Studies on the Transport and Cellular Distribution of Vitamin A in Normal and Vitamin A-deficient Rats with Special Reference to the Vitamin A-binding Plasma Protein*

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SUMMARY

The retinol binding protein (RBP) and thyroxine-binding prealbumin were isolated from rat serum. Under physiological conditions RBP and prealbumin form a protein complex. Rat RBP exhibited characteristics very similar to those previously encountered for the human and monkey counterparts, including the association constant to prealbumin ($8 \times 10^4 \text{ M}^{-1}$). Thyroxine-binding prealbumin in rat is in contrast to its human counterpart the major thyroxine carrying plasma protein. Its molecular weight differs from that of the human protein (51,000 and 62,000, respectively) in spite of its similar binding constant for thyroxine.

The role of the vitamin A-transporting protein complex was investigated with respect to the tissue distribution of [3H]-vitamin A in normal and vitamin A-deficient rats. It was demonstrated that the hepatocytes which stored virtually all liver vitamin A could rapidly mobilize newly administered vitamin, and the retinol occurred exclusively bound to RBP. The mobilization of liver vitamin A was unaffected by prior actinomycin D treatment of the rats, which suggests that newly synthesized RBP requires retinol for its release from the hepatocytes.

An investigation was also undertaken of the role of the kidney as a storage organ for vitamin A. Kinetic measurements showed that the kidney received most of its vitamin A from RBP in contrast to the liver which gets its main supply from the chylomicrons. Fractionation of kidney cortex cells on a discontinuous silica gel gradient indicated that most vitamin A resided in tubular cells. The vitamin-containing cell fractions were shown to possess the property to take up RBP in vivo. These results suggest that some vitamin A may reach the kidney by a process of glomerular filtration followed by tubular reabsorption.

The basic features for the intestinal uptake and transport of vitamin A are now well understood. From the intestines, retinylesters are transported together with the chylomicrons to the liver, the main storage site for vitamin A (cf. Reference 1). Detailed studies on the enzymatic processing of vitamin A in the intestinal cells and in the liver have been published (2-6). Results on the distribution of vitamin A among hepatocytes and Kupfer cells within the liver are, however, conflicting (cf. Reference 7).

Goodman et al. (8) have in an elegant study shown that newly absorbed vitamin A is present in second greatest abundance in the kidneys. No detailed information is, however, available concerning the uptake or the cellular distribution of the vitamin in this organ.

Recent studies by Goodman and co-workers (9) and in this laboratory (10, 11) have shown that vitamin A in human blood is transported by a small plasma protein, the retinol-binding protein. Under physiological conditions RBP is firmly bound to the tetrameric thyroxine-binding prealbumin (9-14). RBP contains a single binding site for retinol (9-11) and each prealbumin subunit appears to have independent binding sites for thyroxine and RBP. Under physiological conditions virtually all RBP is saturated with retinol and complexed to prealbumin (15, 16).

Previous studies have shown that in plasma of humans suffering from vitamin A deficiency the levels of serum RBP, vitamin A, and prealbumin are substantially lowered and a high degree of correlation exists for the amounts of RBP and prealbumin over a 10-fold concentration range (17). Similar findings have also been reported in liver disease (16). These results warrant a detailed study of the in vivo regulation of the synthesis and degradation of the individual components constituting the vitamin A-transporting protein complex. Since the two-protein transporting system for vitamin A occurs in humans as well as in monkeys (18), it was reasonable to assume that a similar system is present also in other vertebrates.

To obtain a suitable animal model for future studies on the biosynthesis of RBP and prealbumin, now well under way in this laboratory, as well as on the metabolism of vitamin A in target cells, we describe in the present communication basic characteristics for the transporting system of vitamin A in normal

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1 The abbreviations used are: RBP, retinol-binding protein; $d$, density.
2 S. F. Nilsson and P. A. Peterson, manuscript in preparation.
and vitamin A-deficient rats. During the course of this work
the prealbumin-RBP complex of the rat was isolated and char-
acterized. The time course of the distribution of radioactively
labeled vitamin A in liver, kidney, and blood following intrave-
nous administration has been studied. In addition, data are
given for the relative distribution of vitamin A among fric-
tionated liver and kidney cells.

**EXPERIMENTAL PROCEDURE**

**Materials**

Serum for the isolation of RBP and prealbumin was obtained
from blood collected from the abdominal aortas of male Sprague-
Dawley rats. The serum was processed immediately or stored
frozen until further processing. Retinol and retinylacetate were
purchased from Sigma Chemical Co. Radioactive [11,12-3H] reti-
nylacetate (specific activity 0.25 mCi per mg) was a kind
gift by Dr. O. Wiss, Hoffmann-La Roche Inc., Basle. Carrier-
free [125I] and [125I]thyroxine were obtained from Amersham
(UK). Highly purified guanidine HCl was obtained from Heico
(Delaware Water Gap, Pennsylvania). All other reagents used were
of the highest quality available.

**Methods**

**Preparation of Animals**—Weanling (46 to 54 g) male Sprague-
Dawley rats were made vitamin A-deficient by use of the USP
11 rat test diet (Nutritional Biochemicals, Cleveland, Ohio).
The animals were housed in individual hanging wire cages in a
room with automatic lighting from 8 a.m. to 8 p.m. Normal
control animals were fed a commercial laboratory chow (An-
ticex, Stockholm, Sweden). Both groups of animals had free
access to food and drinking water.

The normal and vitamin A-depleted animals did not differ in
growth rate until after 6 weeks. The vitamin A-depleted rats
continued to grow during 10 weeks on the diet, then the body
weights were constant for approximately 1 week to subsequently
decline. All animals in this study were used for experiments
after 8 to 12 weeks on the deficient diet except when specifically
stated otherwise. The depleted rats were in apparently good
health up to the 10th week, although overt signs of vitamin A
deficiency were apparent at the end of this time period.

Intravenous injections into the rats were accomplished either
through the jugular vein or through the tail vein. Blood was
usually obtained from the abdominal aorta from animals to be
exsanguinated. Serial blood samples were collected from the
tail vein.

