

The Metabolic Availability of Vitamin A Is Decreased at the Onset of Diabetes in BB Rats^{1,2}

Jing Lu, Walter T. Dixon, Andrew T. C. Tsin* and Tapan K. Basu³

Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, AB, Canada T6G 2P5 and *Division of Life Sciences, The University of Texas at San Antonio, San Antonio, TX 78249-0662

ABSTRACT Streptozotocin (STZ)-induced diabetic rats have been associated with an impaired metabolic availability of vitamin A (retinol). This study was undertaken to investigate whether Biobreeding (BB) rats, in which diabetes mellitus resembling human type I diabetes develops spontaneously, respond the same way at the onset of diabetes. Weaning diabetes-prone (BBdp) and normal (BBn) BB rats consumed NIH-07 nonpurified diet ad libitum until 120 d of age. Plasma and hepatic concentrations of retinol and its carriers, retinol-binding protein (RBP) and transthyretin (TTR) were lower in diabetic BB (BBd) rats than in BBn rats. In parallel with RBP, the abundance of mRNA was lower in the liver of BBd rats. Furthermore, the status of zinc, an important factor for the synthesis of RBP, was also disturbed in BBd rats, as indicated by lower circulatory levels and greater urinary excretion. To determine whether the biochemical evidence of vitamin A deficiency in BBd rats could be reversed, BBdp rats were fed a diet supplemented with vitamin A either alone or in combination with zinc. None of these treatments increased plasma vitamin A concentration. The hepatic abundance of RBP mRNA was significantly greater, whereas circulatory RBP concentrations were unaffected by vitamin A plus zinc supplementation. Overall, these results suggest that impaired metabolic availability of vitamin A, possibly caused by its decreased transport from hepatic stores, is another metabolic derangement associated with type I diabetes. *J. Nutr.* 130: 1958–1962, 2000.

KEY WORDS: • vitamin A • type I diabetes • retinol-binding protein • zinc • BB rats

Vitamin A is an essential nutrient for growth, cell differentiation, reproduction and vision. It is provided in the diet mainly as the retinyl ester, which is hydrolyzed to retinol in the intestine. In the enterocytes, the free retinol is reesterified and incorporated into chylomicrons, which transport it to the liver, where it is stored predominantly in its ester form (Blaner and Olson 1994). When required, retinyl esters are rehydrolyzed to retinol, which is then carried by retinol-binding protein (RBP)⁴ into the circulation. In the blood, the retinol/RBP complex further binds to transthyretin (TTR), at a ratio of 1:1:1 (Soprano and Blaner 1994), and is then transported to the target cells. Circulatory levels of retinol are regulated homeostatically over a wide range of dietary intakes.

Impaired metabolic availability of vitamin A has been identified in human subjects with type I diabetes, as evidenced by decreased levels of plasma retinol and RBP, accompanied by increased urinary excretion of the RBP (Basu et al. 1989, Dubrey et al. 1997). The metabolism of Zn, an important

factor for the synthesis of vitamin A carrier proteins, has also been shown to be disturbed in the presence of diabetes as indicated by hyperzincuria (Cunningham et al. 1994, Heise et al. 1988). Studies involving streptozotocin (STZ)-induced diabetic rats have shown that circulatory levels of retinol as well as 11-*cis*-retinal concentrations in the retina of the eye, an important component of rhodopsin, are reduced in diabetes, whereas the hepatic storage of vitamin A is markedly elevated (Tuitoek et al. 1996c). Insulin treatment of these rats normalized the metabolic availability of vitamin A, whereas vitamin A supplementation in the diet did not (Tuitoek et al. 1996a and 1996c), indicating a linkage between vitamin A status and insulin secretion in diabetes. Indeed, vitamin A has been reported to be required for normal insulin secretion (Chertow et al. 1987).

