

Metabolism of retinol-binding protein and vitamin A during hypervitaminosis A in the rat

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Abstract Vitamin A is normally transported in plasma as retinol bound to a specific protein, retinol-binding protein (RBP). Detailed studies were conducted to examine the effects of excess vitamin A on the plasma concentration and metabolism of RBP, and to obtain information about vitamin A transport in the hypervitaminotic state. Two separate experiments were conducted. In the first (Study I, 99 days), plasma RBP and vitamin A levels were compared in three groups of rats fed 0.14 mg (control), 7.3 mg (group 2), or 41 mg (group 3) of vitamin A per day. After day 50 of the study, the administration of excess vitamin A to hypervitaminotic rats (groups 2 and 3) was discontinued and the rats were allowed to recover from vitamin A toxicity. In the second, shorter experiment (Study II), serum vitamin A and RBP levels were compared in control and hypervitaminotic (34 mg of retinyl acetate per day) rats. The rats in this study were also given [³H]retinyl acetate daily to determine the distribution of retinyl esters and retinol between the lipoprotein and nonlipoprotein protein fractions of plasma. In both studies, administration of large, excessive doses of vitamin A resulted in substantial and significant decreases in the levels of serum RBP. Excessive doses of vitamin A produced fatty liver in the rats, in association with a normal (group 2, Study I) or with a decreased (group 3, Study I) level of RBP in the liver. It is possible that excess vitamin A leads to decreased rates of RBP synthesis in, and of RBP secretion from, the liver. Administration of excessive doses of vitamin A also resulted in elevations of serum vitamin A levels, which were mainly due to large increases in the circulating levels of retinyl esters. In the hypervitaminotic rats, most of the serum vitamin A, and virtually all of the retinyl esters, was found in association with the serum lipoproteins of hydrated density less than 1.21. These results demonstrate that the serum lipoproteins play an important role in the transport of the vitamin A that accumulates in serum in hypervitaminosis A. We suggest that the toxic manifestations of hypervitaminosis A occur when vitamin A circulates in plasma and is presented to membranes in a form other than bound to RBP. Plasma lipoproteins may nonspecifically deliver vitamin A to biological membranes and hence lead to vitamin A toxicity.

Supplementary key words retinol · retinyl esters · vitamin A toxicity · lipoproteins · plasma · liver · plasma transport · fatty liver

It is well established that chronic excessive intake of vitamin A in animals brings about distinct hypervitaminot-

ic, toxic manifestations (1–3). These manifestations include resorption of cartilage and bone (leading to bone fragility and fractures) (4, 5), hemorrhages and secondary vitamin K deficiency (6), epithelial abnormalities (1), changes in cerebrospinal fluid pressure (7, 8), and congenital malformations (1). Hypervitaminosis A induces fatty liver and elevations in plasma free fatty acid levels in the rat (9, 10).

Considerable information is available about the mechanism of the toxic effects of vitamin A upon tissues. Studies both in vivo and in vitro have demonstrated that excess vitamin A results in increased lability of biological membranes (11–14). This effect of the vitamin is believed to be due to its surface-active properties, because retinol is a highly surface-active compound (15) that is “membrane-seeking” and potentially membranolytic. In particular, excess vitamin A has been shown to lead to increased synthesis and release of lysosomal enzymes, and these hydrolases have been shown to be critically involved in the effects of vitamin A on cartilage and limb-bone rudiments (11, 14, 16, 17).

Vitamin A is normally transported in plasma as retinol bound to a specific transport protein, retinol-binding protein (RBP) (18, 19). Recent studies of vitamin A deficiency in the rat demonstrated that such deficiency specifically affects the level of RBP in plasma and results in a substantial decrease in circulating RBP concentration (20, 21). Vitamin A deficiency appears to interfere primarily with the secretion, rather than with the synthesis, of RBP by the liver, so that the deficient liver contains an expanded pool of apo-RBP that can be released rapidly into the plasma, as holo-RBP, when vitamin A becomes available (20–22).

Retinol bound to RBP does not appear to manifest its surface-active effects on biological membranes. Thus, in a recent study, retinol bound to RBP failed to influence the extracellular matrix of chick limb-bone rudiments grown in organ culture (23). These observations suggested that the mode of transport of vitamin A may be an important

Abbreviations: RBP, retinol-binding protein.

determinant of the development of the manifestations of hypervitaminosis A.

We now report detailed studies of the effects of excess vitamin A on the plasma concentration and metabolism of RBP in the rat. Information about the transport of vitamin A in hypervitaminotic rats is also presented.

METHODS

Experimental design

Two separate studies were carried out.

