Regulation of Retinol-binding Protein Metabolism by Vitamin A Status in the Rat*  

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SUMMARY  

Retinol circulates in rat plasma bound to a specific protein, retinol-binding protein (RBP). A radioimmunoassay for rat RBP was developed with a double antibody precipitation technique. The immunoassay accurately measures RBP in amounts of 0.5 to 3 ng per assay tube. There was no significant difference in the immunoreactivity of apo-RBP as compared to holo-RBP. Using this assay, a study was conducted to examine the effects of vitamin A depletion and deficiency, and of repletion, on the level of serum RBP, in order to explore the role of nutritional vitamin A status in the regulation of RBP metabolism. Weaning rats were divided into five groups, each fed a vitamin A-deficient diet with or without supplements as follows: Group 1, control: supplemented with vitamin A; Group 2, pair-fed control: supplemented with vitamin A but with food intake matched to that of the deficient rats; Group 3, deficient: no vitamin A supplementation; Groups 4 and 5, retinoic acid: supplemented, after the initial depletion period, with a modest daily dose (14 or 28 µg) of retinoic acid. The rats were studied for 75 days. In the deficient group the serum vitamin A levels decreased gradually during the first 25 to 30 days of the study, to levels of about 2 µg/100 ml. Serum RBP levels also declined during the induction of vitamin A deficiency, with a time course similar to that seen for vitamin A, but with a lag of about 3 days (from 50 ± 4 µg per ml on Day 3 to 20 ± 2 µg per ml at Day 27, and then more slowly to 13 ± 2 µg per ml at Day 75). After 3 to 4 weeks most of the circulating RBP was present as the apoprotein, not containing a molecule of bound retinol. The results obtained with the retinoic acid-fed rats, who continued to grow normally, were identical with those of the deficient rats. In contrast both the pair-fed and ad libitum control groups exhibited no major changes in either serum vitamin A or RBP, throughout the entire study.  

Liver homogenates were immunoreactive, and generated immunoassay curves which were indistinguishable from those obtained with pure rat RBP. The level of immunoreactive RBP in the livers of deficient rats was 4 times (p < 0.001) that in the livers of control rats. When vitamin A was administered orally to deficient rats on Day 53, a very rapid increase in serum RBP level, from a mean of 14 to 50 µg per ml, was seen within 5 hours (the first time interval sampled). These findings suggest that vitamin A deficiency primarily interferes in some way with the secretion, rather than with the synthesis, of RBP by the liver, and that the deficient liver contains a pool of previously formed apo-RBP which can be released rapidly into the serum, as holo-RBP, when vitamin A becomes available.  

Vitamin A circulates in plasma in both man and the rat as retinol bound to a specific protein, retinol-binding protein (1, 2). The isolation and partial characterization of rat RBP is reported in the companion paper (1). Rat RBP has α₁ mobility on electrophoresis and a molecular weight of approximately 20,000. The properties of rat RBP resemble those of human plasma RBP in many ways. The two proteins have nearly identical ultraviolet absorption and fluorescence spectra, and have fairly similar amino acid compositions, both with a rather high content of aromatic amino acids. In contrast, rat and human RBP are immunologically distinct, and do not cross-react with each other.  

In plasma, rat RBP circulates in the form of a protein-protein complex, with an apparent molecular weight of approximately 60,000 to 70,000. The protein (prealbumin-2) with which RBP interacts has an electrophoretic mobility slightly greater than that of rat serum albumin, and an apparent molecular weight of approximately 45,000 to 50,000. Prealbumin-2 may also represent a major transport protein for thyroid hormone in the rat.  

We now report the development of an accurate, sensitive, and specific radioimmunoassay for rat RBP. Using this immunoassay a study was carried out to examine in detail the effects of vitamin A depletion and deficiency on the level of serum RBP in serum, and on certain other aspects of RBP and vitamin A metabolism in the rat. This study provides information about the role which nutritional vitamin A status plays in regulating the production and secretion of RBP by the liver.  

EXPERIMENTAL PROCEDURE  

Radioimmunoassay for Rat RBP—Rat plasma RBP was isolated and an antirat RBP antiserum was prepared in a rabbit as  

