Decreases in retinol and retinol-binding protein during total parenteral nutrition in rats are not due to a vitamin A deficiency

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Abstract Perfusion feeding in rats induced a decrease in circulating retinol despite an adequate supply of vitamin A. We studied the effect of total parenteral nutrition (TPN) on the retinol specific carrier in rat, analyzing holo-RBP (bound to retinol) and apo-RBP (without retinol) in serum and in liver. Vitamin A-sufficient (A^+) and -deficient (A^-) rats were characterized in terms of vitamin A and RBP status and then perfused (TPN-A^+ and TPN-A^-) or orally pair-fed (O-A^+ and O-A^-) with vitamin A. In A^- rats, a decrease in serum retinol (2.6-fold) and an increase in apo-RBP was concomitant with a massive accumulation of RBP in the liver. In TPN-A^- rats, both circulating RBP and liver total RBP were decreased. In TPN-A^+ rats, there was a decrease in circulating retinol (2.4-fold) in parallel to a decrease of serum and liver RBP protein and mRNA. We provide evidence that infused retinyl palmitate was not responsible for serum retinol and RBP decrease and that retinol depletion was not due to vitamin A deficiency. Whatever the vitamin A status, TPN may induce in rats a down-regulation of hepatic RBP synthesis, which may, at least partially, explain the alteration of retinol and RBP in serum.—**Lespine, A., B. Periquet, S. Jaconi, M-C. Alexandre, J. Garcia, J. Ghisolfi, J-P. Thouvenot, and G. Siegenthaler.** Decreases in retinol and retinol-binding protein during total parenteral nutrition in rats are not due to a vitamin A deficiency. *J. Lipid Res.* 1996. **37:** 2492–2501.

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Vitamin A (retinol) is transported from the liver stores to target tissues by a specific transport protein of 21 kDa: the retinol-binding protein (RBP) (for a review see ref. 1). RBP is secreted in the plasma bound to retinol: holo-RBP, in a molar ratio of 1/1 (2). In humans with a normal vitamin A status, most RBP circulates bound to retinol (holo-RBP) whereas only 2% was found free from retinol (apo-RBP) (3). The holo-RBP strongly interacts with transthyretin (TTR), also called prealbumin, a complex that reduces the glomerular filtration and renal catabolism of RBP. The liver parenchymal cells are the major site for RBP synthesis (4). However, expression of RBP has been found in numerous extrahepatic tissues (5–7). The RBP level in plasma is highly regulated and remains constant except in extreme vitamin A status. Vitamin A deficiency alters circulating RBP and blocks its secretion from the liver (2, 8), and large oral doses of vitamin A in rats resulted in a decrease of serum and liver RBP (9). Moreover, a depressive effect on serum retinol has been reported after retinoic acid injection or feeding (10, 11), after 2,3,7,8-tetrachlorodibenzo-*p*-dioxin treatment (12), and after total parenteral nutrition (13, 14). By contrast, in acute renal failure, serum retinol was increased, associated with an increase in circulating holo- and apo-RBP (15–17). Such abnormalities with serum retinol and RBP are certainly of different origins, depending on the pathology being considered or the model studied.

Total parenteral nutrition (TPN) is an intravenous supply of nutrients and micronutrients used for patients who are unable to tolerate oral feeding. Little is known about the metabolism of nutritional compounds during TPN, and controversial data exist concerning the fate of vitamin A when given intravenously. Studies in humans receiving TPN reveal the difficulty to define adequate vitamin A needs and formulation during intravenous feeding (18). In adults after long term TPN, low serum retinol was reported (13). Studies in preterm infants showed that, in spite of an adequate amount of vitamin

Abbreviations: A^+, vitamin A-sufficient rats; A^-, vitamin A-deficient rats; ALT, alanine aminotransferase; apo-RBP, retinol-binding protein without retinol; AST, aspartate aminotransferase; holo-RBP, retinol-binding protein bound to retinol; HPLC, high performance liquid chromatography; O-A^+ and O-A^-, control per os rats; RBP, retinol-binding protein; TPN, total parenteral nutrition; TPN-A^+ and TPN-A^-, rats intravenously fed.

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TABLE 1. Comparison of diets provided to per os and intravenously fed rats

Variable	Before Experiment		During Experiment	
	A^+ Rats	A^- Rats	Perfused Diet	Oral Diet
Energy supply ($kJ \cdot kg^{-1}$ rat $\cdot day^{-1}$)	1166	1192	1180	1166
Nutrient distribution (% of total energy)				
Lipid	12	12	12	12
Protein	67	67	67	67
Glucose	21	21	21	21
Retinyl palmitate (μmol RE $\cdot kg^{-1}$ rat $\cdot day^{-1}$)	1.66[a]	0	1.33	1.66[a]

Before the experiment the rats were fed per os during 4 weeks with a solid semisynthetic diet. During the experiment perfused rats (TPN-A) received the nutritive solution for 5–7 days. Pair-fed controls (O-A) received orally a solid semisynthetic diet supplying the same amount of energy and vitamin A.

