Effect of vitamin A status at the end of term pregnancy on the saturation of retinol binding protein with retinol¹⁻³

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ABSTRACT
Background: Vitamin A (retinol), which is required for normal fetal development and successful gestation, circulates in the blood bound to a specific protein, the retinol binding protein (RBP). Little is known about the transport and metabolism of this complex protein or about retinol status during normal human pregnancy.

Objective: The aim of this study was to assess retinol status and transport modalities of retinol in well-nourished women with normal pregnancies, a population poorly investigated compared with pathologic and malnourished pregnant women.

Design: The maternal blood and cord blood concentrations of retinol, vitamin E, β-carotene, RBP, and transthyretin of pregnant French women at term (n = 27) were measured and compared with values from a nonpregnant control group (n = 27). In addition, holo-RBP (retinol bound), apo-RBP (retinol free), and total protein were assessed in both groups to enable the hemodilution occurring during pregnancy to be taken into consideration and to evaluate the extent of saturation of RBP with retinol.

Results: Healthy pregnant women at term had normal serum circulatory amounts of retinol, vitamin E, binding proteins, and β-carotene. However, they had less binding of retinol to RBP (holo-RBP: 49.9% in pregnant women, 54.0% in cord blood, and 77.5% in the control group).

Conclusion: The results of this study suggest that retinol homeostasis and transport are modified during normal human pregnancy.

INTRODUCTION
Vitamin A (retinol) compounds are fat-soluble micronutrients that are critical for many functions, including vision, reproduction, growth, and regulation of cell proliferation and differentiation (1, 2). Therefore, vitamin A is essential for successful gestation and is supplied by the mother to her fetus (3, 4). Obstetric abnormalities [fetal malformations, intrauterine growth retardation (IUGR), and spontaneous abortion] were observed during gestation in animals subjected to hypovitaminosis A (5–7) or hypervitaminosis A (8). In addition, long-term hypovitaminosis A affects female and male reproductive tracts, resulting in reversible sterility (9, 10). Women with low dietary vitamin A intakes have difficulty becoming pregnant, mainly because of the effect of vitamin A deficiency on their health status (11–13). In addition, their infants, who have low concentrations of vitamin A at birth, have a higher risk of morbidity, possibly because of an impaired immune status (14). In addition, human obstetric disease states (IUGR and placental disruptions) may be associated with abnormal maternal blood retinol concentrations (15, 16). Acute hypervitaminosis A during the embryogenic period of human pregnancy may result in teratologic effects (8, 17, 18). These considerations suggest that there should be a thorough regulation of vitamin A metabolism between the mother, the placenta, and the fetus during pregnancy.

Vitamin A status and metabolism have been poorly investigated in well-nourished women with normal pregnancies compared with the large number of studies in pathologic and malnourished pregnant women (19–24). However, healthy pregnant women undergo important physiologic adaptations that might influence retinol concentrations, especially an increase in blood volume (25).

The aim of the present study was to determine the concentrations and circulating quantities of retinol and β-carotene (the main provitamin A carotenoid) in maternal blood and in arterial and venous cord blood (a classic reflection of fetal status) at term in normal pregnancy and to compare them with values from nonpregnant women. Because retinol circulates in blood as a ternary complex associated with retinol binding protein (RBP) and transthyretin, the concentrations of these proteins were measured, as was the extent of saturation of RBP with retinol.
SUBJECTS AND METHODS

Subjects

Thirty pregnant women were recruited from the Department of Obstetrics and Gynecology of Hotel-Dieu Hospital, Clermont-Ferrand, France, when they arrived for normal delivery. All subjects gave their written, informed consent before participation in accordance with the ethical standards of the responsible institutional committee on human experimentation. Exclusion criteria included multiple pregnancies, disease states during pregnancy (pre eclampsia, fetal malformation, IUGR, infection, and hepatic or digestive disease), preterm labor, and vitamin A supplementation. Twenty-seven healthy, nonpregnant women who had undergone analytic screening constituted the control group. These women had volunteered for various experimental protocols at the Human Nutrition Research Center, Auvergne. The control group (29.5 ± 3.2 y) was age-matched with the pregnant group (29.7 ± 5.4 y). The control group did not take supplements and their smoking habits were similar to those of the pregnant group: 9 pregnant women smoked and 8 control women smoked. The absence of pregnancy was certified by immunologic measurement of β-chorionic gonadotropin. The women in both groups were of the same socioeconomic background (middle class) and race (white).

