Tissue distribution and subcellular localization of retinol-binding protein in normal and vitamin A-deficient rats

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Abstract Levels of retinol-binding protein (RBP), the plasma transport protein for vitamin A, were measured by radioimmunoassay in sera and in a large number of tissues from both normal and vitamin A-deficient rats. The tissues included liver, kidney, fat, muscle, brain, eye, salivary gland, thymus, lung, heart, intestine, spleen, adrenal, testes, thyroid, and red blood cells. The RBP levels in tissues other than serum, liver, and kidneys varied from 12 μg/g of tissue for normal spleen to an undetectable level in red blood cells. Much of the RBP in the tissues with low levels may have been due to residual serum in the samples. In general, except for liver, RBP levels were lower in tissues from vitamin A-deficient rats than in those from normal rats. In normal rats, the liver, kidney, and serum levels were 30 ± 4 (mean ± SEM), 151 ± 22, and 44 ± 3 μg/g, respectively. In vitamin A-deficient rats, the liver RBP level was about three times the normal level whereas the kidney and serum levels were about one-fifth the normal values. When normal liver homogenates were fractionated by centrifugation, 67% of the RBP was recovered in the microsomal fraction and only 9% was found in the soluble 105,000 *g* supernate. In contrast, 76% of the RBP in homogenates of normal kidneys was in the soluble fraction. Similar results were obtained with deficient livers and kidneys. Incubation with deoxycholate released the liver RBP into the soluble fraction. RBP is produced in the liver and removed from the blood by the kidneys. The levels of RBP in normal and deficient liver, serum, and kidney appear to reflect the relative rates of RBP secretion and turnover.

Supplementary key words plasma protein · liver · kidney · serum · microsomes

Retinol-binding protein (RBP), the plasma transport protein for vitamin A, has been isolated from the serum of man (2), the cynomolgus monkey (3), the rat (4, 5), the dog (6), and the pig (7). In all of these species, RBP is a small protein, of about 20,000 molecular weight, which binds one molecule of retinol per molecule of RBP. Human, monkey, and rat RBP circulates in plasma in the form of a protein–protein complex together with a larger protein with prealbumin mobility; in dog and pig plasma, RBP also circulates mainly as a complex of higher apparent molecular weight. The association constant for the interaction between human prealbumin and RBP is about 10^6 (8). RBP appears to be synthesized in the liver and to be released into the plasma as holo-RBP (containing bound retinol) (9). Sensitive radioimmunoassays have been developed for both human (10) and rat (11) RBP. Each of these immunoassays is highly order-specific (6).

The studies reported here were designed to explore the role of various body tissues in the metabolism of RBP. The levels of RBP in different tissues of both normal and vitamin A-deficient rats were determined by radioimmunoassay. The subcellular location of RBP was also examined in the tissues with the highest levels of RBP.

MATERIALS AND METHODS

Tissue distribution of RBP

Male weanling rats of the Holtzman strain were divided into two groups of five rats each. One group was fed a vitamin A-deficient diet (11) for a period of 80 days. After 35 days the rats had serum vitamin A levels below 3 μg/dl, as determined by previously described methods (11). The second group was fed a diet containing 2.4 μg of vitamin A (as retinyl esters) per g of diet, but it was otherwise identical with the deficient diet. On day 80 of the study, the rats were anesthetized with ether, and as much blood as possible was drawn from the abdominal aorta. A washout perfusion of the entire animal was then carried out by slowly injecting 100 ml of 0.9% NaCl into the left ventricle of the heart in order to flush blood from the body tissues. The small intestine was removed and the contents were washed out with 50 ml of 0.9% NaCl. The following tissues were

Abbreviations: RBP, retinol-binding protein; DOC, deoxycholate.

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then removed, rinsed in 0.9% NaCl, and blotted dry: liver, kidneys, heart, adrenals, lungs, thymus, spleen, epididymal fat pads, testes, submaxillary salivary glands, thyroid, eyes, brain, and the central portion of the right gastrocnemius muscle. The blood was allowed to clot, and the serum was removed after centrifugation at 900 *g* for 30 min at 4°C. Red blood cells were obtained from blood samples collected in heparinized syringes from different rats fed identical diets. The red cells were washed twice with heparinized 0.9% NaCl and then suspended in a volume of distilled water equal to the volume of removed plasma. All samples were stored at −20°C until assayed.

