The Effects of Chylomicron Vitamin A on the Metabolism of Retinol-binding Protein in the Rat*

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SUMMARY

Vitamin A-deficient rats have low levels of retinol-binding protein (RBP) in serum and elevated levels of RBP in their livers. Chylomicrons containing newly absorbed vitamin A were injected intravenously into vitamin A-deficient rats, and the levels of vitamin A and of RBP in serum were determined on samples collected serially from individual rats. Data were also obtained on liver immunoreactive RBP concentrations with samples obtained at death. Chylomicrons were used so that the vitamin could be administered physiologically, in the form in which it is normally absorbed. After the injection of chylomicrons containing vitamin A a rapid increase in the serum levels of RBP and of vitamin A occurred, with maximal levels observed at 2 to 4 hours. The magnitude of the response was directly related to the amount of vitamin A given, in the dose range 0 to 17 μg of vitamin A. In an initial study of the dose range of 0 to 32 μg, a maximal response was obtained with doses of 16 μg or greater. Substantial increases in serum RBP levels were observed soon after chylomicron clearance, by 45 min after chylomicron injection. Livers were obtained 2 hours after chylomicron injection in rats given graded amounts of vitamin A. The dose-response relationship of the increase in serum RBP was mirrored by a complementary dose-related decrease in the level of RBP in the liver. Release of RBP from liver into serum, which was a function of the amount of vitamin A given, apparently occurred. Rats pretreated with either puromycin or cycloheximide also showed a rapid and substantial rise in serum RBP and vitamin A levels, after the injection of vitamin A. The results indicate that the increased level of RBP in serum after vitamin A injection mainly represents the release of previously formed RBP from an existing pool in the liver, rather than representing newly synthesized protein. The secretion of RBP by the liver is regulated efficiently by the availability of vitamin A for the formation of the retinol-RBP complex.

We have recently reported a study of the regulation of retinol-binding protein metabolism by nutritional vitamin A status in the rat (1). Vitamin A normally circulates in plasma in both man and the rat as retinol bound to a specific transport protein, retinol-binding protein (2, 3). Rat RBP has a molecular weight of approximately 20,000, α1 mobility on electrophoresis and circulates in plasma in the form of a protein-protein complex with apparent molecular weight of 60,000 to 70,000 (3). The properties of rat RBP resemble those of human plasma RBP in many ways.

Vitamin A deficiency specifically affects the level of RBP in serum and results in a substantial decrease in the level of RBP (1). When weanling rats were fed a vitamin A-deficient diet, serum vitamin A levels decreased during the first 30 days to about 2 μg/100 ml. Serum RBP levels also declined during the induction of vitamin A deficiency, to about one-fourth of the concentration found in control rats. The decreased RBP levels found in vitamin A deficiency were not secondary to reduced calorie or protein intake or to general ill health, as indicated by results obtained with pair-fed control and with retinoic acid-supplemented rats. Moreover, there were no differences in the concentration of total serum protein between vitamin A-deficient and control groups.

Liver homogenates were immunoreactive in the radioimmunoassay for rat RBP and generated immunoassay curves which were indistinguishable from those obtained with pure RBP (1). The level of immunoreactive RBP in the livers of deficient rats was four times (p < 0.001) that in the livers of control rats. When vitamin A was administered orally to deficient rats, a very rapid increase in serum RBP level, from a mean of 4 to 56 μg per ml, was seen within 5 hours (the first time interval sampled). The findings suggested that vitamin A deficiency primarily interferes 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.

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1 The abbreviation used is: RBP, retinol-binding protein.
We now report detailed studies of the effects of newly absorbed vitamin A, administered in the form of lymph chylomicrons, on the levels of RBP in serum and liver. These studies support and extend our previous conclusions, and provide considerable new information about the role which vitamin A plays in regulating the production and secretion of RBP by the liver.