**Electrophoresis**—Preparative zone electrophoresis was per-
formed in blocks (19) of Pevikon C-870 (Kema Nord AB, Stock-
holm, Sweden) as previously described (20). Sodium barbital,
Tris-glycine buffer, pH 8.3, in the running gel, 0.1
M NaCl. The samples were diluted to the appropriate
concentrations and dialyzed in the cold against two changes of
the solvent. Standard 12-mm double sector cells with sapphi-

**Ultracentrifugation**—Sedimentation coefficients and molecular
weights were estimated at 20° in a Spinco model E analytical
ultracentrifuge, equipped with an RTIC temperature control
unit and an electronic speed control device. All experiments
were conducted in 0.02 M Tris-HCl buffer, pH 7.4, containing
0.15 M NaCl. The samples were diluted to the appropriate
concentrations and dialyzed in the cold against two changes of
of the solvent. Standard 12-mm double sector cells with sapphi-

**Amino Acid Analyses**—Quantitative amino acid analyses
were carried out as described by Spackman *et al.* (33). The pro-
tin samples (0.5 to 1.0 mg) were hydrolyzed in 6 N HCl at 110°
for 24 hours. Chromatography was carried out on a Jeol-6AM
fully automatic amino acid analyzer (Jeol Co., Tokyo, Japan).
Tryptophan was measured spectrophotometrically (34).

**Preparation of 125I-labeled Protein**—Iodination of highly pu-
rified RBP and prealbumin was accomplished with 125I using the
iodine monochloride technique of McFarlane (35). The labeled
proteins were freed from excess iodine by passage over a Seph-
adex G-25 column (14 × 0.6 cm) equilibrated with 0.02 M
Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl. There was
always less than 0.7 atom of iodine per protein molecule in the
final products. The labeling efficiency was routinely on the
order of 70% and the obtained specific activity of the labeled protein was 10 to 100 μCi per mg. Over 98% of the radioactivity of all preparations was precipitable with 10% trichloroacetic acid.

**Gel Chromatography in 6 m Guanidine HCl**—Reduced and alkylated proteins usually behave as linear random coils in 6 m guanidine HCl (36). Accordingly, their elution from a gel chromatography column equilibrated in this medium is according to size, i.e. the length of the polypeptide chain. The subunit structure of reduced and alkylated prealbumin and RBP labeled to size, i.e. the length of the polypeptide chain. The subunit structure of reduced and alkylated prealbumin and RBP labeled to size, i.e. the length of the polypeptide chain. For instance, the elution of the prealbumin subunits and RBP monomeric forms from Sepharose 6B was identical when equilibrated with 6 m guanidine HCl. The same marker proteins as previously employed were used to calibrate the column (cf. Reference 37). All proteins were reduced with 0.1 M dithiothreitol (Mann) for 1 hour and alkylated with 0.3 M iodoacetic acid (Schuchardt, Munich, Germany) for 1/2 hour prior to application. The elution volumes of the proteins were determined by weighing the effluent. Fractions of about 0.9 g were collected at a flow rate of 5 g per hour.

**Fluorescence Measurements**—All fluorescence measurements were carried out with an Amino-Bowman spectrophotometer. Unless otherwise stated, the fluorescence measurements were performed at ambient temperature (22 ± 2°C) in a buffer composed of Tris-HCl (0.02 M) and NaCl (0.15 M) adjusted to pH 7.4.

Association constants for the binding of RBP and thyroxine, respectively, to prealbumin were measured by estimation of the prealbumin protein fluorescence on complex formation. The optical densities were never allowed to exceed 0.1 at the excitation wavelength in the two sets of experiments, rendering inner filter effects negligible. In both experiments, a constant concentration of a prealbumin solution was used to which various amounts of either RBP or thyroxine were added to obtain the desired molar ratios. The contribution of RBP to the emission at 335 nm was subtracted from the measured values by using blanks of RBP with appropriate concentrations. Details of the experimental procedure have been published (13, 14). Complete emission spectra were recorded at each observation.

**Vitamin A Assay**—Vitamin A was extracted from liver samples according to the method of Bligh and Dyer (39). The content of vitamin A in the chloroform extract was estimated by the trifluoroacetic acid procedure of Dugan et al. (40). The recovery of vitamin A from the extracted liver samples was better than 95% as indicated by the amount of radioactivity obtained in the chloroform layer after extraction of liver [H]vitamin A.

Quantitative determinations of vitamin A in serum were accomplished by a fluorometric procedure (41).

**Determinations of Serum RBP**—Serum concentrations of RBP were determined employing a single radial immunodiffusion technique (42). Due to the low amounts of RBP present in serum of vitamin A-deficient rats, the immunodiffusion plates had to be dried and stained with Coomassie blue (Mann) prior to recording.

The antiserum used was raised in a rabbit by footpad injection of highly purified rat RBP. The immunization schedule was the same as earlier (43). Highly purified rat RBP was used as the standard in the immunological quantitations.

**Liver Cell Fractionation**—Liver cells were prepared and separated by centrifugation in colloidal silica gel essentially as described earlier (44). The livers were perfused in situ through the vena cava with 100 ml of 0.1 m NaCl followed by 100 ml of 0.15 m NaCl containing 27 mg sodium citrate. The liver was subsequently removed, blotted on filter paper, and a portion (usually 1 to 3 g) was minced. Dispersion of the liver cells was performed by subjecting the minced liver to a solution of 40 ml of 16% polyethylene glycol (PEG 4000, Union Carbide Corp., Chicago, Ill.), adjusted to pH 7.4, in a modified Potter-Elvehjem homogenizer furnished with a loose fitting, cone-shaped rubber pestle. The dispersed liver cell solution, obtained by seven strokes with the pestle, was subsequently filtered through a nylon sieve to remove cell aggregates and connective tissue.