All of the tissues were vigorously homogenized in a Potter-Elvehjem homogenizer equipped with a Teflon pestle (100 strokes with pestle rotating at 1500 rpm). Except for adrenals and thyroids, the tissues were homogenized in 3 vol (w/v) of a 0.25 M sucrose, 0.025 M KCl solution. The adrenals and thyroids were homogenized in 0.5 ml of the above solution. The homogenates were centrifuged at 2000 *g* for 20 min at 4°C. The supernates were decanted and saved for assay. The precipitates were washed with 2 ml of the sucrose–KCl solution and recentrifuged. The wash was discarded, and each precipitate was suspended in sufficient sucrose–KCl to bring the total volume to 10 ml. Both the supernates and the washed precipitates were assayed directly for RBP by radioimmunoassay after suitable dilution with barbital–albumin buffer (10, 11). With adipose tissue, the sample of the supernate was taken from the aqueous layer beneath the lipid layer.

We have not been able to release more RBP by sonication of the 2000 *g* precipitates from liver, kidney, or intestine when the precipitates were prepared after extensive homogenization as described above. We do not have comparable data for other tissues.

The samples were assayed for immunoreactive RBP by a modification of the previously described radioimmunoassay (11). The control rabbit serum was omitted from the incubation mixture, and only the anti-rat RBP antiserum was added to the incubation mixture at a final dilution of 1:15,000 (v/v). After incubation in the dark at 4°C for 3–5 days, the immunoglobulin was precipitated with polyethylene glycol, 6000–7500 mol wt (Matheson Coleman & Bell, Norwood, Ohio), as suggested by Desbuquois and Aurbach (12). To the 0.5 ml of incubation mixture in each tube, 1.5 ml of a freshly prepared mixture composed of 8 parts human plasma, 48 parts of a 50% (w/v) solution of polyethylene glycol, 20 parts water, and 75 parts barbital–albumin assay buffer (10) was added. After mixing with a Vortex mixer, the tubes were centrifuged at 8000 *g* at 4°C for 15 min. The supernates were immediately aspirated, and the precipitates were assayed for ^{125}I in a Packard model 5219 Auto-Gamma counter. Calculations were done on a Wang model 700A programmable calculator by the logit–log method (13).

Using this modification of the radioimmunoassay, the displacement curves for serum, for whole liver and whole kidney homogenates, and for isolated liver microsomes were all identical to that obtained with purified serum RBP. When RBP standards were incubated in 0.005% sodium DOC, the displacement curve was also indistinguishable from the curve obtained without DOC.

Experiments were also conducted to determine whether liver or kidney homogenates contain materials that might interfere with the immunoassay. Rat serum of known RBP content was added to homogenates of liver or kidney. The observed recovery of the added serum RBP was 96% with the liver and 99% with the kidney homogenate.

Subcellular distribution of RBP

For this study, kidneys and livers were obtained from both normal and vitamin A-deficient rats at various times between 40 and 100 days on the diet. The rats were stunned and decapitated. The livers were flushed by the perfusion of about 30 ml of 0.9% NaCl through the portal vein. Each kidney was flushed with 10 ml of saline injected into the renal artery. As soon as possible the livers and kidneys were diced into 0.5-cm squares and then homogenized by four gentle strokes of a loose-fitting Teflon–glass Potter-Elvehjem homogenizer (pestle rotating at 200 rpm) with 4 vol (w/v) of a buffer containing 0.5 M sucrose, 0.1 M Tris–HCl (pH 7.6), 0.01 M $MgCl_2$, and 1% dextran. The homogenate was filtered through eight layers of cheesecloth and diluted so that 1 g of liver or kidney was present in 16 ml of homogenate. 20 ml of each homogenate was centrifuged at 400 *g* at 4°C for 10 min. The precipitates were saved, and the supernates were transferred to new centrifuge tubes and centrifuged at 10,000 *g* at 4°C for 10 min. The precipitates of the 10,000 *g* centrifugation were also saved. Aliquots of the 10,000 *g* supernates were centrifuged at 105,000 *g* in a 40.3 rotor of a Beckman Spinco model L ultracentrifuge at 4°C for 1 hr. Both the supernates and the precipitates were saved. All precipitates and supernates were assayed for RBP.

After liver RBP was found to be particulate (see Results), the effect of 1% sodium DOC was examined. A freshly prepared liver homogenate was divided into two parts. One part served as a control and sodium DOC was added to the second to a final concentration of 1% (w/v). The homogenates were centrifuged at 105,000 *g* for 90 min, and both the supernates and the precipitates were assayed for RBP.

