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The mechanism of uptake of retinol by plasma-membrane vesicles

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The mechanism of retinol uptake by human placental brush-border membrane vesicles was investigated using initial-velocity studies of [^{3}H]retinol uptake from the [^{3}H]retinol–RBP (retinol-binding protein) complex. The process was rapid and time- and temperature-dependent. The uptake was specifically reversed by the addition of native or apo-RBP, but not by serum albumin. By contrast, uptake of free [^{3}H]retinol was temperature-independent, partially reversible and showed no requirement for a specific protein for reversibility. Treatment of membrane vesicles with *p*-chloromercuribenzenesulphonate (PCMBS), which inhibited ^{125}I-RBP binding, also inhibited the uptake of retinol from RBP, but the uptake of free retinol was unaffected. Addition of PCMBS after the attainment of steady-state uptake equilibrium abolished the binding of RBP, but did not affect the retinol already taken up from RBP. The results suggest that binding of RBP to its specific receptor is obligatory for the subsequent delivery of retinol to the membrane. Since the studies were carried out on isolated membrane vesicles, endocytosis of RBP is most unlikely to be involved in the placental transport of retinol. A double-reciprocal plot of initial velocity versus [^{3}H]retinol–RBP concentration gave an apparent K_m of 116±13 nM. Transthyretin decreased the rate of uptake of [^{3}H]retinol from RBP without substantially altering the steady-state uptake levels, suggesting that membranes take up retinol from uncomplexed RBP. High-pressure gel-filtration chromatography showed that [^{3}H]retinol is largely transferred to a membrane component with an apparent molecular mass of 125 kDa.

INTRODUCTION

Vitamin A is essential for normal growth and function in a number of tissues. Foetal development is one particularly sensitive process. Vitamin A deficiency, for example, can lead to resorption of the foetus, whereas excessive intake can cause teratogenic changes, especially during the critical periods of organ and limb development (Moore, 1971). Transport of vitamin A from mother to foetus is believed to be highly regulated, with little alteration in foetal vitamin levels, despite large changes in maternal intake. Retinol-binding protein (RBP), the plasma transport protein for retinol, has been implicated in the process (Goodman, 1984), but the mechanism is unclear. There have been at least two reports (Takahashi *et al.*, 1977; Ismadi & Olson, 1982) which suggested that retinol reaches the foetus mainly by transplacental transport of the maternal retinol–RBP complex. In the preceding paper (Sivaprasadarao & Findlay, 1988) we reported the existence of a protein receptor for RBP on human placental brush-border membranes which represent the main barrier between the maternal and foetal circulations. The present paper addresses the role played by this membrane-bound receptor for RBP in the process of retinol transfer from mother to foetus.

In other tissues there is equal uncertainty about the mechanism of retinol uptake. Some workers (Rask & Peterson, 1976; Chen & Heller, 1977) have suggested that retinol transfer from RBP to the target cell occurs without any cellular uptake of apo-RBP. Others (Blomhoff *et al.*, 1985) support the internalization of holo-RBP by receptor-mediated endocytosis. A third possibility is that retinol, being hydrophobic (Lan *et al.*, 1984; Hamilton & Cristola, 1986), might simply partition between RBP in the serum and the cell membrane without any involvement of membrane receptors. This latter process undoubtedly can occur, since there is evidence to suggest that free retinol is not only taken up by cells, but elicits specific biological effects.

This may not represent the normal biological mechanism, however, and the studies described here were undertaken to delineate more clearly the steps involved in the delivery of retinol from RBP to cell membranes. The information which results is probably applicable to all tissues containing the receptor for RBP.

MATERIALS AND METHODS

Materials

Human plasma RBP and TTR were prepared as previously described (Rask *et al.*, 1971; McGuire & Chytil, 1980). Carrier-free Na^{125}I was purchased from Amersham International. All-*trans*-[15-^{3}H(n)]retinol (20 Ci/mmol) was obtained from New England Nuclear. Crystalline human serum albumin, bovine β-lactoglobulin, egg albumin, γ-globulins, myoglobin, emulphogene BC-720, PCMBS sodium salt and trypsin (9235 units/mg) were purchased from Sigma Chemical Co. Soya-bean trypsin inhibitor (7756 units/mg) was from Calbiochem. Dibutyl phthalate was from Aldrich Chemical Co., and dinonyl phthalate was purchased from BDH. Human placenta was obtained immediately after delivery from Leeds General Infirmary, Leeds, U.K.

Abbreviations used: h.p.g.f.c., high-pressure gel-filtration chromatography; PBS, phosphate-buffered saline (10 mM-sodium phosphate/150 mM-NaCl, pH 7.4); PCMBS, *p*-chloromercuribenzenesulphonate; RBP, retinol-binding protein; TTR, transthyretin.

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Methods

Preparation of human placental membrane vesicles. Brush-border membrane vesicles were prepared from freshly obtained human placenta as described by Booth *et al.* (1980). Membranes were stored in portions at −20 °C. Before use, they were thawed, incubated at 37 °C for 15 min, then washed with the assay buffer twice, by centrifugation and resuspension at 4 °C.

Preparation of [^{3}H]retinol complexes. Preparation of [^{3}H]retinol complexes with RBP, human serum albumin and bovine β-lactoglobulin was as described by Horwitz & Heller (1973).

Assay of [^{3}H]retinol uptake from RBP. Assay of [^{3}H]retinol uptake from the [^{3}H]retinol–RBP complex by the placental membrane vesicles was performed by using an oil-centrifugation method adapted from that described by Cuatrecasas & Hollenberg (1976). A 200–300 μg (up to 2 mg of protein/ml) portion of membrane protein was incubated with (3–5) × 10^4 d.p.m. (approx. 3–5 pmol) of [^{3}H]retinol–RBP in 150 μl of assay buffer (0.1% ovalbumin in PBS) at 37 °C for 15 min. At the end of the incubation, the tubes were chilled and a 100 μl aliquot of the mixture was transferred to chilled 0.4 ml-capacity polypropylene centrifuge tubes ('microeppendorfs'; Sarstedt 72/701) containing 200 μl of a 3:2 mixture of dibutyl phthalate and dinonyl phthalate layered over 50 μl of 5% (w/v) sucrose in PBS. The tubes were spun for 2 min at 12000 *g*. Non-specific uptake was determined by performing incubations in the presence of at least a 100-fold molar excess of unlabelled RBP. Portions of the supernatant were removed for radioactivity counting. The tubes were then frozen in a solid-CO_2/acetone bath and cut at the oil/sucrose interface. The pellets were incubated in 200 μl of 10% (w/v) SDS at room temperature overnight, and their radioactivity was determined by using a Kontron Intertechnique liquid-scintillation counter. Specific binding was calculated from the radioactivity content of the pellets as well as of the supernatants.