[a]Taking into account that intestinal absorption of vitamin A in rats was 80% (ref. 22), oral diet contained 20% more vitamin A.

A perfused in the liquid diet during TPN, the serum retinol and liver reserves of vitamin A remained low (19). Animal models have been developed to study the fate of vitamin A during intravenous feeding and conflicting data have been reported, certainly due to differences in TPN protocols (14, 20). No experimental data are available concerning the origin of the abnormalities in vitamin A metabolism occurring during TPN.

We have developed an experimental model of parenteral feeding in the rat in order to follow the metabolism of intravenously administrated vitamin A. In our model, vitamin A was supplied as retinyl palmitate combined with lipid emulsions as this form is more stable (21) and supposed to be closer to the physiological carrier, the chylomicron remnants, compared to the unstable retinol formulation: MVI paediatric (Multi Vitaminic Infusion). We confirmed our previous observations that TPN in rats induced a rapid and dramatic decrease in circulating retinol (14). The goal of the present study was to analyze the mechanisms involved in the serum retinol decrease. As patients undergoing TPN often have impaired intestinal absorption, leading in some cases to vitamin A deficiency and most of the time to low vitamin A status, rats used in this study are representative of vitamin A-deficient rats (A^-) and vitamin A-sufficient rats (A^+). We studied how TPN altered serum retinol levels, and investigated the relation with total RBP decrease in serum and in liver, together with the change in RBP profile in serum.

MATERIAL AND METHODS

Animals and diet

Weaning male Wistar rats were maintained at 25°C with a 12-h light–dark cycle and given free access to food and water. From their arrival to 4 weeks, rats were assigned to a semisynthetic diet purchased from UAR laboratory (Villemoisson-sur-Orge, France). In the A^+ group, rats received a diet supplemented with 15.6 μmol retinyl palmitate $\cdot kg^{-1}$ of diet. In the A^- group, rats received a vitamin A-deficient diet. The vitamin A content of each diet was analyzed before use: food was digested overnight in 10% potassium hydroxide and retinol was extracted and measured by HPLC as described in the following paragraph.

Total parenteral nutrition

Rats from A^- and A^+ groups were either perfused (TPN-A^- and TPN-A^+), or orally fed (O-A^- and O-A^+). The rats were anesthetized (Ketamine, 2.5 mg $\cdot kg^{-1}$) and surgery was performed in aseptic conditions. A Silastic catheter (Sigma Medical, Nanterre, France) was inserted into the right jugular vein, and exteriorized at the nape of the neck via a subcutaneous tunnel. The rats were maintained 24 h under continuous infusion of 0.9% saline via volumetric infusion pumps. Flow rate was initially 1.0 ml $\cdot hr^{-1}$ and progressively increased to 2.4 ml $\cdot hr^{-1}$. During that time the rats had access to vitamin A-deficient food. From the second day, they were not allowed to drink or eat. They were perfused with a liquid diet (228 ml $\cdot kg^{-1} \cdot day^{-1}$) made with the following solutions, per 100 ml of liquid diet: glucose 50%: 28.3 ml, Hyperamin 30%: 33.8 ml, Endolipid 20%: 5.9 ml (Bruneau, Paris, France), Nonan for trace elements, 2.9 ml (Aguettant, Lyon, France), Vitalipid Infant: 3.6 ml and Soluvit: 1.2 ml (Kabi Vitrum, Stockholm, Sweden). Total caloric intake was 280 kcal $\cdot kg^{-1}$ rat $\cdot day^{-1}$, with an energy distribution for glucose–protein–lipid of 65:23:12 (**Table 1**), reproducing the per os diet. The nutritive solution was prepared under sterile conditions and changed every 48 h. In our perfusion system, we used Vitalipid as retinyl palmitate source, combined with Endolipid 20%. Such a formulation was shown to be stable in the perfusion system (21). More-

TABLE 2. Effect of TPN on serum biochemical parameters and body weight in A^- and A^+ rats

Rats	Protein	Creatinine	AST	ALT	Body Weight
	$g \cdot l^{-1}$	$\mu mol \cdot l^{-1}$	*IU*	*IU*	*g*
A^+	53.5 ± 0.8 (4)	43.4 ± 1.4 (8)	120.7 ± 12.7 (8)	55.2 ± 6.6 (8)	221 ± 4 (15)
TPN-A^+	50.1 ± 3.0 (4)	47.0 ± 2.5 (5)	107.5 ± 23.5 (5)	82.2 ± 50.7 (5)	233 ± 8 (13)
O-A^+	55.0 ± 1.08 (4)	42.7 ± 3.3 (3)	100.0 ± 7.0 (3)	44.0 ± 3.5 (3)	254 ± 3 (15)
A^-	56.0 ± 2.2 (2)	36.2 ± 1.1 (6)	77.8 ± 5.4 (6)	62.2 ± 9.8 (6)	204 ± 7 (13)
TPN-A^-	51.4 ± 1.9 (5)	53.4 ± 5.3 (7)	65.6 ± 23.8 (5)	185.6 ± 71.9 (5)	225 ± 6 (7)
O-A^-	53.6 ± 0.8 (9)	41.8 ± 2.2 (9)	121.2 ± 20.0 (9)	55.1 ± 4.2 (9)	229 ± 8 (9)