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1 The abbreviation used is: RBP, retinol-binding protein.
described in the companion paper (1). Purified rat RBP was iodinated with \( ^{125} \)I with a modification of the Chloramine-T method as described by Yalow and Berson (3). A solution of very high specific activity carrier-free \( ^{125} \)I (Isotope Serve, Cambridge Nuclear Corp., Cambridge, Mass.), 6 \( \mu l \) (1.6 mCi), was added with a 10-\( \mu l \) syringe (Hamilton Co., Whittier, Calif.) to 40 \( \mu l \) of 0.25 m potassium phosphate buffer, pH 7.4. To the \( ^{125} \)I solution were added 20 \( \mu l \) of a solution of rat RBP (1 mg per ml in phosphate buffer), then 30 \( \mu l \) of Chloramine-T (3.5 mg per ml in phosphate buffer) and, finally, 40 \( \mu l \) of sodium metabisulfite (4.8 mg per ml in phosphate buffer). KI, 200 \( \mu l \) (10 mg per ml in phosphate buffer) was added, the solution mixed and applied to a Sephadex (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) G-50, fine, column (0.8 \( \times \) 16 cm) previously washed with 1 ml of bovine serum albumin (20 mg per ml) in 0.07 M barbital buffer, pH 8.6. The reaction vial was washed with a second 200-\( \mu l \) portion of KI solution and the wash was added to the column. The protein was eluted with 0.07 M barbital buffer; fractions of 0.5 ml were collected in vials containing 10 to 15 mg of bovine serum albumin each, and each vial was assayed for \( ^{125} \)I. Under these conditions the specific activity of the iodinated RBP was roughly of the order of 20 \( \muCi \) per \( \mug \).

The vial containing the eluted labeled RBP was placed on ice, 1 drop of 5% bromphenol blue was added and the solution mixed. Iodinated RBP was isolated by vertical starch gel electrophoresis for 17 hours (140 volts, 10 ma) at 4\( ^\circ \) in 0.3 M borate buffer, pH 8.6, according to the method of Smithies (4). \( ^{125} \)I-RBP was localized by autoradiography, and the appropriate starch zone removed and placed in a disposable syringe packed with glass wool and previously washed with sodium barbital buffer 0.05 M, pH 8.5, containing 1% bovine serum albumin. The starch was minced with a knife, compressed in the syringe, and the eluate collected in vials containing 3 drops of 25% bovine serum albumin in barbital buffer.

The radioimmunoassay procedure was similar to that previously described for human RBP (5), except that the final dilution of antirat RBP antiserum was usually 1:15,000 (v/v); at this dilution 60 to 70% of \( ^{125} \)I-RBP was bound to antibody in the absence of added unlabeled RBP. The mixture was incubated in the dark at 4\( ^\circ \) for 3 days. Each tube was then mixed with 0.1 ml of goat antirabbit \( \gamma \)-globulin antiserum (Cappel Laboratories Inc., Downington, Pa.) and incubated overnight at 4\( ^\circ \). Centrifugation, radioassay, and calculations were performed as described previously (5). With each immunoassay run the following controls were included: (a) a tube with the specific anti-RBP antiserum at a 1:40 (v/v) final dilution, which resulted in approximately 90% precipitation of \( ^{125} \)I; (b) a tube with control antiserum alone, which produced 1 to 2% precipitation of \( ^{125} \)I; and (c) a tube with an excess of unlabeled RBP (usually 50 ng), which produced a 90 to 95% displacement of \( ^{125} \)I from the antibody. A stock solution of rat RBP, 1 mg per ml in deionized water, was diluted with standard assay buffer to make working standard solutions ranging from 1 to 400 ng per ml. The concentration of RBP in the stock solution was determined from its absorbance at 280 nm, assuming an \( E_{1%}^{1cm} \) of 19 (1).

Fig. 1 shows the curve describing the displacement of rat \( ^{125} \)I-RBP from antibody by increasing amounts of unlabeled rat RBP. The curve was sigmoid in appearance on a semilogarithmic plot, with the most sensitive portion of the curve corresponding to the addition of 0.5 to 3 ng of rat RBP per assay tube. When pooled plasma from normal rats was diluted with standard barbital-albumin buffer (5) and assayed at various dilutions simultaneously with standard RBP, the immunoassay curve was identical in shape with the curve obtained with pure RBP (Fig. 1). Thus, RBP in whole plasma displaced \( ^{125} \)I-RBP from antibody in a manner quantitatively identical with the displacement obtained with pure RBP. The recovery of rat RBP, the intra-assay (within assay) and interassay (between assay) agreement, were comparable with the corresponding values reported for the radioimmunoassay for human RBP (5). Because of the higher specific radioactivity of the labeled rat RBP, as compared to the previously reported labeled human RBP (5), the sensitivity of the immunoassay for rat RBP is approximately an order of magnitude greater than that of the reported immunoassay for human RBP.

Experimental Design-One hundred thirty-one weanling male rats, suitable for vitamin A research, were obtained from the Holtzman Co., Madison Wis. (weight range 48 to 67 g). The rats were housed in individual hanging wire bottom cages in an air-conditioned room with a mean temperature of 22\( ^\circ \). All rats had free access to food and water except the animals in the paired group. Upon their arrival the rats were randomly assigned to either a vitamin A-deficient diet (see Table I) (72 rats) or an identical control diet containing 6 \( \mug \) of vitamin A (as retinyl esters) per g of diet (59 rats). Both diets were prepared fresh weekly. During the first 2 weeks animals which did not gain weight or which showed other signs of disease (three control and six deficient rats) were killed. All animals were weighed two times a week throughout the study.

The rats within each of the two groups (deficient and control) were randomly divided into two subgroups. One subgroup of each treatment group was assigned to a slaughter experiment...