Assay of free [^{3}H]retinol uptake. Uptake of free [^{3}H]retinol by the placental membrane vesicles was performed in 0.1 ml of assay buffer containing 0.3–0.5 pmol of [^{3}H]retinol and 100–200 μg of membrane protein. [^{3}H]retinol was added as an ethanolic solution such that the final concentration of ethanol was kept below 2%. After incubation at 22 °C for 15 min, membrane-bound [^{3}H]retinol was separated from free [^{3}H]retinol by centrifugation (4 °C at 25000 *g* for 10 min). The pellet was washed twice with the assay buffer and counted for radioactivity as described above. Non-specific uptake was evaluated in parallel incubations by using membranes denatured by prior incubation at 65 °C for 4 h as described by Stremmel *et al.* (1985).

Assay of ^{125}I-RBP binding. Radioiodination of RBP and assay of ^{125}I-RBP binding to the placental membrane vesicles was carried out as described previously and in the preceding paper (Sivaprasadarao & Findlay, 1987, 1988).

Protein determination. The protein contents of the membranes were assayed as described by Markwell *et al.* (1978), with bovine serum albumin as a standard.

Data analysis. The kinetic data was analysed by computer using a non-linear least-squares curve-fitting program (Numerical Algorithms Group Subroutine EØ4GDF).

H.p.g.f.c. This was carried out using LKB TSK G-3000 SW resin. The column (300 cm × 7.5 cm) was equilibrated in 20 mM-sodium phosphate/0.1 M-NaCl, pH 7.4, containing 0.1% emulphogene BC-720 where appropriate. A 200 μl portion of the sample was injected and the column developed with the appropriate buffer at a flow rate of 0.2 ml/min. The column was calibrated using Blue Dextran (void volume) bovine γ-globulins (M_r 160000), serum albumin (M_r 67000), ovalbumin (M_r 45000), myoglobin (M_r 17000) and tyrosine (column volume).

RESULTS

Time and temperature-dependence of retinol uptake

When placental microvilli were incubated with [^{3}H]retinol–RBP, specific uptake of [^{3}H] retinol was seen.

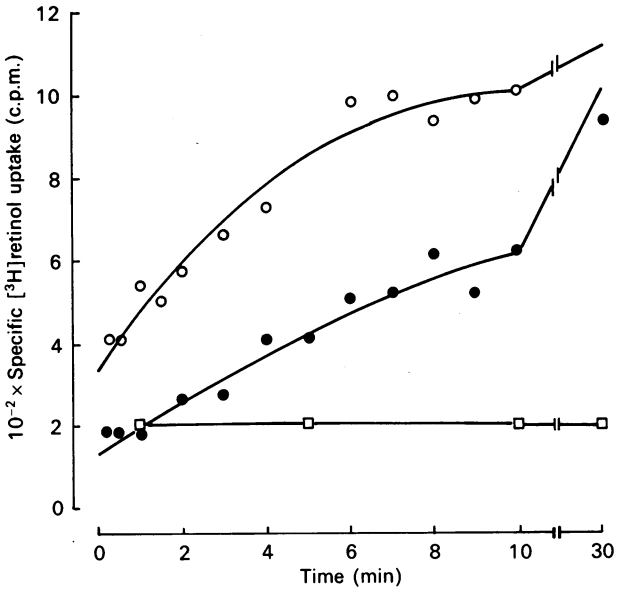

Fig. 1. Kinetics of RBP-bound [^{3}H]retinol uptake by placental membrane vesicles

Uptake of [^{3}H]retinol was initiated by the addition of membranes (0.5 mg of protein/ml) to 20 nM-[^{3}H]-retinol–RBP in the assay buffer at 0 °C (□) and 37 °C in the absence (○) and the presence (●) of 200 nM-TTR (2.0 ml total volume). At the times indicated, 100 μl portions were removed on to the top of oil/sucrose cushions, and rapidly chilled in a solid-CO_2/acetone bath for 2 s to terminate the reaction. The samples were stored on ice and subsequently centrifuged at 4 °C before determination of the radioactive content of membranes and supernatant. Non-specific-uptake experiments, performed in the presence of 5 μM unlabelled RBP, was subtracted from the total uptake to give the specific component. The data fitted best (correlation coefficient 0.98 for ○ and 0.99 for ●) the equation:

$$x_t = x_\infty - (x_\infty - x_0)e^{-kt}$$

where x_∞, x_0 and x_t are binding at equilibrium, zero time and time t respectively, and k is the pseudo-first-order rate constant. The values of k for ○ and ● were 0.209 min^{-1} and 0.083 min^{-1} respectively.

