Transthyretin (TTR) acts physiologically in the transport of retinol in the circulation. We previously reported the generation and partial characterization of TTR-deficient (TTR−) mice. TTR− mice have very low circulating levels of retinol and its specific transport protein, retinol-binding protein (RBP). We have examined the biochemical basis for the low plasma retinol-RBP levels. Cultured primary hepatocytes isolated from wild type (WT) and TTR− mice accumulated RBP in their media to an identical degree, suggesting that RBP was being secreted from the hepatocytes at the same rate. In vivo experiments support this conclusion. For the first 11 h after complete nephrectomy, the levels retinol and RBP rose in the circulations of WT and TTR− mice at nearly identical rates. However, human retinol-RBP injected intravenously was more rapidly cleared from the circulation (t1/2 = 0.5 h for TTR− versus t1/2 > 6 h for WT) and accumulated faster in the kidneys of TTR− compared with WT mice. The rate of infiltration of the retinol-RBP complex from the circulation to tissue interstitial fluids was identical in both strains. Taken together, these data indicate that low circulating retinol-RBP levels in TTR− mice arise from increased renal filtration of the retinol-RBP complex.

The predominant retinoid in the fasting circulation is retinol (1, 2). All circulating retinol is bound to its specific plasma transport protein, the 21-kDa retinol-binding protein (RBP)1 (1, 2). RBP, which is synthesized and secreted primarily by hepatocytes, is the sole specific transport protein for retinol in the circulation (1, 2). The secretion of RBP is strongly stimulated by its association with retinol, which alters the conformation of the protein (1, 2). In blood, RBP is found as a 1:1 protein-protein complex with a 55-kDa serum protein, transthyretin (TTR) (1, 2). Association with TTR is proposed both to facilitate RBP release from its site of synthesis in the endoplasmic reticulum (2) and to prevent renal filtration of RBP (1). Delivery of retinol to cells through the circulation by the RBP-TTR complex is the major pathway through which cells and tissues acquire retinol. It is generally accepted that cells and tissues acquire the retinoic acid they need for regulating gene expression via intracellular oxidation of this retinol to retinoic acid (3).

We previously reported the targeted disruption of the mouse gene for TTR (4, 5). Although TTR-deficient (TTR−) mice have no immunoreactive TTR, they appear normal and are viable and fertile (4). Yet, these mutant mice show marked biochemical differences when compared with wild type (WT) mice in parameters associated with retinoid transport and metabolism (4, 5). The plasma retinol and RBP levels in TTR− mice are very low, ~5% of those observed in WT mice (4, 5). These retinol levels correspond to those seen in severely vitamin A-deficient animals that are near death (4, 5). Nevertheless, despite these low circulating levels of retinol-RBP, total retinol (retinol + retinyl ester) levels in tissues of TTR-deficient mice are similar to those in tissues of age-, sex-, and strain-matched WT mice (4, 5). These apparently contradictory observations raise two important questions for understanding the physiologic role(s) of TTR in living animals and the retinoid-related physiology of the TTR-deficient mice. The first of these is the basis of the low circulating retinol-RBP levels. Second, how do the tissues of TTR− mice acquire and maintain normal total retinol levels? We now report data that provide an answer for the first of these questions and some insight into the second.

**EXPERIMENTAL PROCEDURES**

**Materials**—Dulbecco’s modified Eagle’s medium (DMEM), Medium 199, Krebs buffer, penicillin/streptomycin, gentamicin, fetal bovine serum, and trypsin were purchased from Life Technologies, Inc. Collagenase type IV, collagen type I, soybean trypsin inhibitor, and HEPES were purchased from Sigma. Fatty acyl chlorides were obtained from Nu-Chek-Prep, Inc. (Elysian, MN). [3H]Retinol, [35S]methionine, and Na251I were purchased from PerkinElmer Life Sciences. Hexane, methanol, chloroform, methane chloride, acetonitrile, and benzene, all of HPLC grade or grades of comparable purity, were purchased from Fisher. Authentic all-trans-retinol was a kind gift of Dr. Christian Eckhoff of Hoffmann-La Roche. All other chemicals and supplies were purchased from standard commercial suppliers.