Study I. 30 weanling male rats (weight range 51–63 g) were obtained from the Holtzman Co., Madison, Wis. The rats were housed in individual wire-bottomed cages in a room with a mean temperature of 22°C. All rats had free access to food and water. The rats were fed a purified vitamin A-deficient diet (20) supplemented with 4 I.U. (1.2 µg) of vitamin A (as retinyl esters) per gram of diet. When the rats reached a mean weight of 110 g, they were randomly assigned to three groups (10 rats in each group) and were then fed the vitamin A-deficient diet supplemented as follows.

Group 1. Control. The rats in this group were given orally 0.14 mg of vitamin A in oil daily.

Group 2. Hypervitaminotic. This group received by mouth daily 4 drops of vitamin A concentrate (ICN Nutritional Biochemicals, Cleveland, Ohio) containing 7.3 mg of retinyl esters.

Group 3. Hypervitaminotic. This group of rats was given orally 1 drop of vitamin A concentrate containing 1.8 mg of retinyl esters until day 25, after which the rats were given twice daily 0.3 ml of vitamin A concentrate by oral needle (41.2 mg of retinyl esters per day).

All animals were weighed twice a week throughout the study and their daily food intake was determined.

After day 50, the rats in groups 2 and 3 were not given vitamin A, whereas the control group continued to receive 0.14 mg of vitamin A every day. The experimental period was 99 days. Blood samples were collected in heparinized syringes from the subclavian venous plexus on days 1, 2, 8, 16, 23, 27, 31, 36, 43, 50, 53, 63, 71, and 93 by the method of Phillips, Stafford, and Stuet (24) as described previously (20). The blood samples were collected 24 hr after a dose of vitamin A and after the rats were starved overnight. Samples of approximately 0.8 ml of blood were collected till day 23, after which 1.8-ml samples were collected.

On day 50, five rats in each group were killed. The remaining rats were killed on day 99. Prior to killing, the rats were anesthetized with diethyl ether, and blood was collected from the abdominal aorta. The livers were flushed with 0.9% NaCl solution, blotted, and rapidly froz-

en with acetone and dry ice. Liver and plasma samples were stored at -20°C until assayed.

Study II. 32 rats 55 days old were obtained from the Holtzman Co. (weight range 230–250 g). The rats were housed as in Study I and were fed the same vitamin A-deficient diet (20) supplemented with 0.14 mg of retinyl acetate in 1 drop of cottonseed oil daily. When the rats reached a mean weight of 300 g, they were randomly assigned to two groups (16 rats in each group).

Control group. Each rat in this group was given daily by mouth 0.14 mg of retinyl acetate (Sigma Chemical Co., St. Louis, Mo.) in 1 drop of oil.

Hypervitaminotic group. Each rat in this group was given orally 34.4 mg of retinyl acetate daily in 7 drops of oil, the oil drops being given at two intervals. Daily the rats in both groups were also given orally 0.67 µCi (1.75 µg) of [³H]retinyl acetate in 1 drop of oil. The experimental period was 23 days. The animals were weighed every 2 days and the feces of the individual rats were collected separately every 3 days. Blood samples of approximately 2.5 ml each were collected from the subclavian venous plexus on the following days of the experiment: 0, 1, 5, 8, 12, and 18. Unlike in Study I, blood samples were collected between 4 and 8 hr after the administration of retinyl acetate, and the rats were not starved overnight. On day 8, eight rats in each group were killed 5 hr after receiving retinyl acetate. On day 23, the remaining rats were killed approximately 24 hr after receiving vitamin A.

Vitamin A and RBP assays

Samples of livers were extracted with diethyl ether by the method of Ames, Risley, and Harris (25), and the vitamin A contents of the extracts were determined by the trifluoroacetic acid method of Dugan, Frigerio, and Siebert (26). The serum samples were assayed for total vitamin A by the fluorometric method of Thompson et al. (27) as described previously (20). The RBP levels in serum and liver samples were determined by a modification of the previously described radioimmunoassay (20). Instead of using a second antibody, polyethylene glycol (6000–7500 daltons) was used to precipitate the antibody-bound ¹²⁵I-labeled RBP (28). Calculations were done on a Wang 700A programmable calculator by the logit-log method (29).

In this publication the term vitamin A is used to include both retinol and retinyl esters, whereas retinol refers to unesterified vitamin A alcohol only. The amounts of vitamin A in biological samples are expressed as the equivalent weights of retinol.

Separation of retinyl esters and retinol by column chromatography on alumina

Serum (0.2 ml) was mixed with 1.0 ml of 50% ethanol and 2.5 ml of hexane in an amber 15-ml glass-stoppered

centrifuge tube using a vortex mixer. The tube was then centrifuged in a clinical centrifuge for 1 min. The hexane layer was withdrawn to an amber 25-ml flask. The aqueous layer was extracted once more with 2.5 ml of hexane. The hexane extracts were pooled and were evaporated to dryness under a stream of nitrogen at about 40°C. The residue was dissolved in 0.5 ml of hexane and chromatographed on 0.25 g of 10% (w/v) water-deactivated alumina (Woelm neutral). A Pasteur pipet was used as the column. Retinyl esters and retinol were eluted successively using 10 ml each of hexane and 20% (v/v) diethyl ether in hexane. Fractions of 5 ml each were collected, and retinyl esters and retinol present in each fraction were assayed spectrofluorometrically by the method of Thompson et al. (27). The separation of retinyl esters and retinol by this procedure was found to be very good, and the recovery was 90%.