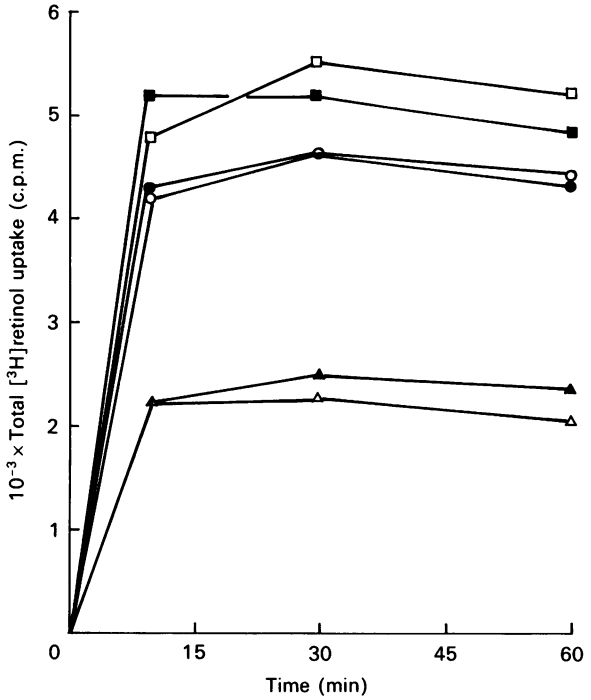

Fig. 2. Effect of time and temperature on the uptake of free [^{3}H]retinol

Membranes (1.0 mg of protein/ml) were incubated with 5.2 nM-[^{3}H]retinol in the absence (○,●) or presence (□,■) of 7.5 μM-unlabelled retinol at 37 °C (open symbols) or 0 °C (closed symbols). Triplicate aliquots (100 μl) removed at the time points indicated were assayed for bound [^{3}H]retinol. Parallel assays were performed using membranes heated at 65 °C for 4 h (△,▲).

This uptake was time- and temperature-dependent (Fig. 1). At 0 °C, equilibrium was reached within 1–2 min, but equilibrium uptake amounted to only about 20% of that at 37 °C, where equilibrium was reached in about 10 min. The uptake was biphasic when analysed by a non-linear least-squares computer program. The uptake data at 37 °C fitted best the equation:

$$x_t = x_\infty - (x_\infty - x_0)e^{-kt}$$

[see the preceding paper (Sivaprasadarao & Findlay, 1988) for kinetic analysis]. The results may be explained in terms of a rapid binding of the [^{3}H]retinol–RBP complex to the membrane vesicles at both 0 and 37 °C. This is followed, but at 37 °C only, by slower uptake of [^{3}H]retinol from membrane-bound [^{3}H]retinol–RBP. The resultant apo-RBP is displaced by [^{3}H]retinol–RBP in the medium.

By contrast, the uptake of free [^{3}H]retinol was temperature-independent (Fig. 2). When free [^{3}H]retinol was incubated with membrane vesicles, at least 60% of the added radioactivity was taken up by the membrane vesicles very rapidly both at 0 °C and 37 °C. No decrease in the total binding, however was observed when incubations were carried out in the presence of a large excess (7.5 μM) of unlabelled retinol. This conventional way of using a large excess of unlabelled ligand to determine the non-specific uptake or binding is obviously inappropriate in the case of a hydrophobic substance such as retinol. Any specific binding would be obscured, particularly when the number of binding sites is limited.

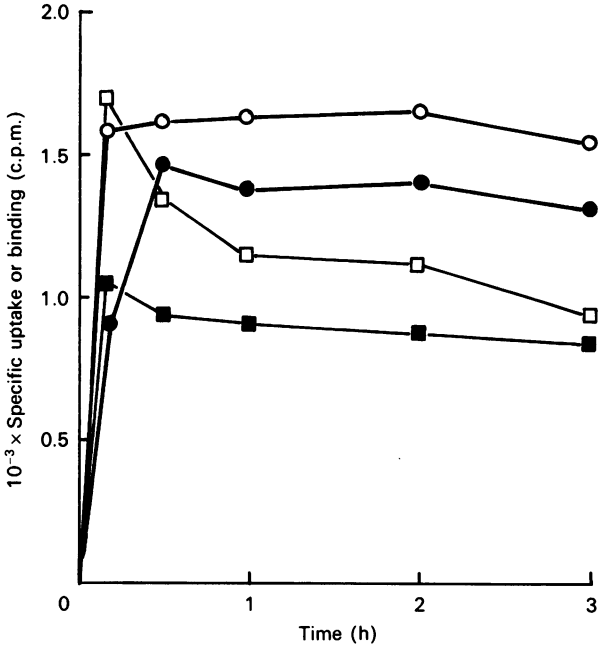

Fig. 3. Effect of TTR on the time course of ^{125}I-RBP binding and [^{3}H]retinol uptake

Specific binding of ^{125}I-RBP (□,■) and [^{3}H]retinol uptake (○,●) at time t after addition of membranes (1.0 mg of protein/ml) to 6 nM-^{125}I-RBP or 20 nM-[^{3}H]retinol–RBP at 37 °C in the presence (●,■) or absence (○,□) of 10-fold molar excess of TTR were determined as described under 'Methods'.

For this reason, the indirect approach involving protein denaturation used by Stremmel *et al.* (1985) for the determination of non-specific binding of fatty acids to the liver plasma membranes was adopted. After incubation of membrane vesicles at 65 °C for 4 h, binding was reduced by more than 50% (Fig. 2). The protein contents of the pelleted membranes were not altered.

Effect of TTR on RBP binding and retinol uptake

Fig. 3 illustrates the influence of a 10-fold molar excess of TTR over RBP on the time course at 37 °C of ^{125}I-RBP binding as well as uptake of [^{3}H]retinol from the [^{3}H]retinol–RBP complex.

Equilibrium binding of ^{125}I-RBP, although inhibited to a significant extent, did not show any decline with time in the presence of TTR. In the absence of TTR, however, ^{125}I-RBP binding, declined by over 40% in 3 h [the preceding paper (Sivaprasadarao & Findlay 1988)]. In contrast, steady-state uptake levels of [^{3}H]retinol showed no decline over this period of incubation, and was little altered by the presence of TTR. However, the kinetic data for uptake presented in Fig. 1 (●) show that, in the presence of TTR, the uptake by placental microvilli of [^{3}H]retinol bound to RBP reaches the steady-state level much more slowly. The half-time for uptake in the presence of TTR (8.4 min) is about 2.5-fold higher than that obtained in its absence (3.3 min).