Dietary intake of vitamin A during pregnancy

To assess the dietary intake of vitamin A and carotenoids, we used a self-administered, semiquantitative food-frequency questionnaire derived from the one described by Russel-Briefel et al. (26). The pregnant and control groups were asked weekly to indicate the average frequency of consumption of 39 food items and of vitamin supplements. Information about the typical serving size of each food item was obtained by using pictures of servings for which corresponding weights had been validated (27). The 39 food items collectively accounted for >95% of the vitamin A and carotenoid intake in the French population (J Ireland-Ripert, Centre Informatique sur la Qualité des Aliments, Paris, personal communication, 1991). The questionnaire was given to the women on their admission to the hospital and was completed before discharge from the hospital. We took care that women in the pregnant group had not eaten unusual foods (ie, items not included on the list of 39 items) that may be major sources of vitamin A. Women in the control group filled in this questionnaire when they visited the research center to have their blood sampled. The daily dietary intake of vitamin A was calculated by using GENI (Micro 6, Nancy, France).

Sample collection and treatment

When each woman arrived at the hospital for delivery, 5 mL venous maternal blood was collected before active labor began. The women had been fasting for >11 h, except for 3 who had eaten a light meal 3–5 h earlier. After delivery, a piece of placental tissue was immediately frozen at −80°C. Arterial and venous cord vessels were located by trained midwives using morphologic and color indicators, and arterial and venous cord blood were collected separately. The serum was prepared by clotting red cells for 4 h in the dark (2 h at room temperature and 2 h at 4°C) and then centrifuging the samples at 1000 × g for 10 min at 4°C. Serum was separated into aliquots and frozen at −80°C until analyzed. The control group provided blood samples after fasting overnight and the samples were processed as described for the pregnant group.

Vitamin A, vitamin E, and β-carotene assays

Vitamin A (all-trans retinol and retinyl palmitate), vitamin E (α-tocopherol), and all-trans β-carotene were determined from assays of 0.5 mL thawed serum or 1 g placental tissue, as described previously, with some minor modifications (28, 29). An equal volume of ethanol containing internal standards (retinyl laurate, tocopheryl acetate, and echinencene for retinol, vitamin E, and β-carotene, respectively) was added to each sample. The samples were then extracted twice with 2 volumes of hexane. After evaporation to dryness, the extract was dissolved in 250 μL of a mixture of dichloromethane and methanol (35:65, by vol) and dispatched equally into 2 injection vials. The compounds were analyzed by reversed-phase HPLC on a Waters (Milford, MA) apparatus equipped with a 600 pump, a 710 automatic injector, and a 996 diode-array detector and controlled by MILLISENIUM 2.1 (Millipore Waters Chromatography, Millipore, France). For retinol and α-tocopherol (vitamin E) measurements, the samples were eluted on a Nucleosil 250 × 4.6-mm C18 column (Interchim, Montluçon, France) by using pure methanol as the mobile phase (2 mL/min); the detection was performed at 325 and 292 nm. β-Carotene was separated after elution of the sample on a Zorbax 250 × 4.6-mm C18 column (Interchim) with a mixture (1.8 mL/min) of acetonitrile, dichloromethane, and methanol (70:20:10, by vol) and detected at 450 nm. Identification was based on coelution with authentic standards and ultraviolet light–spectrum comparisons. Quantification involved internal standardization and dose-response curves established with authentic standards.

RBP, transthyretin, and total blood protein measurements

Total RBP and transthyretin were measured by using nephrometric kits on a nephelometer (model BN 100; Behring SA, Marburg, Germany). Total blood proteins were measured by using the Biuret method (Hitachi 717; Boehringer Mannheim Diagnostics, Mannheim, Germany) (30). Retinol, β-carotene, vitamin E, RBP, and transthyretin concentrations were divided by their paired total protein concentrations to account for hemodilution.

Holo-RBP and apo-RBP measurements

Holo-RBP (retinol bound) and apo-RBP (retinol free) in serum were assessed by using polyacrylamide gel electrophoresis (PAGE) immunoblotting analysis. Whole serum was subjected to vertical-slab nondenaturing PAGE (7.5% acrylamide), in which the release of retinol from holo-RBP was shown to be insignificant (<2%) (31). The proteins were separated according to their electrophoretic mobilities (with respect to their net charge and molecular weight) and were transferred onto a nitrocellulose sheet. Both holo- and apo-RBP immunoreactive bands were visualized by using a rabbit anti-human RBP serum diluted to 1:200 (Behring) and biotinylated goat anti-rabbit immunoglobulins, avidin-bound peroxidase (ABC kit, Vector Laboratories, Burlingame, CA), and diaminobenzidine (Sigma-Aldrich Corp, Saint Quentin Fallavier, France) as substrate. The holo- and apo-RBP percentages were determined after densitometry of the membrane with a CD8 camera (Sony, Kyoto, Japan) and were analyzed with PICLAB software (Rage, Marseille, France). The delipidized serum (containing only apo-RBP) was prepared by mixing 1 volume of human serum with 2 volumes of 1-butanol and diisopropyl ether (80:20, by volume) for 4 h at room temperature. The
mixture was then centrifuged at 1000 × g for 10 min at 4°C and the organic solvent layer was discarded.