Most studies linking vitamin A metabolism and diabetes have been carried out in STZ-induced diabetic rats. STZ is a nitrosourea derivative that is toxic to the liver and kidney (Perloff et al. 1995), the two major sites involved in vitamin A metabolism. The possibility that vitamin A metabolic derangement is partially a reflection of STZ toxicity rather than due solely to diabetes cannot be excluded. Hence, to understand vitamin A metabolism in type I diabetes, this study was undertaken to investigate the metabolic availability of vitamin A in BB rats, which develop diabetes spontaneously and are suitable animal models for human type I diabetes (Like et al. 1982, Parfrey et al. 1989). Feeding the NIH-07 diet, which consists of unrefined, natural ingredients and produces the

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³ To whom correspondence should be addressed.

⁴ Abbreviations used: BBd, diabetic biobreeding; BBdp, biobreeding diabetes prone; BBn, biobreeding normal; BSIREH, bile salt-independent retinyl ester hydrolases; BSDREH, bile salt-dependent retinyl ester hydrolases; cRBP, cellular retinol-binding protein; RBP, retinol binding protein; STZ, streptozotocin; TTR, transthyretin.

highest incidence of diabetes in BB rats (Scott 1994), we examined vitamin A status, including its carrier proteins at the onset of diabetes. In addition, the zinc status, which is important in the maintenance of vitamin A homeostasis, was also determined.

MATERIALS AND METHODS

Animals and diets. Diabetes-prone Biobreeding (BBdp) and normal (BBn) rat dams, originally from Health Canada (Animal Resources Division, Health Protection Branch, Ottawa, Canada), were obtained from the Department of Agricultural, Food and Nutritional Science, University of Alberta breeding colony. Animals were housed in a temperature- and humidity- controlled room with a 12-h light:dark cycle. All studies were reviewed and approved by the University of Alberta Animal Welfare Committee.

This study consisted of two experiments. In Experiment 1, the levels of vitamin A and its carrier proteins were determined at the onset of diabetes. Weaning (21-d-old) litter- and gender-matched BBdp and BBn rats ($n = 12/\text{group}$) were given free access to the NIH-07 diet (Zeigler Brothers, Gardmers, PA) and water up to 120 d of age. The NIH-07 diet contains (g/kg diet): protein 215; carbohydrate 514; fat 52; fiber 32; water 125. After diabetes was diagnosed (see below), the diabetic rats and their paired BBn rats were killed within 24 h by carbon dioxide asphyxiation.

In Experiment 2, the effects of vitamin A supplementation, either alone or in combination with zinc, on the metabolic availability of vitamin A were examined. BBn rats fed the NIH-07 diet were used as controls (group 1). Weanling (21-d-old) BBdp pups from each litter were evenly assigned to three treatment groups of 12 rats each. Group 2 received the NIH-07 diet only. Groups 3 and 4 received the basal diet supplemented with retinyl palmitate (18.5 $\mu\text{g/g}$ diet) either alone or in combination with zinc (180 $\mu\text{g/g}$ diet), respectively. All rats in BBdp groups were killed within 24 h after the diagnosis of diabetes. By 120 d of age, BBdp rats that had not developed diabetes were also killed.

Food intake and body weight were monitored throughout the studies. BBdp rats >50 d old were tested for glycosuria by Testape (Eli Lilly, Indianapolis, IN) three times each week. Glucose levels were determined in blood samples taken from the tail vein by Glucometer II (Ames Miles, Toronto, Canada). Diabetes was diagnosed on the basis of glycosuria $>2+$ and subsequently hyperglycemia (blood glucose >11 mmol/L). Rats were killed after overnight food deprivation. Blood was collected in heparinized tubes. To avoid light-induced oxidation of vitamin A, separated plasma was protected from light and stored at -20°C . Livers were removed, cleaned, immersed in liquid nitrogen and stored at -72°C for later analysis.

