structural damage to the protein, so as to form the retinol-free apo-RBP. This method has been used to explore some of the factors which affect the interaction of retinol with RBP. A method has also been developed for the recombination of apo-RBP with retinol, to again form the holoprotein. The specificity of the binding of retinol to RBP was explored with this method, by determining whether compounds other than all-*trans*-retinol would interact with and form a complex with apo-RBP. Some of these findings have been reported in preliminary form (6).

Abbreviations: RBP, retinol-binding protein; PA, prealbumin; HSA, human serum albumin; TLC, thin-layer chromatography.

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METHODS

Isolation of RBP and PA

Human plasma RBP and PA were isolated by a sequence of procedures described in detail in our previous publications (1, 3, 4, 7). As described previously (3), RBP so isolated is microheterogeneous on disc gel electrophoresis, and it consists of a mixture of two forms of the holoprotein (containing bound retinol), together with some retinol-free apoprotein. The purified RBP used in these studies usually comprised 20–30% apoprotein.

Extraction of retinol from RBP to form apo-RBP

A two-phase extraction method was developed for the removal of retinol from RBP, so as to form apo-RBP from holo-RBP. In this method, an aqueous solution of RBP and an equal volume of heptane are shaken together in an amber flask under nitrogen, in a metabolic shaking incubator (Research Specialties Co., Richmond, Calif.). During the shaking, retinol is gradually extracted from the RBP and into the heptane phase. The results of a typical experiment are shown in Fig. 1. In this experiment, 13 ml of a solution of RBP in potassium phosphate buffer, 0.02 M, pH 7.4, and 13 ml of *n*-heptane were shaken at 23°C under nitrogen in a 125-ml amber Erlenmeyer flask at a rate of 100 strokes/min. Before extraction, the aqueous RBP solution had the absorption spectrum shown in the upper right-hand panel of Fig. 1, whereas the heptane phase was devoid of ultraviolet absorbance. After 6 hr of gentle shaking, approximately 78% of the retinol was extracted into the heptane phase, which showed a retinol absorption peak as in the lower left-hand panel of Fig. 1. The 330-nm absorbance peak of RBP, due to the retinol bound to the protein, was decreased by a comparable amount (Fig. 1, lower right-hand panel).

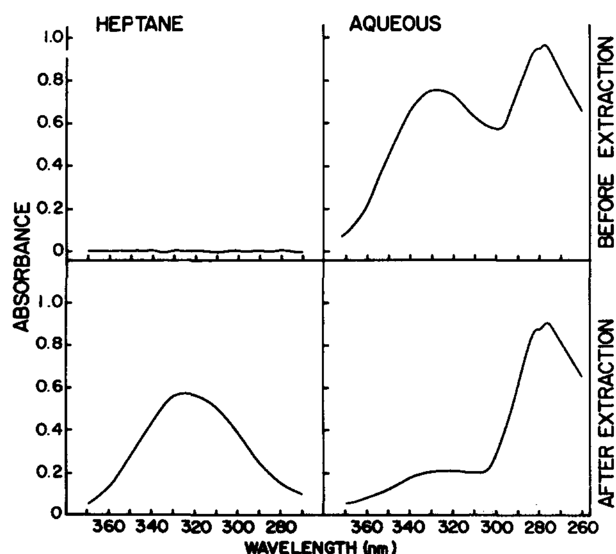

FIG. 1. Production of apo-RBP from holo-RBP by extraction of retinol from RBP with heptane.

During the course of a 2-yr period this extraction method was used to prepare apo-RBP from RBP isolated from plasma on eight different occasions. In these eight extractions, an average of 78% (range 75–81%) of the retinol was extracted from the RBP, as judged by the decrease in absorbance at 330 nm relative to that at 280 nm, and the average recovery of absorbance at 280 nm in the aqueous phase after extraction was 88% (range 84–93%). The recovery of retinol (as judged by absorbance at 326–330 nm) in the heptane plus the aqueous phases was 100–104%.

Recombination of extracted (apo-) RBP with retinol

A method was developed for the recombination of extracted (apo-) RBP with retinol, so as to again form holo-RBP. The method used was based upon the procedure originally developed by Avigan for the addition of labeled cholesterol to plasma lipoproteins (8). In this method, an aqueous solution of extracted RBP is shaken gently with Celite (Johns-Manville Products) covered with a thin film of retinol, in an amber flask under nitrogen at room temperature. A 50–75-times molar excess of retinol (as compared with RBP) is used. During the shaking some of the retinol becomes associated with the apo-RBP to form holo-RBP. At the end of the incubation period the Celite is removed by centrifugation and the aqueous phase is subjected to gel filtration on a small column of Sephadex G-100 (Pharmacia Fine Chemicals Inc., Piscataway, N.J.). The column fractions containing RBP are then pooled and subjected to further study.