Standard rats were fed 4 weeks with a semisynthetic diet with or without vitamin A (15.6 µmol retinyl palmitate per kg diet), the maximal energy intake being 1200 kJ · kg^{-1} · day^{-1} (A^- and A^+). TPN-A^- and TPN-A^+ rats were perfused during 5–7 days. During the same period of time as the perfused animals, per os controls received orally a semisynthetic solid diet (O-A^- and O-A^+, see Table 1). Data are expressed as mean ± SEM; n, number of animals in parentheses. AST, aspartate aminotransferase; ALT, alanine aminotransferase.

over, we have checked that liposoluble vitamins (A and E) in the nutritive solution were stable and a loss of only 15% was noted in our experimental conditions. The liquid diet supplied 1.33 µmol retinyl palmitate · kg^{-1} · day^{-1}. Rats were maintained under intravenous feeding during 5 to 10 days.

In parallel, pair-fed control rats (O-A^- and O-A^+) received per os a semisynthetic diet containing exactly the same amount and distribution of energy, trace elements, and vitamins provided to rats during perfusion. Control rats received per os 1.66 µmol retinyl palmitate · kg^{-1} · day^{-1}. Considering that the intestinal absorption of retinyl palmitate was 80% (22), they received 20% more than perfused animals. In previous experiments, control per os rats were submitted to a surgical operation similar to that performed on the perfused animals (20). They were anesthetized, the jugular vein was cleaned, and the subcutaneous tunnel was perforated. Those sham-operated rats recovered rapidly from the surgery and presented the same retinol and RBP profile as non-operated rats. Consequently, we did not perform surgery on control rats.

Animal health evaluation

Biochemical parameters and body weights were monitored in rats before and after experiment. Proteins, creatinine, alanine aminotransferase, and aspartate aminotransferase were analyzed in serum on a Hitachi 717 (Hitachi, Tokyo, Japan) in the biochemistry laboratory at the Purpan Hospital (Toulouse, France). These parameters revealed that the main kidney and liver functions were not altered during vitamin A deficiency nor during TPN (**Table 2**). As expected vitamin A deficiency in rats induced a lower growth rate, and at the time the experiment started, A^+ rats weighed 221 ± 4 g versus 204 ± 7 g for A^- animals (Table 2). During TPN body weight increased at an average rate of 3 g per day in both groups.

Urine was collected every day during perfusion and analyzed for proteins, glucose, cetonic bodies, blood, pH (Ames reagent strips, Miles Laboratory, Elkhart, IN). These parameters remained in the normal range during the experimental procedure.

Blood and liver collection

Rats were slightly and rapidly anesthetized with ether and blood was immediately collected by abdominal aorta. Serum was obtained after 15 min centrifugation at 2000 *g*. Livers were collected on ice, cleaned of blood, weighed, and homogenized as described below.

Vitamin A extraction and analysis

Extraction and analysis of vitamin A from serum and liver was performed according to Periquet et al. (23). Briefly, liver was homogenized with a Potter-Elvehjem homogenizer, in 9 volumes of buffer (0.25 M sucrose; 0.05 M Tris-HCl, pH 7.6; 0.025 M KCl; 0.005 M $MgCl_2$). Serum or liver homogenate was mixed with one volume (v/v) of ethanol solution containing 10 mg · l^{-1} retinyl acetate, used as an internal standard. Samples were extracted with 5 volumes of n-hexane. After centrifugation, 500 µl of the upper phase was evaporated under a nitrogen stream and subdued light, dissolved in methanol, and injected into HPLC system. High-performance liquid chromatography was carried out on a Philips Model 4100 programmable liquid chromatography system. Column was a Spherisorb ODS 2, 3 µm, 25 cm (Chromato-Sud, Bordeaux, France). Chromatography was performed at 50°C with methanol as solvent. The detecting wavelength was 325 nm.

RBP analysis

Liver was homogenized in a buffer supplemented with 5 mM EDTA, 1 mM phenyl methyl sulfonyl fluoride, 2 mM dithiothreitol, 10 μM aprotinin, and 20 μM leupeptin. Proteins were assayed using the method of Lowry et al. (24). For circulating RBP analysis, serum was run on PAGE, 7.5% acrylamide. These nondenaturing conditions have been extensively used to separate holo- from apo-RBP in several studies and this procedure was shown to be reproducible and quantitative (3, 17). Proteins from liver homogenate were separated on SDS-PAGE, 15% acrylamide. These denaturing conditions had to be used for liver RBP detection in order to facilitate further immunoblotting analysis (17). Several amounts of liver homogenate were run in parallel and the linearity of the band intensity was obtained for a gel loading between 0.1 and 0.3 mg total proteins. This technique allowed us to detect a 21 kDa band corresponding to total liver RBP dissociated from retinol: apo-RBP. Previous works have shown that this form represented 90% of total RBP in human liver (3). RBP bands were visualized with rabbit anti-rat RBP serum and horseradish peroxidase-labeled goat anti-rabbit IgG using diaminobenzidine as substrate. Band intensity of immunodetected proteins was read with a Microtech II HR scanner and analyzed with the NIH-Imager program.