**EXPERIMENTAL PROCEDURE**

**Vitamin A-defficient Rats**—Fifty-two male weanling rats of the Holtzman strain were fed a vitamin A-free diet to deplete their vitamin A stores as described previously (1). Depletion of the animals' vitamin A stores was estimated from the serum vitamin A levels of the individual rats. Two experiments were carried out. The first (Study I) employed 12 rats, chosen at random, which had been fed the deficient diet for 36 days. These rats had a mean body weight of 237 g. The remaining rats were each given 30 μg of retinoic acid per day in order to maintain them in good health with a normal rate of growth (1). We have previously observed that retinoic acid-supplemented rats are indistinguishable from unsupplemented vitamin A-deficient rats with regard to RBP levels in serum and liver and with regard to the response of serum RBP to oral vitamin A (1). Retinoic acid supplementation was discontinued 5 days before the second experiment (Study II) was carried out. Study II was conducted 26 days after Study I; at this time the rats weighed approximately 290 g each.

**Chylomicrons**—Polyethylene cannulae were implanted into the thoracic ducts of Sprague-Dawley rats below the diaphragm by a modification of the method of Bollman, Cain, and Grindlay (4), as previously employed in this laboratory in other studies of vitamin A metabolism in the rat (5-7). The rats were kept in restraining cages and given access to a solution of 5% glucose and 0.45% NaCl. One day after surgery the rats were given either peanut oil alone, with a negligible vitamin A content, or a solution of 1.5 mg of retinol in 0.5 ml of peanut oil. The oil meals were given by gastric intubation under light ether anesthesia. Chyle was collected for periods of from 8 to 24 hours.

Chyle was layered under a 0.9% NaCl solution and centrifuged at 25,000 rpm for 25 min. The centrifuge tubes were allowed to stand at 4°C for 4 to 5 hours and were then centrifuged to yield three additional chylomicron preparations containing, respectively, 1.3, 2.9, and 4.5 μg of vitamin A per ml, all with the same total lipid concentration of 3.67 mg per ml. The “vitamin A” chylomicron suspension contained 17.3 μg of vitamin A per ml. The “control” chylomicron suspension contained less than 0.03 μg of vitamin A per ml. Portions of the two chylomicron suspensions were then mixed together to provide three additional chylomicron preparations containing, respectively, 1.3, 2.9, and 4.5 μg of vitamin A per ml, all with the same total lipid concentration of 3.67 mg per ml.

Five groups of four rats each were employed in the first phase of Study II. Each rat in each group was injected via the tail vein with 1 ml of one of the five chylomicron preparations; a different chylomicron preparation was injected into each group of rats. Blood samples of approximately 0.5 ml each were collected from the subclavian venous plexus by the method of Phillips and Avigan (10); a more complete description of this technique was given in our previous publication (1). In order to reduce the number of samples to be collected serially within a few hours from each rat, the first blood samples were collected on the day prior to the injection of the chylomicrons. The results obtained with these samples were designated as zero-time values. Samples were collected at 1 and at 5 hours after the injection of chylomicrons. Five of the rats were killed at 5 hours after chylomicron injection; the rest of the rats were killed at 27 hours. Prior to killing, blood was collected from the abdominal aorta of each rat while under ether anesthesia. The livers were flushed with 0.9% NaCl solution prior to their removal. Tissues were rapidly frozen and stored at -20°C. All blood samples were allowed to stand at 4°C for 5 hours and were then centrifuged for 30 min at 1800 rpm. Serum samples were collected and stored at -20°C.

**Study II**—Two preparations of washed chylomicrons were obtained from thoracic duct cannulated rats. One preparation contained newly absorbed vitamin A, and the other (“control”) preparation was obtained from a rat fed peanut oil without vitamin A. The preparations were each diluted with 0.9% NaCl solution to the same total lipid concentration of 3.67 mg per ml. The “vitamin A” chylomicron suspension contained 17.3 μg of vitamin A per ml. The “control” chylomicron suspension contained less than 0.03 μg of vitamin A per ml. Portions of the two chylomicron suspensions were then mixed together to provide three additional chylomicron preparations containing, respectively, 1.3, 2.9, and 4.5 μg of vitamin A per ml, all with the same total lipid concentration of 3.67 mg per ml.