Determination of vitamin A. Plasma and liver vitamin A were assayed by HPLC as described (Tuitoek et al. 1996c). Chromatography was performed on a LC-18 (15.0 cm \times 4.6 mm) (Supelco, Mississauga, ON) reverse-phase column with 3- μm packing, with a mobile phase consisting of methanol/water (95:5, v/v). Detection was carried out by UV absorption at 325 nm. Quantification was obtained by the injection of known amounts of retinol; calculations with the use of a Shimadzu Class-VP Chromatography data system (Shimadzu, Columbia, MD). The standard added to plasma, taken through the extraction procedure and injected into the HPLC, resulted in the recovery of 82–88%.

Radioimmunoassay. Plasma and liver RBP and TTR were assayed by RIA (Tuitoek et al. 1996b). Liver samples were homogenized in 19 volumes of 250 mmol/L sucrose with a Polytron homogenizer (Brinkmann, Westbury, NY) at speed 5 for 15 s and then diluted with an equal volume of an aqueous Triton X-100 solution (20 g/L) to release the proteins trapped in subcellular organelles. The plasma samples and Triton X-100-treated liver homogenates were diluted appropriately with assay buffer (50 mmol/L Tris-HCl containing 10 g bovine serum albumin/L, pH 8.6). After the addition of ^{125}I -labeled proteins, assay buffer and anti-rat antiserum for RBP or TTR, all samples were incubated in the dark at 4°C for 3 d. After being mixed with goat anti-rabbit γ -globulin antiserum for RBP and polyethylene glycol for TTR, all samples were incubated at 4°C to precipitate the antibody-bound ^{125}I -protein; this was collected by

centrifugation at $2800 \times g$ for 15 min at 4°C . The precipitates were washed with barbital albumin buffer, and the wash supernatants were combined. The precipitates representing antibody-bound RBP- ^{125}I or TTR- ^{125}I and the wash supernatants representing free protein were assayed for ^{125}I separately in a Packard 500C AutoGamma Counter (Packard Instrument, Meriden, CT). After calculation of the bound/free ratios of RBP- ^{125}I or TTR- ^{125}I , the amounts of the proteins were determined from the standard curve. To determine the molar concentrations of RBP and TTR, molecular weights of 21422 and 54892, based on the amino acid sequence of rat RBP and TTR, respectively, were used.

RNA isolation and Northern blot analysis. Total RNA was isolated from the liver samples using TriZol (Gibco BRL, Burlington, Canada). RNA quantification and quality determination were carried out by UV spectrophotometry at 260 and 280 nm. Total RNA (15 μg) was analyzed by Northern blotting after electrophoresis in 10 g/L agarose gel and transferred to a MSI Nitropure nitrocellulose membrane (MSI Laboratories, Westborough, MA) as described by Reimer et al. (1997). The 28S and 18S ribosomal RNA bands were used to check the integrity of RNA and compensate for any loading discrepancies. The radioautograms were quantified using GS-670 imaging laser densitometry (BioRad Laboratories, Mississauga, Canada); values were normalized to the 28S and 18S ribosomal RNA bands. Using Random Primers DNA Labeling System (Life Technologies, Burlington, Canada) the cDNA probe for rat RBP was labeled with [^{32}P] dATP (3000 Ci/mmol, Amersham Canada, Oakville, Canada).

Determination of zinc. Zinc levels in the plasma, liver and urine were determined using flame atomic absorption spectrophotometry (Perkin Elmer 4000, Norwalk, CT) with a zinc standard (Fisher Scientific, Edmonton, AB, Canada) as described by Butrimovitch (1977), Luterotti (1992) and Kielerich (1980), respectively. All glassware was rinsed with a 20% (v/v) nitric acid solution followed by deionized water to avoid any contamination.

Determination of plasma total cholesterol and triglyceride. The plasma samples were analyzed for total cholesterol and triglyceride using enzymatic kits obtained from Sigma Biochemicals (St. Louis, MO; Catolog # 7921 and 336, respectively).