**Mice**—For our studies, we employed WT 129SV mice and mice lacking immunoreactive TTR (TTR− mice) from the same genetic background. These TTR− mice were created by targeted gene disruption in ES cells, as we have described previously (4). These ES cells were allowed to colonize blastocysts obtained from crosses of WT 129SV male and female mice. Thus, the inbred 129SV TTR− mice we used for our studies are from a homogeneous genetic background. Retinoid-related parameters of TTR-deficient mice from the 129SV strain have not been reported previously. We earlier have characterized TTR− mice from the outbred MF-1 genetic background (4, 5).
All mice were maintained in a specific virus and pathogen free "barrier" facility prior to use in the studies. The animals were allowed ad libitum access to a standard rodent chow diet (Purina Products, Richmond, VA) and water. The room housing the mice was maintained on a standard 12-h light-dark cycle.

Preparation of Mouse Isolated Liver—For hepatocyte isolation, mouse livers were perfused in situ through the portal vein at the rate of 10 ml/min, first with Krebs buffer (NaCl, 6.75 g/liter; NaHCO3, 2 g/liter; KCl, 0.345 g/liter; KH2PO4, 0.158 g/liter; MgSO4, 0.287 g/liter; gentamicin, 10 mg/liter; and glucose, 1.0 g/liter) for 4 min followed by medium 199 containing 0.25 mg/ml collagenase type IV and 10 nM soybean trypsin inhibitor for 12 min. After perfusion, the liver, with the capsule intact, was quickly excised from the body cavity and transferred to a sterile Petri dish. After opening the capsule, the liver digest was resuspended in medium 199 and filtered through a fine nylon mesh to remove undigested materials. Hepatocytes were separated from nonparenchymal cells and debris by centrifugation at 20 x g for 3 min at room temperature. The hepatocyte-containing pellet was washed with DMEM containing glucose (4.5 g/liter), sodium pyruvate (0.110 g/liter), NaHCO3 (3.7 g/liter), penicillin (200 units/ml), streptomycin (0.050 g/liter), 20 mM HEPES, pH 7.4, and 10% fetal calf serum. Routinely, greater than 85% of the isolated cells excluded trypan blue. The yield of isolated mouse hepatocytes averaged \( \sim 20 \times 10^6 \) hepatocytes/liter.

Isolated hepatocytes were plated at 5 x 10^6 cells per 100-mm plastic dish in DMEM containing glucose (4.5 g/liter), sodium pyruvate (0.110 g/liter), NaHCO3 (3.7 g/liter), penicillin (200 units/ml), streptomycin (0.050 g/liter), 20 mM HEPES, pH 7.4, and 10% fetal calf serum. The plastic tissue culture dishes had been coated prior to hepatocyte plating with collagen type I, according to the supplier's instructions. This culture medium was changed for fresh medium after incubation for 4 h at 37 °C in a humidified atmosphere of 5% CO2, 95% air. At this time, most hepatocytes have attached to the collagen-coated plates and have started to assume a flattened morphology. Hepatocyte cultures were incubated at 37 °C in 5% CO2, 95% air overnight prior to the start of an experiment. The hepatocytes isolated and maintained according to these procedures remained viable in culture for at least 3 days.

At the start of our experiments to assess the effects of TTR deficiency on RBP synthesis and secretion, hepatocytes were washed three times with PBS and fresh DMEM containing glucose (4.5 g/liter), sodium pyruvate (0.110 g/liter), NaHCO3 (3.7 g/liter), penicillin (200 units/ml), streptomycin (0.050 g/liter), 20 mM HEPES, pH 7.4, and supplemented with either 10% fetal calf serum, 5% TTR-deficient mouse serum, or 5% TTR-deficient mouse serum supplemented with 1 μg of human TTR per ml of culture medium. The cells were incubated at 37 °C for 12 h to assess RBP synthesis and secretion. After removal of the medium, cultured hepatocytes were washed 3 times with 5 ml of ice-cold PBS. The media and cells were stored at -70 °C for up to 4 weeks prior to assay of media and cellular levels of RBP by radioimmunoassay.