Effect of PCMBS

Table 1 examines the effects of PCMBS on ^{125}I-RBP binding and [^{3}H]retinol uptake. Treatment of the membrane vesicles with PCMBS completely prevented both the binding of ^{125}I-RBP and the uptake of

Table 1. Effect of PCMBS on the binding of ^{125}I-RBP and uptake of free and RBP-bound [$_3$H]retinol

Membranes were preincubated with (A) or without (B) PCMBS for 15 min at 37 °C. Then they were adjusted to the indicated temperatures, where incubations were performed with ^{125}I-RBP (4 nM), [^{3}H]retinol–RBP (6.3 nM) or [^{3}H]retinol (15 nM) for 15 min. At the end of equilibrium, PBS or PCMBS were added to (A) and (B) respectively and incubated for 15 min at 37 °C. Concentrations of PCMBS and membranes after all additions were 2 mM and 0.5 mg/ml respectively. Parallel incubations (control) using buffer in place of PCMBS were simultaneously performed to determine total binding. Non-specific binding for (*a*)–(*c*) was determined in the presence of 2 μM-RBP. Non-specific binding for (*d*) was obtained by using heat-denatured membranes. All values were corrected for non-specific binding and expressed as a percentage of control.

Addition	Temperature of equilibration (°C)	Amount bound or taken up (% of control) (A)+ PCMBS	(B)− PCMBS
(*a*) ^{125}I-RBP	22	0.5	0
(*b*) [^{3}H]Retinol–RBP	37	0	85.5
(*c*) [^{3}H]Retinol–RBP	0	0	0
(*d*) [^{3}H]Retinol	37	127	120

[^{3}H]retinol from RBP. When PCMBS was added to membranes which had already attained steady-state equilibrium, the binding of ^{125}I-RBP was completely abolished, but the extent of loss of [^{3}H]retinol from the membranes depended on the temperature at which steady-state eqilibrium had previously been established. Whereas the steady-state retinol uptake equilibrium at 0 °C was completely abolished, that at 37 °C was only slightly inhibited. On the other hand, PCMBS caused some increase in the uptake of free retinol irrespective of when the inhibitor was added.

Reversal of uptake by the addition of native RBP

Fig. 4 shows that the addition of a large excess of native RBP to membrane vesicles in steady-state equilibrium with [^{3}H]retinol–RBP, followed by incubation at 37 °C, resulted in almost complete loss of membrane-associated [^{3}H]retinol ($t_{\frac{1}{2}}$ approx. 6 min). This reversal process is temperature-dependent. No significant loss of membrane retinol was noticed when the reversed reaction was allowed to proceed at 0 °C.

Reversal of uptake by dilution

Membranes were allowed to reach steady-state equilibrium by incubating at 37 °C with [^{3}H]retinol–RBP. They were then separated from this incubation medium, and resuspended in buffer alone or in buffer containing apo-RBP or defatted human serum albumin. These were then incubated at 37 °C and the time course of the remaining membrane-bound radioactivity was monitored. The results (Fig. 5*a*) show that dilution with buffer alone caused little or no decrease in the membrane-associated [^{3}H]retinol. On the other hand, the presence of apo-RBP resulted in rapid and complete loss of retinol from the membrane vesicles. Serum albumin, even at a

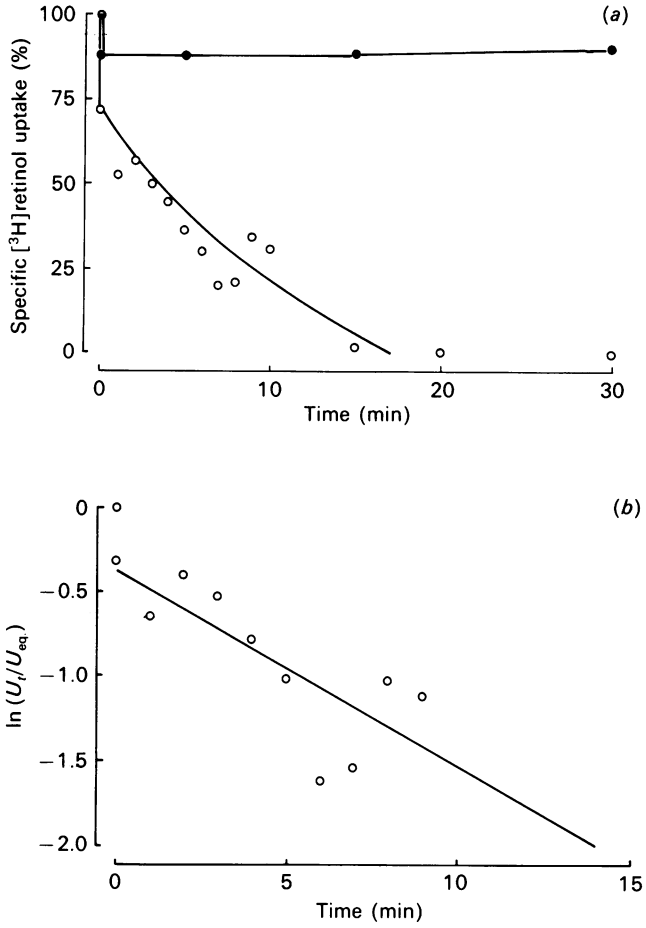

Fig. 4. Kinetics of efflux of [^{3}H]retinol from placental microvilli

(*a*) The uptake of [^{3}H]retinol was brought to steady state by incubating membranes (1.0 mg of protein/ml) with [^{3}H]retinol–RBP (50 nM) at 37 °C for 15 min and the equilibrium uptake ($U_{eq.}$) was determined. Efflux of [^{3}H]retinol from the membranes was initiated at 37 °C (○) or 0 °C (●) by the addition of 4 μM unlabelled RBP. Portions (100 μl) removed at the indicated times were rapidly chilled in a solid-CO_2/acetone bath to terminate the efflux. Specific uptake remaining at time t (U_t) was plotted as percentage of initial value ($U_{eq.}$) against t. (*b*) Gives the first-order fit of the data at 37 °C for the first 9 min of efflux. The standard error of the fit was 0.033, and the slope was 0.115 ± 0.033 min^{-1}.

10-fold higher concentration than apo-RBP, was not only very slow but much less efficient, in reversing the uptake process.

By contrast, when similar reversal studies were performed with membranes loaded with free retinol, apo-RBP was only marginally more efficient in inducing the release of retinol from the membrane than buffer alone or defatted serum albumin (Fig. 5*b*).