**Statistical analysis**

Mean values for arterial and venous cord blood were calculated and compared with the paired values. Mean (±SD) values were calculated for all variables. Group comparisons were made by using Student’s t test, either paired (to compare maternal with arterial or venous cord serum and arterial with venous cord serum) or unpaired (to compare serum from the control group with that from the pregnant group). Spearman’s rank-order correlation test was used to assess the relations among the variables. Statistical procedures were performed by using STATVIEW (Abacus Concepts, Inc, Berkeley, CA). For all of the studies, the criterion for significance was P < 0.05.

**RESULTS**

**Newborns and dietary intake during pregnancy**

Three of the 30 pregnant women recruited to the study were excluded from the final analysis (Table 1): 2 because they had hypotrophic newborns (birth weight < 10th percentile) (32) and 1 because she took vitamin A supplements during pregnancy [1200 retinol equivalents (RE)/d]. All of the newborns from the remaining mothers were healthy and their morphometric values were within the standard range for French newborns (32). Growth, development, and postpartum changes were normal for all mothers and newborns on the basis of a medical examination that occurred in the 7 d after delivery. The dietary intake of vitamin A (preformed retinol plus provitamin A carotenoids) was not significantly different between the pregnant group and the control group (2150 ± 1170 compared with 1691 ± 1110 RE/d, respectively) and was well above the French daily recommended intake (1000 RE) (33).

**Maternal and cord blood concentrations of retinol, β-carotene, and vitamin E concentrations at term**

Maternal and cord blood concentrations of retinol, β-carotene, and vitamin E are shown in Table 2. Retinyl palmitate was not detectable in the cord blood of any of the women and was measurable in the serum of only 2 women, in whom it reached 46 and 34 μmol/L. These women had eaten a meal in the 5 h before the blood sampling; thus, retinyl palmitate likely came from the newly absorbed vitamin A. The women did not show any peculiarity in any other variables measured.

There were no significant differences between arterial and venous cord blood in any of the variables measured. Therefore, data on arterial and venous cord blood were combined into a single group called “cord blood.” We found significant differences in concentrations of retinol, vitamin E, and β-carotene between cord blood and maternal blood, which were 2-fold, 5-fold, and 14-fold higher, respectively, in maternal blood than in cord blood.

Maternal serum retinol and vitamin E concentrations were significantly lower in the pregnant group than in that of the control group. When the retinol, β-carotene, and vitamin E concentrations in both groups were expressed on the basis of the circulating protein amounts, there was no significant difference between the groups.

Retinol is a hydrophobic molecule transported in blood, bound to its specific carrier protein (RBP), which itself forms a soluble ternary complex with transthyretin. Both of these proteins were measured in maternal, cord, and control blood (Table 2). As with the other variables, maternal RBP and transthyretin concentrations were significantly different from their cord blood counterparts and from the control data. When we expressed the values on the basis of circulating proteins, however, these differences between the pregnant group and the control group disappeared. Mean retinol and β-carotene concentrations in the placenta were 0.023 ± 0.008 and 0.004 ± 0.001 μmol/g tissue, respectively. Retinyl palmitate was detected in placental tissue in only one sample.

**Retinol saturation coefficient of RBP in maternal and cord blood at term**

There were no significant differences in molar ratios of retinol to RBP between the pregnant group and the control group or between arterial and venous cord blood. However, cord blood values were significantly different from maternal values (Table 2). In addition, we studied the retinol saturation coefficient of RBP by measuring the percentages of holo- and apo-RBP (Figure 1). As expected, holo-RBP was the major form of RBP in the control group (Table 2). No degraded forms of RBP were observed in the control group or in the pregnant group (Figure 1). There were major differences in the percentages of holo- and apo-RBP between the pregnant group (49.9% and 46.5%, respectively) and the control group (77.5% and 19.7%, respectively).

