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Transfer and Metabolism of Retinol by the Perfused Human Placenta

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ABSTRACT. The transfer and metabolism of retinol by human placenta was investigated using an *in vitro* perfusion system with independent maternal and fetal circulations. ^{3}H-retinol bound to albumin added to the maternal perfusate was rapidly taken up and concentrated by the placenta to levels 16.5 ± 5.28 times the maternal perfusate. Approximately 8% of the retinol retained in the placenta was esterified. No metabolites were detected in the perfusates. Perfusion of placenta with retinol bound to retinol-binding protein (RBP) reduced the placental concentration to 4.4 ± 1.72 times the maternal concentration and eliminated evidence of metabolism. The transfer rate of RBP:^{3}H-retinol was less than that of albumin:^{14}C-retinol when measured concurrently in three experiments (clearances 0.11 *versus* 0.75 mL/min, 0.21 *versus* 1.7 mL/min, and 0.29 *versus* 0.48 mL/min, respectively). Transfer of the radioactive retinol was more rapid than ^{125}I-RBP or albumin, indicating that retinol was transferred independently of the proteins. The transfer index of retinol (clearance retinol:clearance L-glucose) was 0.73 ± 0.085 compared to 2.1 ± 0.36 for thiamin and 3.4 ± 0.95 for riboflavin, both water-soluble vitamins with active transport systems. The retinol transferred to the fetal perfusate is not bound to RBP, as demonstrated by gel filtration chromatography and chromatography on a transthyretin affinity column, despite the availability of RBP in the cord serum added to the perfusate. The endogenous retinol in the cord serum is bound to RBP. The conclusions derived are that RBP binding reduces the accumulation of retinol in the placenta and the transfer to the fetus, that retinol is transferred intact to the fetal circulation where immediate binding is not to RBP, and that redistribution to RBP occurs subsequently, possibly in fetal liver. (*Pediatr Res* 32: 195–199, 1992)

Abbreviations

RBP, retinol-binding protein
TTR, transthyretin
RBP:retinol, retinol bound to retinol-binding protein
albumin:retinol, retinol bound to albumin
TCA, trichloroacetic acid

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The importance of vitamin A to the developing mammalian fetus has been amply demonstrated in the experimental animal. Both deficiencies and excesses are teratogenic (1, 2). The pertinence of these observations to the human has recently become tragically evident. Retinoic acid administered to the pregnant female for the treatment of acne has caused severe congenital abnormalities in the newborn infant (3).

Despite its obvious importance, little is known about the placental transfer of retinoids. The question has been approached in the sheep (4) and monkey (5), but the placental role is difficult to deduce from studies in the intact animal and extrapolation of results to the human is uncertain. Measurements have been made in the human of maternal and cord retinol levels (6) that are interesting but shed little light on placental functions. Torma and Vahlquist (7) have studied the uptake of vitamin A and RBP by fragments of human placenta *in vitro* and speculated concerning the transplacental process. The model, however, does not permit differentiation of events at the maternal surface of the placenta from those at the fetal surface.

In the present study, we have used the technique of *in vitro* perfusion of a human placental lobule. By establishing independent "maternal" and "fetal" circulations, it was possible to study the transplacental transfer of retinol, the principal circulating form of vitamin A. *In vitro*, retinol circulates bound to a specific serum protein, RBP, which in turn complexes with TTR (8, 9). We have investigated the placental transfer and metabolism of retinol bound to RBP and compared the results to retinol bound nonspecifically to serum albumin. The comparison has provided insights into the physiologic role of RBP and the biochemical mechanism through which retinol is taken up by the tissues.

MATERIALS AND METHODS

Perfusion technique. Term placentas of clinically normal pregnancies were obtained after delivery by cesarean section or by vagina. Independent maternal and fetal circulations were established to a placental lobule as previously described (10). Flow rates approximated 12–15 mL/min on the maternal side and 4–6 mL/min on the fetal side. In the "closed" experiments, the perfusate was recirculated through a 70-mL reservoir. The perfusions were performed under reduced lighting, as were all procedures with retinol, because of its light sensitivity.