Ultracentrifugation of serum

Plasma lipoproteins were separated from proteins with hydrated density greater than 1.21 by ultracentrifugation at density 1.21 (30). The serum samples used for this experiment were obtained from rats in Study II (day 23 of the study). Six rats each from the control and hypervitaminotic groups were used. 2.5 ml of fresh serum from each rat was adjusted to density 1.21 with a solution of KBr, and lipoproteins were separated as a single fraction by centrifugation for 48 hr at 105,000 *g* in the Ti-50 rotor of a Beckman L2-65B ultracentrifuge. The centrifuge tube was sliced in the clear zone below the floating lipoprotein layer to obtain two fractions comprising the lipoproteins (density less than 1.21) and the other serum proteins with density greater than 1.21.

Retinyl esters and retinol present in the floating lipoprotein fraction and in the "bottom" nonlipoprotein fraction were separated on an alumina column as described above. 2.0 ml of the "bottom" fraction and 0.8 ml of the floating lipoprotein fraction were used for the separation of retinyl esters and retinol.

Other methods and materials

Red cell osmotic fragility was determined with a Fragiligraph (Kalmedic Instruments, New York) at 37°C on fresh samples of rat heparinized blood. The samples of blood were diluted 1:30 in an isotonic NaCl solution buffered to pH 7.4 with 0.1 M sodium phosphate, and an aliquot of the suspension was used to determine the red cell osmotic fragility.

Fat in the liver was assayed gravimetrically by the following method. A weighed sample of liver (approximately 2.0 g) was ground with anhydrous sodium sulfate and extracted with 50 ml of diethyl ether. The ether extract was dried to constant weight.

The liver homogenates were analyzed for total protein

by the method of Lowry et al. (31), standardized against serum albumin.

Feces of each rat, collected every 3 days, were separately extracted by homogenizing with ethanol-acetone 1:1 (v/v) in a Waring blender and adjusting the volume of the homogenate to 50 ml. The homogenate was then centrifuged at 2000 rpm for 20 min, and 0.2 ml of the supernate was assayed for radioactivity.

For assay of radioactivity, samples were evaporated to dryness under nitrogen, and the residues were each dissolved in 15 ml of 0.5% diphenyloxazole in toluene. The samples were assayed in a Packard liquid scintillation counter, model 3365. When appropriate, corrections for quenching were made by use of an automatic external standard of ^{226}Ra . The counting efficiency of this system was about 30% for ^3H .

Statistical significance was assessed by using Student's *t* test (32) with a Wang model 700A calculator with programs prepared for that instrument. Data reported in the tables and figures are mean \pm SEM values for the various groups of rats.

The [11,12- ^3H]retinyl acetate (specific radioactivity, 380 $\mu\text{Ci}/\text{mg}$) was a generous gift of Hoffmann-La Roche, Basle, Switzerland. The labeled retinyl acetate was freshly purified on a 10% (w/v) water-deactivated alumina column before it was administered to the animals.

RESULTS

Effects on growth rate

Fig. 1 shows the mean growth rates of the rats in each of the three dietary treatment groups in Study I. The control rats (group 1) grew well and were healthy throughout the study. The growth rates of the rats in all three groups were comparable until day 26 of the study. After this time the rats in group 2 showed a slightly lower growth rate that was not significantly lower than that of the control rats; these rats (group 2) appeared healthy throughout the experimental period. In group 3, when the dose of vitamin A was increased to 41 mg/day (day 25) the rats ceased to grow and exhibited characteristic signs of hypervitaminosis A ($P < .01$ for body weight compared with controls; see Fig. 1). The clinical signs characteristic of chronic vitamin A toxicity (1) that were observed were reduced food intake and depressed growth, alopecia of the head, thickening of the skin, occasional bleeding from the nose, and weakness or partial paralysis of the legs. Unlike the control rats, the hypervitaminotic rats were aggressive and not docile on being handled. When excess vitamin A administration was discontinued (day 50), the rats slowly recovered and then continued to grow until the end of the study.