The liver cells were fractionated in a discontinuous density gradient of colloidal silica (Ludox HS, 52.4% v/v, Du Pont de Nemours, Wilmington, Delaware) adjusted to pH 7.5 with HCl. Centrifuge tubes were layered with the following solutions: Solution 1: 16 ml of 15.5% silica sol (d = 1.09); Solution 2: 15 ml of 17.5% silica sol (d = 1.10); Solution 3: 20 ml of 20% silica sol (d = 1.12); and Solution 4: 15 ml of 34% silica sol (d = 1.20). All solutions contained in addition 12% polyethylene glycol. On top of the gradient, 31 ml of the cell suspension containing 12% silica sol and 12% polyethylene glycol (final density 1.075 g per ml), were carefully layered. Centrifugation was performed for 40 min at 800 × g. The content of the centrifuge tube was emptied by pumping 52.4% silica sol into the bottom of the tube and 3-ml fractions were collected from the top (45). Each one of the fractions was washed twice with 16% polyethylene glycol to remove the silica and the cells were recovered by centrifugation for 10 min at 800 × g. Finally, the cells in each fraction were suspended in 0.15 m NaCl. The entire procedure was carried out at room temperature. Prior to washing, densities of the fractions were measured in a density gradient column prepared from kerosene and carbon tetrachloride (46).

**Fractionation of Kidney Cells**—Kidney cell suspensions were prepared with collagenase and hyaluronidase (47) and the cells were fractionated by centrifugation on gradients of colloidal silica. The kidneys were perfused in situ by injections into the abdominal aorta (approximately 1 cm below the orifices of the renal arteries) of 100 ml of ice-cold calcium-free Hanks' balanced salt solution containing 0.5 mg per ml of collagenase (Type 1 from Clostridium histolyticum, Sigma) and 1.0 mg per ml of hyaluronidase (type I from bovine testes, Sigma). The kidneys were removed and freed from their capsule, and the kidney cortex was dissected out. The cortex was minced and incubated in 10 ml of the perfusion solution for 60 min at 37°C in a rocking water bath (100 oscillations per min). The incubation solution was then passed through a nylon sieve and the liberated cells were recovered by centrifugation at 150 × g for 2 min after washing in calcium- and glucose-free Hanks' balanced salt solution.

The cells from two kidneys were suspended in 10 ml of a solution of 12% silica sol containing 15% polyethylene glycol and the resulting suspension was layered on top of a discontinuous density gradient consisting of: (a) 25 ml of 3% silica sol and 15% polyethylene glycol (d = 1.04); (b) 25 ml of 9% silica sol and 15% polyethylene glycol (d = 1.07); (c) 25 ml of 11% silica sol and 15% polyethylene glycol (d = 1.07). Fractionation was performed for 40 min at 800 × g. The fractions were cultured at room temperature. Prior to centrifugation, densities of the fractions were measured in a density gradient column prepared from kerosene and carbon tetrachloride (46).

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3 I. Berggård and H. Pertoft, manuscript in preparation.
The material containing radioactivity was in both cases quantitatively estimated with use of the diphenylamine method. In preparations of greater purity, protein was usually measured by reading the absorbance at 280 nm. DNA was measured by the method of Lowry et al. (49) with human serum albumin as the external standardization system. When necessary, appropriate quench corrections were performed with the channel ratio method.

Samples containing iodine isotopes were counted in a Gamma...
portions of [\textsuperscript{125}I]thyroxine-containing rat serum were serially chromatographed on a column (110 × 7 cm) of Sephadex G-200 equilibrated with 0.02 M Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl. The column was eluted with a flow rate of 120 ml per hour and fractions of 30 ml were collected. The eluted protein showed a similar distribution as that shown in Fig. 1. Virtually all vitamin A fluorescence appeared in a single peak eluted somewhat later than albumin (cf. Fig. 1.). The distribution of [\textsuperscript{125}I]thyroxine was superimposed on that containing the retinol fluorescence. Accordingly, fractions containing radioactivity and exhibiting retinol fluorescence were pooled and concentrated.

First DEAE-Sephadex Chromatography Step—The concentrated protein constituting the RBP fraction obtained from the Sephadex G-200 chromatography step was subjected to chromatography, after exhaustive dialysis against the starting buffer, on a DEAE-Sephadex column (40 × 7 cm) equilibrated with 0.05 M Tris-HCl buffer, pH 7.4, containing 0.05 M NaCl. The applied material, containing 25,000 mg of total protein, was eluted with a 5,000-ml linear gradient from 0.05 M to 0.20 M NaCl in 0.05 M Tris-HCl buffer, pH 7.4. The resulting chromatogram exhibited one dominating protein peak, eluted at 0.15 M NaCl. Retinol fluorescence and [\textsuperscript{125}I]thyroxine radioactivity appeared together on the frontal part of the main protein peak. Fractions containing radioactivity were combined and concentrated.

Second DEAE-Sephadex Chromatography Step—Preliminary electrophoretic experiments in agarose gels showed that a further resolution of RBP and protein-bound [\textsuperscript{125}I]thyroxine from the major contaminants could be achieved at pH 9.0. Accordingly, the concentrated protein fraction containing RBP and [\textsuperscript{125}I]thyroxine (2250 mg of total protein) was subjected to chromatography on a column (60 × 3 cm) of DEAE-Sephadex equilibrated with 0.05 M Tris-HCl buffer, pH 9.0, containing 0.1 M NaCl. Prior to application the material was dialyzed against three changes of the starting buffer. Elution was performed with a 2000-ml linear NaCl gradient from 0.1 to 0.2 M in the pH 9.0 buffer. The retinol fluorescence, which appeared in two equally large, distinct but partly overlapping peaks, was well separated from the bulk of the eluted protein. Most of the unrelated protein appeared later than RBP. The [\textsuperscript{125}I]thyroxine radioactivity emerged from the column in a single peak coinciding with the earliest eluted retinol-containing material. It was thus obvious that RBP had resolved into two components, and fractions corresponding to each of the two peaks were separately pooled and concentrated. The two fractions will henceforth be termed RBP-I and RBP-II according to the order of their elution positions.

Second Gel Chromatography on Sephadex G-200—Each of the two fractions (RBP-I and RBP-II) obtained from the second DEAE-Sephadex chromatography step was separately subjected to gel chromatography on a column of Sephadex G-200 (130 × 2 cm) equilibrated with 0.02 M Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl. The RBP-I fraction gave rise to a single protein peak but the retinol fluorescence and the [\textsuperscript{125}I]thyroxine, again coinciding, were eluted in the latter part of this protein peak, clearly indicating that the RBP in this fraction was still contaminated with unrelated protein. Accordingly, fractions containing retinol fluorescence and [\textsuperscript{125}I]thyroxine were pooled and concentrated.