Perfusate. The basic perfusate consisted of Earle's buffered salt solution equilibrated against O_2-CO_2, 95–5%, pH 7.4, with 150 mg/dL of D-glucose and mixed amino acids in physiologic concentration. In the experiments with albumin-bound retinol, 10 g/L BSA was added to the perfusate with ^{3}H-retinol ~20 000 cpm/mL and retinol 1.7 μmol/L. Ten g/L albumin in the perfusate solubilizes the retinol and provides sufficient binding to prevent absorption to the perfusion tubing and apparatus similar to previous experience with vitamin D (11). Binding was accomplished by incubating ^{3}H-retinol with BSA for 1 h. For the RBP experiments, ^{3}H-retinol was bound to RBP by incubation

at 37°C for 1 h or at 5°C overnight, while rotating gently. The RBP:retinol was added to perfusate containing 20% maternal serum.

Preparation of samples for analysis. Perfusate samples were added directly to scintillant to determine radioactivity. If there was an insufficient concentration of counts for chromatographic analysis, samples were first concentrated with an Amicon-B15 concentrator (Amicon Corp., Beverly, MA) for gel filtration analysis or by extraction with hexane for reverse phase chromatography. Throughout the analytical procedures, retinol was protected against oxidation by the addition of butylated hydroxytoluene, 6.1 mM (1 mg/mL), or α-tocopherol, 100 μg.

At the end of the perfusion, 2–4 g of villous tissue was dissected from the perfused placenta and homogenized in 10 volumes of methanol:hexane (1:9). The extraction was repeated and both extracts combined and evaporated under vacuum. The residue was dissolved in 3 mL hexane containing 6.1 mM butylated hydroxytoluene, transferred to a small tube, and taken to dryness. The residue was dissolved in 0.5–1.0 mL of methanol and passed through a 0.22-μm filter, and 100 to 500 μL were analyzed by HPLC.

Chromatographic analysis. Reverse phase HPLC was used to identify retinoic acid, retinol, and the retinyl esters. Separation was on a Waters RPC-18 column (Waters Associates, Milford, MA) with a UV detector at 325 nm. The mobile phase was 80% acetonitrile in 1% ammonium acetate with a flow rate of 1 mL/min. Retinol and retinoic acid, 100 μg, were added to the sample before injection to serve as standards, the former eluting at 7 min and the latter at 4.5 min. For the retinyl esters, 100% methanol was the mobile phase, retinyl palmitate was the standard, and elution time was 13.4 min (12).

High performance gel filtration was performed to separate materials according to molecular size (13). Samples were injected into a Waters TSK 2000 SW column equipped with a UV or fluorescence detector. The mobile phase was 0.1 M monobasic potassium phosphate titrated to pH 7.4 and 0.05% sodium azide. Elution times were 14.4 min for albumin and 20.1 min for RBP.

A TTR-sepharose affinity column was used to identify RBP-bound retinol. The column was equilibrated at room temperature in 50 mM tris-chloride, 150 mM NaCl, and 0.05% NaN_3 at pH 7.4. Samples were applied and recycled through a 2-mL reservoir for 2 h at room temperature. The column was washed with 22 mL of buffer to remove materials not bound to the column and then the bound RBP was eluted with 30 mL H_2O at pH 8.0. Under these conditions, approximately 50% of the RBP in the sample equilibrated with and was retained by the column.

Normal phase chromatography was carried out on two Waters 5 μm Resolve Silica columns linked in series and eluted with 0.4% diethyl ether in hexane flowing at 1.2 mL/min, using a previously described procedure for the separation of the geometric isomers of retinol (14).

Clearances. Clearance (mL/min) was calculated as previously described (10).

Materials. Perfusates were prepared by GIBCO (Grand Island, NY) according to specifications. Retinol [11,12(n)]^{3}H-vitamin A alcohol, 60 Ci/μmol; inulin-^{14}C-carboxylic acid, 9 mCi/μmol; and L-1-^{14}C-glucose, 40–60 mCi/μmol, were purchased from Amersham (Arlington Heights, IL). The retinoids were obtained from Sigma Chemical Co. (St. Louis, MO) and the HPLC solvents from Fisher Scientific Co. (Pittsburgh, PA). If indicated, the ^{3}H-retinol was further purified by reverse-phase chromatography with α-tocopherol as antioxidant.

RBP was purified to homogeneity from human serum by conventional chromatography as previously described (15). Human ^{125}I-RBP was prepared by the lactoperoxidase method according to standard procedures (16). The TTR:sepharose affinity column was prepared by cross-linking 10 mg of purified TTR (15) to 5 mL of CNBr-activated sepharose 4B (LKB-Pharmacia Biochetechnology Inc., Piscataway, NJ) following the instructions provided by the manufacturer.