H.p.g.l.c. (TSK G-3000 SW) of the medium, into which most of the radioactivity was released from membranes previously loaded using the [^{3}H]retinol–RBP complex, revealed that most of the [^{3}H]retinol appeared in the column volume when buffer alone was used for the reversed reaction. When serum albumin was present in the buffer, the radioactivity was distributed between two peaks corresponding to serum albumin and free retinol.

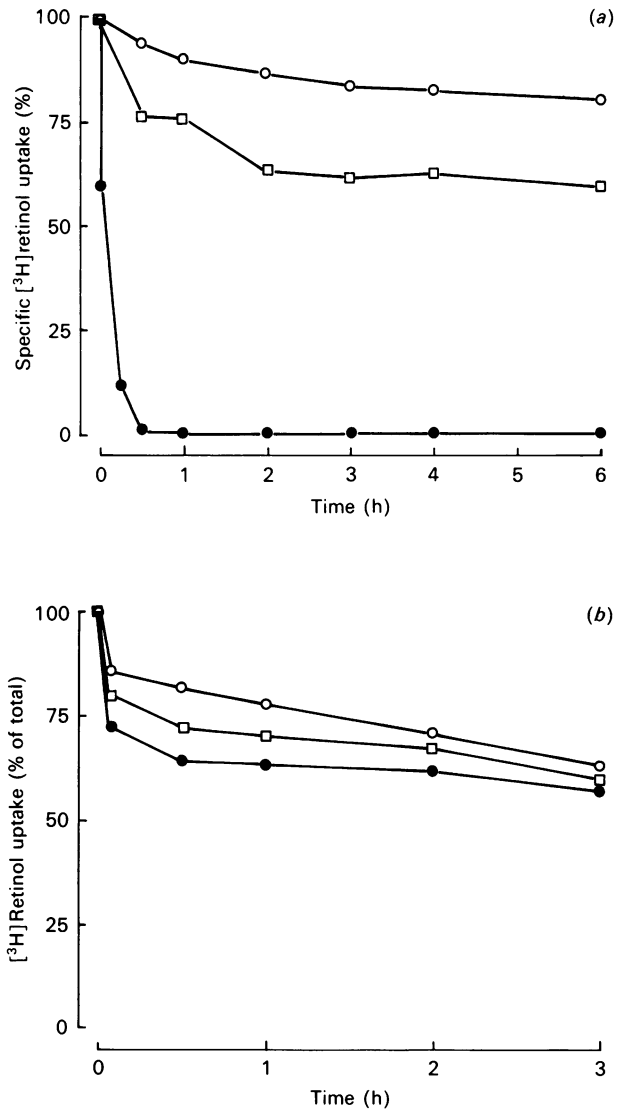

Fig. 5. Reversibility of [^{3}H]retinol uptake by dilution

Membrane vesicles (1 mg of protein/ml), after incubation with (*a*) [^{3}H]retinol–RBP (64 nM) or (*b*) free [^{3}H]retinol (6.25 nM) to equilibrium, were separated from the rest of the medium by centrifugation and suspended in buffer alone (○), 2.5 μM-RBP (●) or 25 μM-serum albumin (□).

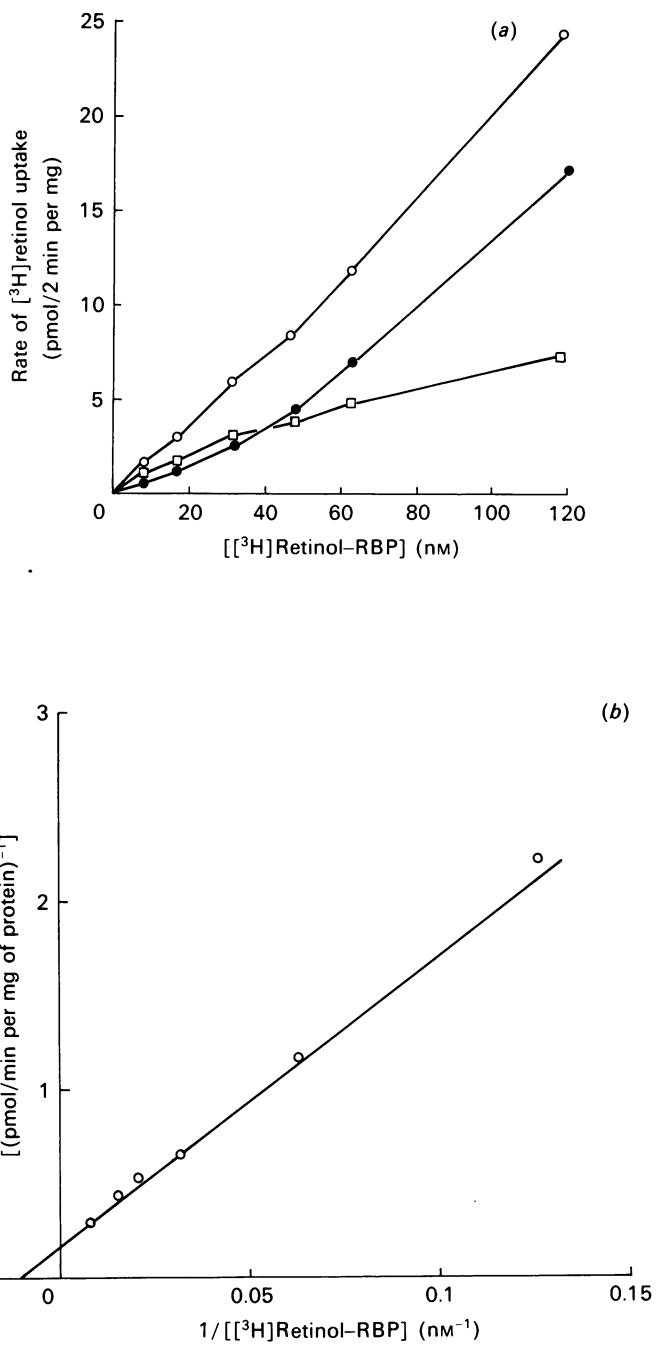

Fig. 6. Concentration-dependence of the rate of total and non-saturable uptake of [^{3}H]retinol uptake from [^{3}H]retinol–RBP