The RBP-II fraction was resolved into two protein peaks on Sephadex G-200. The earliest eluted material appeared in the same position as the protein peak obtained for the RBP-I fraction, whereas the second peak was eluted considerably later. The retinol fluorescence also appeared in two positions. About 30% of the fluorescence was eluted with its maximum in the tail part of the first protein peak, corresponding to the elution position for RBP-I, whereas the remaining fluorescence coincided with the second protein peak. Fractions comprising the last eluted material containing retinol were pooled and concentrated. This material constituted highly purified free RBP, as will be shown below.

Zone Electrophoresis in Barbital Buffer—The concentrated RBP-I fraction obtained after the second gel chromatography on Sephadex G-200 was subjected to zone electrophoresis in barbital buffer, pH 8.6, ionic strength 0.05. This low ionic strength was chosen due to the fact that interaction of the human prealbumin and RBP is very sensitive to low ionic strength.

After completed electrophoretic separation, three well resolved protein peaks were apparent. The peak with the highest anodal mobility was the only one containing appreciable amounts of [\textsuperscript{125}I]thyroxine as revealed by radioactivity measurements. The peak with the lowest anodal mobility comprised all of the retinol fluorescence. Accordingly, fractions constituting the first and the third peaks were separately pooled and concentrated. It will be demonstrated below that the two fractions contained highly purified thyroxine-binding prealbumin and free RBP, respectively.

Purity and Characteristics of Prealbumin and RBP—The purity of prealbumin and RBP was assessed by polyacrylamide gel electrophoresis in Tris-glycine buffer, pH 8.9. It can be seen in Fig. 2 that prealbumin exhibited a single protein zone with high anodal mobility, whereas RBP-II displayed a total of five bands. The same electrophoretic distribution was also encountered for RBP-I. Only two of the five RBP bands exhib-
FIG. 3. Chromatography on Sephadex G-75 of a mixture of highly purified rat prealbumin and RBP II. The column (63 x 1.5 cm) was equilibrated with 0.02 M Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl. The sample contained 1.6 mg of total protein. The distribution in the effluent of RBP was determined by measuring the specific retinol fluorescence. The arrow indicates the elution position of free RBP.

The specific retinol fluorescence (demonstrated prior to protein staining). Although this result may indicate that the RBP fractions were contaminated with unrelated protein, a more likely explanation is that rat RBP exhibits micro-heterogeneity on polyacrylamide gel electrophoresis analogous to that found for its human counterpart (11).

Additional support for the purity of RBP was obtained when a mixture of prealbumin and RBP was chromatographed on a column of Sephadex G-75 equilibrated with 0.02 M Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl. It can be seen in Fig. 3 that RBP was quantitatively eluted together with prealbumin, clearly demonstrating that prealbumin and RBP under physiological conditions appear as a protein complex. Furthermore, the figure demonstrates that RBP is highly purified, since no protein could be detected in the elution position of free RBP. The generated prealbumin-RBP complex displayed on polyacrylamide gel electrophoresis the sum of the patterns obtained for the individual proteins establishing that all five RBP protein zones were the result of micro-heterogeneity.

Some physical properties for prealbumin and RBP were determined and are summarized in Table II.

Sedimentation velocity analyses were carried out at protein concentrations of 0.02% to 0.08%. Both prealbumin and RBP behaved as single components within this concentration range. The sedimentation constant (s_{20,w}) for prealbumin was 3.0 S and for RBP 2.1 S.

Stokes molecular radius was determined by gel chromatography on a calibrated column of Sephadex G-100. The values obtained for the prealbumin-RBP complex, prealbumin, and RBP were 32 A, 28 A, and 19 A, respectively. This demonstrates that the rat prealbumin-RBP complex is smaller than the human prealbumin-RBP complex (38 A).

The frictional ratios were calculated from the sedimentation coefficients and the molecular weights obtained from equilibrium ultracentrifugations. The frictional ratios of prealbumin and RBP were 1.18 and 1.22, respectively, values in accordance with a globular structure, provided the proteins have "normal" hydration.

The molecular weights of prealbumin and RBP were estimated by sedimentation equilibrium ultracentrifugation. Fig. 4 shows the resulting graphs for prealbumin and RBP when in e was plotted versus r^2. The measured molecular weights were 51,200 for prealbumin and 20,500 for RBP, and the linear relationships obtained in the plots indicated homogeneity for the two examined preparations. The molecular weights measured by

| TABLE II |
| --- | --- | --- |
| Some properties of rat RBP and prealbumin |
| |
| **RBP** | **Prealbumin** |
| Sedimentation coefficient, s_{20,w} | 2.1 S | 3.0 S |
| Stokes molecular radius, A | 32 A | 28 A |
| Diffusion constant, D_{20,w}, cm^2/s | 9.2 | 6.8 |
| Frictional ratio | 1.22 | 1.18 |
| Molecular weights |
| By sedimentation equilibrium | 20,500 | 51,200 |
| By sedimentation diffusion | 21,000 | 51,000 |
| By gel chromatography | 19,000 | 12,700 |

* Estimated by analytical gel chromatography. The values are given as 10^{-7} cm^2 s^{-1} for the apparent diffusion constants. The molecular weights were calculated using Svedberg's equation combining gel chromatography data with the sedimentation constants.

* Estimated in 6 M guanidine hydrochloride on reduced and alkylated proteins.
sedimentation equilibrium ultracentrifugation and those computed from the sedimentation constants and the Stokes' radii were in good agreement (cf. Table II).

In order to investigate the possibility of a subunit structure for prealbumin and RBP, the two proteins were separately subjected to gel chromatography on a column of Sepharose 6B equilibrated with 6 M guanidine hydrochloride. The column was calibrated with reference proteins as described under "Experimental Procedure." The reduced and alkylated RBP appeared slightly after human RBP, indicating a molecular weight of approximately 19,000. Prealbumin appeared somewhat earlier than the human β2-microglobulin, which has a molecular weight of 11,800. From the elution position of prealbumin a molecular weight of 12,700 could be calculated. Without reduction and alkylation, prealbumin appeared exactly at the same elution position. It thus appears that prealbumin is composed of 4 subunits. It is also evident that the subunits are not held together by covalent interactions since the subunit structure is apparent without reduction and alkylation. In analogy with findings for the human and the monkey prealbumins, it may be inferred that the rat prealbumin is composed of four apparently identical polypeptide chains.