In a typical experiment, 150 mg of Celite was added to a 25-ml amber Erlenmeyer flask containing 1 mg of retinol dissolved in 4 ml of light petroleum ether (bp 38–53°C). The petroleum ether was evaporated completely under a stream of nitrogen while swirling the contents of the flask by hand, so as to produce dry Celite covered with a thin film of retinol. 3 ml of a solution containing 1.2 mg of apo-RBP in 0.02 M potassium phosphate buffer, pH 7.4, was added and the flask was then shaken at a rate of 80 strokes/min in a metabolic shaking incubator for 5 hr. The contents of the flask were centrifuged for 5 min at 2000 rpm, and the slightly yellow and slightly turbid aqueous phase was applied to a column of Sephadex G-100, 45 × 1.4 cm (bed volume approx 70 ml), in a cold room at 4°C. Elution was carried out with 0.02 M phosphate buffer, pH 7.4, 0.2 M NaCl at a rate of 6 ml/hr; 3-ml fractions were collected. The results of gel filtration are shown in Fig. 2. RBP was eluted in fractions 10–14, with elution volume approximately 35 ml; these fractions displayed the typical fluorescence of holo-RBP (3, 9). Following the RBP peak there appeared a larger

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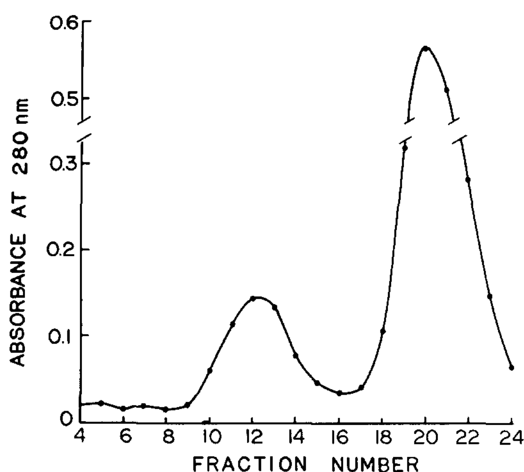

FIG. 2. Recombination of extracted (apo-) RBP with retinol. The figure shows the absorbance, at 280 nm, of the fractions eluted after gel filtration on a column of Sephadex G-100. Fractions 10–14 were fluorescent and contained RBP. For further details see text.

peak of UV-absorbing but nonfluorescent material (fractions 18–23 in Fig. 2). Experiments with ^{14}C-labeled retinol have demonstrated that these later fractions apparently contain oxidized retinol, dispersed or dissolved in the aqueous phase. The RBP-containing fractions (fractions 10–14, Fig. 2) were pooled, and the resulting solution was concentrated by ultrafiltration using a Diaflo UM-1 membrane (Amicon Corp., Lexington, Mass.); the absorption spectrum of the concentrated solution was determined with a Beckman DB spectrophotometer.

Recombination of extracted (apo-) RBP with compounds other than retinol

The procedure just described was used to determine whether compounds other than all-*trans*-retinol would interact with, and form a complex with, apo-RBP. The compounds studied included β-carotene, retinyl acetate, retinal, retinoic acid, 13-*cis*-retinol, 11,13-di-*cis*-retinol, phytol, and cholesterol. For each compound studied, one or more experiments were conducted using the amounts of materials and experimental conditions exactly as described above for a "typical experiment." Thus, in each experiment, 4 ml of a solution of the compound in light petroleum ether was added to 150 mg of Celite, and the experiment was conducted as described above. For the study with retinoic acid, the retinoic acid was added dissolved in diethyl ether, and the ether was evaporated as described to yield Celite coated with a thin film of retinoic acid.

In several instances (see below) radioactively labeled compounds were used in the recombination studies. In these instances, a small portion of the pooled RBP-containing fractions (after Sephadex G-100 gel filtration) was extracted with $CHCl_3$–CH_3OH 2:1 (v/v), and the lipid extract so obtained was assayed for radioactivity. Portions of the G-100 column fractions just preceding and just following the RBP peak were also extracted and assayed for radioactivity, so as to obtain base line values for the level of radioactivity (if any) in the column effluent. The base line values (always very low) were subtracted from the values found in the RBP "pool." When a significant amount of radioactivity was found in the pooled RBP fractions, another portion of the RBP "pool" was extracted and the extract chromatographed on a column of alumina (10) so as to determine how much of the radioactivity found in the RBP "pool" was present as the unchanged compound under study. The major portion of the RBP "pool" was concentrated by ultrafiltration and its absorption spectrum determined; for studies with radioactive compounds a portion of the concentrated RBP "pool" was also extracted and assayed for radioactivity. The extent of association of each labeled compound with apo-RBP was calculated from the amount of RBP in the RBP "pool," and from the specific radioactivity of the compound and the amount of radioactivity found as that compound in the RBP "pool" (both after gel filtration and after concentration by ultrafiltration). Where possible, the extent of association was also estimated from the change in the absorption spectrum of RBP, relative to that of the original apo-RBP. The spectral changes were determined by calculation of difference spectra, as described below.