Five groups of four rats each were employed in the first phase of Study II. Each rat in each group was injected via the tail vein with 1 ml of one of the five chylomicron preparations; a different chylomicron preparation was injected into each group of rats. Blood samples of approximately 0.5 ml each were obtained from the subclavian venous plexus of two rats in each group at 15 min, 45 min, 4 hours, 8 hours, and 16 hours after chylomicron injection. These rats were killed 27 hours after chylomicron injection. As in Study I, zero-time values were obtained from samples collected on the day prior to the injection of chylomicrons. The other two rats in each group were killed at 2 hours after chylomicron injection. At the time of slaughter the rats were stunned and decapitated. The blood was collected and allowed to clot. The livers were flushed with an ice-cold 0.9% NaCl solution injected through the portal vein, rapidly frozen in Dry Ice-acetone, and stored at -20°C until assayed.

In the second phase of Study II, carried out concurrently with the first phase of the study, the effects of protein synthesis inhibitors were examined. Eight rats were each injected intraperitoneally with an NaCl solution of a protein synthesis inhibitor 1 hour prior to the intravenous injection of 1 ml of the
chylomicon suspension containing 17.3 μg of vitamin A per ml. Each of two rats received each of the following four treatments: puromycin dihydrochloride (Grade II, Sigma), 15 mg or 30 mg; or cycloheximide (Sigma), 0.75 mg or 2.25 mg. Serial blood samples were collected from the puromycin-treated rats at 45 min, 4 hours, and 8 hours after the injection of chylomicon, and the rats were slaughtered at 27 hours. Blood samples were obtained from the cycloheximide-treated rats 45 min after the injection of chylomicon. By 4 hours these rats appeared moribund, lying in their cages and breathing shallowly and irregularly. One rat died while the venous blood sample was being collected at this time. Accordingly, since death appeared imminent, the other three rats were slaughtered at this time (4 hours), and blood and liver samples were obtained as described above.

RESULTS

Study I—The first study was designed to determine whether the injection of chylomicon containing vitamin A would result in a rise in the level of serum RBP, and if so to obtain information about the time course of the response and about its relationship to the amount of vitamin A injected. The results are shown in Fig. 1. Comparable results were obtained with two rats given 22 μg of vitamin A in 1 ml of whole chyle and with three rats given the same amount of vitamin A in 1 ml of washed chylomicon. This indicated that the serum RBP response to newly absorbed vitamin A was due to the vitamin A-containing chylomicon and not to some other component of chyle.

Within 1 hour after the injection of chylomicon containing vitamin A, the serum RBP levels of the deficient rats rose to concentrations similar to those usually observed in control rats (1). The rapid rise in serum RBP concentration was accompanied by a similar rise in serum vitamin A level. The magnitude of the response appeared to be dose related. Maximal levels of serum RBP (60 to 70 μg per ml) at 1 hour after chylomicon injection were obtained with doses of 16 μg or greater of vitamin A. Between 5 and 27 hours the serum concentrations of RBP declined in a manner similar to that previously observed in response to a single oral dose of vitamin A (1).

When liver homogenates were assayed for RBP a definite trend was observed; the amount of immunoactive RBP in the liver at 5 or 27 hours was generally inversely related to the dose of vitamin A given. More detailed data on liver RBP levels are presented below.