Statistical analysis. Statistical analyses were performed by SAS computer program (Version 6.12, SAS Institute, Cary, NC). The level of significance was set at $P < 0.05$. A paired Student's t test was used to determine the differences between age-paired diabetic biobreeding (BBd) and BBn rats for Experiment 1. For Experiment 2, data were analyzed by two-way ANOVA that included the effect of gender. If no effect of gender was found, groups classified according to different treatments were compared using one-way ANOVA. Significant differences between groups were compared using Student Newman Keuls test procedure.

RESULTS

All BBdp rats were monitored regularly for hyperglycemic characteristics including elevated levels of glucose in plasma (>11 mmol/L) and urine ($>2+$). Once these rats were hyperglycemic, they were considered BBd rats. BBd rats exhibited characteristic signs of diabetes, including elevated blood and urinary glucose, water intake and the volume of daily urinary excretion. No significant differences in the mean body weight gain, food intake and liver weight among groups in either experiment were observed.

In Experiment 1, BBd rats were characterized by hyperlipidemia (Fig. 1) in addition to hyperglycemia. Plasma triglyceride and total cholesterol concentrations of BBd rats were significantly greater than those of BBn rats. The onset age of diabetes among BBdp rats was 89 ± 4.4 d. The BBd rats had lower concentrations of plasma retinol in parallel with its carrier proteins, RBP and TTR, in both plasma and the liver compared with those of BBn rats (Table 1). The hepatic concentrations of free retinol were also lower in BBd rats, whereas the total vitamin A levels were not significantly different from controls. Abundance of the hepatic RBP

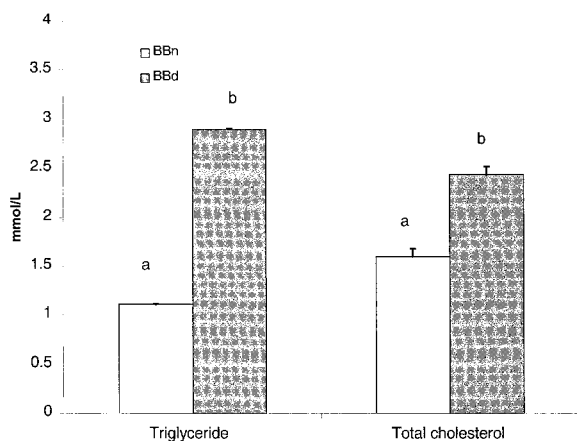


FIGURE 1 Plasma triglyceride and total cholesterol concentrations of age-paired normal (BBn) and diabetic BB (BBd) rats. Results are expressed as mean \pm SEM ($n = 6$). Within each variable, bars with different letters are significantly different, $P < 0.05$.

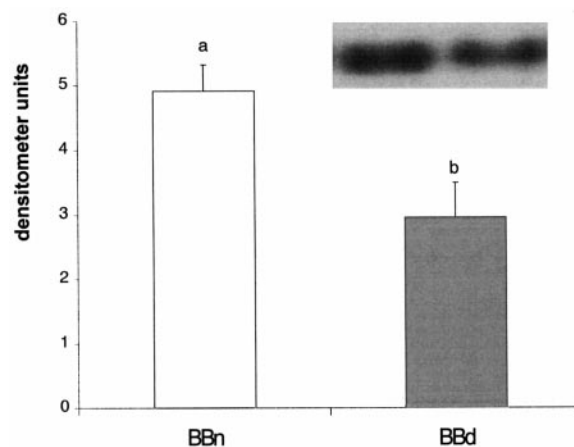


FIGURE 2 Quantification of hepatic retinol-binding protein (RBP) mRNA of age-paired normal (BBn) and diabetic BB (BBd) rats by Northern blotting. Data are normalized by 28s and 18s ribosomal RNA. Bars are means \pm SEM ($n = 6$). Bars with a different letter are significantly different, $P < 0.05$.

mRNA was also lower in BBd rats (Fig. 2). In addition, the BBd rats exhibited biochemical evidence of zinc deficiency as indicated by a lower plasma concentration accompanied by a higher urinary excretion of this trace element (Fig. 3). Liver zinc concentrations, however, were not affected in BBd rats.