RNA isolation and northern blot analysis

Total RNA was isolated from liver using the Chomczynsky and Sacchi procedure (25). RNA separation and hybridization procedures were similar to those described by Tsutsumi et al. (7). In brief, total RNAs (20 μg) were run on agarose 1.2%/ formaldehyde 1.1% gel electrophoresis and transferred to a nylon membrane. Staining of the nylon membrane was performed with methylene blue and the comparison of the 28 S and 18 S ribosomal RNAs allowed us to conclude that the RNA loads were similar. The cDNA for rat RBP was an Eco R1 insert of 546 bp in pGem4, kindly provided by Dr. William S. Blaner (Columbia University, New York). Specific RBP mRNA were hybridized with ^{32}P-probe prepared with [^{32}P]dCTP (ICN Pharmaceuticals, Inc., Irvine, CA) by random primed labeling using a commercial kit (Boehringer-Mannheim, Germany). Blots were washed twice in 2 × SSC, 0.1% SDS at 42°C, 20 min. Detection was performed by exposition to XAR-2 film at −80°C using an intensifying screen.

Statistical analysis

The Bartlett test was used to test homogeneity of variance of the two starting groups (A^- and A^+). When variances were homogeneous, mean differences were compared by Student's *t* test. Differences among the six groups (A^-, A^+, TPN-A^-, TPN-A^+, O-A^- and O-A^+) were evaluated by analysis of variance (one-way ANOVA). When ANOVA indicated statistically significant differences ($P < 0.05$), mean values were ranked by the Tukey's test.

RESULTS

Effect of vitamin A deficiency on vitamin A status and RBP levels

We showed in A^+ rats that RBP circulated mainly as holo-RBP (90%), whereas apo-RBP represented only 10% of total RBP (**Fig. 1A,** lanes 1, 2, 3, three different rats). Then, vitamin A was detected in serum only as retinol at a concentration of 1.51 ± 0.17 μmol · l^{-1} for A^+ rats (**Table 3**).

In our conditions of vitamin A deficiency, total RBP tended to decrease in serum (NS, **Table 4**). In the meantime, the pattern of circulating holo- and apo-RBP was significantly modified (Fig. 1A, lanes 4, 5, 6, three different rats) with a major increase in apo-RBP and a decrease in holo-RBP ($P < 0.001$ and $P < 0.05$, respectively, Table 4). This was associated with a decrease in circulating retinol from 1.51 ± 0.17 to 0.59 ± 0.14 μmol · l^{-1} ($P < 0.001$, Table 3).

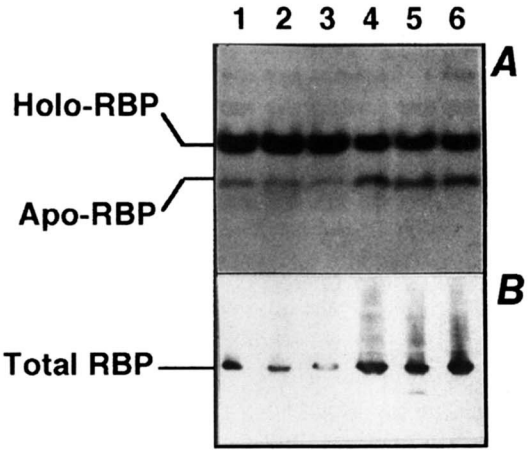

Fig. 1. Immunoblotting analysis of serum and liver RBP from standard rats. A: PAGE-immunoblotting of serum holo-RBP and apo-RBP: sera from A^+ rats (lanes 1, 2, 3, three different animals) or A^- (lanes 4, 5, 6, three different animals) were run on PAGE in 7.5% acrylamide. Electrophoresis performed in such nondenaturing conditions allowed us to detect the form of RBP bound to retinol: holo-RBP and the form free from retinol: apo-RBP. B: SDS-PAGE immunoblotting of liver RBP: livers of A^- rats (lanes 1, 2, 3) or A^+ rats (lanes 4, 5, 6) were homogenized and supernatant was run on SDS-PAGE in 15% acrylamide. These denaturing conditions allowed us to detect total RBP.

TABLE 3. Effect of TPN on serum retinol levels

Rats	Retinol	n
	$\mu mol \cdot l^{-1}$	
A^+	1.51 ± 0.17	17
TPN-A^+	0.63 ± 0.11[a]	11
O-A^+	1.42 ± 0.20	11
A^-	0.59 ± 0.14[a]	15
TPN-A^-	0.64 ± 0.11[a]	10
O-A^-	1.37 ± 0.10[b]	10

Rats were fed for 4 weeks with a semisynthetic diet with (A^+) or without (A^-) vitamin A, and then were placed on TPN with a solution containing 1.33 μmol retinyl palmitate · kg^{-1} · day^{-1} for 5–10 days (TPN-A^- and TPN-A^+). Per os control rats were pair-fed and received solid food supplying 1.66 μmol retinyl palmitate · kg^{-1} · day^{-1} (O-A^- and O-A^+). Blood was drawn 8 h after TPN or fasting for retinol measurement. Data are expressed as mean ± SEM of 10–17 experiments.