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**Fig. 1.** The radioimmunoassay standard curve for rat RBP. The figure shows the displacement of \( ^{125} \)I-RBP from antibody by purified RBP and by dilutions of normal rat plasma. The points for plasma were determined by first calculating the mean value for the level of RBP in the sample of plasma from the several assays carried out on that sample. The expected amount of RBP added per assay tube was then calculated from the amount of diluted plasma added. In the figure these values for the amount of RBP are plotted against the observed bound to free ratios for the corresponding samples.

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**Table I.** Characteristics of the experimental diets...

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**Graph.**...
with five rats from each of the deficient and control groups slaughtered on the 3rd, 7th, 14th, 20th, 25th, and 48th days of the study. Prior to killing, the rats were anesthetized with ether and blood was collected from the abdominal aorta. The livers were removed, rinsed with 0.9% NaCl solution, and blotted before rapid freezing with acetone and Dry Ice. Liver and serum samples were stored at -20°C for 8 hours.

Technique for Venipuncture from Subclavian Venous Plexus—We are grateful to Dr. W. A. Phillips of The Upjohn Co. for showing us this useful technique. This method has been very briefly referred to in an earlier publication by Phillips and Avigan (7). One-milliliter plastic disposable syringes equipped with disposable needles, No. 23 gauge x 1 inch, were used both for blood collection and later as centrifuge tubes. The rats were securely held by the paws on their backs with all four legs extended, and their heads drawn to one side at an angle of about 45° from the midline. The needle was inserted at the division of neck and shoulder, over the clavicle and below the pectoralis muscle, at an angle of about 30° from the midline. The needle was withdrawn slowly and very slight suction applied until blood appeared in the syringe. After the syringe was partly filled, pressure was applied to the plunger with the thumb and forefinger, and the needle removed. The pressure was maintained for 30 sec or longer if required for the bleeding to stop. The metal shaft of the needle was broken off, and the hole sealed with Duco cement. After standing for 2 to 3 hours at 4°C, the plungers of the syringes were removed, and the resulting tubes centrifuged at 1800 rpm for 30 min. The serum from 0.5 to 0.7 ml of blood can be removed easily from these slender tubes. No differences in growth rate or state of health were observed in the rats bled sequentially in this manner as compared to those which were not bled. Sera were stored at -20°C until assayed for RBP and vitamin A. Because of the very large number of samples collected, not all samples were assayed. At the end of the study, all of the serum samples collected from two rats in each of the five treatment groups were assayed for RBP and vitamin A. Sets of samples collected on only certain of the days were then assayed, in order to provide statistically valid data for the changes with time seen in each of the five groups of rats.

Vitamin A Assay—Samples of liver were extracted with 2.2 ml of chloroform-methanol (2:1, v/v) and ether by the method of Ames et al. (8), and the vitamin A content of the extracts was determined by the tri fluorocarboxylic acid method of Dugan, Frigerio, and Siebert (9). Samples of some of the livers were also assayed for vitamin A by the fluorimetric-saponification method of Thompson et al. (10); excellent agreement between methods was found in all cases.

The serum samples were assayed for vitamin A by the fluorimetric method of Thompson et al. (10); all volumes of reagents were reduced by one-half in order to carry out the assay with 0.1 ml of serum. All glassware used in these assays was washed with 50% ethanol or hexane before use in order to remove fluorescent contaminants.

Liver RBP Assay—Liver homogenates were prepared by homogenizing samples of freshly thawed liver with 3 volumes (v/w) of 0.25 M sucrose, 0.025 M KCl solution in a Potter-Elvehjem homogenizer. The homogenates were centrifuged at 2000 x g for 20 min at 5°C, and the resulting supernatants were used directly in the radioimmunoassay for rat RBP. The precipitates, sedimented at 2000 x g for 20 min, were found to contain negligible amounts of immunoreactivity in the radioimmunoassay, and were discarded. Liver homogenates and homogenate fractions were analyzed for total protein by the biuret method (11).

Other Procedures—Gel filtration of serum on columns of Sephadex G-100 was carried out as described previously (2) and in the companion paper (1). Total serum protein concentrations were estimated by the method of Lowry et al. (12), standardized against bovine serum albumin. Measurements of absorbances and absorption spectra, and fluorescence measurements, were made as described in the companion paper (1).

For serum samples, the percentage saturation of RBP with retinol was calculated with a molecular weight of 20,040 for rat RBP (the value obtained by sedimentation equilibrium analysis in the analytical ultracentrifuge (11)). For this calculation, all of the serum vitamin A was assumed to be present as retinol bound to RBP.

Statistical significance was assayed by using Student t test (13), with a Wang model 700A electronic calculator with programs prepared for that instrument.