Rat prealbumin and RBP were separately subjected to measurements of their circular dichroism. Fig. 5 depicts the circular dichroism spectra for prealbumin and RBP in the ultraviolet region. It is evident from the figure that prealbumin exhibited a strong ellipticity at about 213 nm. This trough is quite similar to that found for human and monkey prealbumins although the magnitude is somewhat smaller. Calculations indicate that the prealbumin structure contained at the most 10 to 20% α structure. No α helix content could be compatible with the observed ellipticity distribution, and it is probable that 80 to 90% of the structure is composed of some unordered yet rigid structure.

RBP exhibited a more complex circular dichroism. It is evident from Fig. 5 that there is a positive ellipticity around 320 nm, followed by a negative trough with minimum at 270 nm. A new band exhibiting positive ellipticity is apparent at 240 nm which may be due to disulfide bridges, and this band is followed by the main negative ellipticity. The complex over-all spectrum for RBP is strikingly similar to that recorded for the human counterpart, although the magnitudes of the individual bands differ.

The fluorescent properties of prealbumin and RBP were investigated. RBP had in addition to its absorption band at 280 nm a band with maximum at 330 nm due to the presence of retinol. On excitation of the protein at these two wave lengths, two types of fluorescence were obtained: one with maximum at 335 nm caused mainly by the protein tryptophyl residues, and the other at 470 nm caused by the vitamin. By excitation at 280 nm, prealbumin exhibited a single fluorescence with maximum at 335 nm.

The prealbumin fluorescence at 335 nm undergoes dramatic changes with addition of RBP containing retinol. Fig. 6 shows the progressive quenching of the prealbumin fluorescence on increments of the molar ratio of RBP to prealbumin. It is evident that interpolation of the linear regions of the quenching curve intersects at a 1:1 molar ratio of the two proteins. In this region the tryptophan fluorescence of prealbumin is considerably quenched.

The finding that the prealbumin fluorescence is partially quenched on complex formation with retinol-containing RBP made investigations of the affinity between the two proteins possible. Furthermore, the binding of thyroxine to prealbumin is also accompanied by a quenching of the tryptophan fluorescence suitable for use in evaluation of the binding constant. Fig. 7 shows two Scatchard plots resulting from the binding of RBP and thyroxine, respectively, to prealbumin. The apparent 

![Fig. 5. Circular dichroism spectra for rat RBP (---) and prealbumin (-----) in the ultraviolet region.](http://www.jbc.org/)

![Fig. 6. Effects on the rat prealbumin fluorescence emission at 335 nm (excitation at 280 nm) at various molar ratios of RBP to prealbumin.](http://www.jbc.org/)

![Fig. 7. Binding of rat RBP (○) and thyroxine (□) to rat prealbumin determined by fluorescence quenching in 0.02 M Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl. The data were plotted according to Scatchard (38). ϕ is the molar ratio of bound RBP or thyroxine to prealbumin, and c the concentration of free RBP or thyroxine, respectively.](http://www.jbc.org/)
emission spectrum (excitation at 280 nm) exhibited a quenched presence of 1,8-anilinonaphthalene sulfonic acid, the prealbumin thalene sulfonic acid alone in aqueous solution at the same concentration. The emission maximum of 1,8-anilinonaphthalene sulfonic acid was increased approximately 100-fold compared to that of 1,8-anilinonaphthalene sulfonic acid to a solution of rat prealbumin the fluorescence of 1,8-anilinonaphthalene sulfonic acid was increased approximately 100-fold compared to that of 1,8-anilinonaphthalene sulfonic acid alone in aqueous solution at the same concentration. The emission maximum of 1,8-anilinonaphthalene sulfonic acid, 510 nm, underwent a marked hypsochromic shift to 460 nm in the presence of prealbumin. Furthermore, in the concentration. The emission maximum of 1,8-anilinonaphthalene sulfonic acid did not cause further enhancement of fluorescence, apparently indicating saturation of the 1,8-anilinonaphthalene sulfonic acid-binding sites of prealbumin. Extrapolation of the initial slope of the curve to the plateau yielded an intersection of 0.87 mole of 1,8-anilinonaphthalene sulfonic acid per mole of prealbumin. The titration data (Fig. 8A) were fitted to yield an association constant ($K_{asso}$) of $3 \times 10^4$ M$^{-1}$. The solid line in the figure is the theoretical binding curve for this $K_{asso}$ value.

When thyroxine was added in small increments to a solution of prealbumin containing saturating amounts of 1,8-anilinonaphthalene sulfonic acid, the 460 nm fluorescence decreased progressively. The quenching was almost complete when 1 mole of thyroxine was added per mole of protein, confirming the existence of one high affinity thyroxine-binding site per molecule (cf. Fig. 8B). Assuming that 1,8-anilinonaphthalene sulfonic acid competes with thyroxine for the binding to prealbumin, the association constant for the thyroxine-prealbumin interaction could be calculated. The value obtained ($2.7 \times 10^5$ M$^{-1}$) was in good agreement with the data obtained by measuring the thyroxine quenching of the prealbumin protein fluorescence (see above).

Amino Acid Composition of Prealbumin and RBP—Due to scarcity of material only single analyses of prealbumin and RBP were accomplished. The results, shown in Table III, suggest

![Graph](http://www.jbc.org/)

**Fig. 8. A**, graph of relative fluorescence at 460 nm versus the molar ratio of 1,8-anilinonaphthalene sulfonic acid to prealbumin. Circles are data and the line was calculated assuming a single binding site with $K_{asso}$ of $3 \times 10^4$ M$^{-1}$. Excitation was at 390 nm. **B**, graph of relative fluorescence at 460 nm versus the molar ratio of thyroxine to prealbumin. Excitation was by light at 390 nm. Portions of a thyroxine solution were added to prealbumin and 1,8-anilinonaphthalene sulfonic acid.
the presence of 113 amino acid residues in prealbumin, assuming
that it is composed of 4 identical subunits, and 174 residues in
RBP. These values for RBP are, of course, somewhat inexact
since they were calculated on the basis of a single 24-hour hy-
drolysis value only. Thus, leucine, isoleucine, and valine are
most probably too low, especially if they occur as nearest
neighbors in the primary structure. The values for cysteine and
methionine may also have yielded too low values, since they
were not determined after prior performic acid oxidation. In
spite of these uncertainties, it appears that there exists a rea-
sible agreement in amino acid composition between rat preal-
bumin and human prealbumin as well as between RBP from the
two species.