Labeled and unlabeled compounds

[6,7-$^{14}C_2$]Retinyl acetate (SA 20.6 μCi/mg), [6,7-$^{14}C_2$]-retinoic acid (SA 23.0 μCi/mg), and β-[15,15′-$^3H_2$]-carotene (SA 143 μCi/mg) were the generous gifts of Hoffmann-La Roche, Basel, Switzerland. The [^{14}C]-retinoic acid was analyzed by solvent partition (11) just before use; 98% of the ^{14}C partitioned as a long-chain fatty acid, indicating a probable purity of at least 98%. The labeled β-carotene was diluted with unlabeled β-carotene and purified by chromatography on a small column of Al_2O_3 (10) just before use; after chromatography the concentration was determined from the absorbance at 470 nm, using 2280 as the value for $E^{1\%}_{1cm}$. [6,7-$^{14}C_2$]Retinal was synthesized from [6,7-$^{14}C_2$]retinyl acetate by saponification of the retinyl acetate to form retinol, followed by oxidation with MnO_2 (12) and purification of the product retinal by chromatography on a small column of Al_2O_3 (10).

Unlabeled β-carotene, retinyl acetate, retinal, retinol, and retinoic acid were purchased from Eastman Organic Chemicals, Rochester, N.Y. The values for $E^{1\%}_{1cm}$ used to determine concentrations in solution in benzene were: retinol and retinyl acetate (with regard

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to its retinol content), 1625 at 330 nm; retinal, 1528 at 370 nm; and retinoic acid, 1258 at 368 nm. 13-*cis*-Retinol and 11,13-di-*cis*-retinol were generous gifts of Hoffmann-La Roche.

[4-^{14}C]Cholesterol (SA 22.7 mCi/mmole, Volk Radiochemical Co., Burbank, Calif.) was purified by TLC on silica gel G, with benzene–ethyl acetate 5:1 as solvent (13), just before use. Unlabeled cholesterol (Nutritional Biochemicals Corp., Cleveland, Ohio) was purified through its dibromide as described by Fieser (14), followed by two crystallizations from 95% ethanol.

^{14}C-labeled phytol was the generous gift of Dr. Joel Avigan. The labeled phytol had been prepared from a hydrolysate of algae grown with $^{14}CO_2$, and had been purified by repeated TLC after the addition of carrier unlabeled phytol. The specific radioactivity was estimated by Dr. Avigan as being approximately 1–1.5 μCi/mg. The specific activity was determined in our laboratory by preparing a solution of the labeled phytol and assaying it for ^{14}C and for its content of phytol (mass). The concentration of phytol in the solution was estimated by gas–liquid chromatography of measured portions, after formation of the trimethylsilyl (TMS) ether derivative. Gas–liquid chromatography was carried out with a Barber-Colman model 15 apparatus, using an 8-ft column packed with Gas-Chrom Q coated with 1% SE-30 as liquid phase, at 174°C and 25 psi. The TMS ether of phytol emerged from the column as a peak with retention time of 34 min. The amount of phytol mass in the solution of labeled phytol was determined from the size of the phytol-TMS ether peak compared with peaks obtained with known amounts of pure unlabeled phytol (Nutritional Biochemicals) under identical conditions. The specific activity of the [^{14}C]phytol was thus determined as 1.95×10^6 dpm/mg. The labeled phytol was purified by TLC on silica gel G with benzene–ethyl acetate 4:1 just before use; 80% of the ^{14}C was recovered in the phytol band (R_F approx 0.6), from which the phytol was eluted with $CHCl_3$.

Other methods

Difference spectra of various RBP preparations (as compared with apo-RBP) were calculated in order to determine the differences between the spectrum of each RBP preparation and that of extracted (apo-) RBP. The measured absorption spectra were first normalized by setting the absorbance at 280 nm at 1.0, and the resulting normalized spectrum of apo-RBP was subtracted from that of the given RBP preparation under study.

Radioassay was carried out by dissolving samples in 15 ml of 0.5% diphenyloxazole in toluene, followed by assay with a Packard Tri-Carb liquid scintillation counter. Significant quenching was not observed.

RESULTS

Parameters affecting retinol extraction to form apo-RBP

The rate of extraction of retinol from RBP and into the heptane phase increased as the speed of shaking was increased. Only a limited range of shaking speeds (approximately 70–120 strokes/min) was found to be suitable for most studies. At slower speeds the rate of retinol extraction was too low to be useful for most (particularly for preparative) purposes; at higher speeds some of the protein was apparently damaged, as indicated by spectral changes and loss of 280 nm absorbance from the aqueous phase.

At a given speed of shaking, the rate of extraction of retinol from RBP was greater as the temperature was increased from 4°C to 37°C. In one experiment, two flasks, one at 4°C and one at 37°C, were shaken for 1 hr at 80 strokes/min and then for 2 hr at 93 strokes/min. The cumulative extraction of retinol from the flask at 4°C was 5% after 1 hr and 14% after 3 hr; the extraction of retinol from the flask at 37°C was 15% after 1 hr and 50% after 3 hr.

