Retinol Uptake from Retinol-Binding Protein (RBP) by Liver Parenchymal Cells in Vitro Does Not Specifically Depend on Its Binding to RBP†

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ABSTRACT: The uptake characteristics of both the retinol and retinol-binding protein (RBP) moieties of the retinol–RBP complex by liver parenchymal cells (PC) in vitro were studied to assess whether retinol uptake is mediated by a cell-surface receptor for RBP. At 37 °C as well as 4 °C, [^{3}H]retinol uptake from [^{3}H]retinol–RBP showed a time-dependent increase, and was not saturable at concentrations exceeding the physiological concentration by more than a factor of 2 (3 μM). Uptake of [^{3}H]retinol was not inhibited by a 10-fold molar excess of unlabeled retinol–RBP. Cell association of ^{125}I-RBP at 37 and 4 °C was low and showed no time dependence. In addition, the association of ^{125}I-RBP was not saturable at concentrations up to 3 μM. These data do not support the existence of a cell-surface receptor for RBP on rat liver PC. The uptake of [^{3}H]retinol from RBP was also compared to the uptake of retinol from cellular retinol-binding protein (CRBP) and lactoglobulin. Uptake characteristics of [^{3}H]retinol from CRBP and lactoglobulin were similar to that of [^{3}H]retinol from RBP. Furthermore, a similar percentage of the [^{3}H]retinol taken up by PC was metabolized into retinyl esters, irrespective of its carrier. These data suggest that the uptake of retinol and its subsequent metabolic processing do not depend on binding to RBP. The low level of cell association of ^{125}I-binding proteins was not due to uptake, degradation, and secretion of ligand by PC. This suggests that retinol is dissociated from its binding protein before uptake by PC.

The liver plays a central role in retinoid (vitamin A and its analogs) metabolism and storage. Dietary retinoid is taken up by hepatic parenchymal cells (PC)[1] as chylomicron remnant retinyl ester, which upon uptake is hydrolyzed to retinol (Hendriks et al., 1987). The resulting retinol is either transported to the fat-storing cell (FSC) (also called stellate cell, lipocyte, and Ito cell), through some uncharacterized process, for reesterification and storage or secreted into the blood bound to retinol-binding protein (RBP) for delivery to target organs (Hendriks et al., 1987). In the circulation, the retinol–RBP complex is bound to another plasma protein, transthyretin (TTR), and thus circulates as a retinol–RBP–TTR complex. Plasma concentrations of retinol–RBP are highly regulated and average around 2 μM in healthy individuals (Blaner, 1989).

It is still unclear how the uptake of retinol by the various peripheral cell types is facilitated and regulated. The uptake of both retinol and RBP by tissues and cells has been studied extensively (Blaner, 1989). The existence of a specific cell-surface receptor for RBP has been postulated to exist on the surfaces of testicular interstitial cells (Bhat & Cama, 1979; McGuire et al., 1981), Sertoli cells (Shingleton et al., 1989), retinal pigment epithelial cells (Heller, 1975; Pfeffer et al., 1986), intestinal mucosal epithelial cells (Rask & Peterson, 1976; Sege & Peterson, 1978), F9 teratocarcinoma cells (Eriksson et al., 1986), placental brush borders (Sivaprasadarao & Findlay, 1988a,b; Törmä & Vahlquist, 1986), human keratinocytes (Törmä & Vahlquist, 1984; Forsum et al., 1977), and on hepatic PC and FSC (Gjoen et al., 1987; Senoo et al., 1990). To date, no convincing purification and characterization of such cell-surface receptors for RBP have been reported, no specific antibodies are available, and no immunocytochemical localization of the putative RBP cell-surface receptor has been reported. Although recently, a study describing the partial characterization of a bovine retinal pigment epithelial receptor for RBP in microsomal preparations has been reported (Båvik et al., 1991). However, it is unclear from this recent study whether the RBP receptor described is present as an intracellular (perhaps involved in the secretion of newly synthesized RBP from the cells) receptor or as a cell-surface receptor (Båvik et al., 1991).

Some investigators have questioned, on the basis of model studies and the physical properties of the retinol–RBP complex, whether a cell-surface receptor for RBP is necessary for the uptake of retinol from the RBP molecule (Noy & Xu, 1990a; Fex & Johannesson, 1987). Some data contradicting earlier published reports regarding the existence of RBP receptors on cell surfaces have also appeared in the recent literature. Creek and colleagues (Creek et al., 1989; Hodam et al., 1991) were not able to observe specific binding of ^{125}I-RBP to the surface of keratinocytes, thus suggesting the lack of an RBP receptor on these cells. Other data from human breast epithelial carcinoma cells and human hepatoma cells have indicated that retinol uptake in these different cell types proceeds in a manner which appears to be independent of an RBP cell-surface receptor (Randolph & Ross, 1991). Thus, at present, the existence of cell-surface receptors for RBP is

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[1] Abbreviations: PC, parenchymal cell; FSC, fat-storing cell; RBP, retinol-binding protein; TTR, transthyretin; CRBP, cellular retinol-binding protein; BSA, bovine serum albumin; PBS, phosphate-buffered saline; TCA, trichloroacetic acid; TC, tyramine cellobiose; LRAT, lecithin: retinol acyltransferase.

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controversial and a matter in need of much further research.