[a] $P < 0.001$ compared to A^+ rats.

[b] $P < 0.001$ compared to A^- rats.

In the liver of A^- rats, a massive accumulation of total RBP was observed (Fig. 1B, lanes 4, 5, 6, three different rats). At that stage of deficiency, retinyl esters in rat liver were 0.39 ± 0.02 nmol per g tissue (compared to 0.51 ± 0.02 μmol · g^{-1} in A^+ livers), and no retinol was detected (compared to 5.6 ± 0.2 nmol · g^{-1} in A^+ livers), reflecting an almost complete depletion of hepatic vitamin A.

Effect of intravenous feeding on vitamin A-deficient rats

When A^- rats, with low levels of circulating and hepatic vitamin A, were supplied intravenously with retinyl palmitate by TPN, the level of circulating retinol remained low (Table 3), even after 10 days of perfusion. By contrast, in O-A^- rats serum retinol was restored to a normal value shortly after per os supply of retinyl palmitate. It is noteworthy that the main circulating form of vitamin A was retinol in all experimental conditions, as animals were starved before serum analysis.

TABLE 4. Pattern of holo- and apo-RPB in standard A^+ and A^- rat serum

Rats	Holo-RPB	Apo-RPB	Total RPB
A^+ (n = 8)	1075 ± 83 (89.3 ± 1.2%)	129 ± 22 (10.7 ± 1.2%)	1204 ± 61
A^- (n = 6)	578 ± 151[a] (53.6 ± 6.9%)	401 ± 33[b] (46.4 ± 6.9%)	979 ± 162

Rats were fed for 4 weeks with either a vitamin A-containing diet (15.6 μmol retinyl palmitate · kg^{-1} · day^{-1}, A^+) or with a vitamin A-deficient diet (A^-). Holo- and apo-RBP were measured in serum by PAGE immunoblotting under nondenaturing conditions. Values were obtained by scanning the intensity of the immunodetected band. Values in parentheses are the relative proportion of holo- and apo-RBP. Results are expressed in arbitrary intensity units and are mean ± SEM of 6–8 animals.

[a] $P < 0.05$; [b] $P < 0.001$ comparing A^+ and A^- animals.

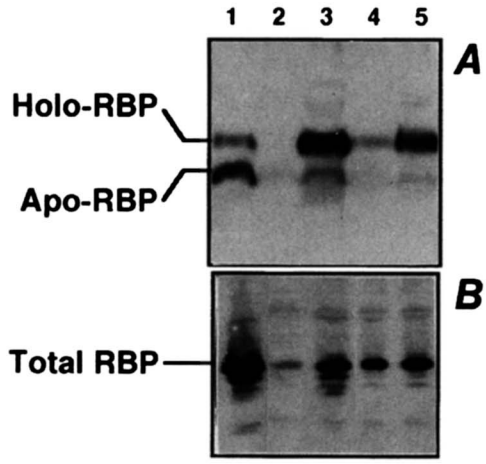

Fig. 2. Immunoblotting analysis of serum and liver RBP of A^- rats: effect of TPN supplying vitamin A. A^- rats were either submitted to TPN including vitamin A or fed with a solid semisynthetic diet with a similar amount of vitamin A. A: Holo-RBP and apo-RBP in serum: sera run on PAGE, 7.5% acrylamide as described in Fig. 1; lane 1, A^- rats; lane 2, TPN-A^-, 5 days of perfusion; lane 3, O-A^-, oral pair-fed control; lanes 4 and 5, experiments similar to 2 and 3, but with 7 days of TPN. B: RBP in liver: liver homogenates were run on SDS-PAGE in 15% acrylamide as in Fig. 1B. Two experiments were representative of 9: lane 1, A^- rats; lane 2, TPN-A^-; lane 3, O-A^-; lanes 4 and 5 same experiment as 2 and 3.

The pattern of serum RBP in O-A^- (**Fig. 2A,** lanes 3 and 5, two experiments), became rapidly similar to A^+ rats, presented in lanes 1, 2, 3 of Fig. 1A. We confirmed that the starting A^- rats used for this experiment had mainly circulating apo-RBP (Fig. 2A, lane 1). In TPN-A^- rats, a decrease in circulating total RBP was observed, the most altered form being apo-RBP (Fig. 2A, lanes 2 and 4). Levels of total RBP correlated significantly with the concentration of circulating retinol ($r = 0.95$, $n = 9$).