The effect of pH on the rate of extraction of retinol from RBP is shown in Fig. 3. Retinol was extracted more

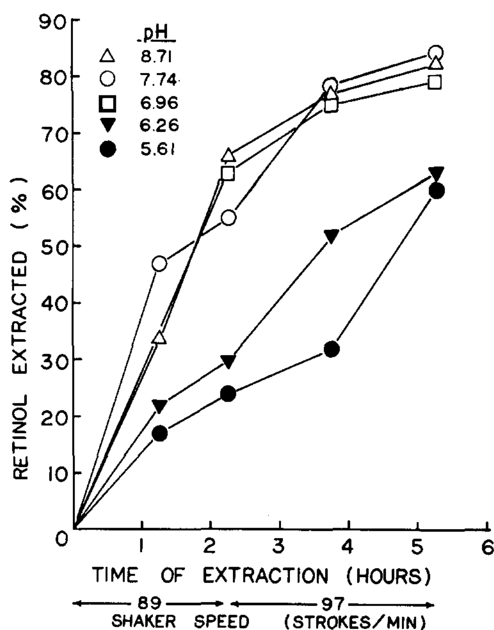

Fig. 3. Effect of pH on the rate of extraction of retinol from RBP. Five flasks were prepared, each containing 1 mg of RBP dissolved in 3 ml of 0.1 M buffer at pH's ranging from 5.61 to 8.71 (see the figure). Potassium phosphate buffer was used in all flasks except for the one at pH 8.71, in which Tris–HCl buffer was used. 3 ml of heptane was added to each flask, and the flasks were flushed with nitrogen, sealed, and shaken at 23°C for the time and at the speeds shown in the figure. The percentage extraction of retinol was determined at intervals by measuring both the absorbance of retinol in the heptane phase (peak 326 nm) and the decrease in absorbance of retinol bound to RBP (peak 330 nm) in the aqueous phase.

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slowly at pH 5.6–6.2 than in the pH range 6.9–8.7. In another experiment, two flasks containing RBP at pH 7.0 (potassium phosphate buffer, 0.1 M) and at pH 10.0 (sodium carbonate buffer, 0.1 M), respectively, were shaken together; retinol was extracted more rapidly from RBP at pH 10 than from RBP at pH 7. If we assume that the relative rate of retinol extraction is inversely related to the stability of the retinol–RBP complex, then these data indicate that the retinol–RBP complex is most stable in the lower portion of the pH range 5.6–10.

Effect of PA on the extraction of retinol from RBP

Fig. 4 shows the results of two experiments carried out to determine the effect of addition of PA (to form the RBP–PA complex) on the rate of extraction of retinol from RBP. In the first experiment (Fig. 4, right) the rate of retinol extraction from RBP alone was compared with the rate of its extraction from the RBP–PA complex formed by mixing together solutions of purified RBP and PA (RBP + PA) and with the rate of retinol extraction from the RBP–PA complex as isolated directly from plasma ("D" preparation; see Ref. 1). The rate of removal of retinol from the RBP–PA complex was markedly less than the rate of removal from RBP alone. In the second experiment (Fig. 4, left) 0.5 mg of RBP was added to either 1.5 mg of PA (to form the RBP–PA complex) or 1.5 mg of HSA (with which RBP does not form a complex), and the solutions were shaken together, each with an equal volume of heptane, as indicated. In this experiment also, the rate of extraction of retinol from the RBP–PA complex was markedly less than the rate of removal from RBP which was not present as a protein–protein complex. These findings strongly suggest that the interaction of retinol with RBP is stabilized by the formation of the RBP–PA complex.

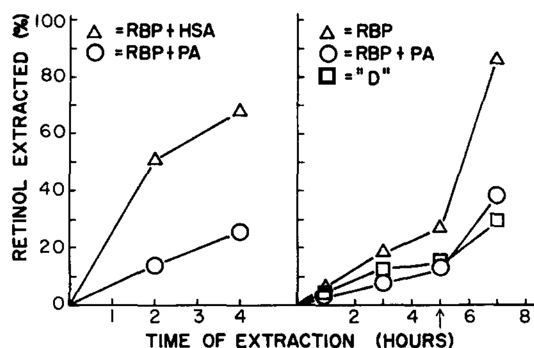

FIG 4. Effect of PA on the extraction of retinol from RBP. In the experiment shown in the right-hand panel, three flasks were prepared containing the following protein(s) dissolved in 3 ml: (*1*) RBP, 0.8 mg of RBP; (*2*) RBP + PA, 0.5 mg of RBP + 1.5 mg of PA; (*3*) "D," 2.2 mg of the "D preparation" (see Ref. 1), containing the undissociated RBP–PA complex isolated from plasma, with an approximately 1.5-times molar excess of PA over RBP. 5 ml of heptane was added to each flask, and the flasks were flushed with nitrogen, sealed, and shaken at 5°C at a rate of 75 strokes/min for 5 hr and then at 95 strokes/min for 2 hr. The arrow along the abscissa indicates the time when the speed of shaking was increased. In the experiment shown in the left-hand panel, two flasks were prepared containing: (*1*) RBP + PA, 0.5 mg of RBP + 1.5 mg of PA; and (*2*) RBP + HSA, 0.5 mg of RBP + 1.5 mg of HSA, each in 3 ml of 0.02 M sodium phosphate buffer, pH 7.4, 0.2 M NaCl. 3 ml of heptane was added to each flask, and the flasks were flushed with nitrogen, sealed, and shaken at 23°C at 93 strokes/min for 2 hr and then at 97 strokes/min for 2 hr. The percentage of retinol extracted was calculated from the measured decrease in absorbance at 330 nm, relative to that at 280 nm, in the aqueous phases.