To identify whether vitamin A deficiency in BBd rats is due to genetic or disease factors, vitamin A concentrations in the plasma and the liver were examined in BBdp rats that had remained diabetes free to 120 d of age and had been fed the NIH-07 diet (Experiment 2). No significant differences were observed in BBdp rats compared with BBn rats (data not shown).

After vitamin A supplementation for up to 3 mo, the liver total vitamin A concentrations were markedly greater in BBd rats, whereas the plasma concentrations of vitamin A and its carrier proteins, including RBP and TTR, did not differ from BBd rats fed the basal NIH-07 diet (Table 2). Although supplemental vitamin A plus zinc intake did not affect plasma vitamin A, this treatment increased the abundance of RBP mRNA in the liver of the BBd rats (Fig. 4), whereas liver RBP concentrations were unaffected (Table 2).

Supplementation of vitamin A and its combination with zinc did not improve the diabetes-associated low plasma zinc

concentrations of BBd rats (Table 2). Urinary zinc concentrations, however, were markedly greater in rats supplemented with zinc and vitamin A than in the group given vitamin A alone. Urinary zinc in BBd rats fed the basal diet did not differ from either supplemented group.

DISCUSSION

The presence of hyperglycemia affects vitamin A metabolism as indicated by decreased levels of plasma retinol and its carrier proteins in the plasma and the liver at the onset of diabetes in BB rats. These results are in agreement with the previous studies involving type I diabetic patients (Basu et al. 1989) and STZ-induced diabetic rats (Tuitoek et al. 1996b and 1996c), in which the plasma levels of retinol, RBP and TTR were all reduced markedly. The plasma and hepatic vitamin A concentrations in those BBdp rats without diabetes are similar to those of control rats, suggesting that vitamin A deficiency in BBd rats is due primarily to hyperglycemia. To determine whether the vitamin A deficiency in BBd rats could be normalized, they were fed a diet supplemented with vitamin

TABLE 1

Vitamin A and its carrier proteins concentrations in age-paired (normal BB) BBn and diabetic BB (BBd) rats¹

| | BBn | BBd | <i>P</i> -value ² |
|--|-------------------|-------------------|------------------------------|
| Plasma vitamin A, $\mu\text{mol/L}$ | 1.10 \pm 0.17 | 0.70 \pm 0.16 | 0.01 |
| Free retinol, $\mu\text{mol/g liver}$ | 0.161 \pm 0.037 | 0.056 \pm 0.014 | 0.01 |
| Liver total vitamin A, ³ $\mu\text{mol/g liver}$ | 1.23 \pm 0.17 | 1.08 \pm 0.18 | 0.33 |
| Plasma RBP, ⁴ $\mu\text{mol/L}$ | 4.26 \pm 0.91 | 2.72 \pm 0.43 | 0.04 |
| Plasma TTR, $\mu\text{mol/L}$ | 8.08 \pm 1.29 | 6.03 \pm 1.03 | 0.04 |
| Liver RBP, nmol/g liver | 2.18 \pm 0.22 | 1.29 \pm 0.18 | 0.009 |
| Liver TTR, nmol/g liver | 1.58 \pm 0.32 | 0.81 \pm 0.07 | 0.03 |

¹ Results are expressed as means \pm SEM, ($n = 6$).

² Paired Student's *t* test was used to determine differences between group means.

³ Liver total vitamin A includes free retinol + retinyl esters.

⁴ Abbreviations: RBP, retinol-binding protein; TTR, transthyretin.