### Table I

**Composition of vitamin A-deficient diet**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin-free casein*</td>
<td>18.0</td>
</tr>
<tr>
<td>Salt mixture Bernhart-Tomarelli R (6)*</td>
<td>4.0</td>
</tr>
<tr>
<td>Cellulose (Alpha-Cel)*</td>
<td>2.8</td>
</tr>
<tr>
<td>Peanut oil*</td>
<td>5.0</td>
</tr>
<tr>
<td>Corn starch*</td>
<td>34.0</td>
</tr>
<tr>
<td>Vitamin mix*</td>
<td>34.0</td>
</tr>
<tr>
<td>Glucose, anhydrous-</td>
<td></td>
</tr>
<tr>
<td>Cornstarch*</td>
<td></td>
</tr>
<tr>
<td>Peanut oil*</td>
<td></td>
</tr>
<tr>
<td>Glucose, anhydrous-</td>
<td></td>
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</tbody>
</table>

* ICN Nutritional Biochemicals, Cleveland, O.

1. The vitamin-free casein was refluxed with 24 volumes (w/v) of 0.1% HCl in ethanol for 8 hours. The extracted casein was washed with ethanol and dried first on a Buchner funnel and then in an oven at 50°C for 8 hours.


3. The vitamin mix provided the following per kg of diet: vitamin D, 2,200 I.U.; vitamin E, 110 mg; ascorbic acid, 1 g; inositol, 110 mg; choline chloride, 1.65 g; menadione, 49.5 mg; p-aminobenzoic acid, 110 mg; niacin, 100 mg; riboflavin, 22 mg; pyridoxine-HCl, 22 mg; thiamine-HCl, 22 mg; calcium pantothenate, 66 mg; folic acid, 2 mg; biotin, 440 μg; and vitamin Bi1, 22.5 μg.

4. The metal shaft of the needle was broken off, and the hole sealed with Duco cement. After standing for 2 to 3 hours at 4°C, the plungers of the syringes were removed, and the resulting tubes centrifuged at 1800 rpm for 30 min. The serum from 0.5 to 0.7 ml of blood can be removed easily from these slender tubes. No differences in growth rate or state of health were observed in the rats bled sequentially in this manner as compared to those which were not bled. Sera were stored at -20°C until assayed for RBP and vitamin A. Because of the very large number of samples collected, not all samples were assayed. At the end of the study, all of the serum samples collected from two rats in each of the five treatment groups were assayed for RBP and vitamin A. Sets of samples collected on only certain of the days were then assayed, in order to provide statistically valid data for the changes with time seen in each of the five groups of rats.

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6. Liver RBP Assay—Liver homogenates were prepared by homogenizing samples of freshly thawed liver with 3 volumes (v/w) of 0.25 M sucrose, 0.025 M KCl solution in a Potter-Elvehjem homogenizer. The homogenates were centrifuged at 2000 x g for 20 min at 5°C, and the resulting supernatants were used directly in the radioimmunoassay for rat RBP. The precipitates, sedimented at 2000 x g for 20 min, were found to contain negligible amounts of immunoreactivity in the radioimmunoassay, and were discarded. Liver homogenates and homogenate fractions were analyzed for total protein by the biuret method (11).

7. Other Procedures—Gel filtration of serum on columns of Sephadex G-100 was carried out as described previously (2) and in the companion paper (1). Total serum protein concentrations were estimated by the method of Lowry et al. (12), standardized against bovine serum albumin. Measurements of absorbances and absorption spectra, and fluorescence measurements, were made as described in the companion paper (1).
RESULTS

Comparison of Holo- with Apo-RBP in Radioimmunoassay—As described in the companion paper (1), RBP isolated from rat serum was microheterogeneous on disc gel electrophoresis, and consisted of a mixture of two forms of the holoprotein, together with a smaller amount of the retinol-free apoprotein. Holo-RBP and apo-RBP, separated from each other by preparative polyacrylamide gel electrophoresis (1), were each separately studied by radioimmunoassay. The results shown in Fig. 2 show that purified rat holo- and apo-RBP showed identical immunoreactivities in the radioimmunoassay.

Fig. 2 also shows the radioimmunoassay curves generated with samples of serum from control and from vitamin A-deficient rats. As indicated below, most of the RBP in control rat serum was present as the holoprotein, whereas almost all of the RBP present in deficient rats was present as apo-RBP. There were no significant differences in the immunoreactivities of the RBP present in control and deficient sera. Moreover, the curves generated with these samples of serum were identical in shape with those generated by pure holo- or apo-RBP (Fig. 2). These findings indicate that holo- and apo-RBP are immunologically indistinguishable in biological samples as well as when studied as pure proteins.