**Table 1**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
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<tr>
<td>Age (y)</td>
<td>29.7 ± 5.4[^1^]</td>
</tr>
<tr>
<td>Pregnancy duration (d)</td>
<td>276 ± 9</td>
</tr>
<tr>
<td>Weight gain during pregnancy (kg)</td>
<td>14.2 ± 4.2</td>
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<td>Parity</td>
<td></td>
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<tr>
<td>1</td>
<td>10</td>
</tr>
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<td>≥2</td>
<td>17</td>
</tr>
<tr>
<td>Smoking status during pregnancy (cigarettes/d)</td>
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</tr>
<tr>
<td>&gt; 3</td>
<td>9</td>
</tr>
</tbody>
</table>

[^1^] All data for the mothers were collected during the last routine antenatal medical examination (eighth month); all data for the newborns were collected immediately after birth. 

[^2^] ± SD.
As described previously (40), retinol concentrations were lower in the pregnant group than in the control group of nonpregnant women. However, plasma volume expansion is known to occur during pregnancy (25), resulting in decreased concentrations of stable markers such as total blood proteins. This hemodilution phenomenon was cited recently as one of the explanations for the decrease in maternal concentrations of retinol during pregnancy (40). By correcting the retinol values by using the paired protein concentrations, we established that the corrected amounts of retinol were not significantly different between the 2 groups. This implies that the same quantities of retinol were present in the blood of pregnant nonpregnant women, suggesting that the same absolute amounts were circulating in the pregnant group but at a lower concentration than in the control group. Similarly, vitamin E, RBP, and transthyretin concentrations were lower in the pregnant group in this study and others (41–43). The biological consequences of a lower concentration of the same quantity of vitamins or binding proteins are unknown. It might be that some steps of the metabolism or transfer of these compounds, such as the binding to a receptor or to an active enzymatic site, are conditioned by the concentration more than by the total quantity in the whole blood.

The corrected amounts of total RBP in maternal blood at term were not significantly different from the raw amounts in control blood. However, the percentages of holo- and apo-RBP in arterial and venous cord blood, \( P < 0.05 \) (Student’s \( t \) test).

**DISCUSSION**

In the present study, we measured several variables linked to vitamin A status in maternal and cord blood of French women with normal pregnancies and adequate vitamin A status. Generally, we found retinol and \( \beta \)-carotene concentrations equal to or higher than those found in malnourished pregnant women or women with disease states during pregnancy (5, 6, 22–24, 34–38). Any differences could be explained by differences in socioeconomic conditions or dietary practices. However, our data are consistent with data from the few studies performed in healthy, well-nourished pregnant women (39).
only 2 cases. First, during vitamin A deficiency in rodents (44), an increase in serum apo-RBP (with a decrease in total RBP) was reported when the serum retinol concentration was low as a result of a vitamin A–deficient diet. In our study, the pregnant group had normal vitamin A intakes and the amounts of retinol and total RBP in their serum, after correction for hemodilution, were similar to those of the control group; therefore, these women were not considered to be vitamin A deficient. Second, increased apo-RBP was reported in human chronic renal failure (45–47), together with an increase in serum total RBP and retinol, whereas serum transthyretin remained normal. According to the authors of these reports, this increase in serum total RBP and retinol was due to a decrease in the glomerular filtration rate (GFR) and an impairment in tubular reabsorption. In contrast, in the pregnant group, corrected total RBP, transthyretin, and retinol in serum were similar to those of the control group, whereas serum apo-RBP was higher. In addition, clinical and biological data for the pregnant group in the present study did not indicate the occurrence of proteinuria. However, we cannot exclude the possibility that some pregnant women could have had incipient and biologically nondetectable microalbuminuria. Furthermore, the GFR increases dramatically during normal pregnancy (48), whereas it decreases during chronic renal failure. Consequently, the increased apo-RBP in the pregnant group cannot be explained by renal failure during pregnancy or by vitamin A deficiency. Thus, our results suggest that pregnancy is a physiologic situation in which apo-RBP concentrations can increase.