Recombination of extracted (apo-) RBP with retinol

The results of a typical experiment are shown in Fig. 5. After recombination with retinol, the absorption spectrum of RBP was indistinguishable from that of RBP freshly isolated from plasma. This is illustrated even more clearly in Fig. 6, which shows the calculated difference spectra of RBP as isolated from plasma, and of RBP which had been extracted and then recombined (reassociated) with retinol, as compared with extracted (apo-) RBP. The difference spectra of the two species of holo-RBP were indistinguishable from each other. Fig. 6 also shows, for comparison, the absorption spectra of retinol in solution in benzene and in heptane. The difference spectrum for RBP presumably represents the absorption spectrum of retinol bound to RBP (and in aqueous solution). Fig. 6 shows that the absorption spectrum of retinol bound to RBP (the RBP difference spectrum) has the same peak maximum position (at 330 nm) as the absorption spectrum of retinol in benzene, but that the absorption spectrum of retinol bound to RBP is otherwise intermediate between the spectra of retinol in benzene and in heptane.

The recombination procedure usually results in the reassociation of apo-RBP with almost the identical amount of retinol which had been previously removed from the RBP extraction. During the course of a year, five recombination experiments were carried out with

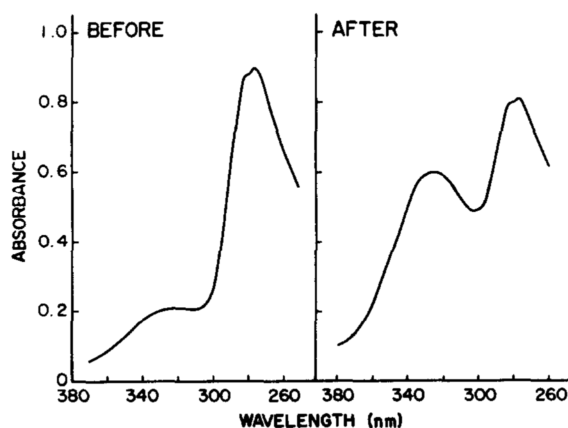

FIG. 5. The absorption spectrum of extracted (apo-) RBP before and after recombination with retinol.

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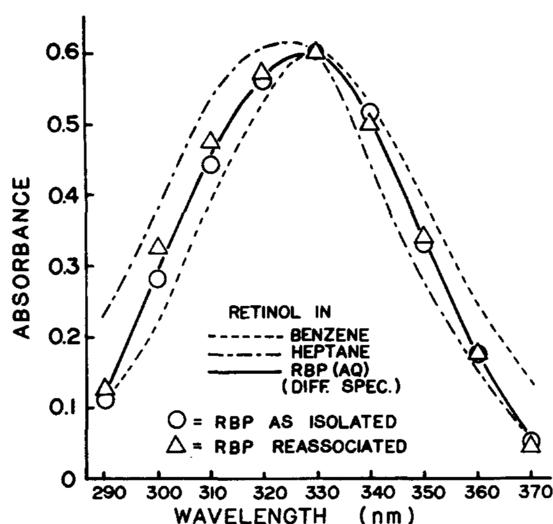

FIG. 6. The difference spectrum of holo-RBP (representing the difference between the spectra of holo-RBP and apo-RBP). The points represent the calculated values for the difference spectrum (compared with extracted RBP) of RBP isolated from plasma, and of RBP which had been extracted and then reassociated with retinol. A single solid curve is drawn for these two sets of points. The two broken curves show the absorption spectra of retinol in solution in heptane and in benzene, respectively.

all-*trans*-retinol under the conditions described in the Methods section for a "typical experiment." The apo-RBP preparations used in these experiments had had an average of 77% (range 75–79%) of the bound retinol removed by extraction with heptane. In the five recombination experiments, an average of 95% (range 87–106%) of the amount of retinol which had been extracted with heptane was reassociated with the RBP as a result of the recombination procedure.

Association of extracted (apo-) RBP with compounds other than all-*trans*-retinol

A series of experiments was carried out to determine whether compounds other than all-*trans*-retinol would interact with, and form a complex with, apo-RBP. Identical conditions and procedures were employed in all these experiments, as described in the Methods section. It was assumed that the amount of a given compound which would bind to apo-RBP under these conditions, as compared with the amount of retinol which would bind, would serve as a measure of the affinity of apo-RBP for the given compound, relative to its affinity for retinol.