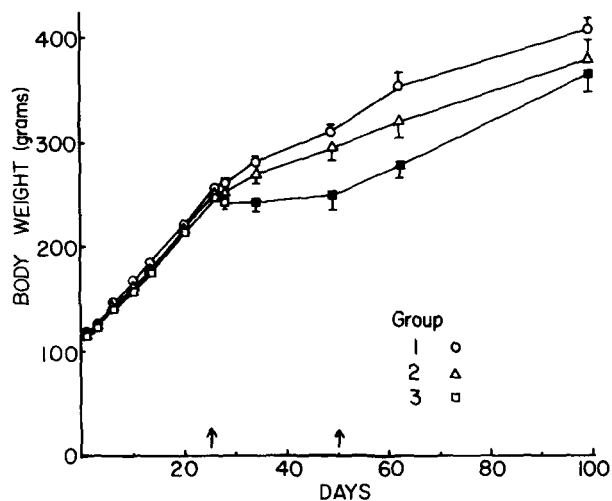


Fig. 1. Mean growth rates of the rats in each of the three different vitamin A treatment groups in Study I. Group 1, control (\circ); group 2, 7.3 mg of vitamin A/day (Δ); group 3, 1.8 mg and then 41.2 mg of vitamin A/day (\square). Mean \pm SEM values are shown for the rats in each group (10 rats per data point prior to day 50, 5 rats per point for days 62 and 99). The first arrow (at day 25) represents the point at which the dose of vitamin A to the rats in group 3 was increased from 1.8 to 41.2 mg/day. The second arrow (at day 50) represents the time when the vitamin A dose was discontinued in groups 2 and 3. The solid squares represent values in group 3 that were significantly different from the corresponding control (group 1) values ($P < 0.01$ except for day 99, where $P < 0.05$). In addition, at day 49 the values for groups 2 and 3 differed significantly from each other ($P < 0.01$).

In Study II, the rats in the hypervitaminotic group (34 mg/day) also ceased to grow while on the high dose and lost a mean of 10 g in 22 days. These rats also exhibited clinical signs of vitamin A toxicity.

Plasma vitamin A concentrations

The mean plasma vitamin A levels of the rats in each of the three dietary groups of Study I are shown in Fig. 2 (upper panel). The control rats (group 1) showed a slight increase in plasma vitamin A levels, to about 65–70 $\mu\text{g}/\text{dl}$, during the first 2–3 weeks of the study; the control levels then declined and plateaued in the range of 40–60 $\mu\text{g}/\text{dl}$. A steep increase in the plasma vitamin A level was seen in the rats of group 2 (7.3 mg of vitamin A/day). The mean vitamin A level in group 2 rose to $103 \pm 6 \mu\text{g}/\text{dl}$ at day 16; after that period the vitamin A level slowly declined although the animals continued to receive daily the same large dose of vitamin A. When the administration of vitamin A was discontinued (day 50), in group 2 rats the plasma vitamin A level reached the normal (control) level within 2 days.

Generally similar results were observed in the group 3 rats. On the initial intermediate dose of vitamin A (1.8 mg/day), plasma vitamin A rose to moderately elevated levels (Fig. 2). When the dose was raised to 41 mg/day, the plasma vitamin A levels rose rapidly to a mean of $102 \pm 11 \mu\text{g}/\text{dl}$ and then declined despite the continuation of

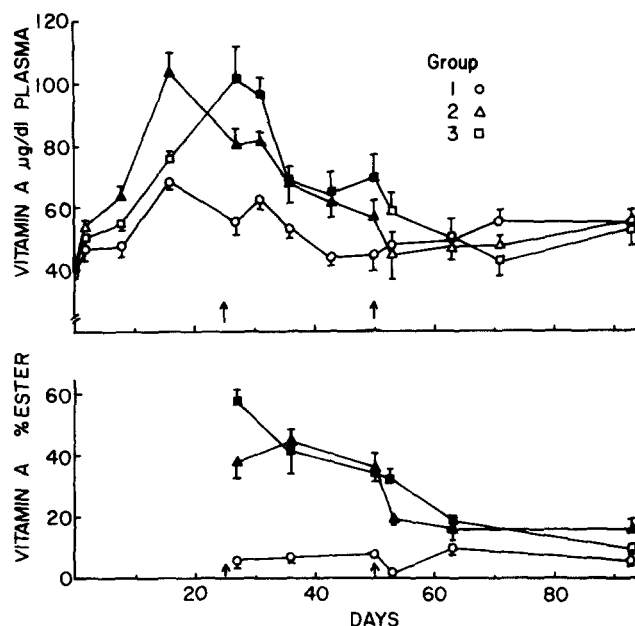


Fig. 2. Upper panel, effects of hypervitaminosis A on plasma vitamin A levels in Study I; lower panel, effects of hypervitaminosis A on percentage of retinyl esters in plasma in Study I. Mean \pm SEM values are shown. See legend to Fig. 1 for identification of groups and definition of the two arrows. In the lower panel, the data prior to day 27 could not be obtained because only 0.8-ml blood samples were collected from the rats during that period. The solid symbols represent values in groups 2 and 3 that were significantly different from the corresponding control (group 1) values. In the upper panel, $P < 0.01$ except for group 3 on days 36 and 43 and group 2 on day 50, when $P < 0.05$. In addition, on days 8, 16, and 31 the values for groups 2 and 3 (upper panel) differed significantly from each other ($P < 0.025$). In the lower panel, $P < 0.01$ except for group 3 on day 50 and group 2 on days 63 and 93, when $P < 0.05$. On days 27 and 53 the values for groups 2 and 3 (lower panel) differed significantly from each other ($P < 0.01$). In the upper panel, there were 10 rats per data point through day 50 and 5 rats per point thereafter; the lower panel represents 5 rats per data point.

the high daily dose. When the vitamin A dose was discontinued, plasma vitamin A levels decreased further and became normal within a few days.