**TABLE 3**
Spearman’s rank correlations for study variables

<table>
<thead>
<tr>
<th></th>
<th>Rol MB</th>
<th>Holo MB</th>
<th>Apo MB</th>
<th>Holo CB</th>
<th>Apo CB</th>
<th>Rt MB</th>
<th>TTR MB</th>
<th>β-Car MB</th>
<th>β-Car CB</th>
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</thead>
<tbody>
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<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>0.59</td>
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<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>0.7</td>
<td>0.53</td>
</tr>
<tr>
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<td>NS</td>
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<td>NS</td>
<td>0.66</td>
<td>NS</td>
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</tr>
<tr>
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<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>0.79</td>
<td>NS</td>
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<tr>
<td>Holo CB</td>
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<td>NS</td>
<td>NS</td>
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<td>NS</td>
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<tr>
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<td>Rt MB</td>
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<tr>
<td>TTR MB</td>
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<td>NS</td>
<td>NS</td>
<td>NS</td>
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<td>β-Car MB</td>
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<td>NS</td>
<td>NS</td>
<td>NS</td>
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<td>β-Car CB</td>
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As reported previously, no significant differences were detected between arterial and venous cord blood for any of the variables. Therefore, arterial and venous data were combined into a single group called “cord blood.” Only correlations > 0.4 (P < 0.05) are reported. Rol, retinol; MB, maternal blood; CB, cord blood; RBP, retinol binding protein; Holo, percentage of holo-RBP; Apo, percentage of apo-RBP; Rt, retinol-RBP molar ratio; TTR, transthyretin; β-Car, β-carotene.
In some studies, it was reported that abnormal quantities of total RBP (ie, 60–2380 μg/L) are excreted in the urine of pregnant women (49, 50) because of the increase in the GFR during pregnancy (48). It is also known that after holo-RBP delivers its retinol to target tissues, the resulting RBP—devoid of retinol (apo-RBP)—shows a lower affinity for transthryatin and is rapidly excreted in the urine (51). In our study, we showed that the percentage of apo-RBP in serum was higher in the pregnant group than in the control group, which agrees with the findings of other studies.

Using apo- and holo-RBP, we estimated that retinol was actually bound to only 0.0156 g RBP/L (49.9% of 0.031 g/L) in the pregnant group compared with 0.032 g/L (77.5% of 0.042 g/L) in the control group. Similar analyses were made for cord blood samples. This result implies that the molar ratio of retinol to holo-RBP was 1.05, 1.34, and 1.72 for control blood, cord blood, and maternal blood, respectively. The fact that one molecule of RBP binds only one molecule of retinol correlates well with the molar ratio found in our control group (1.05). In contrast, the ratios of 1.34 and 1.72 could not be explained by our current understanding of RBP. For example, these ratios imply that 0.53 μmol retinol/L was circulating in the maternal blood not bound to RBP. The results of solubility studies indicate that retinol cannot be free in serum at a concentration > 0.06 μmol/L (52). Moreover, an increase in free retinol would result in a higher urinary loss of retinol and predispose the woman to a vitamin A deficiency at term, a situation that has not been reported under conditions similar to those of our study.

Two main hypotheses might explain our findings of higher apo-RBP concentrations and retinol–holo-RBP molar ratios in the cord blood of the pregnant group than in the blood of the control group in the face of the solubility and renal-filtration results. The first is that retinol might be bound to another still-uncharacterized protein, as suggested previously by Sklan et al (53, 54). The second is that pregnancy and in utero life alter the affinity of RBP for retinol. This lower affinity, possibly due to the known higher GFRs in pregnant women than in nonpregnant women or to an interaction of RBP with placental factors in both the mothers and the neonates, could lead to an easier dissociation of the retinol from its binding site on RBP under in vitro conditions during the analytic procedures of sample preparation and electrophoretic separation.

The fact that vitamin E and β-carotene were 5- and 14-fold more concentrated, respectively, in maternal blood than in cord blood may have been due to lower tocopherol and β-carotene transport capacity in newborns than in their mothers, as suggested previously for tocopherol (39, 55). Although no specific membrane receptor has been found for these compounds, the transfer of the compounds could occur by passive diffusion or binding to lipoproteins, for which a receptor is present on placental membranes (56, 57). In contrast, as described by others (21), retinol was only twice as concentrated in the maternal blood as in the cord blood. Using perfused placentas, Dancis et al (58) showed that retinol (bound to RBP) was transferred across the placenta quickly, totally, and without metabolism. It is now established that placental membranes possess a specific receptor for RBP involved in the retinol transfer through membranes (59). These placental characteristics, combined with the importance of vitamin A in fetal development, could explain the relatively high transfer rate for retinol between maternal and fetal blood.

As reported previously, no significant differences were found between arterial and venous cord blood concentrations for any of the variables studied, suggesting a balance between placental and fetal vitamin A homeostasis (41). However, we measured concentrations that may not reflect the dynamic exchanges potentially occurring in the fetal blood or in the placental circulation.

In conclusion, our findings show that well-nourished, healthy pregnant women have a normal vitamin A status at term. However, changes in the saturation rate of RBP strongly suggest a physiologic adaptation of vitamin A metabolism during pregnancy, which could be related to the delivery of vitamin A to the fetus via the placenta. An understanding of the mechanisms of this transfer is needed so that the involvement of vitamin A in obstetric pathologies such as IUGR can be investigated further.

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