A single, fairly large preparation of apo-RBP was used in this set of studies. Before extraction with heptane the RBP had an absorbance ratio (330 nm/280 nm) of 0.793; after extraction with heptane the ratio had decreased to 0.196, indicating that 76% of the retinol was removed by extraction. The amount of retinol removed was a mean of 0.54 mole/mole of RBP. Small-scale recombination studies with all-*trans*-retinol (see "typical experiment" in Methods section) were carried out at the beginning, in the middle, and at the end of the entire series of association studies. Almost identical results were obtained in these three studies, which were carried out at intervals of several weeks. In these three experiments, the absorbance ratios (330 nm/280 nm) of RBP after recombination with retinol were 0.76, 0.74, and 0.75, respectively, indicating that an average of 0.50, 0.48, and 0.49 mole of retinol had combined and formed a complex with each mole of RBP in the extracted RBP preparation. These data illustrate the quantitative reproducibility of the results which can be obtained with the recombination procedure, and indicate that the preparation of apo-RBP did not change with regard to its affinity for retinol during the course of these studies.

The results of the association experiments with compounds other than retinol are summarized in Table 1. Two alcohols (cholesterol and phytol) which are not carotenoid derivatives with vitamin A activity were studied. Detectable amounts of cholesterol were not bound by apo-RBP. In contrast, the branched-chain polyisoprenoid alcohol, phytol, did bind to a slight extent to apo-RBP, although the affinity of RBP for phytol was only approximately 8% of that for retinol.

Of the carotenoid vitamin A compounds studied, β-carotene was only minimally bound to RBP; the affinity of RBP for β-carotene was less than that for phytol. The affinity of RBP for retinyl acetate and for retinal was about one-third its affinity for retinol. Very close agreement was obtained for the amounts of retinyl acetate and of retinal bound as calculated from the radio-

TABLE 1. Association of apo-RBP with compounds other than retinol

Compound	Amount Bound to RBP: From Radioactivity Data	Amount Bound to RBP: From RBP Absorption Spectrum	Relative Affinity for RBP[a]
	moles/mole RBP		
All-*trans*-retinol		0.49[b]	1.00
β-Carotene	<0.01		<0.02
Retinyl acetate	0.16	0.16[c]	0.33
Retinal	0.18	0.16, 0.17[d]	0.35
Retinoic acid	0.43	0.42[e]	0.87
Cholesterol	<0.002		<0.01
Phytol	0.04		0.08

[a] Relative to all-*trans*-retinol which was given the value 1.00.
[b] Mean of three values (0.48, 0.49 and 0.50; see text).
[c] Calculated from the increase in absorbance at 330 nm, relative to that at 280 nm, as compared with apo-RBP, using the value 1625 for $E^{1\%}_{1cm}$ for the retinol content of retinyl acetate, at 330 nm.
[d] Calculated from the increase in absorbance at 370 nm, relative to that at 280 nm, as compared with apo-RBP, using the value 1528 for $E^{1\%}_{1cm}$ at 370 nm. The two values listed are the results of two experiments, one with labeled and one with unlabeled retinal.
[e] See text.

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activity data and from the analysis of the absorption spectra.

Under the conditions employed, retinoic acid bound to apo-RBP almost as effectively as did retinol. In this experiment, a portion of the concentrated RBP "pool," obtained after gel filtration and ultrafiltration, was extracted with $CHCl_3$–CH_3OH 2:1, and the total lipid extract was then separated into neutral and acidic lipids by solvent partition (11). 96% of the recovered ^{14}C was found in the acidic lipid fraction (as would be expected for retinoic acid). The amount of retinoic acid bound to RBP (see Table 1) was calculated from the amount of acidic lipid radioactivity so found.

The difference spectrum of the concentrated RBP solution containing bound retinoic acid had its peak maximum located at 330 nm (Fig. 7). The shape of the RBP–retinoic acid difference spectrum differed, however, from that of holo-RBP containing bound retinol (see Fig. 7). The peak maximum of the RBP–retinoic acid difference spectrum was located at a lower wavelength than that seen for the absorption spectrum of retinoic acid dissolved in any of a variety of organic solvents (Fig. 7 and Table 2). The difference spectrum also differed markedly from the absorption spectrum of potassium retinoate, in solution in 0.1 M phosphate buffer pH 7.5, which displayed a very broad absorption spectrum with a peak at approximately 360–365 nm. Thus, retinoic acid bound to RBP behaves, with regard to the position of its absorption peak maximum, more like retinol than like retinoic acid in solution in various solvents. As indicated above, however, the solvent partition data clearly established that the bound material was

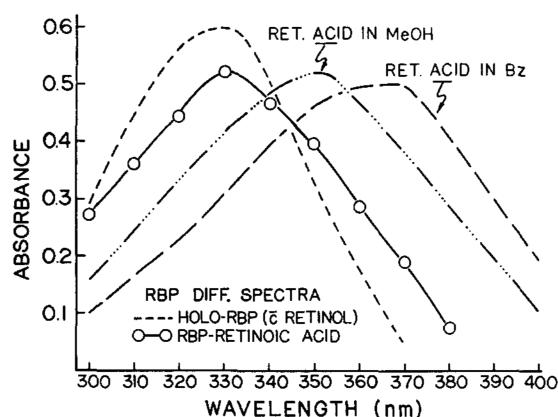

FIG. 7. The difference spectrum of the RBP–retinoic acid complex. Also shown are the absorption spectra of retinoic acid in solution in benzene and in methanol, and the difference spectrum of holo-RBP containing bound retinol. The latter spectrum (broken curve) is reproduced from the data shown in Fig. 6. The retinoic acid spectra, and the difference spectrum of the RBP–retinoic acid complex, have been adjusted to similar peak heights for visual presentation. The spectra should be compared with regard to the position and shape of the peaks, but not with regard to their height (absorbance).