Lane 1 in Fig. 2B showed the accumulation of RBP in A^- rat liver. In liver from O-A^- rats, RBP was decreased to a normal value (Fig. 2B, lanes 3 and 5). In TPN-A^- (Fig. 2B, lanes 2 and 4, showing two similar experiments representative of nine) liver total RBP was decreased to a larger extent than in O-A^- rats, their oral pair-fed controls. Minor immunoreactive bands appearing under RBP were degradation products that can be easily avoided by adding antiproteases to the homogenization buffer. Upper band intensities were attenuated and the apparent molecular weight did not correspond to RBP aggregates, suggesting a nonspecific binding of the antibody. **Table 5** shows five similar experiments, comparing vitamin A and RBP status in O-A^- and TPN-A^-. A high variability of RBP decrease was observed in serum. These results revealed that a strong decrease in hepatic RBP during TPN was associated with a profound change in serum retinol and with a

TABLE 5. Effect of TPN on vitamin A status and RBP levels in A^- rats

Experiment	Serum Retinol	Liver Retinol	Liver Retinyl Esters	Rate of Decrease of RBP: In Serum: Holo-	In Serum: Apo-	In Liver
	$\mu mol \cdot l^{-1}$	$nmol \cdot g^{-1}$	$nmol \cdot g^{-1}$		*-fold*	
Exp. 1						
TPN-A^-	0.10	2.11	25.0	14	7	4
O-A^-	1.16	6.12	32.5			
Exp. 2						
TPN-A^-	0.28	1.93	67.9	4	none detected	1.4
O-A^-	1.16	4.64	115.6			
Exp. 3						
TPN-A^-	0.24	7.04	91.7	3	1.3	1.1
O-A^-	1.55	14.08	75.9			
Exp. 4						
TPN-A^-	0.77	2.74	106.7	nd	nd	1.2
O-A^-	1.27	2.81	38.3			
Exp. 5						
TPN-A^-	0.74	11.62	64.8	1.5	1.5	no change
O-A^-	1.62	12.32	79.6			

Vitamin A in serum and in liver was measured by HPLC. Results are expressed as µmol or nmol retinol or as nmol retinyl esters per liter serum or per gram liver. RBP in plasma and in liver was detected by PAGE and SDS-PAGE immunoblotting analysis, respectively. Bands were scanned with a densitometer; nd, not determined.

decrease in liver retinol, the threshold being when serum retinol went below 0.35 µmol · l^{-1}.

Effect of intravenous feeding on vitamin A-sufficient rats

In TPN-A^+, retinol in serum was reduced to 0.63 ± 0.10 µmol · l^{-1} (Table 3). In parallel, total RBP was decreased to the same extent as retinol, the ratio of control /TPN being 1.65 ± 0.20 for total RBP, and 1.98 ± 0.28 for retinol, mean ± SEM of 5 experiments. There was a strong correlation between serum retinol levels and total RBP in TPN-A^+ rats (r = 0.94, n = 6). **Figure 3A** shows, on SDS-PAGE, that total circulating RBP was decreased during TPN (lane 1, one experiment representative of 5). In parallel, nondenaturing conditions were performed and revealed that TPN affected both apo- and holo-RBP to the same extent (not shown), inducing the almost complete disappearance of apo-RBP.

In liver from A^+ rats, TPN altered RBP content (Fig. 3B, lane 1, one experiment representative of 3) as already observed in A^- rats. A cDNA probe specific for rat RBP mRNA was used to detect the amount of RBP mRNA in A^+ livers. **Figure 4** reveals that the steady state of specific RBP mRNA was decreased after TPN (lane 1 and 3) compared to per os fed animals (lane 2 and 4, two experiments). Two bands were detected on the northern blot, using low stringency conditions to wash the hybridized filter. However, thus far, only one mRNA transcript has been described for RBP and the presence of these two bands could be due to a residual RNA base-pairing, formed during our experimental procedure.

Intravenous feeding without retinyl palmitate

To determine the involvement of retinyl palmitate in the alteration of serum retinol and RBP levels noticed during TPN, A^+ rats were placed on TPN as described above but retinyl palmitate was omitted from the liquid diet. In those rats, serum retinol concentration was dramatically decreased to 0.28 ± 0.03 µmol · l^{-1} (n = 4) together with both forms of serum RBP (**Fig. 5A,** lane 1, one experiment representative of 4). In parallel, their

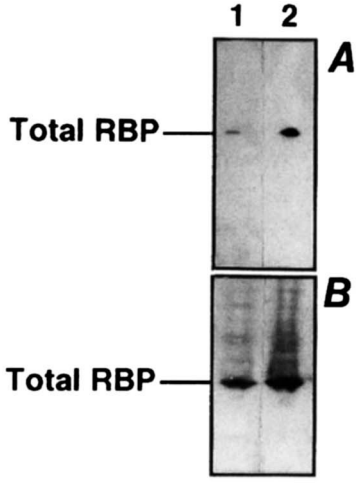

Fig. 3. Immunoblotting analysis of serum RBP from TPN-A^+ rats. A^+ rats were submitted to 5 days of TPN containing vitamin A. A: sera were run on SDS-PAGE, 15% acrylamide in order to detect total RBP. B: livers were homogenized and total RBP was revealed on SDS-PAGE immunoblotting analysis, one experiment representative of 5. Lane 1, TPN-A^+ rats; lane 2, O-A^+ rats.