The results obtained after chromatographic separation of retinyl esters and retinol in plasma, for all three groups of rats, showed that the increased total plasma vitamin A levels in the hypervitaminotic rats were due to increases in retinyl ester levels. The levels of retinol (nonesterified) in control and hypervitaminotic animals were comparable at all times and remained fairly constant in the range of 30–50 $\mu\text{g}/\text{dl}$ in all three groups throughout the study. Fig. 2 (lower panel) shows the percentage of total plasma vitamin A present as retinyl esters in each of the three groups of rats. As expected, less than 10% of plasma vitamin A was present as retinyl esters in the control group (group 1) throughout the study. During the period when the plasma levels of vitamin A were elevated in the hypervitaminotic rats (groups 2 and 3), the percentage of total vitamin A present as retinyl esters was markedly elevated, to about

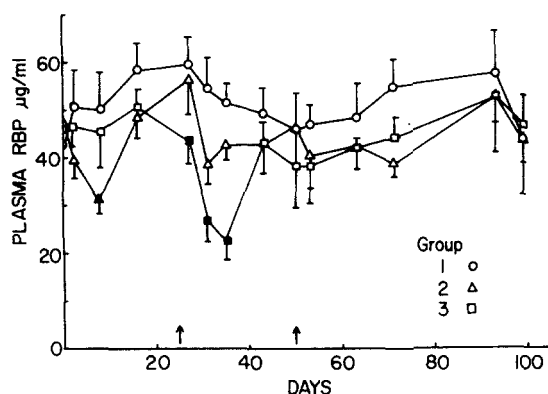


Fig. 3. Effects of hypervitaminosis A on plasma RBP levels in Study I. Mean \pm SEM values are shown, representing 10 rats per data point through day 50 and 5 rats per point thereafter. See legend to Fig. 1 for identification of groups and definition of the two arrows. The solid symbols represent values in groups 2 and 3 that were significantly different from the corresponding control (group 1) values. In addition, at day 35 the values for groups 2 and 3 differed significantly from each other ($P < 0.01$).

40% or higher. When the high vitamin A dose was discontinued in groups 2 and 3 (after day 50), the observed decreases in total vitamin A levels were accompanied by decreases in the percentage of retinyl esters, to the range of 10–20% of total plasma vitamin A.

In Study II, blood samples were collected between 4 and 8 hr after administration of a dose of vitamin A. Very high levels of serum total vitamin A were observed in the hypervitaminotic rats, with the mean values ranging from 125 to 250 $\mu\text{g}/\text{dl}$. It is likely that a substantial portion of these very high levels represented newly absorbed vitamin A being absorbed into the body from the intestinal tract. Analysis of the feces indicated that about one-third of the administered ^3H -labeled vitamin A was excreted in the feces by the hypervitaminotic rats. As expected, the serum vitamin A levels of the control rats remained fairly constant (range of mean values between 42 and 56 $\mu\text{g}/\text{dl}$) throughout Study II.

Plasma RBP concentrations

Fig. 3 shows the mean plasma RBP levels of the rats in each of the three dietary treatment groups of Study I. The control group showed a small rise in RBP level during the first 2–4 wk of the study. In general, in the control group the RBP levels remained fairly stable and were in the range of 40–60 $\mu\text{g}/\text{ml}$ throughout the study.

Within 2 days after beginning the daily dosage of 7.3 mg vitamin A (group 2), the mean plasma RBP concentration decreased to 39 $\mu\text{g}/\text{ml}$, and by day 8 it had declined to 31 $\mu\text{g}/\text{ml}$, the lowest RBP level observed in this group. After day 8, the mean RBP concentration in this group increased and by day 25 was slightly below, but not significantly different from, the control level. After day 25, plasma RBP levels in the group 2 rats fluctuated somewhat but were generally a little below, although not sig-

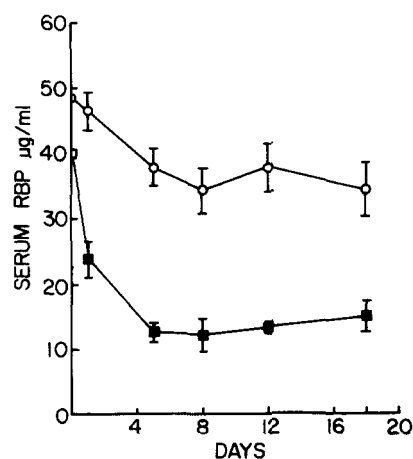


Fig. 4. Effects of hypervitaminosis A on serum RBP levels in Study II. Mean \pm SEM values are shown, representing eight rats per data point. Control, O; hypervitaminotic, ■. The solid symbols represent hypervitaminotic values significantly ($P < 0.005$) different from control values.

nificantly different from, the control levels and were in the range of about 40–50 $\mu\text{g}/\text{ml}$ for the remainder of the study.