RESULTS

Transfer and Metabolism of Albumin-Bound Retinol. ^{3}H-retinol was incubated with serum albumin at 37°C for 1 h, which permitted protein-binding as demonstrated by TCA precipitation. The albumin-bound retinol was added to the maternal perfusate, which contained 10 g/L serum albumin, and recirculated for the duration of the perfusion. The fetal perfusate, which also contained 10 g/L serum albumin, was kept open (two experiments) or recirculated (four experiments).

Placental uptake. Uptake of ^{3}H-retinol from the maternal perfusate was very rapid during the first 20 min and continued at a slower rate throughout the perfusion. At the end of the perfusion, the concentrations of ^{3}H-retinol in the placenta and in the maternal perfusate were measured. The concentration in the placenta was 16.5 ± 5.28 times that in the perfusate (Table 1).

In five experiments, the albumin-bound ^{3}H-retinol was added to the fetal perfusate that was recirculated. The ratio of the concentrations of ^{3}H-retinol in the placenta to the fetal perfusate was 16.0 ± 4.79, suggesting that similar mechanisms were involved in uptake and concentration of retinol from the maternal and fetal perfusate.

Metabolism. The maternal and fetal perfusates were analyzed by reverse phase HPLC. No radioactive metabolites were detected. In four experiments, the placenta was extracted at the completion of the perfusion and also analyzed. Over 90% of the radioactivity coeluted with retinol; 8, 4, 7, and 11% eluted with retinyl esters. Retinoic acid was not detected.

Placental transfer. Transplacental clearances of ^{3}H-retinol were calculated (Table 2). ^{14}C-inulin was included in these experiments to provide a simultaneous measure of diffusion of a large (5-kD), water-soluble molecule. Retinol was transferred 10-fold more rapidly than inulin (10.2 ± 2.58). Albumin, to which the retinol was bound, is larger than inulin (60 kD), with a permeability constant across the perfused placenta of 1.5×10^{-3} cm^3/min/g as compared to 6.0×10^{-3} cm^3/min/g for inulin (17). It is evident that retinol had to be released from albumin before or during transport to permit its more rapid transfer.

Transfer and Metabolism of RBP-Bound Retinol. Transfer of RBP. Retinol circulates in maternal and fetal serum bound to RBP. The possibility that retinol was transferred to the fetus as RBP:retinol was initially addressed. RBP:retinol was iodinated with ^{125}I and added to the maternal perfusate with ^{14}C-inulin. The maternal and fetal perfusates were recirculated for 120 min in two experiments.

Table 1. *Uptake of maternal retinol by placenta**

	Albumin:retinol		RBP:retinol	
Experiment no.	Duration of perfusion (min)	[Pl]/[M]†	Duration of perfusion (min)	[Pl]/[M]†
1	100	18	60	5.2
2	60	17	180	5.7
3	120	24	120	3.3
4	120	14	180	5.9
5	100	8	120	1.4
6	120	18	60	4.6
Mean ± SD		16.5 ± 5.28		4.4 ± 1.72

* ^{3}H-retinol bound to albumin or to RBP was added to the maternal perfusate, which was recirculated. The fetal perfusate was kept open or closed without detectable effect on the placental concentration. At the completion of perfusion, the concentration of radioactive retinol in the placenta (counts/min/g) was compared with that in the maternal perfusate (counts/min/mL). The placenta removes and concentrates retinol more effectively when bound to albumin than when bound to RBP ($p < 0.001$).

† [Pl]/[M] = counts/min/g placenta:counts/min/mL maternal perfusate.

Table 2. *Transfer of albumin-bound retinol**

Experiment no.	Clearance (mL/min)	Clearance ratio (retinol:inulin)
1	0.30	15
2	0.16	
3	15.0†	9
4	0.15	8
5	1.2	10
6	0.37	9
Mean ± SD	0.44 ± 0.39	10.2 ± 0.32

* Albumin:^{3}H-retinol was added to the maternal perfusate with inulin-^{14}C. The maternal perfusate was recirculated through the placenta and transfer into the nonrecirculated fetal perfusate was measured. The transfer rate of retinol bound to albumin greatly exceeded that of inulin, a smaller molecule than albumin (5 *vs* 60 kD).

† This deviant value was excluded from the mean but not from the retinol:inulin ratios.