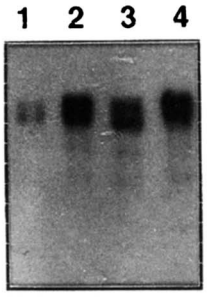

Fig. 4. Northern blot analysis of RBP mRNA in A^+ liver. Liver total RNA was prepared from A^+ liver and were hybridized with cDNA probe of rat RBP. Two experiments were performed: lanes 1 and 3, TPN-A^+; lanes 2 and 4: O-A^+.

pair-fed controls received per os a vitamin A-deficient diet during 5 days, and no change in circulating retinol or RBP was noticed (Fig. 5A, lane 2). Figure 5B represents the RBP from liver of those rats under TPN (lane 1). A strong decrease in RBP was observed, whereas in liver from rats fed a vitamin A-deficient diet per os, RBP remained normal (Fig.5, lane 2).

DISCUSSION

In this study we confirmed that vitamin A deficiency in rats induced a decrease in total RBP in serum, associated with an accumulation of liver RBP. In parallel, we noticed in A^- rats a pronounced increase of apo-RBP in serum (Fig. 1 and Table 4). Furthermore, after vitamin A per os feeding of A^- rats, we showed that the recovery of serum retinol level (Table 3) was associated not only with a normalization of total liver RBP but also with a normalization of holo- and apo-RBP profile in serum (Fig. 2). The increase in apo-RBP and the accumulation of RBP in the liver during vitamin A deficiency certainly play a major role in the rapid mobilization of newly absorbed retinol. Previous works have shown that vitamin A deficiency did not affect hepatic RBP biosynthesis (5, 26), but altered hepatic RBP secretion (27). Furthermore, RBP was found to accumulate in the endoplasmic reticulum of hepatocytes, due to a defect in intracellular transport of the newly synthesized RBP between the reticulum endoplasmic and Golgi apparatuses (28, 29). This blockage was reversed by per os vitamin A feeding (30).

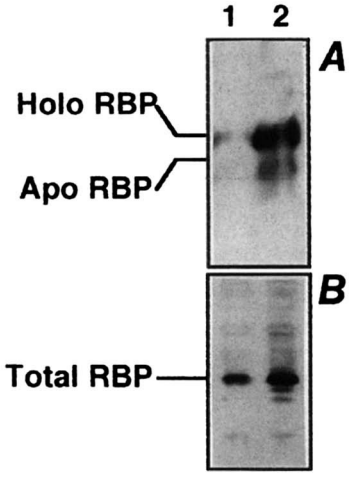

Fig. 5. Immunoblotting analysis of serum and liver RBP from A^+ rats: effect of TPN without vitamin A. Experiment was done as in Fig. 2 on A^+ rats. A^+ rats were submitted to TPN for 5 days in the absence of vitamin A. Their pair-fed controls received a solid diet deficient in vitamin A for the same period. A: Holo-RBP and apo-RBP in serum: serum was run on PAGE immunoblotting, 7.5% acrylamide. One experiment was representative of 4. Lane 1, A^+ after TPN without vitamin A; lane 2, orally pair-fed control. B: RBP in liver: liver supernatants were run on SDS-PAGE immunoblotting, 15% acrylamide. Lane 1, A^+ rat after TPN without vitamin A; lane 2, A^+ rat pair-fed 5 days per os with vitamin A-deficient diet.

When vitamin A was given by the intravenous route to A^- rats, we showed that apo- and holo-RBP amounts were dramatically lowered in the serum and RBP was decreased in the liver to a lower extent than in A^+ rats or in O-A^- rats (Fig. 2). When TPN was performed in A^+ rats, a dramatic decrease in serum retinol was correlated to a decrease in total serum RBP (Fig. 3), corresponding to a massive decrease of both holo- and apo-RBP. **Table 6** summarizes the effects of TPN performed in A^+ rats and A^- rats on retinol and RBP in serum and in liver and points out that TPN-A^+ and TPN-A^- had similar RBP and retinol profiles. It has been suggested by Gerlach and Zile (16) that circulating apo-RBP might act as a feedback signal favoring the release of retinol into the circulation. The loss of apo-RBP during TPN might be involved in the decrease in serum retinol. Low levels of vitamin A have been described in patients submitted to TPN (13). Then a vitamin A deficiency was suspected. Several reports have mentioned an important loss of vitamin A occurring during the perfusion, due to adherence to the tubing and/or the photodegradation (31). In these studies vitamin A was provided as retinol in an aqueous multivitamin infusion (MVI). We used here retinyl palmitate complexed with lipid emulsions, this formulation being shown to be more stable and not absorbed into the PVC tubing (21, 32). Our results clearly show that the mechanisms by which retinol and RBP were diminished in serum during continuous intravenous feeding were distinct from those involved in the vitamin A deficiency.