Dietary Treatment and Growth Rate—Fig. 3 shows the mean growth rates of the rats in each of the five dietary treatment groups. The rats fed the deficient diet and supplemented with 28 μg of retinoic acid per day (Group 5) appeared in excellent health and exhibited a growth rate equivalent to the rats eating the control diet on an ad libitum basis (Group 1). In contrast, the rats on the deficient diet alone (Group 3) ceased to grow after 50 to 60 days and were showing slight weight loss by the 75th day of the study. The daily growth rate of the deficient rats first became different from the controls after 28 to 31 days on the deficient diet. As noted below, this corresponded fairly closely to the time when vitamin A virtually disappeared from serum. In addition to the retarded growth rate, these rats exhibited other signs of vitamin A deficiency (14). When bleeding the deficient rats extreme care had to be taken to avoid injury to their paws while they were being held immobile. This problem, of susceptibility to injury, did not exist with the other four groups. The pair-fed rats had a retarded growth rate equivalent to the deficient rats, but appeared in a much better state of general health.

Serum RBP: Effects of Vitamin A Deficiency—The serum vitamin A concentration of the rats on the vitamin A-deficient diet decreased steadily during the first 25 to 30 days of the study (Fig. 4). The small amount of fluorescent material which was present in serum extracts after 30 days had an excitation maximum of 315 nm, which is typical for serum triglycerides rather than for vitamin A (332-nm uncorrected excitation maximum in hexane). The actual vitamin A levels were probably lower than the values of about 2 μg/100 ml of serum as estimated by the fluorometric method (and shown in Fig. 4).

The serum level of RBP also declined substantially during the
first 30 days of the study, particularly during the interval between Days 15 and 25. The time course of the RBP decline was fairly similar to that of vitamin A but with a slight lag of approximately 3 days (see Fig. 4). Unlike vitamin A, however, RBP did not disappear from serum. After approximately 40 days on the deficient diet the level of RBP became relatively stable at about 12 to 15 µg per ml, representing about 25 to 30% of the level of RBP seen on the 3rd day. Almost all of the RBP circulating at this time was present as apo-RBP, as indicated in Fig. 4 by the very low values for the percentage saturation of RBP with retinol. In contrast, in control rats and during the first few days of depletion (see Fig. 4) most of the RBP present in serum was found as holo-RBP, with average RBP percentage saturation values of 60 to 70%. The percentage saturation of RBP with retinol declined markedly during the period 10 to 30 days, because of the greater percentage decrease in vitamin A than in RBP levels during this time.

**Serum RBP in Control and Retinoic Acid-supplemented Rats**—The rats which were maintained on the control diet ad libitum (intake, 80 to 120 µg of vitamin A per day) maintained relatively constant serum vitamin A levels throughout the 75-day study (mean, 62 ± 3 µg of vitamin A per 100 ml of serum). During the first 2 weeks of the study the serum RBP levels rose to about 70 µg per ml and then remained at approximately this level during the rest of the study (Fig. 5). During the study the liver vitamin A stores of ad libitum fed control rats rose to a mean of 1700 µg of vitamin A per liver (see below).

The control rats which were pair-fed with the deficient rats had serum RBP (see Fig. 5) and vitamin A levels which were not significantly different from the ad libitum control animals. The decreased serum RBP level of the deficient rats was therefore not an effect of a reduced food intake.

Retinoic acid supplementation was started on the 25th day of the study, after the vitamin A stores of the rats on the deficient diet were exhausted. As indicated above, rats fed the deficient diet supplemented with retinoic acid appeared in good health and exhibited equivalent (28 µg per day retinoic acid) or only slightly reduced (14 µg per day) growth rates, as compared to ad libitum fed controls. Administration of retinoic acid at either 14 or 28 µg per day did not affect the serum RBP levels (Fig. 5). Identically low serum RBP levels were found in the retinoic acid-supplemented rats as in the vitamin A-deficient rats. These data indicate that the depression of serum RBP levels seen in deficient rats was not a secondary phenomenon, due to some aspect of poor health (e.g., bacterial infection) associated with vitamin A deficiency.

**Circulating Form of RBP**—As reported in the companion paper (1), rat RBP normally circulates in serum as a protein-protein complex, together with prealbumin-2. A study was conducted to determine whether the apo-RBP present in serum of deficient rats was also present in the form of a protein-protein complex, or as free, uncomplexed RBP. Samples of serum from control and from vitamin A-deficient rats were chromatographed on columns of Sephadex G-100, and the elution volume of immunoreactive RBP determined. As shown in Fig. 6, RBP in serum from a deficient rat had elution volume comparable to that of RBP in normal rat serum. This study indicates that most of the RBP (mainly apo-RBP) in vitamin A-deficient rats circulated in the form of a protein-protein complex, presumably with prealbumin-2.

**Effect of Vitamin A Status on Total Serum Protein**—The total serum protein level was estimated on serum samples obtained on the 3rd, 40th, and 75th days of the study. Each of the groups showed an increase in total serum protein as the study progressed, but no significant differences were observed between the various dietary treatment groups (see Table II).

**Effects of Repletion of Vitamin A-deficient Rats with Vitamin A**—Within 5 hours (the first time of sampling) after an oral dose of 24 µg of vitamin A the serum vitamin A level rose to 62 ± 5 µg/100 ml (mean ± S.E.M.). After 29 hours the serum vitamin A stabilized at 41 ± 4 µg/100 ml (mean ± S.E.M.) and remained near this level for the next 15 days, at which time the rats were killed.