(*a*) [^{3}H]Retinol uptake was initiated by the addition of various concentrations of [^{3}H]retinol–RBP complex to membrane vesicles (0.5 mg of protein/ml) previously treated with buffer alone (○, total) or 5 mM-PCMBS (●, non-saturable) for 5 min at 37 °C. After being incubated for exactly 2 min at 37 °C, the tubes were chilled in a solid-CO_2/acetone bath and uptake was determined as described under 'Methods'. The specific uptake rate (□) was calculated by subtracting the non-saturable uptake from the total. (*b*) Double-reciprocal plot of specific [^{3}H]retinol uptake. The straight line was the best fit obtained by the least-squares analysis of the uptake data from (*a*). The estimates of K_m and $V_{max.}$ were 116±13 nM and 6.9±0.5 pmol/min per mg of vesicle protein respectively.

Radioactivity released into the apo-RBP containing buffer, however, appeared mostly in a peak coincident with RBP (results not shown).

Dependence of retinol uptake on the [^{3}H]retinol–RBP concentration

Saturability in the rate of uptake of the process was tested by incubating membrane vesicles with various concentrations of the [^{3}H]retinol–RBP complex under initial-rate conditions (37 °C, 2 min). Subtraction of the non-saturable component, determined in the presence of PCMBS, from total uptake, showed that a saturable uptake process was taking place (Fig. 6*a*). From the Lineweaver–Burke plot of the uptake data (Fig. 6*b*) a K_m of 116±13 nM and a $V_{max.}$ of 6.9±0.5 pmol/min per mg of membrane protein were calculated.

Table 2. (*a*) Specificity of [^{3}H]retinol uptake and (*b*) uptake of [^{3}H]retinol from various binding proteins

(*a*) Membranes (0.5 mg/ml) were incubated with 10 nM-[^{3}H]retinol–RBP for 15 min at 37 °C in the presence of indicated concentrations of the competing proteins. Specific uptake of [^{3}H]retinol was then assayed.

Addition (competing protein)	Concn. (μM)	Specific uptake (%)
None	–	100
Holo-RBP	5	0
Apo-RBP	5	7
TTR	5	35*
Human serum albumin	20	102
β-Lactoglobulin	20	106

* 90% when incubation was for 60 min at 37 °C.

(*b*) Membranes (1.0 mg/ml) were incubated with the various [^{3}H]retinol–protein complexes for 30 min at 37 °C. Non-specific uptake was determined with parallel incubations in the presence of at least a 200-fold molar excess of appropriate binding proteins loaded with unlabelled retinol.

Complex	Input (c.p.m./assay)	Uptake (c.p.m.) Total	Uptake (c.p.m.) Non-specific
[^{3}H]Retinol–RBP	11286	4672±178	2200±94
[^{3}H]Retinol–albumin	7360	1415±36	1351±71
[^{3}H]Retinol–β-lactoglobulin	2210	443±28	421±30

Trypsin-sensitivity

Treatment of membrane vesicles with 0.2 mg of trypsin/ml for 20 min at 37 °C destroyed 65% of specific retinol uptake, suggesting that the receptor mediating the uptake of retinol from RBP is a protein. Under these conditions 50% of ^{125}I-RBP binding was also destroyed.

Trypsin treatment of the membrane vesicles also abolished 65% of the total free retinol binding when 5 nM-[^{3}H]retinol was used instead of the retinol–RBP complex. Heat treatment of the membranes at 65 °C for 4 h has previously been shown to abolish over 60% of the retinol binding (Fig. 2). The results therefore suggest that heat-sensitive protein-binding sites are present in the membrane and that the use of heat-inactivated membranes for the determination of non-specific binding is valid.

Specificity

The specificity of the uptake process was examined by testing the ability of various proteins to inhibit the uptake of [^{3}H]retinol from RBP (Table 2*a*). Except for RBP and its apo form, none of the other proteins was inhibitory. The inhibition observed with TTR at early incubation times disappeared at later time points. Moreover, no specific uptake was observed when [^{3}H]retinol was presented as a complex with other retinol-binding proteins such as human serum albumin and bovine β-lactoglobulin (Table 2*b*).

The specificity of free retinol-binding sites in the membranes could not be tested because of the difficulty