On the initial dose of 1.8 mg of vitamin A per day, the group 3 rats showed RBP levels that were slightly below, but not significantly different from, the control levels. When the vitamin A dose was increased to 41 mg/day on day 25, the effects on serum RBP levels were marked; they declined substantially, to a mean of 22 $\mu\text{g}/\text{ml}$ by day 35. After this, serum RBP levels rose towards normal, to a mean level of 43 $\mu\text{g}/\text{ml}$ on day 43, despite the continued administration of the high dose of vitamin A. Beyond day 50 the serum RBP levels of the rats in groups 2 and 3 were very similar throughout the remainder of the study.

A marked decrease in the serum RBP level in response to excessive intakes of vitamin A was also observed in Study II (Fig. 4). The mean serum RBP level of the hypervitaminotic rats in this study declined to 12.7 $\mu\text{g}/\text{ml}$ on day 5 and remained in the range of 13–15 $\mu\text{g}/\text{ml}$ throughout the study. A rise towards normal in the serum RBP level was not observed in the hypervitaminotic rats in Study II; this difference from Study I may reflect the differences in the experimental designs of the two studies.

The unesterified retinol-to-RBP molar ratios of the serum collected on days 1, 5, and 8 were markedly higher in the hypervitaminosis A rats (1.81 ± 0.46) than in the control rats (0.50 ± 0.06). Because each molecule of RBP can bind only one molecule of retinol, the rats receiving the toxic dose of vitamin A clearly had a large portion of their unesterified retinol circulating in a form other than bound to RBP.

Liver vitamin A and total fat content

Hypervitaminotic rats were able to store very large amounts of vitamin A in their livers (Table 1). The mean liver vitamin A content at day 50 of both groups of hy-

TABLE 1. Effect of hypervitaminosis A on liver vitamin A content

| Treatment Group (Study I) | Day 50 (50 days on treatment) | Day 99 (49 days off treatment) |
|--------------------------------|-------------------------------|--------------------------------|
| <i>mg of vitamin A/liver</i> | | |
| Group 1 (control) ^a | 4.7 ± 0.1 | |
| Group 2 (hyper A) ^b | 102 ± 5 ^d | 83 ± 5 |
| Group 3 (hyper A) ^c | 98 ± 7 ^d | 83 ± 7 |

Values are means ± SEM for five rats.

^a 0.14 mg of vitamin A/day.

^b 7.3 mg of vitamin A/day.

^c 1.8 and then 41 mg of vitamin A/day.

^d *P* < 0.001 compared with control.

pervitaminotic rats in Study I was close to 100 mg/liver. Although there was a more than fivefold difference in cumulative vitamin A intake in group 3 compared with group 2 rats, the liver vitamin A storage in both of these groups was approximately the same. The results suggest that the liver has a finite capacity for vitamin A storage, beyond which vitamin A reserves cannot be increased. After discontinuing vitamin A administration for 49 days, the rats in groups 2 and 3 still had huge reserves of vitamin A (83 mg) in their livers.

Hypervitaminosis A produced fatty livers in both groups of rats in Study I. **Table 2** shows the effects of vitamin A toxicity on total liver fat content. The livers of the hypervitaminotic rats contained approximately double the amount of total fat found in the controls. Moreover, there was only a slight decrease in liver fat after vitamin A administration was discontinued for 49 days.

Liver RBP

Very large doses of vitamin A (group 3 rats of Study I) resulted in a significant decrease in the liver RBP level, to about one-half that seen in control rats (**Table 3**). In contrast, the rats given the smaller dose of vitamin A (group 2, 7.3 mg of vitamin A/day) showed liver RBP levels that were similar to those of the controls. When the high vitamin A dose was discontinued, liver RBP levels rose; by

TABLE 2. Effect of hypervitaminosis A on total liver fat content

| Treatment Group ^a (Study I) | Day 50 (50 days on treatment) | Day 99 (49 days off treatment) |
|--|-------------------------------|--------------------------------|
| <i>%^b</i> | | |
| Group 1 (control) | 3.0 ± 0.2 | 3.6 ± 0.1 |
| Group 2 (hyper A) | 5.7 ± 0.3 ^c | 5.5 ± 0.3 ^c |
| Group 3 (hyper A) | 6.8 ± 0.3 ^{cd} | 5.3 ± 0.4 ^e |

^a See Methods and footnotes to Table 1 for details.