TABLE 2. Absorption spectrum of retinoic acid in solution in various solvents

Solvent	Absorption Peak Maximum[a]
	nm
Benzene	368
$CHCl_3$	364
Hexane	360
Heptane	360
Isooctane	360
Acetone	352
Isopropanol	350
Ethanol	350
Methanol	350

[a] Approximate value (±2–3 nm).

retinoic acid and not retinol. Furthermore, the shape of the RBP–retinoic acid difference spectrum resembled the spectrum of retinoic acid more than that of retinol (see Fig. 7). The amount of retinoic acid bound to RBP was calculated from the difference spectrum by assuming that the bound retinoic acid had a value for $E^{1\%}_{1cm}$ of 1258 at its peak (at 330 nm) (i.e., the same $E^{1\%}_{1cm}$ as retinoic acid in solution in benzene at 368 nm). The amount of retinoic acid bound as calculated in this manner (0.42 mole/mole RBP) agreed closely with the amount bound as calculated from the radioactivity data (0.43).

Experiments were also conducted to assess whether two isomers of retinol, 13-*cis*-retinol and 11,13-di-*cis*-retinol, would bind to apo-RBP. These two experiments were carried out together. At the end of the recombination procedure the recoveries of RBP in the concentrated RBP "pools" were 74% and 79%, respectively. The absorption spectra of these two samples of RBP showed significant increases (compared with apo-RBP) in absorbance in the range 290–340 nm, relative to the absorbance at 280 nm. These findings indicated that each of the two retinol isomers had associated with apo-RBP to some extent. The difference spectra of each of the two RBP–retinol isomer complexes showed a peak located at approximately 310 nm. The absorption spectrum of 11,-13-di-*cis*-retinol in solution in benzene showed a peak with maximum located at 316 nm; with ethanol as solvent the peak maximum was at 311 nm. The absorption spectrum of 13-*cis*-retinol in benzene showed a peak at 334 nm; with ethanol or hexane as solvent the peak was located at 328 nm. These data conform to those reported in the literature (15). Thus, the difference spectrum of the complex of RBP with 11,13-di-*cis*-retinol was located at a spectral position very near to that of the absorption spectrum of 11,13-di-*cis*-retinol itself in solution in organic solvents. In contrast, the difference spectrum of the complex of RBP with 13-*cis*-retinol was located at a position approximately 20 nm lower than the absorption spectrum of 13-*cis*-retinol alone. These data suggest that the configuration of 13-*cis*-retinol may be altered

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when the molecule binds to RBP, and may resemble 11,13-di-*cis*-retinol to some extent when bound. The extent of binding of each retinol isomer was estimated from the difference spectra by assuming that the values for $E^{1\%}_{1cm}$ for each retinol isomer bound to RBP were the same as the values for each compound alone in solution in ethanol (1686 at 328 nm for 13-*cis*-retinol, and 908 at 311 nm for 11,13-di-*cis*-retinol [15]). With this assumption, it was estimated that the relative affinity of RBP for the 11,13-di-*cis* isomer was almost (80–90%) comparable to that for all-*trans*-retinol, whereas the affinity of RBP for the 13-*cis* isomer was somewhat less (approximately 50% of that for all-*trans*-retinol).

DISCUSSION

These studies were undertaken in order to explore some of the major features of the interaction of retinol with RBP. As reported here, methods were developed for the removal of retinol from RBP, so as to form the retinol-free apoprotein, and for the recombination of apo-RBP with retinol to again form the holoprotein. The two-phase extraction procedure for the production of apo-RBP results in the removal of retinol from RBP and its extraction into the heptane phase. The extraction procedure does not interfere with the ability of the protein to interact with retinol, since the apo-RBP is fully capable of again binding retinol to form holo-RBP.

The two-phase extraction procedure was used to explore some of the factors which affect the interaction of retinol with RBP, by assuming that the relative ease with which retinol was extracted from RBP was inversely related to the stability of the retinol–RBP complex. The retinol–RBP complex was most stable in the lower portion of the pH range 5.6–10. Two experiments were conducted to examine the effect of the formation of the RBP–PA complex on the interaction of retinol with RBP. The rate of removal of retinol from the RBP–PA complex was markedly less than the rate of its removal from RBP alone. These results strongly suggest that the interaction of retinol with RBP is stabilized by the formation of the RBP–PA complex. A similar conclusion has recently been presented by Peterson (16). This conclusion is also supported by the results of recent studies employing fluorescence spectroscopy.[1] The RBP–PA interaction may hence serve to stabilize and further protect the retinol bound to RBP. In addition, the protein–protein interaction protects the RBP molecule by preventing its glomerular filtration and hence the loss of RBP via urinary excretion or renal catabolism, or both.