It may thus be concluded that most of the physicochemical
and chemical parameters examined for the rat proteins appear
to be similar to those of the human counterparts.

**Kinetics and Tissue Distribution of Vitamin A in Normal and Vitamin A-deficient Rats**

Following the isolation of the specific plasma carrier protein
for vitamin A in the rat (see above) a study was undertaken to
investigate the tissue distribution of vitamin A in normal and
vitamin A-deficient rats. Since it is understood that RBP plays
a key role in the distribution of the vitamin, quantitative im-
nunological estimations of RBP were performed in vitamin A
depletion and deficiency.

**Tissue Distribution of [3H]Vitamin A—Lipoprotein-bound**

[3H]retinylacetate was injected intravenously into normal and
vitamin A-deficient rats. The animals were killed at different
time intervals following the administration of vitamin A. The
amounts of radioactivity present in liver, kidney, and serum
were measured and expressed as percentages of the injected
radioactive dose. The data obtained are summarized in Fig. 9.
It is evident from the figure that the liver contained its maximal
amount of radioactive vitamin A already at the earliest time
studied (15 min after administration). In normal rats the liver
was only slowly depleted of the radioactive vitamin; at 16 hours
it still retained two-thirds of its maximal content. In vitamin
deficiency, the initial liver content of [3H] was similar to that found
for the normal rats, but the radioactivity declined rapidly and
only about one-third of its initial content was retained at 16
hours.

In blood the [3H]vitamin A radioactivity declined in an ex-
ponential fashion for normal rats, whereas in vitamin A-deficient
animals a rebound of the radioactive vitamin was apparent.
Following a rapid decay during the first hour after administra-
tion, the levels increased to a peak value around 3 hours to
subsequently decline again. This rebound effect was due to
elimination from serum of [3H]retinylacetate, bound to the lip-
oproteins, during the first phase, and reappearance of [3H]retinol,
bound to RBP, in the second phase. This was demonstrated by
subjecting serum, obtained at the indicated times, to gel
chromatography on Sephadex G-200. During the early time
intervals a considerable amount of [3H] radioactivity appeared
close to the void volume, whereas at 2 hours after the adminis-
tration and subsequently virtually all radioactivity appeared in
the elution position of the prealbumin-RBP complex (cf. Fig. 1).

The deposition of [3H]vitamin A in the kidney increased steadly
with time both in normal and deficient rats. The rela-
tive magnitude of the content of [3H] radioactivity in kidneys of
the two groups of animals was, however, different. More than
twice as much [3H]vitamin A was recovered in the kidneys of
the deficient animals compared to the normal ones. It may

**FIG. 9. Distribution of [3H] vitamin A in liver, blood, and kid-
ney of normal (○) and vitamin A-deficient (●) rats at different
times after the intravenous administration of [3H]retinylacetate.**

thus be concluded that newly administered vitamin A is more
easily mobilized from the vitamin A-deficient liver than from
the normal liver. It also seems likely that the increased content
of RBP-bound [3H]retinol in plasma of vitamin A-deficient rats
compared to normals reflects the differences encountered in
content of [3H] radioactivity in the kidneys of the two groups
of animals.

**Cellular Distribution of [3H]Vitamin A in Liver—Dispersed**

liver cells were separated by centrifugation in colloidal silica
gel. Cells were obtained from normal and vitamin A deficient
rats at different time intervals after the intravenous administra-
tion of [3H]retinylacetate (cf. Fig. 9). The relative distribution
of the liver cells following centrifugation was very similar for
normal and vitamin A-deficient cells. It was noted, however,
that in all fractions the normal cells contained approximately
20% more protein per mg of DNA than those of vitamin A-defi-
cient animals. A typical separation of normal liver cells by
centrifugation in colloidal silica gel is depicted in Fig. 10. Four
fractions of cells, banding at different densities, were obtained.
Fig. 11 shows typical cells obtained in the various fractions.
Approximately 70% of the total liver cells banded at densities
of 1.08 to 1.10. These cells had a larger particle diameter than
the cells present at higher densities. The figure shows that there
seems to be an enrichment of binuclear cells at the lowest density.
It has earlier been shown that the three top fractions mainly
contain hepatocytes (44). At the highest density (1.12 < d < 1.20)
the Kupffer cells were recovered, occasionally contaminated
with minor amounts of free nuclei.

The relative distribution of [3H]vitamin A in the four fractions
of cells was identical within experimental error regardless of
whether the cells were obtained from normal or vitamin A-defi-
cient rats, although, of course, the total radioactivity differed
significantly (cf. Fig. 9). It was shown above that the total
amount of [H]vitamin A in livers of vitamin A-deficient rats declined considerably with time. The relative distribution of the radioactivity in the four cell fractions obtained was, however, unchanged during the time period studied (15 min to 16 hours). The [H]vitamin A radioactivity was present in greatest abundance in the two cell fractions of lowest density (Fig. 9 and Table IV). About equal amounts of radioactivity were recovered in these two fractions despite approximately twice the content of DNA in Fraction II (cf. Table IV). It is evident from the table that Fractions III and IV contained only minor amounts of both the total DNA and the total radioactivity. The specific radioactivity (per mg of DNA) was 5- to 25-fold higher in the hepatocyte fractions (I to III) than in the Kupffer cell fraction. The Kupffer cell fraction only contained about one-tenth of the total DNA recovered in spite of the fact that the Kupffer cells are known to constitute approximately one-third of the total liver DNA (52-54). Making allowance for this discrepancy the Kupffer cells would still contain less than 3% of the total liver vitamin A (the corrected figure, based on the value 0.8% in Table IV, is accordingly 2.4%). Furthermore, when doses of vitamin A 100 times exceeding those commonly employed in the present study were administered intravenously to normal and deficient rats, no more than 3% of the total liver content of vitamin A could be recovered in the Kupffer cell fraction. Thus, it may be concluded that virtually all vitamin A in the liver is present in the hepatocytes.