Study II—This experiment was designed to be a much more detailed and more precisely controlled study of the dose-response relationship between the amount of vitamin A injected and the rise in serum RBP level. All rats received the same amount of chylomicon total lipid (3.67 mg) in the same volume (1 ml), so that the only variable was the amount of newly absorbed vitamin A contained in the chylomicon injected. The amount of vitamin A injected varied from 0 to 17.3 μg, since the latter dose had already been shown in Study I to give a maximal response. The intermediate doses of vitamin A (1.3, 2.9, and 4.5 μg) were small, in order to try to delineate the sensitivity of the RBP response to small amounts of vitamin A. Moreover, serum samples were obtained at more frequent intervals than in Study I.

The serum vitamin A responses to the injection of chylomicon containing varying amounts of vitamin A are shown in Fig. 2. Serum vitamin A levels rose rapidly, reaching their maximal values between 2 and 4 hours after chylomicon injection. The magnitude of the response was directly related to the amount of vitamin A given. The absence of a significant increase in serum vitamin A in the samples obtained at 15 min showed that the chylomicon were cleared from the serum before the rise in serum vitamin A levels began. The increased levels of vitamin A observed can therefore be assumed to reflect increased plasma levels of holo-RBP; this hypothesis is supported by the serum RBP data from these rats.

The changes in serum RBP concentration in response to the injection of chylomicon containing vitamin A were very similar to the changes seen in the levels of serum vitamin A (see Fig. 3).

**Fig. 1.** Serum RBP concentrations of vitamin A-deficient rats injected with chylomicon (or whole chyle) containing varying amounts of newly absorbed vitamin A (Study I). Two rats received “control” chylomicon (0.1 μg of vitamin A by assay) and two rats were given 32 μg of vitamin A. The corresponding data points shown are the mean values for each of these pairs of rats. The data points shown for rats receiving 22 μg of vitamin A represent mean values from five rats, three received washed chylomicon and two received whole chyle. The other data points shown represent values from single rats which were given 4 μg, 8 μg, or 16 μg of vitamin A.

**Fig. 2.** Serum vitamin A concentrations of vitamin A-deficient rats injected with chylomicon containing varying amounts of newly absorbed vitamin A (Study II). Each data point represents the mean of two values obtained with two rats. There was close agreement between the results on the duplicate samples which were averaged to provide each data point. For the 40 pairs of samples represented by the data shown in the figure, paired samples differed from each other by a mean of 4.5 ± 0.5 (S.E.M.) μg of vitamin A/100 ml of serum. The data at 2 and at 27 hours were obtained from rats which were slaughtered; the other data were obtained with samples collected from the subclavian venous plexus. The corresponding serum RBP values are shown in Fig. 3.
Fig. 3. Serum RBP concentrations of vitamin A-deficient rats injected with chylomicrons containing varying amounts of newly absorbed vitamin A (Study II). See Fig. 2 legend for details as to the source of samples. The results obtained with duplicate samples were in close agreement. For the 40 pairs of samples represented by the data shown in the figure, paired samples differed from each other by a mean of 6.1 ± 0.9 (S.E.M.) μg per ml of serum.

Serum RBP levels rose rapidly, also reaching their maximal values between 2 and 4 hours after chylomicron injection. The extent of the rise of RBP level was directly related to the amount of vitamin A given. The serum RBP concentration of the rats receiving 17.3 μg of vitamin A rose to unusually high levels at 2 and 4 hours. The saturation of the RBP with retinol in these 2- and 4-hour samples was relatively low. The observed rises in serum RBP were not the result of RBP contained in the injected chylomicron preparations, since radioimmunoassay of the chylomicron suspensions indicated that the maximal amount injected was 76 ng.

The concentrations of RBP in serum and liver in the rats slaughtered at 2 hours after the injection of chylomicrons containing the graded levels of vitamin A are shown in Fig. 4. The increase in serum RBP was accompanied by a complementary decrease in liver RBP, both of which were generally proportional to the amount of vitamin A administered.