^b Percentage of wet weight of liver recovered as fat (see Methods). Means ± SEM.

^c *P* < 0.001 compared with control.

^d *P* < 0.05 compared with group 2.

^e *P* < 0.005 compared with control.

TABLE 3. Effect of hypervitaminosis A on liver RBP content

| Treatment Group ^a (Study I) | Day 50 (50 days on treatment) | Day 99 (49 days off treatment) |
|--|-------------------------------|--------------------------------|
| <i>ng RBP/mg liver protein</i> | | |
| Group 1 (control) | 207 ± 21 | 195 ± 15 |
| Group 2 (hyper A) | 200 ± 33 | 160 ± 15 |
| Group 3 (hyper A) | 105 ± 16 ^b | 177 ± 9 |

^a See Methods and footnotes to Table 1 for details.

^b *P* < 0.01 compared with control (group 1) and *P* < 0.05 compared with group 2. Differences between other group values not statistically significant.

day 99 the liver RBP levels of group 3 rats were not significantly different from those of the control rats (see **Table 3**).

In Study II, the rats given 34 mg of vitamin A/day also had liver RBP levels that were about one-half the levels observed in the control rats. This was found both in rats killed on day 8 and in those killed on day 23.

Plasma transport of vitamin A in hypervitaminosis A

An ultracentrifugal study was conducted to examine the manner in which retinyl esters and retinol were being transported in plasma in hypervitaminotic compared with control rats. The results of this study are shown in **Table 4**. After ultracentrifugation of samples of hypervitaminotic A rat serum (Study II) at density 1.21, only 16 ± 7% of the serum radioactivity (³H-labeled vitamin A) was recovered in the "bottom" fraction containing the proteins of hydrated density greater than 1.21. The serum lipoproteins (density less than 1.21) contained 84 ± 7% of the total radioactivity. A mean of 88% of the radioactivity in the floating lipoprotein fraction was present in the form of retinyl esters. In contrast, all of the radioactivity found in the nonlipoprotein "bottom" fraction of the hypervitaminotic rats was present as retinol.

TABLE 4. Distribution of ³H-labeled vitamin A in fractions obtained after ultracentrifugation of rat serum adjusted to density 1.21^a

| Treatment Group (Study II) | Density < 1.21 (Lipoproteins) | | Density > 1.21 (Nonlipoproteins) | |
|--------------------------------|-------------------------------|---|----------------------------------|---|
| | Percentage Total Vitamin A | Percentage of Vitamin A as Retinyl Esters | Percentage Total Vitamin A | Percentage of Vitamin A as Retinyl Esters |
| Control ^b | 18 ± 2 | 53 ± 3 | 82 ± 2 | 2.9 ± 1.6 |
| Hyper-vitaminotic ^c | 84 ± 7 | 88 ± 5 | 16 ± 7 | 0 |

^a Values are percentages of total dpm (see Methods). Means ± SEM.

^b 0.14 mg of retinyl acetate/day.

^c 34.4 mg of retinyl acetate/day.

As expected, in the serum of control rats most ($82 \pm 2\%$) of the radioactivity was recovered in the nonlipoprotein "bottom" fraction, where it was present mainly as retinol.

The floating lipoprotein fraction and the "bottom" nonlipoprotein fraction were also assayed for RBP. In control and in hypervitaminotic A animals, 98.5% of the RBP was in the "bottom" fraction containing the proteins of hydrated density greater than 1.21.

Red blood cell osmotic fragility

Red cell osmotic fragility was determined on heparinized blood samples of control and hypervitaminotic rats throughout Study I. No differences were observed between the Fragilograms of hypervitaminotic rats and those of control rats.

DISCUSSION

This study was designed to examine the effects of hypervitaminosis A in the rat on plasma RBP metabolism and to obtain information about vitamin A transport in the hypervitaminotic state. Two separate experiments were conducted. In the first (Study I), plasma RBP and vitamin A levels were compared in three groups of rats fed varying amounts of vitamin A, ranging from daily doses of 0.14 mg (control, group 1) to 7.3 mg (group 2) to 41 mg (group 3). Serial samples of blood were collected from each rat. On day 50 of the study, five rats in each group were killed in order to determine simultaneously vitamin A and RBP levels in both plasma and liver. The administration of excess vitamin A to hypervitaminotic rats (groups 2 and 3) was then discontinued for the rest of the study, and the rats were allowed to recover from vitamin A toxicity. It was anticipated that this experimental design would provide insight into the effects of varying degrees of excessive intake of vitamin A on the metabolism of RBP and vitamin A.