Bilateral Nephrectomy of Mice—Three- to four-month-old female WT and TTR-/- mice were bilaterally nephrectomized to investigate the role of the remaining retinol-binding protein (RBP) in the for e-nephrectomy, the mice were anesthetized by intraperitoneal injection of 100 μl of a solution containing 3 mg of triglyceride and trace [3H]retinyl ester dissolved in 1 ml of corn oil (200–300 g) that had been fasted overnight were administered by gavage of 0.2 ml of a solution of corn oil + olive oil (1:1 v/v) containing 4 mg/ml ethanol (Eastman Kodak Co.) and 40 μCi of [3H]retinyl acetate (PerkinElmer Life Sciences) to which 800 μg of unlabeled retinol (kindly provided by Dr. Christian Echhoff, Hoffmann-La Roche) was added. Within 20 min after administration of the gavage, the rats were anesthetized; the mesenteric lymph duct was cannulated, and chyle was collected on ice, under reduced light, into a sterile 1.5ml tube containing 1 ml of a solution consisting of 1 ml of EDTA/mal saline (0.9% NaCl). Collection continued for periods up to 24 h. During chyle production, rats were provided free access to a solution of 0.9% NaCl, 0.05% KCl, and 5% glucose in water. To isolate chylomicrons, the chyle was overlaid with 0.05% EDTA in saline, pH 7.4, and was spun for 25 min at 100,000 x g and 18 °C. After centrifugation, chylomicrons were aspirated and stored at 4 °C in the dark for periods of up to 4 days prior to use in experiments. Chylomicron triglyceride concentrations were determined using a commercially available kit (Roche Molecular Biochemicals), according to the manufacturer's instructions.

Tissue Uptake of Chylomicron Retinoid—Three-month-old male WT and TTR-/- mice were fasted overnight prior to injection with labeled chylomicrons. The next day, each animal was anesthetized, and 100 μl of rat chylomicrons containing 3 mg of triacylglycerol and trace [3H]retinyl ester were injected s.c. into the right jugular vein. Blood samples were obtained from the lateral tail vein at 5, 10, 30, and 60 min after chylomicron injection. Four animals of each strain were sacrificed 10 and 60 min after chylomicron injection. At the time of sacrifice, blood was taken, and a total body perfusion with ice-cold PBS was performed (as described above). The liver, spleen, kidney, lung, heart, brain, epididymal fat, and skeletal muscle (gastrocnemius) were excised and used for assessing [3H]retinoid uptake.

To assess the levels of [3H]retinoids taken up by tissues from the labeled chylomicrons, tissues were homogenized in 4 volumes of 10 mM sodium phosphate, 150 mM NaCl, pH 7.4 (PBS), using a Polytron homogenizer (Brinkmann Instruments) to give 20% (w/v) homogenates. The tissue homogenates were then extracted with 6 volumes of chloroform/methanol (2:1 v/v). After centrifugation and removal of the lower retinoid-containing chloroform phase, the upper phase was reextracted with 6 volumes of chloroform/methanol (2:1 v/v). The lower chloroform phase from the reextracted tissue was taken and combined with the first chloroform extract in a 20-ml glass liquid scintillation vial and allowed to stand overnight in an ordinary laboratory fume hood. After the chloroform had evaporated, the retinoid-containing liquid lipid was dissolved in 20 ml of Hydrofluor liquid scintillation counting solution (National Diagnostics, Atlanta, Georgia). [3H]counts/min were assayed in a Beckman LS 1800 LSC counter using a quench-correction program for calculation of [3H]disintegrations/min.

Isolation of Lipoprotein Fractions—Very low density lipoprotein (VLDL), low density lipoprotein (LDL), high density lipoprotein (HDL), and of > 1.21 g/ml bottom fractions were isolated by sequential ultracentrifugation of serum samples according to procedures as described by Bronzert and Brewer (10). For isolation of these fractions, pools of mouse serum were used. The serum pools were centrifuged by mixing 300 μl of serum obtained from 10 individual mice of the same strain, age, and sex. The total recovery of retinyl esters that were present in the serum pools when assayed for the VLDL, LDL, and HDL fractions ranged from 83 to 105%. Thus, essentially all of the retinyl
ester present in the circulating fractions of fasting WT and TTR mice could be accounted for by that present in these three lipoprotein fractions.

**HPLC Analysis of Serum and Liver Retinoids**—Serum retinol and liver total retinol (retinol + retinyl ester) concentrations were determined by reverse phase high performance liquid chromatography (HPLC) using a procedure we have described previously (11). Briefly, to an aliquot of serum (or liver homogenates) an equal volume of absolute ethanol containing a known amount of the internal standard retinyl acetate (Sigma) was added. Endogenous retinol and retinyl esters and the internal standard were extracted into hexane. After one backwash with H2O, the hexane extract was evaporated to dryness under a gentle stream of N2. Immediately upon reaching dryness, the retinoid-containing film was dissolved in 40 µl of benzene for injection onto the HPLC. Retinol and retinyl esters were analyzed on a 4.6 × 250-mm 5-µm Beckmann Ultrasphere C18 column (Beckmann Instruments). The mobile phase consisted of acetonitrile/methanol/dichloromethane (70:15:15 v/v/v) delivered at a flow rate of 1.8 ml/min. Retinoids were detected and quantitated by UV absorbance at 325 nm using a Waters PDA 996 photodiode array detector.