The serum RBP level also showed a similar rapid response to vitamin A feeding. Within 5 hours (see Fig. 5) the RBP level rose markedly from a mean of approximately 14 to 26 ± 3 µg per ml. The RBP level decreased somewhat at 29 hours, and then rose and stabilized at a level of about 50 to 62 µg per ml. The values obtained for both the serum vitamin A and RBP levels after repletion of the vitamin A-deficient rats were nearly comparable to those observed in the control rats.

**Liver RBP: Effects of Vitamin A Deficiency**—Livers were obtained from control and vitamin A-deficient rats slaughtered at intervals throughout the study. The rats fed the diet containing 8 µg of vitamin A per g of diet had normal liver vitamin A stores which increased steadily during the study (from 37 ± 2
Fig. 6. Gel filtration on Sephadex G-100 of samples of serum from a control and from a vitamin A-deficient rat. Serum, 3.5 ml, from a rat fed the control diet for 48 days was applied to a column (2.5 x 60 cm; bed volume, 295 ml) of Sephadex G-100. A different column of Sephadex G-100 (2.5 x 69 cm; bed volume, 338 ml) was used to fractionate 2.5 ml of serum from a rat fed the deficient diet for 36 days. Both columns were eluted with 0.02 M sodium phosphate buffer, pH 7.5, containing 0.2 M NaCl. The elutions were conducted at a flow rate of 17 ml per hour. Fractions of 4.7 ml each were collected. Both of the columns had been previously calibrated by the gel filtration of a small amount of purified rat ICP. Absorbance is represented by the solid line, and RBP by the broken line.

**Fig. 7.** Levels of immunoreactive RBP in the livers of control and vitamin A-deficient rats. All values shown are the mean ± S.E.M. for five rats which were slaughtered. Control livers, □—□; deficient livers, ■—■. Also shown are the values for the total vitamin A content of deficient livers (○—○). The much higher values for vitamin A in control livers are not shown (see text).

**Fig. 8.** Displacement of rat $^{125}$I-RBP from antibody by various dilutions of homogenates of liver prepared from control and from vitamin A-deficient rats. The points representing the liver samples were calculated and plotted as described for serum in the legend to Fig. 1.

Effects of vitamin A status on total serum protein levels

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Days on diet</th>
<th>mg protein/ml serum</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
<td>40</td>
</tr>
<tr>
<td>Control (ad libitum)...</td>
<td>50.6 ± 2.0</td>
<td>65.7 ± 1.3</td>
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<tr>
<td>Control (pair-fed).....</td>
<td>66.7 ± 1.0</td>
<td>75.8 ± 4.6</td>
</tr>
<tr>
<td>Deficient...</td>
<td>56.9 ± 2.8</td>
<td>65.8 ± 3.8</td>
</tr>
<tr>
<td>Retinoic acid (28 μg per day)</td>
<td>66.4 ± 2.0</td>
<td>66.0 ± 2.8</td>
</tr>
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</table>

μg (mean ± S.E.M.) at 3 days to 259 ± 26 μg at 14 days to 587 ± 40 μg at 25 days to 1704 ± 277 μg at 48 days. In contrast, after 20 days all of the rats fed the deficient diet were estimated to have a total liver content of less than 1 μg of vitamin A (see Fig. 7). Estimates of less than 1 μg of vitamin A per liver were obtained whether assayed by the direct extraction method or by the saponification and fluorometric method. Since this represents an unreliable range in both assays, the liver reserves of vitamin A can be assumed to have been essentially gone by the 20th day of the study.

When liver homogenates from control or from deficient rats were tested in the radioimmunoassay for rat RBP, considerable amounts of immunoreactivity were observed. Moreover, the immunoassay curves generated by these liver homogenates were identical in shape with the curve obtained with pure RBP (Fig. 8). Thus, liver homogenates contained immunoreactivity which was quantitatively indistinguishable from the immunoreactivity of pure RBP, or of RBP in whole serum.

The level of immunoreactive RBP was low in the livers of control rats throughout the experiment (approximately 0.15 μg of RBP per mg of liver protein, see Fig. 7). Since these livers were not perfused with 0.9% NaCl solution before removal, a small part (approximately 10%) of the RBP found in these livers may have been due to serum contained within the liver. The
concentration of RBP (w/v) in the livers of control rats receiving vitamin A was approximately one-half to one-third of the level found in serum in these rats.

In contrast, the levels of immunoreactive RBP found in the livers of vitamin A deficient rats were higher than the corresponding serum levels, and were considerably higher than the levels of RBP found in the control livers (Fig. 7). In samples of liver obtained during depletion (before Day 20) the levels of immunoreactive RBP were approximately 21 times the levels of control livers (p < 0.01). Coincident with the disappearance of liver vitamin A on about the 20th day of the study, the levels of RBP in the livers of deficient rats increased markedly (p < 0.01 as cf. Day 14 levels) to 4 times the levels in control livers. The RBP concentrations of deficient livers remained at about this level for the remainder of the study. The livers of deficient rats which were supplemented with retinoic acid also contained comparably high levels of immunoreactive RBP.