RESULTS

RBP concentration in tissues

The RBP concentrations in the various tissues of both control and vitamin A-deficient rats are shown in **Table 1.**

TABLE 1. Tissue distribution of RBP in normal and vitamin A-deficient rats

Tissue	Control	Deficient
	μg RBP/g wet wt	
Kidneys	151.4 ± 23.4	31.1 ± 4.1
Serum	44.1 ± 3.1	6.1 ± 1.2
Liver	29.9 ± 4.2	90.3 ± 16.2
Spleen	12.3 ± 2.9	1.5 ± 0.3
Lungs	8.3 ± 2.6	2.1 ± 0.6
Testes	7.3 ± 0.4	3.5 ± 0.7
Adrenals	6.7 ± 1.0	5.2 ± 0.9
Salivary gland	6.2 ± 0.7	3.8 ± 0.9
Heart	5.5 ± 0.9	1.0 ± 0.1
Small intestine	4.8 ± 0.1	2.7 ± 0.3
Thymus	3.4 ± 0.7	2.7 ± 0.5
Eyes	2.8 ± 1.4	0.6 ± 0.2
Muscle	1.7 ± 0.3	0.7 ± 0.1
Brain	0.3 ± 0.1	0.5 ± 0.1
Erythrocytes	<0.01	<0.01

Values are means of five samples ± SEM.

In normal control rats, the serum, liver, and kidneys contained substantial levels of RBP. The normal kidney contained the highest level of RBP observed in any of the tissues we have studied. Of all the tissues analyzed for RBP, only the washed red blood cells failed to displace the ^{125}I-labeled RBP in the radioimmunoassay. All of the other tissues contained some "immunoreactive RBP." Except for the serum, liver, and kidneys, however, the levels of RBP found in the other tissues were rather low. In all of these tissues with low levels of RBP, about 30% (mean ± SEM, 29.5 ± 2.0) of the RBP was associated with the washed 2000 *g* precipitate. In addition to the tissues shown in Table 1, thyroid and adipose tissue were assayed. As some question existed as to the purity of the thyroid homogenates, their data were not included in Table 1. The levels in thyroid were, however, quite low. The large amount of lipid in the adipose tissue caused problems in sampling, but the level was also low and probably of the order of 3 μg of RBP per g of tissue. Much of the RBP observed in tissues other than liver and kidney may represent RBP in serum remaining in the tissue after the limited wash-out perfusion. We believe that this is especially true for spleen.

In the vitamin A-deficient rat, RBP accumulated in the liver whereas the level of RBP in the other tissues declined (Table 1).

Subcellular distribution of RBP

Information about the subcellular localization of RBP in the livers and kidneys is presented in **Table 2.** In the normal livers, two-thirds of the RBP was precipitated with the microsomal fraction, with only 9% of the RBP found in the soluble 105,000 *g* supernate. In contrast, 76% of the RBP in the normal kidney was found in the soluble supernate. The 15% of the RBP observed in the nuclear fraction of both organs (Table 2) can be released by more vigorous homogenization, such as that used in previous studies (9, 11), and probably mainly represents RBP associated with unbroken cells. In contrast, the RBP associated with the microsomal fraction is fairly resistant to release by mechanical means.

The absolute concentrations of RBP in the livers of vitamin A-deficient rats were about three times the levels observed in the livers of control rats, and the absolute amounts of RBP in the kidneys of deficient rats were only one-fifth those found in normal kidneys (Table 1). Nevertheless, the subcellular distributions of RBP in both liver and kidneys of vitamin A-deficient rats were remarkably similar to the distributions observed in the normal rats (see Table 2).

An experiment was conducted to determine whether the RBP associated with the liver microsomal fraction represented RBP nonspecifically bound to the microsomal fraction during the homogenization procedure. Aliquots of ^{125}I-labeled RBP containing 47,000 cpm (about 10–20 ng

TABLE 2. Distribution of RBP after fractionation of liver and kidney homogenates by centrifugation

Fraction	Liver		Kidney	
	Control	Vitamin A-deficient	Control	Vitamin A-deficient
	%		%	
Nuclear	14.8 ± 0.8	15.7 ± 1.4	14.6 ± 1.1	19.6 ± 3.9
Mitochondrial	9.1 ± 0.3	10.0 ± 0.6	8.0 ± 1.4	8.2 ± 1.4
Microsomal	66.9 ± 1.9	58.0 ± 2.1	1.6 ± 0.1	7.3 ± 2.1
Supernatant	9.2 ± 0.8	16.3 ± 1.7	75.8 ± 2.4	64.9 ± 6.7