**Radioimmunoassay (RIA) of Mouse and Human RBP and of Mouse Albumin**—Levels of mouse RBP in tissues and serum and isolated hepatocytes were analyzed using a sensitive and specific RIA procedure (12). This procedure employs sheep anti-retinol serum RBP antiserum and standards consisting of homogeneously purified rat serum RBP. Thus, all mouse RBP concentrations are reported as “rat equivalents.” This RIA procedure was used previously for the analysis of RBP in various mouse tissues and mouse serum (4, 5, 13). Purified human RBP and human serum give no displacement in the mouse RBP RIA. Thus, for some experiments where both human and mouse RBP are present in the sample tissue or serum sample, human RBP does not interfere with the accurate measure of mouse RBP levels.

For some experiments, human RBP was injected into mice. Here, the concentrations of human RBP in tissues and serum were assessed by a sensitive and specific RIA for human RBP (12). This RIA procedure makes use of polyclonal rabbit anti-human serum RBP antiserum and homogeneously purified human serum RBP as standard. Both purified rat serum RBP and mouse serum RBP do not give displacement in this RIA for human RBP. Consequently, the presence of mouse RBP in tissues and serum samples did not interfere with the accurate measure of tissue or serum human RBP levels.

Albumin levels of mouse liver were assessed by RIA using rabbit anti-rat serum albumin antiserum and purified standards of rat albumin (12). Thus, mouse albumin concentrations are reported as rat equivalents.

**Protein Determinations**—A modified Lowry procedure (14) was used for determining protein levels of mouse liver homogenates. The reagents for this assay were purchased from Bio-Rad, and the assay was carried out according to the supplier’s instructions.

**Statistical Procedures**—All data are expressed as means ± 1 S.D. Statistical significance was determined by Student’s unpaired t test (two-tailed). Group differences were rejected as not significant for p > 0.05.

RESULTS

These studies pursue questions raised upon the initial characterization of the phenotype of the TTR−/− mice. Specifically, what is the physiological mechanism(s) responsible for the low circulating retinol-RBP levels? For our present experiments, we used inbred 129SV WT and TTR−/− mice, whereas we had earlier characterized the TTR−/− phenotype in outbred MF-1 mice (4, 5). To link the two studies, we compared various biochemical consequences of the TTR−/− mutation in the two genetic backgrounds. As was the case for outbred MF-1 TTR−/− mice, serum retinol and RBP levels in TTR−/− mice in the 129SV genetic background were very low. For both mouse strains, serum retinol and RBP levels in TTR−/− deficiency were ~5% of those observed for age- and sex-matched WT mice. Thus, in this regard, genetic background does not influence the severity of the TTR−/− phenotype.

Liver total retinol (retinol + retinyl ester) and RBP levels for outbred and inbred WT and TTR−/− mice are shown in Table I. Hepatic total retinol levels in WT and TTR−/− male mice, whether outbred or inbred, were not statistically different (5). Interestingly, female TTR−/− mice from both strains had significantly higher hepatic total retinol concentrations than the corresponding WT controls. Since all of the mice were maintained on the same chow diet, it is unlikely that dietary intake accounts for this difference. Hepatic RBP levels for both male and female 129SV TTR−/− mice were ~3–4-fold greater than those of age- and sex-matched WT controls, whereas the difference in the MF-1 background was only 1.6-fold (Table I (5)). Nevertheless, although genetic background does quantitatively influence the TTR−/− phenotype with regards to hepatic RBP levels, the qualitative effects of TTR deficiency on serum and liver levels of retinol and RBP are similar for outbred MF-1 and inbred 129SV TTR-deficient mice.