Periodic sampling of the fetal perfusate showed a linear increase in concentration of ^{125}I and ^{14}C throughout the perfusion. However, only 10% of the transferred ^{125}I remained TCA precipitable, indicating extensive degradation of RBP during transfer. The maternal RBP remained over 90% TCA precipitable. Within the placenta, 55% of the ^{125}I was no longer TCA precipitable. It was evident that the placenta rapidly degraded the RBP and released the degradation products into the fetal circulation.

The rate of transfer of TCA-precipitable ^{125}I was 0.33 and 0.44 that of inulin in the two experiments. The extensive degradation of RBP during transfer across the placenta and the relatively slow rate of transfer made it very unlikely that retinol bound to RBP was transferred intact to the fetus. That possibility was further eliminated by later experiments described below.

Uptake of RBP. In the next series of experiments, ^{3}H-retinol was incubated with purified RBP at 37°C for 1 h. The RBP:^{3}H-retinol was added to the maternal perfusate to which had been added maternal serum to a concentration of 20%. Binding of ^{3}H-retinol to RBP was confirmed by reverse-phase chromatography of the maternal perfusate before perfusion and after 40 min of perfusion. The fetal perfusate contained 20% cord serum.

The maternal perfusate was recirculated. The fetal perfusate was recirculated in three experiments and kept open in two.

At the completion of the perfusion, the concentration of ^{3}H-retinol in the placenta was much lower than in the experiments with albumin:retinol (Table 1).

Metabolism. No evidence of metabolism of the ^{3}H-retinol was detected by reverse phase chromatography in the maternal and fetal perfusates or in placenta extracts. The retinyl esters observed with the high concentrations of retinol in the placenta after perfusion with albumin:retinol were not evident.

Placental transfer. The effect of RBP binding on the placental transfer of retinol was investigated by adding both RBP:^{3}H-retinol and albumin:^{14}C-retinol to the maternal perfusate. Clearances of the two preparations were simultaneously measured in three experiments. RBP binding reduced the clearance of retinol as compared to albumin:retinol (0.11 *versus* 0.75 mL/min, 0.21 *versus* 1.7 mL/min, and 0.29 *versus* 0.48 mL/min).

It was interesting to compare the placental transfer rate of retinol to those of other nutrients studied with the same technique, particularly the water-soluble vitamins. For this information, RBP:^{3}H-retinol and ^{14}C-L-glucose were added to the maternal perfusate and the placental clearances simultaneously measured. L-Glucose is a water-soluble molecule transferred by membrane-limited simple diffusion. By using the ratio of the clearances of riboflavin to L-glucose, interexperimental variation is reduced and comparison of substrates becomes more meaningful. The ratio of retinol to L-glucose clearance, or transfer index, in five experiments was 0.73 ± 0.085 (Table 3). The transfer indices of thiamin and riboflavin, two water-soluble vitamins previously studied in this laboratory, were 2.1 ± 0.36 and 3.4 ± 0.95, respectively (18, 19).

Table 3. *Transfer rate of RBP-bound retinol*

Experiment no.	Clearance (mL/min)	Transfer index*
1	0.09	0.69
2	0.34	0.81
3	7.7†	0.72
4	0.11	0.82
5	0.12	0.59
Mean ± SD	0.37 ± 0.32	0.73 ± 0.85

* Transfer index = clearance retinol:clearance L-glucose.

† Deviant value excluded from the mean but not from transfer index.