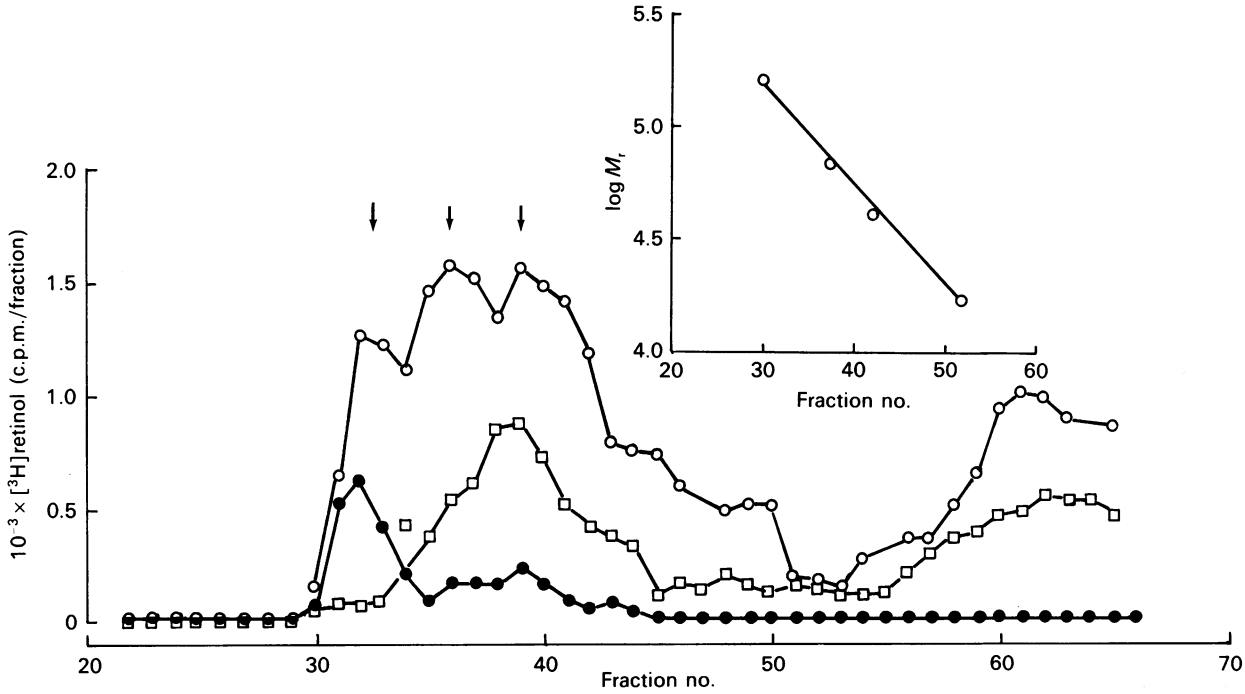

Fig. 7. Gel filtration of solubilized placental membranes after [^{3}H]retinol uptake

Membranes (1.1 mg of protein/ml) were incubated at 37 °C for 15 min with 1.6×10^5 c.p.m. of [^{3}H]retinol–RBP in the absence (○) or presence (●) of a large excess of unlabelled RBP (5 μM). Membranes were then recovered by centrifugation, washed and solubilized in 1 ml of 0.5% emulphogene BC-720 in PBS and centrifuged at 105000 *g* for 1 h. Portions (200 μl) of the clear supernatants were subjected to size fractionation on a TSK G-3000 SW column (300 cm × 7.5 cm) using the same buffer. Fractions (200 μl) were collected at 1 min intervals and counted for radioactivity. The [^{3}H]retinol–RBP complex (□) and [^{3}H]retinol (results not shown) were also run under the same conditions. The column was calibrated as described under 'Methods' (inset).

in distinguishing total binding from non-displaceable binding at high concentrations of the unlabelled retinoid.

H.p.g.f.c.

H.p.g.f.c. analysis of membranes solubilized in emulphogene BC-720 after incubation with [^{3}H]retinol–RBP, showed three radioactive peaks corresponding to molecular masses of about 125, 80 and 60 kDa (Fig. 7). When free or RBP-bound [^{3}H]retinol was run under the same conditions, radioactive peaks appeared at the 80 kDa (data not shown) and 60 kDa positions respectively. Since the micelle molecular mass of emulphogene BC-720 is about 80 kDa, the 80 kDa radioactive species presumably represents mixed micelles of retinol with the detergent (Lichtenberg *et al.*, 1983). The appearance of RBP at the 60 kDa position suggests that RBP forms some kind of oligomer in the detergent solution. The radioactive retinol in the 125 kDa peak is likely to be protein bound. All these peaks were reduced or disappeared altogether when a large excess of unlabelled RBP was included in the incubation medium during [^{3}H]retinol uptake. This suggests that the effects are specific (●) and that the retinol taken up by the membranes becomes protein-bound in particular to a component with high affinity that, under these conditions, chromatographs as a 125 kDa species.

DISCUSSION

The accumulation by placental membranes of [^{3}H]retinol from the [^{3}H]retinol–RBP complex showed the following important differences from the binding of ^{125}I-RBP: (1) the process was highly temperature-dependent; (2) the steady-state level of RBP binding, but not that of retinol uptake, was significantly inhibited by TTR (Fig. 3); (3) prolonged incubation of membranes caused a decline in the equilibrium binding of RBP, but showed little change in the steady-state uptake levels of retinol; (4) analysis by h.p.g.f.c. of membranes solubilized after incubation with [^{3}H]retinol–RBP showed that only a small proportion of the radioactive retinol, originally added as a complex with RBP, was recovered in the RBP peak (Fig. 7). These observations, taken together with the absence of any energy-providing system, suggest that endocytosis of the retinol–RBP complex is unlikely to be the uptake mechanism.

Under physiological conditions, vitamin A, like other hydrophobic substances, could conceivably desorb from its serum binding protein into the aqueous phase and then partition into the membrane bilayer, from where it could be removed by intracellular binding proteins (Brecher *et al.*, 1984). The following observations, however, cannot be explained by such a simple diffusion mechanism: (1) uptake of RBP-bound retinol attains steady-state equilibrium so rapidly (Fig. 1) that it is unlikely to occur by a mechanism that involves a slow aqueous diffusion step; (2) retinol delivered to the membrane by RBP, but not in the free form, is readily and completely removed by the addition of apo-RBP (Fig. 5); (3) PCMBS, a strong inhibitor of RBP binding (Table 1), completely inhibited the uptake of RBP-bound retinol, but not that of free retinol. In addition, free retinol added to the membrane appears associated with at least one membrane component different for that sequestering retinol from [^{3}H]retinol–RBP (results not shown).

Evidence that the binding of RBP to membrane vesicles is obligatory for the subsequent delivery of retinol was provided by the observation that whereas the binding of RBP and the uptake of retinol by the membrane vesicles could be inhibited by *prior* treatment of membranes with PCMBS, addition of PCMBS *after* the attainment of a steady state abolished only the binding of RBP and did not decrease the amount of retinol already taken up from RBP (Table 1). These results therefore provide direct experimental evidence for the mechanism proposed previously for isolated cells (Rask & Peterson, 1976; Chen & Heller, 1977). At some stage in this uptake process it appears that the retinol becomes associated with a high-affinity component which chromatographs as a 125 kDa species.

Takahashi *et al.* (1977) have shown that rat foetal liver, during the early stages of gestation, does not contain RBP, but RBP was nevertheless present in the foetal circulation. It was therefore presumed that transplacental transport of the maternal RBP takes place. Soprano *et al.* (1986*a*) recently reported the presence of mRNA for RBP in the rat yolk sac.

Taken together with the present data, this raises the possibility that circulating RBP in the early foetus might be placental rather than maternal in origin. In this case, maternal RBP may not cross the placenta to deliver the retinol even during the early stages of gestation. Instead, retinol might be transferred from maternal to placental RBP which eventually enters the foetal circulation.