Liver RBP levels are elevated in vitamin A deficiency, resulting from a blockage in the RBP secretory pathway (1, 2). We asked if TTR deficiency likewise inhibited RBP secretion. Primary hepatocytes from WT and TTR−/− mice were isolated and cultured. Since cultured hepatocytes are able to acquire TTR from culture medium (15), we performed these experiments under three different growth conditions. The control medium consisted of DMEM supplemented with 10% fetal calf serum, which we estimate contains ~3–6 µg of bovine TTR per ml of medium. Hepatocytes were also cultured in DMEM supplemented with 5% serum obtained from the TTR−/− mouse or with DMEM supplemented with 5% TTR−/− mouse serum and 1 µg of purified human TTR per ml of medium. Table II shows the rate at which these cells secrete RBP into the medium. RBP accumulated at the same rate in media in both WT and TTR−/− deficient hepatocytes in all three culture conditions. Thus, neither endogenously synthesized nor exogenous TTR influences the rate of RBP secretion from liver parenchymal cells. Consistent with our observations in intact liver (5), TTR-deficient hepatocytes had higher cellular RBP levels than WT cells.

The suggestion that WT and TTR-deficient hepatocytes secrete RBP at equivalent rates is supported by data obtained with nephrectomized WT and TTR−/− mice. Fig. 1 shows serum RBP and retinol levels in age- and sex-matched WT and mutant mice at various times after complete nephrectomy. RBP levels rise in the circulation of WT and TTR−/− mice at nearly identical rates for the initial 11 h (Fig. 1, panel A). Since the kidney is thought to be the major tissue site of RBP catabolism in the body (1), this result implies that RBP is secreted from the liver into the blood at nearly identical rates for the two strains. Like RBP, serum retinol levels (Fig. 1, panel B) initially rise at similar rates for WT and TTR−/− mice and reach a plateau level

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**TABLE I**

Liver total retinol and RBP levels for 3-month-old inbred 129SV and outbred MF-1 wild type and TTR−/− mice

| Strain | n | Retinol | RBP |
|-------|---|---------|-----|
| 129SV |   |   |   |
| Male, TTR−/− | 7 | 443 ± 247 | 38.0 ± 5.6 |
| Male, WT | 8 | 408 ± 276 | 12.4 ± 4.1 |
| Female, TTR−/− | 11 | 459 ± 214 | 37.7 ± 11.6 |
| Female, WT | 10 | 144 ± 111 | 8.5 ± 6.1 |
| MF-1 |   |   |   |
| Male, TTR−/− | 6 | 434 ± 221 | 43.1 ± 2.4 |
| Male, WT | 6 | 436 ± 52 | 25.4 ± 5.2 |
| Female, TTR−/− | 6 | 506 ± 144 | 39.8 ± 4.5 |
| Female, WT | 6 | 230 ± 30 | 25.0 ± 2.3 |

* The n indicates the number of mice used for measurement of each parameter.

+ Purified rat serum RBP was used as the standard in the RIA for mouse RBP; thus, the mouse RBP concentrations represent “µg of rat RBP equivalents.”

+ Significantly different at the 1% level of significance from the level in WT mice of the same strain and sex.
within 12 h. Hepatic RBP levels in both WT and TTR− mice progressively declined in a time-dependent manner, whereas hepatic albumin levels remained constant (data not shown). A decline in hepatic RBP has been reported previously for nephrectomized rats (16, 17). Overall, the data from these in vivo studies are fully consistent with the in vitro cell culture experiments; both indicate that TTR does not play an essential role in RBP secretion.

By having ruled out a defect in RBP secretion, we now asked if the low circulating retinol-RBP levels in TTR− mice were caused by increased renal filtration of retinol-RBP complex, as proposed previously (1). We therefore monitored the serum levels of intravenous-injected human holo-RBP (10 μg of hRBP) in the circulation of WT and TTR− mice. This dose of hRBP increases the circulating retinol-RBP levels of WT mice by ∼40% and cannot, therefore, be considered a true tracer. However, since serum TTR concentrations are usually in 2–3-fold molar excess of those of circulating RBP (1), circulating mouse TTR should bind all the injected retinol-hRBP in WT mice. Human RBP is known to bind rodent TTR (18). Fig. 2, panel A, shows that hRBP was rapidly cleared from the circulation of TTR− but not from WT mice (t1/2 = 0.5 h and t1/2 > 6 h, respectively). Mouse RBP levels in the serum of WT mice also declined slightly over 6 h (Fig. 2, panel B). This decrease may reflect competition between human and mouse RBP for binding to TTR, the unbound RBP being lost from the circulation.