**DISCUSSION**

This study was undertaken in order to obtain information about the role of dietary vitamin A in regulating the metabolism of RBP in the rat. The study was designed to explore in detail the effects of vitamin A depletion and deficiency on the levels of serum RBP, and to determine the time course of such effects in relation to the levels of vitamin A in serum and liver. The effects of nutritional vitamin A status on the levels of immunoreactive RBP in the liver were also examined. Data were, moreover, obtained about the effects of repletion of deficient rats with vitamin A.

In this study, plasma RBP and vitamin A levels were compared in five groups of rats, each fed a vitamin A-deficient diet with or without supplements as follows: Group 1, control: supplemented with an adequate amount of vitamin A; Group 2, pair-fed control: supplemented with vitamin A but with food intake matched to that of the deficient rats; Group 3, deficient: no vitamin A supplementation; Groups 4 and 5, retinoic acid: supplemented, after the initial depletion period, with a modest daily dose (14 or 28 µg for the two groups, respectively) of retinoic acid. The study was conducted for 75 days, during which time the food intake and growth of the rats were individually monitored. Some rats, from different groups, were killed at intervals, in order to simultaneously determine vitamin A and RBP levels in serum and liver. For most rats, serum blood samples were obtained by means of a highly effective venipuncture technique. The effects of vitamin A repletion were studied in some of the deficient and some of the retinoic acid-supplemented animals. It was anticipated that this experimental design would permit us to distinguish the primary effects of vitamin A deficiency from secondary effects due to reduced food intake (by comparison of control, pair-fed control, and deficient groups), or to bacterial infection, or general ill health (by comparison of control, deficient, and retinoic acid groups), or both.

The rats selected for this experiment had only moderate vitamin A stores (15 to 20 µg per g of liver), thus allowing for a rapid onset of vitamin A deficiency. Had adult rats with "high normal" vitamin A stores been used, 4 to 5 months might have been required for depletion (15). The vitamin A-deficient diet described here meets all known requirements for the growing rat except for vitamin A (16). Several vitamins which are not thought to be absolutely required by the rat were included in the diet to avoid extraneous complications (17). Theavitaminosis A produced by this diet should, therefore, have been a pure vitamin A deficiency.

In order to be able to conduct this study it was necessary to develop an accurate method with which to measure the levels of RBP in rat plasma, or tissues, or both. A sensitive and specific radioimmunoassay was developed which accurately measures RBP in the amounts of 0.5 to 3 mg per assay tube. Since human and rat RBP are immunologically distinct, and do not cross-react (1), it was necessary to isolate rat RBP and prepare an antiserum against rat RBP in order to develop this radioimmunoassay. The isolation and partial characterization of rat RBP was reported in detail in the companion paper (1). The radioimmunoassay accurately measures the total amount of RBP present in the sample, and does not distinguish between holo- and apo-RBP. This was shown by the identity of the displacement curves with both purified holo- and apo-RBP, and with serum samples containing predominantly holo- or apo-RBP.

The results presented here clearly show that vitamin A depletion and deficiency specifically affects the level of RBP in serum, and results in a substantial decrease in the level of RBP. In the rats fed the deficient diet, the serum vitamin A levels decreased gradually during the first 25 to 30 days of the study, to levels of about 2 µg/100 ml. The gradual decrease in serum vitamin A level during depletion of liver stores (first 20 days) and for a few days thereafter was observed in every rat which was followed by sequential venipuncture. Serum RBP levels also declined during the induction of vitamin A deficiency, with a time course similar to that seen with vitamin A, but with a lag of approximately 3 days. In contrast to vitamin A, a significant concentration of RBP remained in serum after 75 days on the deficient diet (15 ± 2 µg per ml). This level was equivalent to about 25% of the concentration found in this group of rats on the 3rd day of the study.

The control rats maintained constant serum vitamin A levels throughout the entire study. After an initial small rise the serum RBP levels of the control rats remained approximately constant (64 ± 3 µg per ml). Since one of the symptoms of vitamin A deficiency is a reduced food intake, a pair-fed control group was also studied. The serum vitamin A and RBP levels in the pair-fed and ad libitum control groups were similar. This finding eliminated the possibility that the decreased RBP level in deficient rats was secondary to a reduction in caloric or protein intake.