Mobilization of RBP from Liver—It is well known that on vitamin A depletion the liver and serum levels of the vitamin decrease rapidly. Determinations of vitamin A in liver and serum and of RBP in serum were performed in rats reared on a vitamin A-free diet and in normal control animals. The results are shown in Fig. 12. It is evident from the figure that the content of liver vitamin A decreased rapidly in the rats on the deficient diet.

Table IV

| Liver cell fractions* | I (d = 1.09) | II (d = 1.11) | III (d = 1.12) | IV (d < 1.20) |
|----------------------|--------------|--------------|--------------|--------------|
| [H]Vitamin A         | 47.0 ± 4.9   | 48.0 ± 6.8   | 4.3 ± 1.3    | 0.8 ± 0.1    |
| DNA                  | 28 ± 2       | 40 ± 8       | 15 ± 3       | 11 ± 4       |
| Disintegrations per min of [H] vitamin A per mg of DNA | 45,000 | 26,000 | 7,400 | 1,000 |

* Figures in parentheses denote the density distribution of the cells (cf. Fig. 10).
† Expressed as percentage recovered in the fraction of the total amount recovered in all fractions.
‡ Average of three normal rats injected with 1.7 μCi of [H]retinylacetate. The livers were excised 16 hours after administration.
diet, whereas in the control animals the level of liver vitamin A rose steadily. Serum vitamin A also decreased in concentration in the depleted animals, whereas a slight increase in the serum level of the normal rats was noted. The serum concentration of RBP diminished also in deficiency in spite of a slight elevation with increasing age in the serum of the normal rats. That the effect of vitamin A on the serum levels of RBP is specific was shown by determinations of total plasma protein concentrations in normal and depleted animals at the times indicated in Fig. 12.

No differences were noted between the two groups of animals during the time period studied. The mean levels in both normal and depleted animals increased from 58 mg per ml on the first day to 69 mg per ml on day 48. The data clearly show that the effects of vitamin A depletion are appearing in the order: liver vitamin A, serum vitamin A, and serum RBP, respectively.

The steepest part of the RBP decline curve did not appear until virtually all vitamin A in the liver had been exhausted. It thus seems likely from the above findings that the liver content of vitamin A may regulate the serum level of RBP.

Further investigations were initiated to study the effects of vitamin A repletion on the serum levels of RBP. Groups of vitamin A-deficient rats were injected with three separate doses of retinol and blood was obtained at different times during the following 6 hours. The result is shown in Fig. 13. The concentration of serum RBP increased considerably after repletion with the vitamin. In all three groups of rats, varying in amounts of vitamin A obtained, the maximum RBP value was recorded within 2 hours. The data in the figure indicate that administration of 5 μg of retinol was not sufficient to restore the RBP concentration to normal values, whereas 125 μg, as well as 25 μg, of retinol gave normal and sustained serum levels of RBP during the following 6 hours.

This experiment indicated that vitamin A might induce synthesis of RBP in the liver. To test this idea, actinomycin D was injected into vitamin A-deficient animals 2½ hours prior to the intravenous administration of 125 μg of retinol. Blood was obtained at the same time intervals as previously. Fig. 13 clearly shows that actinomycin D had no or at most minimal effects on the mobilization of RBP from the liver. This unexpected finding indicates that vitamin A most likely does not induce synthesis of RBP.

Cellular Distribution of [3H]Vitamin A in Kidney—According to current concepts, most organs, except for the liver, obtain vitamin A from serum RBP. An experiment was therefore devised to study the cellular distribution of vitamin A in the kidney since this tissue is the second most important storage site for the vitamin.

Dispersed kidney cortex cells, obtained from rats previously injected with lipoprotein-bound [3H]retinylacetate, were separated by centrifugation in colloidal silica gel. Four fractions of cells, banding at different densities, were obtained. The kidney cells exhibited a tendency to aggregate under the conditions employed, and the recovery of cells in the four fractions varied by as much as 20% in different experiments. In spite of this difficulty, the morphological appearance of the cells in the four fractions was quite reproducible. The top fraction always contained the smallest cells, with a diameter of 10 to 15 μ, sometimes contaminated with small amounts of cell debris. In the density range 1.07 to 1.08, approximately 60 to 80% of the cells were banded. The two well resolved fractions at these densities contained considerable amounts of cells with brush borders. The bottom fraction contained large cells with a diameter of 15 to 20 μ. The total recovery of cells from 1 g of kidney amounted to 1 to 2 × 10⁶ cells. [3H]Vitamin A was exclusively present in the two main middle fractions, as shown in Fig. 14, regardless of whether the kidney cells were obtained from normal or deficient rats. Cells in these two fractions contained about 80% of the total [3H]vitamin A of the kidney since the amounts in the marrow represented less than one-fifth of the total.

The same experiment was repeated after 125I-labeled RBP had been administered intravenously into vitamin A-deficient rats. It can be seen in Fig. 14 that the 125I radioactivity was exclu
parts. The most significant difference found is the molecular weight: 51,000 for the rat protein compared to 62,000 for the human prealbumin (56). In spite of this difference, it is highly likely that the rat protein is composed of four apparently identical polypeptide chains in agreement with findings earlier reported for the human protein (56–59). Prealbumin isolated from the rat exhibited on polyacrylamide gel electrophoresis considerably slower mobility than the human protein. This diminished negative charge of the rat prealbumin made the isolation of the rat prealbumin-RBP complex much more difficult than was previously encountered for human or monkey prealbumin-RBP complexes.

The most interesting observation with regard to the vitamin A–transporting protein complex in the rat is its ability to bind thyroxine. This property is also shared by the human and the monkey prealbumins, but in the latter species it is probably only of minor physiological importance since their plasma contains a more efficient thyroxine-binding globulin. It appears, however, that rat prealbumin is the only protein with any significant thyroxine-binding capacity in rat plasma. Thus, vitamin A and thyroxine are transported by the same vehicle in the rat, raising the important possibility that the hormone and the vitamin may exhibit cooperative metabolic interactions (11). There are no reports presently available establishing such an interaction, but it is noteworthy that on vitamin A deficiency the level of RBP in plasma decreases almost 10-fold (see below) concomitantly with a similar decrease in the plasma prealbumin concentration. Vitamin A deficiency in the rat would accordingly cause not only a decreased vitamin A transport capacity but also thyroxine “buffering” ability. It may thus be suggested that some of the effects of vitamin A deficiency are indeed the result of altered thyroxine metabolism. However, further studies are needed to clarify this point.