Kidney levels of hRBP rapidly rose in TTR− mice, reaching a maximum within 5–10 min after retinol-hRBP injection (Fig. 2, panel C) and then quickly declined. Much lower levels of hRBP were seen in the kidneys of WT mice, and these did not change over the period measured (Fig. 2, panel C). Mouse RBP concentrations were low in WT and TTR− mice and did not vary over the 6 h following injection (Fig. 2, panel D).

In contrast to kidney, hRBP concentrations in lung (Fig. 2, panel E), skeletal muscle (Fig. 2, panel F), and liver, heart, and fat (data not shown) of TTR− mice were consistently lower relative to wild type. These data argue against increased infiltration of the retinol-hRBP complex into the interstitial fluid in the absence of TTR.

Despite low serum retinol concentrations, TTR− mice have normal tissue total retinol levels. We asked if the ability to concentrate retinol in tissues reflected a compensatory increase in the clearance rate of chylomicron (dietary) retinoid. We injected rat mesenteric chylomicrons labeled in vivo with [3H]retinyl esters into the circulations of WT and TTR− mice. Blood samples were collected 5, 10, 30, and 60 min after injection, and the levels of chylomicron-associated [3H]retinoid were determined. No differences in chylomicron clearance rates were observed for mutant versus the WT mice (data not shown). At 10 and 60 min after injection, mice from each strain were sacrificed, and tissue [3H]retinoid levels were measured. As expected, most chylomicron retinoid was taken up by the liver (18, 19). There was no significant difference between TTR− and WT mice in liver [3H]retinoid levels at either 10 or 60 min after injection. Moreover, the relative amounts of chylomicron retinoid taken up by extrahepatic tissues were similar in both WT and TTR− mice. Fig. 3 illustrates the tissue distribution of [3H]retinoid 60 min after injection. We conclude that the normal tissue retinol levels in TTR− mice cannot be accounted for by enhanced clearance of postprandial (chylomicron) retinoid.

We next asked if the distribution of retinol among plasma lipoproteins differed in WT and TTR− mice. VLDL, LDL, HDL, and d > 1.21 g/ml bottom fractions were isolated by sequential ultracentrifugation from pools of serum obtained from age- and sex-matched WT and TTR− mice. The concentration of retinol and retinyl esters in each fraction was determined (Table III). Since the mice had been fasted for ∼18 h prior to sacrifice, this retinyl ester does not arise directly from recent dietary intake. For both WT and TTR− mice, all retinol in the serum pools was

### Table II

**Hepatocyte and media concentrations of RBP**

All concentrations are given as the mean ± 1 S.D.

| Culture condition   | n  | Medium RBP | Cellular RBP |
|---------------------|----|------------|--------------|
|                     |    | ng RBP/mg of cellular protein |             |
| 10% fetal calf serum| 8  | 228 ± 43   | 306 ± 83d    |
| TTR                 | 10 | 218 ± 40   | 193 ± 41     |
| WT                  | 6  | 178 ± 31   | 353 ± 77c    |
| 5% TTR− mouse serum | 7  | 202 ± 58   | 239 ± 86     |
| TTR                 | 4  | 180 ± 47   | 290 ± 26d    |
| WT                  | 3  | 158 ± 32   | 209 ± 28     |

* The n indicates the number of independent hepatocyte isolations used in generating these values.

* Medium RBP levels are averaged over 12 h of culture. Thus, this value represents ng of RBP secreted into the medium in 12 h/mg of cellular total protein.

* Since purified rat serum RBP was employed as the standard in the RIA for mouse RBP, these values represent “ng of rat RBP equivalents.”

* Significantly different from the level in WT hepatocytes at the 5% level of significance.

* Significantly different from the level in WT hepatocytes at the 1% level of significance.