The observation that all the [^{3}H]retinol delivered to the membranes by RBP is available for rapid exchange with the retinol of native RBP (Fig. 4), and can be removed by apo-RBP (Fig. 5*a*) suggests that retinol delivered by RBP does not routinely diffuse into the lipid phase of the membrane. The release of retinol from membranes which are sealed right-side-out (Booth *et al.*, 1980) appears to be mediated by the RBP receptor itself, since net release of retinol occurs efficiently only when apo-RBP is provided as the retinol acceptor, i.e. defatted albumin or β-lactoglobulin, which have similar association constants for retinol, appear relatively ineffective in this respect. This is probably the mechanism by which the reported (Ismadi & Olson, 1982; Donoghue *et al.*, 1982) transport of retinol from foetus to mother takes place. Although the significance of this reversal phenomenon is not clear, it offers a possible mechanism whereby recycling of retinol from other extrahepatic tissues into the plasma (DeLuca *et al.*, 1979; Green *et al.*, 1985) occurs. The fact that adequate levels of apo-RBP are present in the plasma (Muto *et al.*, 1972) is also consistent with the suggested mechanism.

Moreover, it indicates a method by which the ratio of apo- to holo-RBP levels in the plasma can regulate retinol distribution among various tissues. For example, in vitamin A deficiency, although holo-RBP levels decrease to almost zero, the apo-RBP level of plasma do not show a similar fall (Muto *et al.*, 1972). Since apo-RBP has poor affinity for TTR, most of it will exist in the free state. As both holo- and apo-RBP bind to the cell-surface receptor, the high ratio of apo- to holo-RBP in the plasma would not only result in the decreased uptake of retinol by most tissues, but actually stimulate the secretion of retinol by extrahepatic tissues. The retinol so liberated might then be used for critically dependent tissues such as the eye and gonads. Thus regulation of the bidirectional movement of retinol across the plasma

membrane, mediated by the RBP receptor, provides an interesting mechanism whereby rapid adjustment in vitamin A distribution can occur in response to changes in nutritional, metabolic and physiological states. It is perhaps relevant that Soprano *et al.* (1986*b*) recently demonstrated the occurrence of RBP mRNA in a number of extrahepatic tissues and speculated that recycling of retinol might involve synthesis of RBP, which after combining with retinol, is secreted into the plasma.

Assuming that the RBP receptor itself is involved in both the uptake and release of the vitamins, the question as to the form in which retinol is received by, or presented from, the cytoplasmic surface of the membrane remains. Cellular RBP and the putative cytosolic RBP are the obvious candidates. Indeed, using isolated pigment-epithelial-cell plasma membranes, Ottonello *et al.* (1987) have recently demonstrated that retinol is transferred from RBP to membrane-bound cellular RBP.

We have previously demonstrated that only free RBP (uncomplexed with TTR) is capable of binding to the receptor [the preceding paper (Sivaprasadarao & Findlay, 1988)]. After delivery of retinol to the target cell, it is now clear that RBP has a diminished affinity for both the receptor [the preceding paper (Sivaprasadarao & Findlay, 1988)] and TTR (Goodman, 1984). As the existing free holo-RBP delivers its retinol to the target cell, a gradual release of more free holo-RBP occurs, owing to a shift to the right of the equilibrium:

$$\text{TTR–RBP} \rightleftharpoons \text{TTR} + \text{RBP}$$

This mechanism is completely consistent with the data (Fig. 1) showing that TTR decreases the rate of retinol uptake without substantially altering the steady-state uptake level. Although it is currently believed that, by complexing with RBP, TTR prevents the loss of RBP through filtration in the kidney (Goodman, 1984), TTR might also play an important role in the regulation of retinol distribution among tissues by controlling the levels of free holo-RBP.

The apparent K_m of the retinol-uptake process is about 116 nM, which is close to the physiological concentration of free RBP, reported to be about 130 nM (Fex & Felding, 1984). The fact that $V_{max.}$ (7.0 pmol/min per mg of protein) is higher than the total number of RBP receptors (approx. 1.5 pmol/mg of protein) reported in the preceding paper (Sivaprasadarao & Findlay, 1988), suggests that a single receptor molecule can successively bind a number of RBP molecules and remove their retinol into the cell. The uptake of retinol is strictly dependent on the binding (delivery) protein (Table 2). Neither β-lactoglobulin nor human serum albumin, both of which can bind retinol, were able to substitute for RBP in retinol delivery. The inability to transport retinol from its β-lactoglobulin complex is particularly interesting in view of the recent report showing close structural similarities between β-lactoglobulin and RBP (Papiz *et al.*, 1986).

Studies on free retinol uptake revealed the presence of a limited number of heart- and trypsin-sensitive sites in the placental membrane vesicles, with moderate affinity (approx. $K_a = 5 \times 10^7 M^{-1}$) for free retinol. Owing to the lipid solubility of retinol, it was not possible to demonstrate competitive displacement by unlabelled retinol and thereby perform specificity studies. However, by using h.p.g.f.c. it was possible to show that [^{3}H]retinol binds in a displaceable manner to a protein with an apparent molecular mass of 54 kDa rather than to the 125 kDA species which appears to be involved in uptake from the [^{3}H]retinol–RBP complex. The relationship between this so-far-uncharacterized species and other suggested proteins (Lotan *et al.*, 1980; Libby & Bertram, 1982; Oreffo *et al.*, 1985; Ottonello *et al.*, 1987) is unknown.

In conclusion our studies indicate that the physiological uptake of retinol from RBP involves membrane-bound protein receptors. The apo-RBP remains outside the cell. Endocytosis and diffusion processes do not appear to be involved. This may represent a mechanism by which many natural hydrophobic effectors, e.g. steroid hormones, fat-soluble vitamins etc., which are transported by specific plasma binding proteins, are delivered to target tissues. It is pertinent that specific cell-surface receptors have recently been reported for corticosteroid-binding globulin (Hryb *et al.*, 1986) and testosterone/oestradiol-binding globulin (Strelchyonok *et al.*, 1984; Hryb *et al.*, 1985).

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