The second experiment (Study II) was designed to confirm and extend some of the results of Study I. In this shorter study, serum vitamin A and RBP levels were compared in control and hypervitaminotic (34 mg of retinyl acetate per day) rats. The rats in both groups were also given [^3H]retinyl acetate daily, in order to simplify the quantitative determination of the distribution of retinyl esters and retinol between the lipoprotein and nonlipoprotein fractions of plasma.

Varying degrees of vitamin A toxicity were observed. The rats fed a moderately excessive dose of vitamin A (7.3 mg/day) grew somewhat less well than did the control rats but appeared clinically healthy. Large excesses of vitamin A (41 or 34 mg/day), however, produced severe manifestations of vitamin A toxicity and cessation of growth.

The results presented here clearly demonstrate that hypervitaminosis A affects the levels of RBP in serum. In both studies, administration of large, excessive doses of vitamin A resulted in substantial and significant decreases in the levels of serum RBP. In the first study, after the decline in RBP levels was observed, continued administration of the large dose of vitamin A (either 7.3 or 41 mg/day) was associated with a gradual rise in RBP levels towards normal. In the second, shorter study, the RBP level remained low for the duration of the study.

The mechanism responsible for the decrease in serum RBP level seen in hypervitaminotic rats is not entirely clear. Excessive doses of vitamin A produced fatty liver in the rats, in association with a normal (group 2, Study I) or with a decreased (group 3, Study I) level of RBP in the liver. It is possible that excess vitamin A leads to decreased rates of RBP synthesis in, and of RBP secretion from, the liver. Further studies will be required in order to answer this question.

Administration of large, excessive doses of vitamin A resulted in elevations of the serum vitamin A levels that were mainly due to large increases in the circulating levels of retinyl esters. On a molar basis, throughout most of the period of high dose administration in Study I, and throughout the entire period of Study II, the levels of total serum vitamin A greatly exceeded the levels of serum RBP. Moreover, during the times when the levels of RBP were depressed, even the levels of serum nonesterified retinol exceeded the levels of RBP. Because the RBP molecule contains one binding site for one molecule of retinol (18, 19), it was apparent that in hypervitaminosis some of the serum retinol and virtually all of the serum retinyl esters must circulate in some form other than in association with (i.e., bound to) RBP.

Accordingly, Study II was designed in part to examine directly the mode of transport of vitamin A in hypervitaminotic rats. In the hypervitaminotic rats, most of the serum vitamin A, and virtually all of the retinyl esters, was found in association with the serum lipoproteins of hydrated density less than 1.21. In these rats, almost all of the lesser amount of vitamin A found in the nonlipoprotein (1.21 "bottom") fraction was present as retinol, presumably bound to RBP. As anticipated, in the serum of control rats most of the vitamin A was recovered in the nonlipoprotein fraction, where it was present mainly as retinol, also presumably bound to RBP. These results demonstrate that the serum lipoproteins play an important role in the plasma transport of the increased amounts of vitamin A (particularly the retinyl esters) that accumulate in serum in hypervitaminosis A.

These findings in the rat have been confirmed in our laboratory by observations on two human patients with vitamin A intoxication.¹ Both patients manifested elevated

¹ Smith, F. R., and DeW. S. Goodman. Unpublished observations.

levels of plasma vitamin A in association with mildly decreased levels of RBP.

The observations reported here have enabled us to develop a hypothesis for mechanisms involved in the development of the pathophysiological manifestations of hypervitaminosis A. Normally, vitamin A is transported in plasma as retinol bound to RBP and is delivered to target tissues in this manner. It is likely that one of the functions of RBP is to serve to prevent retinol from exerting its surface-active properties on the body in a generalized and relatively nonspecific way. Thus, in a recent study, the effects of retinol and of the retinol-RBP complex (holo-RBP) were compared on embryonic skeletal tissue grown in organ culture (23). Retinol that was nonspecifically bound to serum proteins in the organ culture medium caused the degradation of the extracellular matrix of chick limb-bone rudiments. This degradation presumably reflected the effects of retinol on lysosomal membranes, with release of lysosomal hydrolases (14, 17). When, however, retinol bound specifically to RBP was added there was no discernible effect. Moreover, the addition of apo-RBP to medium containing "free" retinol prevented the hypervitaminosis A effects. These results indicate that the retinol-RBP complex probably has sufficient stability to prevent the uptake, by lysosomal or other cell membranes, of the amount of retinol needed for significant changes in the membrane to occur. Thus, RBP may serve to deliver retinol to specific sites of action while protecting biological membranes against the effects of excessive and nonspecific uptake of the vitamin. We suggest that vitamin A toxicity occurs in vivo only when the intake and level of vitamin A in the body are sufficiently high that significant amounts of vitamin A begin to circulate in plasma, and to be presented to membranes, in a form other than bound to RBP. We also suggest that plasma lipoproteins represent the transport form that permits the nonspecific delivery of vitamin A to biological membranes and hence leads to the toxic manifestations of hypervitaminosis A. ■

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