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**Fig. 1.** Effect of time after bilateral nephrectomy on serum RBP (panel A) and retinol (panel B) levels in WT (○) and TTR− (●) mice. Values represent the mean ± S.D. of 3–5 animals per time. If an error bar is not visible, it is smaller than the symbol.
FIG. 2. Effect of time on the levels of human and mouse RBP present in the circulations and tissues of WT and TTR−/− mice after receipt of a bolus intravenous dose of human holo-RBP (10 μg). Panel A, serum levels of human RBP for WT (●) and TTR−/− (○) mice after intravenous injection of the human RBP. Panel B, serum levels of mouse RBP from WT (●) and TTR−/− (○) mice after a bolus intravenous injection of human RBP. Human (panel C) and mouse (panel D) RBP levels in WT (●) and TTR−/− (○) mouse kidney after intravenous injection of human RBP. Human RBP levels in WT (●) and TTR−/− (○) mouse lung (panel E) and skeletal muscle (panel F) after intravenous injection of human RBP. The values represent the mean ± S.D. of 4 mice per time. If an error bar is not visible, it is smaller than the symbol.
recovered solely in the $d > 1.21$ g/ml bottom fraction, with an overall recovery of 85%. This was expected since retinol is bound exclusively to RBP in both WT and TTR$^{-}$ mice (5). The fasting serum for both male and female WT and TTR$^{-}$ mice at three different ages reproducibly contained low levels of retinyl esters. The level of circulating retinyl ester in WT mice (when expressed as retinol equivalents) was always less than 13% and usually less than 5% of the level of circulating retinol. Because circulating retinol levels are low in TTR$^{-}$ mice, a substantial percentage, 18–133%, of the total retinol in these mice is retinyl ester. In both strains most circulating retinyl ester is recovered in the liver-derived VLDL fraction with lesser amounts in the LDL and HDL fractions. No retinyl esters were detected in the $d > 1.21$ g/ml bottom fraction.

![Tissue Distribution of Radioactive Material](image)

**FIG. 3.** Tissue distribution of radioactive material 60 min after intravenous injection of rat chylomicrons (3 mg of triglyceride) containing [3H]retinyl ester (0.2 μCi, 12 nmol) into fasted WT (darkened bars) and TTR$^{-}$ (open bars) mice. Values are expressed as the percentage of total radioactivity recovered in each organ or tissue after normalization for the total recovery of radioactivity and are given as means ± S.D. The total recovery of radioactivity for the two strains was not different. Four animals were analyzed per strain. L, liver; K, kidney; Lu, lung; S, spleen; M, muscle; H, heart; A, adipose tissue; B, brain.

**Tissue Distribution of Radioactive Material**

**TABLE III**

| Strain                  | Retinol (μg/dl) | Retinyl ester (μg retinol/dl) | % Retinyl ester | Retinyl ester distribution (%) |
|-------------------------|-----------------|-------------------------------|----------------|-------------------------------|
|                         |                 |                               |                | VLDL | LDL | HDL | >1.21 bottom |
| 2-Month-old, male TTR$^{-}$ | 1.6             | 0.6                           | 27             | 34   | 38  | 28  | 0           |
| 2-Month-old, male WT    | 29.8            | 0.7                           | 2              | 53   | 34  | 13  | 0           |
| 2-Month-old, female TTR$^{-}$ | 2.1         | 2.8                           | 57             | 62   | 16  | 22  | 0           |
| 2-Month-old, female WT  | 24.4            | 0.6                           | 2              | 42   | 40  | 18  | 0           |
| 6-Month-old, male TTR$^{-}$ | 4.0         | 1.9                           | 32             | 40   | 29  | 31  | 0           |
| 6-Month-old, male WT    | 30.1            | 1.8                           | 6              | 63   | 27  | 10  | 0           |
| 6-Month-old, female TTR$^{-}$ | 1.6         | 1.8                           | 53             | 51   | 16  | 33  | 0           |
| 6-Month-old, female WT  | 18.2            | 2.9                           | 13             | 66   | 18  | 16  | 0           |
| 12-Month-old, male TTR$^{-}$ | 4.2           | 0.7                           | 14             | 44   | 32  | 24  | 0           |
| 12-Month-old, male WT   | 40.9            | 1.2                           | 3              | 88   | 12  | 0   | 0           |
| 12-Month-old, female TTR$^{-}$ | 3.1         | 3.3                           | 52             | 62   | 26  | 12  | 0           |
| 12-Month-old, female WT | 24.4            | 2.6                           | 10             | 58   | 27  | 15  | 0           |

*This column gives the % of the total retinol present in each serum pool that is present as retinyl ester. Thus, this value was calculated by dividing the retinyl ester concentration by the sum of the retinol and retinyl ester (as retinol equivalents) concentrations and converting to a percentage.