The *nuclear* fraction was defined as the material that precipitated after centrifugation at 400 *g* for 10 min. The *mitochondrial* fraction comprised material in the 400 *g* supernate that was precipitated by centrifugation at 10,000 *g* for 10 min. The *microsomal* fraction was the material in the 10,000 *g* supernate that was precipitated by centrifugation at 105,000 *g* for 1 hr. *Supernatant* refers to the 105,000 *g* supernatant fraction. The mean recovery of RBP was 102% in these experiments. Values are means of four samples ± SEM.

of RBP) were added to both the liver and the kidney homogenates before the subcellular fractionation by centrifugation. As shown in **Table 3**, 95% of the ^{125}I-labeled RBP was recovered in the soluble 105,000 *g* supernate in both the liver and the kidney homogenates. Because similar results were obtained with both the liver and the kidney homogenates, this study indicated that the RBP observed in the liver microsomal fraction did not represent soluble RBP nonspecifically bound to the microsomal membranes after disruption of the cell.

Direct centrifugation of liver homogenates at 105,000 *g* for 90 min resulted in 93% of the RBP being precipitated. Incubation of the same samples with 1% sodium DOC released the RBP from the particulate fraction, with excellent recovery obtained in the soluble supernate (**Table 4**). This suggests that the RBP in the liver is associated with a membrane fraction rather than existing as a nascent protein chain attached to the ribosomes (14).

A purified sample of rat liver plasma membranes was generously provided by Dr. David Neville (National Institute of Arthritis and Metabolic Disease, Bethesda, Md.). This sample contained a small amount of "immunoreactive RBP" at a level of 40 ng of RBP/mg of protein. This is about one-third the concentration of RBP found in isolated microsomes. Thus, the plasma membrane does not appear to be a major site of RBP storage in the liver.

DISCUSSION

This study was undertaken to explore the role of various tissues in the metabolism of RBP. RBP levels were measured in a large number of tissues of both vitamin A-deficient and normal rats. We also explored the subcellular localization of RBP in the tissues (liver and kidney) having high levels of RBP.

Of all the tissues examined, only washed red blood cells failed to contain detectable levels of immunoreactive RBP. The other tissues of normal rats fell into three groups, which could be called high, low, and very low according to their content of RBP. Tissues that fell into the very low class contained less than 3 μg of RBP/g of tissue; they were fat, skeletal muscle, thyroid, brain, and eyes. The tissues in the low group, between 3 and 12 μg of RBP/g of tissue, were spleen, lungs, testes, adrenals, salivary gland, heart, small intestine, and thymus. The kidneys, serum, and liver contained substantial levels of RBP. The kidney of the normal rat contained the highest level of RBP (151 μg/g of tissue) of any of the tissues that we have studied. The serum (44 μg/ml) and liver (30 μg/g of tissue) levels were comparable to values we have previously reported (9, 11).

Much of the RBP detected in tissues other than liver and kidney probably represented RBP in serum that was not removed from the tissue by the limited flush through the heart with isotonic saline. It is possible that if the tissues could all have been washed as thoroughly as were the red blood cells some of them would also have shown no detectable level of RBP. On the other hand, it is possible that the small amount of RBP in some of the tissues may be metabolically significant for the particular tissue. Because many of these tissues are known to require vitamin A for maintenance of normal differentiated function, it is evident that RBP must somehow deliver vitamin A to these tissues. The nature of the "delivery" process remains, however, to be defined. RBP may, for example, deliver retinol to binding sites (for retinol and/or for RBP) on the plasma membrane at the cell surface and release retinol at these locations. Alternatively, it is possible that the entire retinol–RBP complex may enter the cell to deliver retinol to its intracellular site(s) of action. Although we favor the former hypothesis, this question needs to be explored. Preliminary reports of tissue components that are capable of binding retinol (15, 16) also suggest that delivery of vitamin A by RBP to the cell surface may be the more likely mechanism.

Approximately one-third of the RBP detected in tissues other than liver and kidney was associated with the 2000 *g* precipitate of the tissue homogenate. It is possible that much of this RBP was bound to the plasma membranes of the cells of these tissues. The significance of the immunoreactive RBP detected in these tissues is at present not clear and represents an interesting area for future research.