The two-phase extraction procedure may perhaps be considered as a model for some of the events which occur during the delivery of retinol to peripheral cells and tissues. Thus, the outer surface of the cell membrane may serve as the interface, similar to the heptane–water interface, across which retinol is extracted during its removal from RBP and entry into the cell. If the conditions at the cell surface favored the dissociation of the RBP–PA complex, removal of retinol from RBP would be facilitated. Retinol delivery may hence involve the collision of a molecule of holo-RBP with the interface of the cell membrane, with concomitant removal of retinol from RBP and transfer into the cell. Specific binding sites for retinol (on the cell membrane or inside the cell, or both) may possibly be involved in this process. The apo-RBP so formed would then circulate and be preferentially filtered in the renal glomeruli, because of its lesser affinity (compared with holo-RBP) for PA, and the apoprotein would thus be subjected to selective catabolism (5). Alternatively, it is possible that retinol delivery to cells may involve the entry of the entire retinol–RBP complex into the cell, with RBP serving to deliver the retinol to its intracellular site(s) of action. Information is not currently available to permit us to choose between these alternatives.

The recombination procedure consists of the exposure of a solution of apo-RBP to Celite coated with a thin film of retinol, followed by the isolation of the RBP by gel filtration on Sephadex G-100. This procedure consistently results in the reassociation of apo-RBP with an amount of retinol almost identical with that previously removed from the RBP by extraction. Since the purified RBP used in the extraction studies comprised 70–80% holo-RBP and 20–30% apo-RBP, it is not clear why the recombination procedure did not result in the saturation of all of the apo-RBP with retinol, and hence in the reassociation of the extracted RBP with more retinol than had been removed by extraction. It is possible that the small proportion of "apo-RBP" present in RBP as isolated from plasma represents structurally altered protein which is no longer able to bind retinol. It is also possible that the "apo-RBP" in freshly isolated RBP has its retinol binding site occupied by a molecule of retinol which has been chemically modified so that it is no longer discernible by UV absorption or fluorescence spectroscopy. Further work will be necessary in order to examine these and other possibilities.

The recombination procedure was employed to examine the specificity of the binding of retinol to RBP. Studies were conducted to explore whether compounds other than all-*trans*-retinol would effectively bind to apo-RBP. It was assumed that the relative amount of a given compound which would bind to apo-RBP under the conditions employed would serve as a measure of the relative affinity of RBP for the given compound.

[1] Goodman, DeW. S., and R. B. Leslie. *Biochim. Biophys. Acta.* In press.

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The results of these studies demonstrate that the binding of retinol to RBP is highly but not absolutely specific. Although cholesterol did not bind to apo-RBP, the protein displayed a slight affinity (approximately 8% of that for retinol) for the branched-chain polyisoprenoid alcohol, phytol. The affinity of RBP for β-carotene was minimal, whereas both retinyl acetate and retinal were bound about one-third as effectively as was all-*trans*-retinol. In contrast, retinoic acid bound to apo-RBP almost as effectively as did retinol. The absorption spectrum of retinoic acid bound to RBP (represented by the "difference spectrum" of the RBP–retinoic acid complex) was located at lower wavelengths than the absorption spectrum of retinoic acid in solution in a variety of polar or nonpolar organic solvents. This finding suggests that the retinoic acid molecule assumes an unusual configuration when it is bound to RBP. It is now well established that retinoic acid can serve in place of retinol to satisfy the nutritional requirements for vitamin A (except for the need for retinol in vision and reproduction), despite the fact that retinoic acid is not converted to retinol in vivo (17–19). The finding that retinoic acid and retinol are almost comparable with regard to their binding by RBP may provide some insight into the role which retinoic acid can play in nutrition. It is, for example, possible that the molecular site of action of vitamin A may consist of a receptor site which can "recognize" either retinol or retinoic acid (similar to the binding site of RBP). The nature of the molecular site(s) of action of vitamin A is not known (except with regard to the role of vitamin A in vision [20]). Recent studies suggest that vitamin A may be involved in the regulation of the metabolism of certain specific carbohydrate-containing compounds and glycoproteins (21, 22).

The studies with two isomers of retinol, 13-*cis*- and 11,13-di-*cis*-retinol, demonstrated that each of the isomers can bind to apo-RBP to some extent. The results of these studies were, however, somewhat uncertain and should be taken only as qualitative assessments. In order to obtain more quantitative information, it would probably be necessary to use labeled isomers of retinol (not now available) as well as to validate the estimates of $E^{1\%}_{1cm}$ used for the compounds when bound to RBP. Despite the limitations of these data, the results suggest that 13-*cis*-retinol binds to RBP about one-half as effectively as does all-*trans*-retinol, and that 11,13-di-*cis*-retinol is more effectively bound than is the 13-*cis* isomer. It is of interest that the structure of 11,13-di-*cis*-retinol is more similar to that of all-*trans*-retinol than is the structure of 13-*cis*-retinol, as assessed from molecular models. These data thus provide further information about the relative specificity of the binding site on RBP for the structure of all-*trans*-retinol.

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