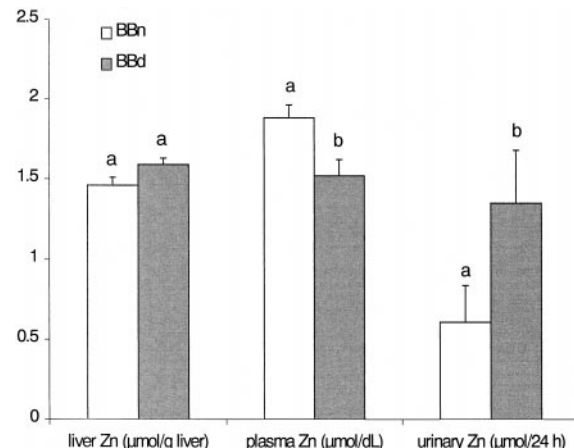


FIGURE 3 Tissue and urinary zinc content of age-paired normal (BBn) and diabetic BB (BBd) rats. Bars are means \pm SEM ($n = 6$). Within each variable, bars with different letters are significantly different, $P < 0.05$.

TABLE 2

Effect of vitamin A supplementation and vitamin A plus zinc supplementation on the zinc, vitamin A and its carrier protein status in diabetic BB (BBd) rats¹

| | BBd (NIH-07) | BBd (Vitamin A supplemented) | BBd (Vitamin A, zinc supplemented) |
|------------------------------------|--------------------------------------|---------------------------------|---------------------------------------|
| | % of BBn ² | | |
| Plasma vitamin A | 64 ± 16.8* | 56 ± 10.8* | 53 ± 5.9* |
| Liver total vitamin A ³ | 85 ± 13.3 ^a | 1300 ± 197.7 ^b | 1270 ± 55.9 ^b |
| Plasma RBP ⁴ | 64 ± 18.9* | 58 ± 14.1* | 57 ± 6.1* |
| Plasma TTR | 81 ± 11.9* | 58 ± 6.8* | 59 ± 3.1* |
| Liver RBP | 88 ± 17.0 | 53 ± 7.5* | 55 ± 13.6* |
| Plasma zinc | 90 ± 5.5 | 83 ± 8.9* | 73 ± 3.3* |
| Liver zinc | 118 ± 7.3 | 97 ± 5.1 | 125 ± 5.6 |
| Urine zinc | 279 ± 78.9 ^a ^b | 210 ± 68.4 ^b | 342 ± 57.9 ^a |

¹ Results are expressed as means ± SEM, $n \geq 4$; means in a row without a common letter differ, $P < 0.05$.

² All values are expressed as percentages of values in biobreeding normal (BBn) rats.

³ Liver total vitamin A includes free retinol + retinyl esters.

* Significant ($P < 0.05$) different from the corresponding values from BBn rats.

⁴ Abbreviations: RBP, retinol-binding protein; TTR, transthyretin.

A, either alone or in combination with zinc. Neither of these treatments, however, altered the reduced plasma levels of vitamin A.

Although the total vitamin A concentrations in the liver were unaffected, the hepatic levels of free retinol were markedly reduced in BBd rats. The transformation of retinyl ester to free retinol in the liver is regulated by the bile salt-dependent BSDREH and bile salt-independent (BSIREH) hepatic retinyl ester hydrolases (Blaner and Olson 1994). BSIREH is thought to be involved in the initial hydrolysis of dietary retinyl esters delivered to the liver from the gut. Because of its high specific activity in vitamin A storage site hepatic stellate cells, BSDREH is believed to be more important for the later retinyl ester hydrolysis and retinol release in the liver than BSIREH (Harrison 1993). Biliary structure and hepatic function are affected in diabetes (Watkins et al. 1995). Diabetic BB rats often have a significantly altered bile flow and biliary secretions of bile acid, cholesterol, phospholipid, sodium, potassium, chloride and bicarbonate (Gonzalez et al. 1992). It is

thus possible that a diabetes-induced modification of bile salt metabolism may account for this impaired vitamin A hydrolysis activity in BBd rats.