**Effects of Protein Synthesis Inhibitors**—In the second phase of Study II, some of the vitamin A-deficient rats were injected with either puromycin dihydrochloride or with cycloheximide; two different doses of each inhibitor were studied. All four rats given cycloheximide were nearly dead by 4 hours after chylomicron injection (5 hours after cycloheximide). These rats were therefore killed at this time and the samples collected. The rats given puromycin were in a much better condition and probably could have survived the treatment. For each inhibitor, virtually the same results were obtained with each of the two doses employed. The results obtained with each inhibitor were therefore pooled for presentation, without regard to dose.

Each of the drug-treated rats was given 1 ml of the chylomicrons containing 17.3 μg vitamin A. The effects of chylomicron injection on the serum levels of vitamin A and of RBP in these rats are shown in Figs. 5 and 6. Also shown in Figs. 5
Effects of injection of chylomicrons containing vitamin A on liver RBP levels of rats treated with protein synthesis inhibitors and of control rats

<table>
<thead>
<tr>
<th>Inhibitor injected</th>
<th>No. of rats</th>
<th>Vitamin A injected</th>
<th>Time after chylomicron injection</th>
<th>Liver RBP a</th>
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</thead>
<tbody>
<tr>
<td>None</td>
<td>2</td>
<td>0</td>
<td>27 h</td>
<td>146 µg/g</td>
</tr>
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<td>2</td>
<td>17.3 µg</td>
<td>2 h</td>
<td>49 µg/g</td>
</tr>
<tr>
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<td>2</td>
<td>17.3 µg</td>
<td>27 h</td>
<td>94 µg/g</td>
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<td>17.3 µg</td>
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<td>4</td>
<td>17.3 µg</td>
<td>4 h</td>
<td>53 µg/g</td>
</tr>
</tbody>
</table>

Note: Mean values for the number of rats shown in the second column. 

These rats were injected, in Study II, with chylomicrons containing no vitamin A and illustrate the high liver RBP levels characteristic of vitamin A-deficient rats (1).

DISCUSSION

These studies were designed to examine in detail the effects of newly absorbed vitamin A on the release and metabolism of RBP by the liver. Chylomicrons containing newly absorbed vitamin A were injected intravenously into vitamin A-deficient rats, and the levels of vitamin A and of RBP in serum were then determined on samples collected serially from individual rats; data were also obtained on liver RBP concentrations. Chylomicrons were used so that the vitamin could be administered in a physiological form. Studies previously conducted in both the rat (5, 7) and in man (11) have demonstrated that dietary vitamin A is normally absorbed via the lymphatic route, almost entirely as retinyl esters, and mainly in association with lymph chylomicrons. It is also well established that the liver plays the major role in the metabolism of newly absorbed vitamin A. After the intravenous injection into rats of chylomicrons containing newly absorbed vitamin A, at least two-thirds of the vitamin A was removed from the vascular compartment by the liver (6).

Previous observations demonstrated that repletion of vitamin A-deficient rats with dietary vitamin A resulted in a rapid rise in the serum level of RBP (1). The results presented here demonstrate that the increased serum RBP concentrations were a response to the vitamin A contained in chylomicrons and not to some other component of chyle. The results delineate both the time course of the response and the relationship between the amount of vitamin A injected and the extent of the rise in serum RBP. After the injection of chylomicrons containing vitamin A, serum RBP levels did not rise during the first 15 min, the period when chylomicron vitamin A was being largely cleared from the vascular compartment (6). Thereafter a rapid increase in the serum levels of RBP and of vitamin A occurred, with maximal levels observed at 2 to 4 hours. The molar ratio of vitamin A to RBP in the serum samples obtained at 2 and 4 hours was approximately 0.3 in all treatment groups. This low molar ratio suggests that either the administration of vitamin A stimulated the release of RBP in excess of that required to bind and transport the vitamin, or that the peripheral tissues of the deficient rats may have been removing the retinol from the RBP quite rapidly, or both. By 27 hours, the molar ratios of vitamin A to RBP in the rats receiving the largest dose of vitamin A in Study II (17.3 µg) were about 0.5.