Upon per os feeding, most of the retinyl palmitate is cleared from the circulation by the liver. However, extrahepatic tissues might also be involved and bone marrow has been shown to play a significant role in retinyl palmitate clearance (33). During TPN, retinyl pal-

TABLE 6. Summary of effects observed during TPN in rats on retinol and RBP

	Serum			Liver	
Rats	Retinol	Holo-RBP	Apo-RBP	Retinol	Total RBP
A^+	↔	↔	↔	↔	↔
TPN-A^+	↘	↘	↘	↘	↘
O-A^+	↔	↔	↔	↔	↔
A^-	↘	↘	↗	↘	↗↗
TPN-A^-	↘	↘	↘	↘	↘
O-A^-	↔	↔	↔	↔	↔

mitate was perfused associated with lipid emulsions (Vitalipid, Kabi Vitrum) in order to mimic endogenous chylomicron remnant particles. However, little is known about retinyl palmitate uptake by the tissues during intravenous feeding. A direct uptake of the lipid emulsions by extrahepatic tissues might provide enough vitamin A to these tissues to cover their needs, rendering vitamin A liver reserves useless. The issue might be that normal transport of retinol was disturbed and we suggest that retinyl palmitate could be a signal that modifies retinol homeostasis. In order to check this point, we performed TPN in the absence of retinyl palmitate. A decrease in serum retinol was reproduced, in parallel to serum and hepatic RBP decrease (Fig. 5), as in perfusion supplying vitamin A. It is interesting to notice that in A^+ rats fed per os during 5 to 10 days with a vitamin A-depleted diet, circulating retinol and RBP were not altered, nor liver RBP or vitamin A reserves. Moreover, studies from our laboratory showed that vitamin A content in extrahepatic tissues was the same during TPN or per os feeding. Altogether these data provide evidence for the presence of a signal other than retinyl palmitate, being involved in retinol and RBP alterations during TPN. However, we cannot also exclude that the interruption of the enterohepatic vitamin A cycle during TPN may be implicated in the disturbance of vitamin A homeostasis.

In transthyretin-knock-out mice (34) and in rats injected intravenously (16) or orally fed (11) with retinoic acid, a decrease in serum retinol has been associated with an increase in circulating retinoic acid. Retinoic acid accumulating in serum might provide a sustained stimulus to the liver, signalling that no more retinol needs to be released (10). Thus, in our model, measurement of retinoic acid might provide interesting information on the origin of the abnormalities observed. Other nutrients, such as glucose or lipids, supplied intravenously during TPN might also be involved in regulating vitamin A. Numerous studies referred to the role of nutrients in the regulation of proteins at genomic levels. For example, activity, synthesis, and mRNA levels of fatty acid synthetase are highly and directly regulated by glucose (35). Such effects of perfused nutrients must be considered and further explored.

We have clearly shown that RBP decrease in the liver is paralleled by a slowdown in RBP translation, due to a decrease in the steady state level of RBP mRNA (Fig. 4). The decrease in RBP occurring during TPN might be due to a transcriptional down-regulation or to an increased degradation rate of liver mRNA.

The acute phase of protein or energy malnutrition (36) and zinc deficiency (37) have been shown to modify vitamin A metabolism affecting hepatic RBP synthesis. In our model, perfused rats received enough energy to increase their body weight, and were supplied with 0.66 mg $\cdot$ kg^{-1} $\cdot$ day^{-1} of zinc. This amount is in the range to maintain zinc in serum (38). However, little is known about zinc requirements during TPN and studies in humans receiving TPN revealed zinc metabolism abnormalities (39, 40). Moreover, Smith (41) has emphasized the metabolic relationship between zinc and vitamin A during inflammatory diseases. Inflammation was reported to induce systemic alterations in trace metal metabolism such as zinc (42), and to alter vitamin A transport and RBP mRNA production (43). Thus, in our model, inflammation response may be a confounding factor influencing RBP synthesis, as suggested by Rosales and Ross (44) in HIV-positive patients. Surgical stress in patients was shown to decrease retinol, RBP, and TTR in serum (45). These effects were reversed 4 days after surgery. In our experiments, TPN was performed over a period of 5 days, this period of time being shown to be necessary to recover from surgery in rats under TPN (46). As infections also induce a decrease of the negative acute phase proteins, we performed bacteriological analysis in serum and tissues of the TPN rats, revealing that they are free from infection. However, we cannot exclude an inflammation of unknown origin.

An increase in RBP utilization by tissues might contribute to the decrease in RBP in serum. A rise in RBP mobilization from liver associated with a decrease in serum RBP should be accompanied by a rise in the utilization or elimination of retinol and RBP. Free fatty acids

have been shown to increase retinol uptake and metabolism in HepG2 and other cell systems (47). During TPN in humans, in situ lipolysis of perfused lipids generates an increase in circulating free fatty acids (48). They may increase uptake of retinol by tissues, induce an accumulation of apo-RBP in serum, and favor its elimination by the kidneys. This could explain, at least partly, the retinol and RBP decrease in serum during TPN.

Intravenous feeding in rats modifies vitamin A metabolism, inducing a strong decrease in circulating retinol, correlated with a decrease in circulating RBP. These effects are observed whatever the vitamin A status of the perfused animals. Moreover, the liver RBP protein and the steady state of RBP mRNA were decreased in perfused rats. RBP pattern in serum and in liver was different from that in vitamin A-deficient animals. Retinyl palmitate circulating continuously during TPN was not involved in the down-regulation of RBP. More studies are needed to elucidate the involvement of other nutrients, such as zinc or lipids, and other mechanisms.

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