When the tissues of vitamin A-deficient rats were assayed for RBP, the liver levels of RBP were found to be elevated to about three times the normal level. This finding is in accord with our previous reports that vitamin A deficiency interferes with the secretion of RBP by the liver (9, 11). In contrast, the serum RBP level of the vitamin A-de-

TABLE 3. Distribution of ^{125}I-labeled RBP added to liver and kidney homogenates

Fraction	Liver	Kidney
	% of total cpm	
Nuclear	1.7	2.0
Mitochondrial	1.2	1.9
Microsomal	2.1	1.2
Supernatant	95.0	94.9

See Table 2 for a definition of the fractions.

TABLE 4. Effect of deoxycholate on the distribution of liver RBP after centrifugation

Fraction	Treatment	
	Control	1% DOC
Supernatant	6.8	97.3
105,000 *g* precipitate	93.2	2.7

Values are mean percentages of total RBP for data from two rats. The mean recovery of RBP in this experiment was 106%.

ficient rats was decreased to 6 μg/ml. A decrease (compared with normal) in RBP level was also seen in all other tissues except brain, which had negligible levels of RBP even in the normal rats. Kidney was the only tissue other than liver that showed a high level of RBP in the deficient rats; however, the RBP level in the kidney was only one-fifth the level observed in kidneys from control rats.

We have previously shown that the large pool of RBP that accumulates in the liver in vitamin A deficiency can be released rapidly into the serum after the injection of chylomicrons containing vitamin A (9). In these experiments, the amount of RBP released from the liver was directly related to the dose of vitamin A given. Because the release of RBP from the liver is blocked in vitamin A deficiency, the finding that all other tissues have reduced levels of RBP in vitamin A deficiency is consistent with the hypothesis that the liver was the source of all of the immunoreactive RBP detected in this study.

After gentle homogenization and differential centrifugation, approximately two-thirds of the RBP in liver homogenates from both normal and vitamin A-deficient rats was found associated with the microsomal fraction and only about 10% with the soluble supernate. The addition of 1% sodium deoxycholate to the liver homogenates quantitatively released the RBP associated with the particulate fractions into the 105,000 *g* supernate. This suggests that RBP is present in liver associated with membranous subcellular structures rather than existing as nascent protein chains still attached to ribosomes.

One of the membrane fractions often involved in the secretion of extracellular proteins (particularly glycoproteins) is the Golgi apparatus. Preliminary experiments have indicated that fractions enriched in Golgi apparatus do contain RBP but that the Golgi does not appear to be the major subcellular location of RBP in the liver in either normal or vitamin A-deficient rats.[2] Tissue localization studies by immunofluorescence methods support the conclusion that the Golgi is not the major site of RBP localization in liver.[3] Examination of a preparation of rat liver plasma membranes also indicated that although a small amount of RBP was detected in the preparation it represents only a minor portion of the total liver RBP. Thus, the preparation of liver plasma membranes contained about 40 ng of RBP/mg of protein. Because plasma membrane protein has been estimated to represent only 5% or less of the total liver homogenate protein (17), the amount of RBP associated with the plasma membranes would be 5% or less of the total liver RBP.

Unlike the case with liver, 76% of the RBP was recovered in the 105,000 *g* supernatant fraction of the kidney homogenate. This difference is consistent with current knowledge of RBP metabolism because kidney RBP presumably represents plasma RBP undergoing glomerular filtration and renal metabolism. Previous studies have indicated that the kidneys normally play an important role in the metabolism of RBP. This occurs because of the relatively small size of the RBP molecule, which permits free, uncomplexed RBP to be filtered readily by the renal glomeruli. Although the proportion of RBP in plasma present in the free state, not as a complex with prealbumin, is normally very small, it is sufficient to permit a significant amount of RBP to be filtered by the glomeruli and metabolized by the kidneys each day. Thus, patients with severe chronic renal disease show markedly elevated plasma concentrations of RBP (18, 19) and a reduced metabolic clearance and turnover rate of RBP (19).

Small proteins such as RBP that undergo glomerular filtration are normally reabsorbed and catabolized by the renal tubules (20). When tubular function is impaired, as in patients with tubular proteinuria, low molecular weight proteins, including RBP, are excreted in the urine in relatively large amounts (21, 22). It is reasonable to assume that the kidney RBP observed here represented RBP that had undergone glomerular filtration and tubular reabsorption. This hypothesis is supported by our observation that kidney lymph[4] (mainly the product of filtration and reabsorption) contained a significant level (12 μg/ml) of RBP. Further support is also provided by the findings with vitamin A-deficient rats. If kidney RBP mainly represents RBP undergoing filtration and metabolism, a low serum RBP level would be expected to be associated with a low kidney RBP level, as observed in this study. The tissue data thus provide support for the metabolic scheme for RBP as formulated.

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