Further evidence that the low serum RBP levels found in the deficient rats was a primary rather than a secondary effect was obtained with the retinoic acid-supplemented rats. Rats which are maintained on a vitamin A-deficient diet supplemented with retinoic acid become blind and sterile, but are otherwise in a good general state of health and grow at a normal rate (18, 19). Retinoic acid differs from other compounds with vitamin A biological activity in that it cannot be converted into retinol (18, 20). In addition, animals accumulate no liver stores of retinoic acid (20) and maintain rather low serum retinoic acid levels (21). The retinoic acid groups were included in the study in order to provide vitamin A-deficient rats in a good state of health (e.g. no major bacterial infection). Since it was possible that retinoic acid itself might influence RBP metabolism, two levels of retinoic acid supplementation were studied, in order to obtain a graded response should such a response exist. A graded growth response has been previously reported for the levels of retinoic acid used in this study (22) and confirmed here. The
retinoic acid supplementation was begun on the 25th day of the study, after the vitamin A stores were exhausted, to prevent a retinoic acid-sparing effect on the liver stores of retinyl ester (20, 23).

The results obtained with the retinoic acid-supplemented rats were indistinguishable from those seen with the unsupplemented deficient rats. These data, coupled with the good health of the deficient rats during the first 50 days of the study, eliminate bacterial invasion as a factor contributing to the low RBP levels. The absence of an effect of retinoic acid on the serum RBP level is consistent with our finding that retinoic acid circulated bound to rat serum albumin (similar to free fatty acids (24)), rather than to RBP.\(^2\)

In contrast to the effects of vitamin A deficiency on serum RBP levels, no differences were observed in the concentration of total serum protein between deficient and control groups. This finding suggests that the effects on RBP were specific, rather than reflecting a general effect of vitamin A deficiency on serum protein metabolism.

Within 5 hours (first time of sampling) after the oral administration of retinyl acetate to deficient rats, their serum RBP and vitamin A levels returned to normal. This very rapid response to vitamin A supports the conclusion that the low serum levels of RBP specifically resulted from the absence of vitamin A in these animals.

Liver homogenates, when tested in the radioimmunoassay, were found to have considerable immunoreactivity, and generated immunoreactivity curves which were indistinguishable from the curves generated by pure rat RBP or by whole serum. Identical immunoreactivity curves were obtained with liver homogenates from control and from deficient rats. In the control rats the level of immunoreactive RBP remained low and constant throughout the experiment, at approximately one-half to one-third the level found in the serum of these rats. Much higher levels of immunoreactive RBP were found in the livers of the vitamin A-deficient rats. Prior to the total depletion of the liver vitamin A stores the rat fed the deficient diet had liver RBP contents which were approximately 2- to 4-fold greater than those of the controls. The liver stores of vitamin A were exhausted at about the 20th day of the study (10 ± 2 µg of vitamin A per liver on Day 14 to <1 µg of vitamin A per liver on Day 20). At this time (between Days 14 and 20) the content of RBP in the liver increased dramatically to a level about 4 times higher than that observed in the controls. This same difference was observed regardless if the liver RBP was expressed as micrograms of RBP per mg of protein, micrograms of RBP per g of liver, or micrograms of RBP per liver. After 20 days on the deficient diet the livers of the deficient rats contained approximately 750 µg more RBP per liver than did the livers of the control rats. We estimate that this amount of RBP was equivalent to or somewhat greater than the amount of RBP which would have been needed to restore the plasma (and extracellular) compartment to a normal level.

The decreased level of serum RBP seen in deficient rats might, theoretically, have been due to either a decreased rate of secretion of RBP into serum by the liver, or an increased rate of removal of RBP from the serum. In order to examine the latter possibility an experiment was conducted to determine the form in which RBP circulated in plasma in deficient rats. RBP normally circulates together with prealbumin, so a protein protein complex with apparent molecular size approximately 3 to 3.5 times that of rat RBP itself (molecular weight approximately 20,000). We have previously reported that in man apo-RBP appears to have a lesser affinity for human prealbumin than does holo-RBP (25, 26). The possibility therefore existed that the apo-RBP circulating in serum of deficient rats was present mainly as free, uncomplexed RBP, and hence was being much more extensively subjected to glomerular filtration and renal catabolism than normal. The gel filtration experiment reported in Fig. 6 ruled out this possibility, by showing that the apo-RBP in deficient rat serum was present mainly in the form of the protein-protein complex.

As already discussed, vitamin A deficiency results in a decreased level of RBP in serum together with an increased level of immunoreactive RBP in liver. The serum level of RBP can, moreover, be rapidly restored to normal by administration of vitamin A. Taken together, these findings suggest that vitamin A deficiency primarily interferes in some way with the secretion, rather than with the synthesis, of RBP to the liver, and that the deficient liver contains a pool of previously formed apo-RBP which is being released from the liver into the serum at a markedly reduced rate. This intrahepatic pool of apo-RBP can, apparently, be released rapidly into the serum, as holo-RBP, when vitamin A becomes available (repletion experiment). The data suggest that, in general, the liver selectively secretes the retinol-RBP complex (holo-RBP) rather than apo-RBP, and that the secretion process may in some way be coupled to and controlled by the factors involved in the formation of the retinol-RBP complex. Further experiments are planned to examine the characteristics of the immunoreactive RBP found in liver in control and deficient rats, and to explore in more detail the mechanisms whereby vitamin A regulates the production and secretion of RBP by the liver.

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