**DISCUSSION**

We reported earlier that the circulating levels of retinol and RBP in TTR$^{-}$ mice is ~5% that of WT (4, 5, 20). Nevertheless, the tissue total retinol (retinol + retinyl ester) levels of the TTR$^{-}$ mice were equivalent to those of WT (5). This work addresses two issues. 1) Why are the circulating retinol and RBP levels low in the mutant mice? 2) How do these mice maintain normal tissue total retinol levels in the face of low serum retinol?

Early reports describing protein-protein interactions of retinol-RBP with TTR tetramer (55 kDa) speculated that association with TTR prevents renal filtration of the small (21 kDa) RBP molecule (1, 2). This hypothesis, although reasonable, had not been verified experimentally. TTR was also suggested to facilitate RBP synthesis/secretion from liver (21–23). Studies with 4-hydroxy-N-phenylretinamide, which disrupts the RBP-TTR complex and markedly lowers circulating retinol-RBP levels, suggested that unbound circulating RBP infiltrates into the interstitial space within tissues (24). In this work, we have tested these possibilities using genetically engineered TTR-deficient mice.

Although hepatic RBP levels are, indeed, significantly elevated in TTR$^{-}$ mice (see Table I), cultured primary hepatocytes isolated from these mice accumulated RBP in the medium at control rates (Table II). Furthermore, this rate was not influenced by TTR in the culture medium. However, consistent with elevated RBP concentrations in intact liver, RBP levels in hepatocytes isolated from TTR$^{-}$ mice were significantly higher than WT controls. Whether TTR deficiency inhibits RBP secretion or whether the accumulation of RBP exceeds the secretion capacity of the hepatocyte remains to be determined. However, the accumulation of intracellular RBP in TTR-deficient hepatocytes is entirely consistent with observations that TTR facilitates RBP synthesis and/or secretion from HeLa cells (21, 22) and from human HepG2 hepatoma cells (23).

Renal failure in humans (1) and complete nephrectomy in experimental animals (16, 17) give rise to significantly elevated circulating levels of both retinol and RBP. Our studies of TTR$^{-}$ and WT mice indicate that circulating RBP levels rise at very similar rates for both strains during the first 11 h following nephrectomy (Fig. 1). Since liver is the major site of RBP
synthesis (1, 2), this in vivo result supports the physiological relevance of our RBP secretion studies in isolated TTR hepatocytes.

We showed that injected human retinol-RBP is much more rapidly cleared from the circulation of TTR-deficient than WT mice. This result demonstrates that TTR reduces the rate of renal filtration of retinol-RBP and explains the low circulating levels of retinol-RBP in TTR mice. Aside from the kidney, tissue hRBP concentrations in the mutant mice declined at rates parallel to the clearance rate of hRBP from the circulation. In addition, apart from the liver where RBP is synthesized and the kidney where RBP is filtered, mouse RBP concentrations are much lower in tissues from TTR - than WT mice. Taken together, these observations do not support the notion that TTR is required to keep circulating RBP from partitioning into the interstitial fluid of tissues.

We have not yet determined how tissues of TTR - mice maintain normal total retinol concentrations in the face of low circulating retinol-RBP concentrations, although several hypotheses are under investigation. First, an alternative retinol delivery pathway may compensate for the loss of RBP. Since lipoproteins transport retinyl esters in the circulation, it is possible that either postprandial lipoproteins (chylomicrons and their remnants) or lipoproteins of hepatic origin (VLDL and LDL) play a more important role in retinol delivery to tissues than RBP. Note that neither rate of clearance of chylomicron retinyl ester nor its delivery to tissues is elevated in tissues than RBP. Taken together, these observations do not support the notion that TTR is required to keep circulating RBP from partitioning into the interstitial fluid of tissues.

It is well established that some retinyl ester is bound to apo E in VLDL and LDLC Fractions (26, 27). Since VLDL is directly secreted by VLDL fraction (26, 27). Since VLDL is directly secreted by hepatocytes and undergoes metabolism in the circulation to VLDL, it is possible that either postprandial lipoproteins (chylomicrons and their remnants) or lipoproteins of hepatic origin (VLDL and LDL) play a more important role in retinol delivery to tissues than RBP. Note that neither rate of clearance of chylomicron retinyl ester nor its delivery to tissues is elevated in tissues than RBP. Taken together, these observations do not support the notion that TTR is required to keep circulating RBP from partitioning into the interstitial fluid of tissues.

Taken together, these observations do not support the notion that TTR is required to keep circulating RBP from partitioning into the interstitial fluid of tissues.

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