The major aim of this study was to investigate the transport of vitamin A to the liver and subsequently to target cells. An apparent difficulty in performing such studies is the number of conflicting reports on the type of liver cells storing the vitamin (cf. Reference 7). An attempt was made, therefore, to establish which cell type in the liver took up the vitamin from the chylomicrons (simulating the postabsorptive state) and which cell type stored the vitamin. Employing liver cell separation on colloidal silica gel gradients, it was unambiguously shown that the hepatocytes took up and stored virtually all vitamin A. Employing liver cell separation on colloidal silica gel gradients, it was unambiguously shown that the hepatocytes took up and stored virtually all vitamin A. This finding is in contrast to that of Popper and Greenberg (60), who concluded that most of the vitamin was stored in the Kupffer cells. We did not observe more than 3% of the administered amount of vitamin A in the Kupffer cells even at early times. The discrepancy between our result and that of Popper and Greenberg is probably explained by the differences in techniques employed. Popper and Greenberg examined liver cell slices by fluorescence microscopy and they noted serious problems of autofluorescence. Recently, the distribution of vitamin A among hepatocytes and Kupffer cells was studied by Linder et al. (61). They found that virtually all vitamin A was present in the hepatocytes, in agreement with the present data.

The hepatocytes could be fractionated into cells of three different densities. The cells with the lowest density contained most of the vitamin. This is interesting since it was noted that cells in this fraction were to a considerable extent binucleated. We would suggest that these cells are the major cell type responsible for RBP and prealbumin synthesis.

The relative distribution of vitamin A among the hepatocytes was invariant with regard to time and total content of vitamin A as demonstrated in normal and vitamin A-deficient livers. The only difference encountered between normal and vitamin
A-deficient livers was the retention time of the administered radioactive vitamin, the radioactivity declining much faster in vitamin A deficiency than in normal conditions. This rapid turnover of vitamin A in the deficient liver was reflected in the plasma level of RBP-bound radioactivity. It was thus noted that considerably higher levels of radioactively labeled vitamin A were noted in the deficient animals than in the control rats. This, of course, does not reflect the total content of plasma vitamin A but indicates that the vitamin is rapidly mobilized from the liver in the deficient state.

In vitamin A deficiency it is well known that the liver becomes exhausted of its vitamin A content, and subsequently diminished plasma levels of retinol are encountered (cf. Reference 1). As expected, the rat plasma RBP concentration decreased considerably on vitamin A deficiency approaching levels 10-fold less than those of normal animals. This decrease of RBP is probably a primary effect of vitamin A depletion since the total plasma protein concentration appeared normal even in severe deficiency. The low plasma levels of RBP would be easily explained assuming that vitamin A affects the synthesis of RBP in the liver. This assumption was, however, most probably disproven since actinomycin D did not inhibit the appearance of RBP in plasma of replenished animals. A number of possible interpretations have bearing on this result. It could conceivably be inferred that vitamin A affects the synthesis of RBP on the translational level and that the messenger RNA for RBP is extraordinarily long lived since actinomycin D affects the transcription rather than the translation of the messenger. A more plausible explanation may be afforded by the recent observation that vitamin A is present in greatest abundance in the Golgi apparatus (62). It is well established that most plasma proteins leave the liver cells through the membranous network constituting the Golgi apparatus. Many plasma proteins get their carbohydrate moieties within this structure and it may well be that RBP attaches its retinol at the same place. In vitamin A deficiency no retinol is available for the newly synthesized RBP, and this could result in the protein not being released into the blood. Administration of the vitamin to the liver cells would accordingly give rise to a rapid increase in the plasma RBP concentration due to release of preformed RBP.

One of the most intriguing facts about the metabolism of vitamin A is the high concentration of the vitamin in the kidney (8). This observation, together with the well known fact that the kidney is the main catabolic site for low molecular weight plasma proteins like RBP, warranted a detailed study of the cellular distribution of the vitamin in the kidney. The present results indicated that more than 80% of the total kidney content of radioactive vitamin A was confined to the cortex. Furthermore, only two out of the four cell fractions recovered after colloidial silica gel gradient centrifugation contained appreciable amounts of the vitamin. On morphological examination of the vitamin-containing cells, it was apparent that a majority of these cells exhibited brush borders.

Kidney cortex cells exhibiting brush borders are known to be the cells responsible for the pinocytic uptake of protein from the glomerular filtrate (cf. Reference 63). Accordingly, in a separate experiment it was demonstrated that 125I-labeled RBP administered to the blood was exclusively confined to the two vitamin A-containing cell fractions in the kidney. It is therefore tempting to suggest that most of the kidney vitamin A reaches its site of deposition by a process involving glomerular filtration and tubular reabsorption. This view gains support from the fact that RBP is a small protein which is catabolized to a considerable extent by the kidney (64). Furthermore, preliminary experiments suggest that retinol, attached to RBP, is released from the protein on its degradation in the tubular cells. The released vitamin seems subsequently to be transported to the liver by lipoproteins. It may thus be concluded that vitamin A recirculates in contrast to its carrier protein.

In a study parallel to this, Goodman and coworkers have isolated prealbumin and RBP from rat plasma and investigated the levels of RBP in liver and plasma in vitamin A deficiency (65, 66). RBP was characterized in some detail and their data are in excellent agreement with ours. They have also found that the plasma levels of RBP decrease on induction of vitamin A deficiency and that the levels can be restored on replenishment of the vitamin, in accord with the present results. In addition, they have made the important discovery that the liver cell content of RBP is approximately 4-fold higher in vitamin A deficiency compared to normal conditions. This observation, together with the present results on actinomycin D-treated rats, clearly indicates that vitamin A deficiency affects the release of RBP from the liver cells.

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Studies on the Transport and Cellular Distribution of Vitamin A in Normal and Vitamin A-deficient Rats with Special Reference to the Vitamin A-binding Plasma Protein

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