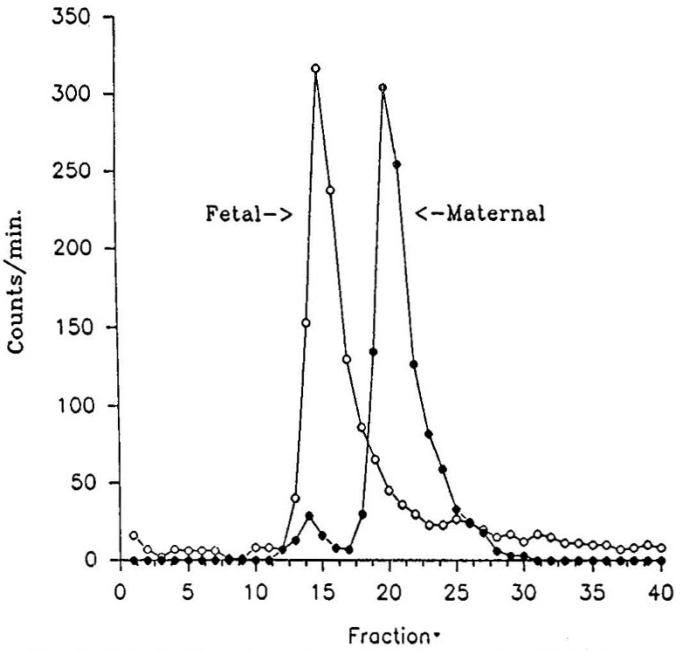

Fig. 1. Gel filtration chromatography of perfusates. RBP:^{3}H-retinol was added to maternal perfusate with 20% serum and recirculated. Fetal perfusate, containing 20% cord serum, was not recirculated. After perfusing for 40 min, the maternal and fetal perfusates were analyzed by HPLC gel filtration. Radioactivity in maternal perfusate eluted with RBP; in fetal perfusate, with materials equal to or greater than 60 kD.

Protein-binding of transferred ^{3}H-retinol. RBP:^{3}H-retinol was added to maternal perfusate containing 20% maternal serum and recirculated for 60 min. The fetal perfusate containing 20% cord serum was not recirculated. After 40 min, an aliquot of maternal perfusate was drawn from the reservoir and the fetal perfusate was collected. Both were analyzed by gel filtration on HPLC (Fig. 1).

With the maternal perfusate, a radioactive peak eluted at 21 min, consistent with RBP:retinol. In contrast, the radioactivity in the fetal perfusate eluted as a peak after 15 min, consistent with molecules of 60 kD and above.

The identity of the protein that binds the retinol was further pursued using a TTR affinity column that selectively binds to RBP. Under the conditions of analysis, approximately 50% of prepared RBP:^{3}H-retinol is retained by the column. The maternal perfusate to which RBP:^{3}H-retinol had been added performed as would be expected (Table 4). The ^{3}H-retinol in the fetal perfusate was not retained by the column, confirming that the transferred ^{3}H-retinol was not bound to RBP.

The binding of the endogenous nonradioactive retinol contained in the sera added to the maternal and fetal perfusates was also investigated using the characteristic fluorescence of RBP:retinol. Retention by the affinity column was slightly higher than that of the laboratory-prepared RBP:retinol and was the

Table 4. *TTR column assay for RBP binding of retinol in maternal and fetal perfusate**

Experiment no.	^{3}H-retinol (% bound)		Endogenous retinol (% bound)	
	Maternal	Fetal	Maternal	Fetal
1	42	<10	69	78
2	54	<10	74	73
3	53	<10	61	74
4	48	<10	49	72

* RBP:^{3}H-retinol was added to the maternal perfusate containing 20% maternal serum and transfer into fetal perfusate with 20% cord serum was investigated. The affinity column retained about half the radioactivity in the maternal perfusate, similar to the results obtained with laboratory-prepared RBP:retinol. None of the transferred ^{3}H-retinol was retained by the column. The endogenous retinol in the maternal and cord sera added to the perfusates was retained equally and at a slightly higher level than the laboratory-prepared maternal perfusate.

same in the maternal and fetal perfusates (Table 4), indicating that the endogenous retinol was bound to RBP in maternal and cord blood.

The difference in binding to the TTR-affinity column between the laboratory-prepared RBP:retinol and that circulating in serum (Table 4) may be explained by the method used for the laboratory preparation. Trace ^{3}H-retinol was incubated with purified RBP, permitting binding to unoccupied sites on the protein and/or interchange with bound retinol. This technique was gentler than removing bound retinol with organic solvents and replacing it with ^{3}H-retinol. Binding of ^{3}H-retinol was demonstrated by TCA precipitation and gel filtration. Binding of tracer, however, may not have been identical to the endogenous retinol.

On reverse phase chromatography, the transferred radioactivity eluted with authentic retinol. The possibility remained that the transferred retinol had been subtly altered by metabolism, sufficient to interfere with RBP binding but not detectable by reverse phase chromatography. Normal phase chromatography provides greater resolution of the isomers of retinol and its metabolites. In two experiments, the transferred radioactivity eluted with authentic retinol. Direct evidence that the transferred ^{3}H-retinol retained its capacity for RBP binding was obtained by extracting the transferred radioactivity from the fetal perfusate with hexane and incubating the extract with purified RBP at 37°C for 1 h. There was complete binding of the extracted radioactivity. Therefore, the transferred ^{3}H-retinol was unaltered and retained its potential for binding to RBP but had not bound to RBP even though the protein was present in the perfusate.