The transport of free retinol from the liver to target cells is accomplished by RBP, which circulates as a 1:1 complex with TTR. Due to the high molecular weight of TTR, its binding to RBP may prevent the glomerular filtration and renal catabolism of RBP. Significantly lower concentrations of these retinol carrier proteins in the plasma and the liver of BBd rats, in parallel with lower RBP mRNA abundance, were observed. The underlying mechanism for these results is not clear. In an early study, a zinc-deficient diet significantly reduced plasma vitamin A, RBP and hepatic RBP concentrations, thus linking zinc status to vitamin A metabolism (Brown et al. 1976). Later experiments demonstrated that hepatic RBP synthesis is dependent on adequate dietary levels of zinc (Smith 1980), and another study has shown that cellular hepatic RBP (cRBP) is dramatically lower in zinc-deficient rats compared with their pair-fed controls with adequate zinc intake (Mobarhan et al. 1992). These results suggest that zinc deficiency can impair hepatic cellular transportation of vitamin A via cRBP and mobilization of vitamin A from the liver via RBP. Type I diabetes is characterized by hyperzincuria, which also was demonstrated in our study. The BBd rats thus had significantly higher urinary and lower plasma concentrations of zinc than those of their nondiabetic counterparts. The hepatic levels of the trace element, however, were unaffected in the presence of hyperglycemia. It should be pointed out that the hepatic content of zinc may not be a reflection of total body zinc because severely zinc-deficient rats have depressed circulatory zinc levels, whereas hepatic concentrations are affected only minimally compared with their pair-fed controls (McClain et al. 1980). It is reasonable to suggest, therefore, that the lower circulatory and hepatic levels of RBP in BBd rats are, at least in part, a metabolic consequence of zinc deficiency. The evidence that reduced abundance of RBP mRNA was improved after zinc plus vitamin A supplementation, but not by vitamin A alone, supports this hypothesis.

In contrast, the plasma and hepatic levels of RBP remained depressed after zinc supplementation despite the improved hepatic abundance of RBP mRNA in BB rats. These results cannot be explained at this time. Nevertheless, zinc deficiency

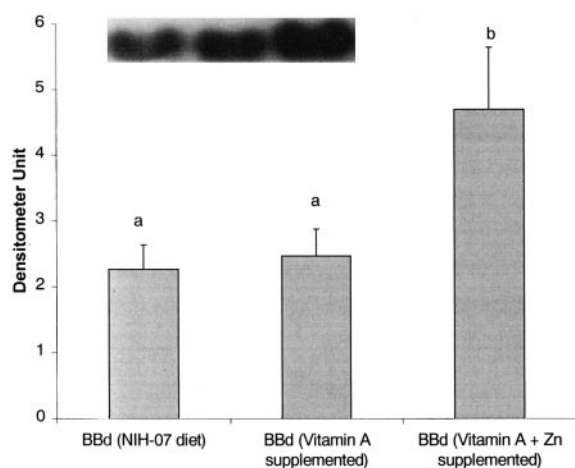


FIGURE 4 Quantification of hepatic retinol-binding protein (RBP) mRNA in diabetic BB (BBd) rats supplemented with vitamin A, and vitamin A plus zinc by Northern blotting. Data are normalized by 28s and 18s ribosomal RNA. Bars are means ± SEM ($n \geq 5$). Bars with different letters are significantly different, $P < 0.05$.

has been reported to be associated with decreased concentrations of RBP and TTR in rat plasma and liver (Bates et al. 1981, Smith et al. 1974, Smith 1980).

In summary, the metabolic availability of vitamin A is impaired in BB rats after the onset of hyperglycemia. The mechanism for this abnormality is not understood. Possible factors may include alteration in hepatic retinyl hydrolase activity, synthesis of retinol carrier proteins and perturbed zinc metabolism. A recent study has suggested that there is a need for vitamin A supplementation in type I diabetic patients with marginal serum retinol levels (Granado et al. 1998). There is little evidence, however, to confirm that such therapy would be of any benefit (American Diabetes Association 1996). In STZ-induced diabetic rats, a 12-fold increase in vitamin A intake did not show any effect on the degree of hyperglycemia and glycosuria (Seifer et al. 1981). In fact, such supplementation appears to enhance the hepatic load of the vitamin (Tuitoek et al. 1996c).

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