The magnitude of the rise of serum RBP, and of serum vitamin A level, was directly related to the amount of vitamin A given, in the dose range 0 to 17 µg vitamin A. A rise in serum RBP level could be observed after the injection of as little as 1.3 µg of vitamin A. In the major study (11), with the exception of the 17.3-µg dose of vitamin A, the amounts of vitamin A given would not have been enough to return the plasma compartment to normal vitamin A levels. From the serum levels of vitamin A achieved after chylomicron injection (Fig. 2), it is evident that most of the injected vitamin A recirculated in the plasma compartment after its initial clearance from the circulation. Since no information is available about the rate of turnover of plasma vitamin A in these rats, a more quantitative statement cannot be made regarding the extent of recirculation.

Several kinds of evidence were obtained which indicated that the rapid rise in serum RBP after chylomicron vitamin A injection mainly represented the release of preformed liver RBP rather than representing newly synthesized RBP. First of all, the very short time interval required for a substantial rise in serum RBP (within 45 min) strongly suggests that the RBP was derived from the expanded pool of liver RBP present in the deficient rats rather than from de novo synthesis. More direct evidence was derived from a study of the levels of serum and liver RBP, in relation to the dose of vitamin A given, in rats slaughtered after 2 hours (Fig. 4). Throughout the vitamin A range studied, the increase in serum RBP was mirrored by a complementary decrease in liver RBP, consistent with a postulated release of RBP from liver into serum.

Additional evidence for the release of preformed RBP from the liver was obtained in the study with protein synthesis inhibitors. Treatment of rats with puromycin or cycloheximide 1 hour before the injection of chylomicrons containing vitamin A did not prevent the occurrence of a substantial rise in the serum
levels of RBP. The levels of serum RBP attained at 4 hours in the inhibitor-treated rats, after injection of 17.3 μg of vitamin A, were comparable to those usually observed in rats fed a diet containing an adequate amount of vitamin A (1). These levels were however, lower than the peak serum RBP levels seen in the rats not treated with protein synthesis inhibitors. This finding suggests that the continued release of RBP from the liver at a maximal rate may require some concomitant de novo biosynthesis of RBP in order to replenish the pool of RBP within the liver. The data obtained concerning the levels of immunoreactive RBP in the livers of the inhibitor-treated rats (Table I) are consistent with this suggestion. Alternatively, the lower peak serum RBP levels found in the inhibitor-treated rats may reflect a lower vitamin A uptake from plasma by the peripheral tissues of the rats treated with protein synthesis inhibitors, and hence a reduced stimulus for RBP release in these rats. This interpretation is suggested by the fact that the peak vitamin A levels of the inhibitor-treated rats were comparable to those of control rats (Fig. 3).

The secretion of RBP by the liver appears to be regulated efficiently by the availability of vitamin A at the liver cell for the formation of the retinol-RBP complex. After the injection of chylomicrons containing vitamin A, a number of processes must occur before holo-RBP containing the newly absorbed retinol can appear in the circulation. These processes include chylomycin clearance and metabolism, uptake of chylomicron retinyl ester by the liver, and retinyl ester hydrolysis to provide retinol. Since the rise in serum RBP level occurred very soon after chylomycin injection, it is evident that these processes must be efficiently “coupled” to the formation of the retinol-RBP complex and to RBP secretion from the liver. Studies currently in progress have shown that almost all of the immunoreactive RBP in liver, in both normal and deficient rats, is found in the particulate fractions of the liver homogenates (12). We suggest that the appearance of retinol in or on the liver cell generates some kind of a “signal” which effectively stimulates both the formation of a complex between the retinol and RBP and the secretion of the resulting holo-RBP molecule into the circulation. The nature of such a “signal” and the manner of its operation are obscure. Its exploration could, however, provide insight into the factors which control vitamin A delivery from the liver to peripheral tissues and may also provide information about factors which regulate the secretion of other plasma proteins as well.

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