The possibility remained that the RBP circulating in fetal plasma was unable to accept the placentally transferred ^{3}H-retinol because it was fully saturated. This was addressed by incubating cord serum with ^{3}H-retinol. Reverse phase chromatography and TTR-affinity chromatography indicated that the ^{3}H-retinol was bound to RBP.

DISCUSSION

Retinol is poorly soluble in water. Several carrier proteins have evolved for transporting retinol through aqueous environments. In the circulation, retinol is transported bound to a specific plasma protein, RBP (8). Cell surface receptors for RBP have been reported on the brush border of human placenta (20). The experiments with albumin-bound retinol demonstrate, however, that neither specific protein binding nor receptor-mediated transport are required for delivery of retinol to the placenta or the fetus.

The mechanisms involved in transport of albumin:retinol appear to be the same for other lipophilic materials when bound to albumin, as suggested by studies of the FFA and of estrogens (21, 22). The ligands are released from the protein to the placenta, bound to placental proteins, and then released to fetal albumin. Uptake and concentration of the ligand in the placenta are consistent with the relative association constants for albumin and placental proteins. The observations on transport can be explained physicochemically, but more complex biologic processes are not excluded.

Under physiologic conditions, retinol is delivered to the placenta bound to RBP. The first steps in transport of retinol to the fetus require release from RBP and uptake into the placenta. Noy and Blaner (23) have proposed a model for the cellular uptake of retinol that can be applied to the placenta. Retinol is progressively released from RBP in the circulation as it enters into equilibrium with the plasma membrane, where it moves from the outer leaflet to the inner leaflet of the membrane (24, 25). Retinol then interacts with cytoplasmic proteins. As with the albumin-bound hydrophobic materials, the concentration of retinol within the cell is determined by the relative binding affinities and available binding sites of the transport protein delivering the retinol and the intracellular binding proteins. Receptor-mediated transport (20) is not a feature of the model proposed by Noy and Blaner.

Placental uptake and transfer of ^{3}H-retinol is reduced when ^{3}H-retinol is bound to RBP as compared to albumin-bound retinol, consistent with the higher avidity of RBP for retinol and suggesting that the primary function of RBP is to control the delivery of retinol to tissues. RBP binding prevents excessive accumulation of a potentially harmful material. Vitamin D binding protein may have a similar function. The placental transfer of 25-hydroxycholecalciferol is sharply reduced when 25-hydroxycholecalciferol is bound to its specific transport protein as compared to transfer when bound to albumin (11).

There is no information concerning the route and mechanisms by which retinol traverses the placenta to reach the fetal circulation. The parallels between placental uptake of retinol and that of FFA suggest that similarities may exist in intracellular transport. A superfamily of structurally related intracellular proteins capable of binding lipophilic materials has been described (26). The members of this family include the fatty acid-binding proteins, cellular retinol-binding protein type I, cellular retinol-binding protein type II, cellular retinoic acid-binding protein, myelin P2, and adipocyte P2 (27). The proteins are located in the cytoplasm and are thought to play a role in the intracellular transport and metabolism of their respective hydrophobic ligands. The fatty acid-binding proteins that have been identified in human placenta appear to be unique isoforms, based on serologic evidence (28). At present, only limited information is available regarding the presence of retinoid-binding proteins in the human placenta. Cellular retinol-binding protein type I mRNA could not be detected by Northern blot analysis in total RNA from human placenta (29), but data concerning the possible presence of other retinoid-binding proteins in the placenta are not available.

The present studies have shown that retinol is transferred into the fetal circulation unchanged by placental metabolism and that the transferred retinol is not bound to RBP. On chromatography (Fig. 1), the transferred ^{3}H-retinol is bound to protein(s) of 60 kD or greater. It is possible that the retinol is secreted bound to a placental protein. The placental synthesis of apolipoprotein E could provide such an opportunity (30). Alternatively, the retinol, on release from the placenta, binds immediately to available lipid carrier proteins such as albumin or lipoproteins. The fetal proteins, fetuin (31) and α-fetoprotein (32), are also potential lipid carriers. This binding is transient, with subsequent transfer to RBP (Table 4) possibly accomplished in the liver.

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