In addition to its role in retinol delivery to peripheral target tissues, RBP has been proposed to mediate retinol transfer from the peripheral target tissues and possibly peripheral retinol stores [such as in adipose tissue (Tsutsumi et al., 1992)] back to the liver (Blomhoff et al., 1985). It has been proposed that this delivery proceeds through a process whereby RBP is internalized by hepatic PC and FSC through receptor-mediated endocytosis (Senoo et al., 1990). The present study was designed to investigate this possibility using physiological concentrations of the retinol–RBP complex and isolated and cultured rat hepatic PC. Both [^{3}H]retinol–RBP and retinol–^{125}I-RBP were used to analyze the uptake characteristics of the retinol as well as the RBP moieties of the retinol–RBP complex. In addition, in order to determine whether uptake of [^{3}H]retinol by PC depends on its binding to RBP, these processes were compared to the uptake of [^{3}H]retinol when nonphysiologically delivered to the PC, bound to either cellular retinol-binding protein (CRBP) or lactoglobulin. Futhermore, the ability of PC to esterify the [^{3}H]retinol taken up from the different [^{3}H]retinol–protein complexes or provided free in solution was investigated.

MATERIALS AND METHODS

Cell Isolation and Cell Culture. Liver parenchymal cells (PC) were isolated from 6-month-old female Brown Norway/Billingham-Rijswijk rats by in situ perfusion of rat liver with 0.05% (w/v) collagenase and were purified by centrifugal elutriation as described previously (Hendriks et al., 1990). Purity was determined using a hemocytometer and always exceeded 99%. Viability was determined using the trypan blue exclusion test and was greater than 95%. PC were cultured and incubated in Dulbecco's modified Eagle's medium (Gibco Limited, Paisley, Scotland) supplemented with 5% heat-deactivated newborn calf serum, 20 IU of penicillin/mL (Seromed, Sanbio, Uden, The Netherlands), 2×10^{-3}% (w/v) streptomycin (Seromed), and 160 IU of insulin/L (Novo Industri BV, Amsterdam, The Netherlands). Cells were cultured at a concentration of 3×10^5 cells per well in a volume of 300 μL on 24-well plates (Costar, Cambridge, MA). Light microscopy showed that more than 95% of the cells was attached to the culture dish after overnight culture. Cell cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

Purification of RBP and CRBP. RBP was purified from rat serum (Blaner & Goodman, 1990), and CRBP was purified from rat testes (Kato et al., 1984). Bovine lactoglobulin was obtained from Sigma Chemical Co., St. Louis, MO.

Preparation of [^{3}H]Retinol–Protein Complexes. A trace amount of [^{3}H]retinol (New England Nuclear, Boston, MA; specific activity 55 Ci/mmol) was added to the retinol–protein complexes and incubated overnight in the dark (4 °C). To remove unbound ligand, activated charcoal was added and the mixture was further incubated for 1 h (4 °C). The mixture was then centrifuged for 10 min at 600g to sediment the charcoal–[^{3}H]retinol complex. Specific activities of the [^{3}H]-retinol–RBP, [^{3}H]retinol–CRBP, and [^{3}H]retinol–lactoglobulin preparations were about 1, 5, and 2.5 Ci/mmol, respectively.

Preparation of Retinol–^{125}I-Protein Complexes. RBP and lactoglobulin were iodinated with Na^{125}I (Amersham, Buckinghamshire, U.K.) using the lactoperoxidase method, essentially as described previously (Roth, 1975). CRBP (5 μg) was iodinated using 0.5 mCi of ^{125}I-Bolton–Hunter reagent (Amersham) according to the instructions of the manufacturer. Free ^{125}I was separated from both ^{125}I-RBP and ^{125}I-lactoglobulin, and ^{125}I-Bolton–Hunter–glycine was separated from ^{125}I-CRBP by gel filtration using a Sephadex G25 column (1.5 × 5 cm) equilibrated with 0.07 M Tris-HCl buffer in 0.07 M NaCl, pH 8.6, containing 2% bovine serum albumin (BSA). To further remove free ^{125}I and ^{125}I-Bolton–Hunter–glycine, radiolabeled proteins were dialyzed 3 times against distilled water. The specific activities were between 100 and 250 Ci/mmol.

Cell Association of Radiolabeled Ligands with PC. Culture medium of PC was aspirated, and 300 μL of culture medium containing the relevant radiolabeled compound was added. The media contained a fixed amount of [^{3}H]retinol-labeled or ^{125}I-protein-labeled retinol–protein complex, or free [^{3}H]-retinol (Amersham, specific activity 57 Ci/mmol), to which a variable amount of unlabeled retinol–protein complex or free retinol (Janssen Chimica, Beerse, Belgium) was added. Radioactivity added per well ranged from 21 000 to 66 000 cpm for the incubations with [^{3}H]retinol–protein complexes and free [^{3}H]retinol, and ranged from 100 000 to 500 000 cpm for incubations with retinol–^{125}I-protein complexes. The cells were incubated at 37 °C in a humidified atmosphere or at 4 °C on ice for the times and at the concentrations indicated. Three separate experiments were performed using [^{3}H]retinol–protein complexes and retinol–^{125}I-protein complexes, and one experiment with free [^{3}H]retinol was performed. Within each experiment, incubations were carried out in duplicate. Incubations were stopped by placing the cultures on ice, and the cells were subsequently rinsed twice with 150 μL of ice-cold phosphate-buffered saline (PBS).

Harvesting of PC after Incubations with [^{3}H]Retinol–Protein Complexes or Free [^{3}H]Retinol, and Determination of [^{3}H]Retinyl Ester Formation. Cells were harvested in 3 × 100 μL of ice-cold PBS using a rubber policeman. A small known aliquot (25–100 μL) of the cell suspension was dissolved in Ready Solve (Beckman, Fullerton, CA) for liquid scintillation counting. Esterification of [^{3}H]retinol after incubations with [^{3}H]retinol–protein complexes and free [^{3}H]retinol was determined in a similar experimental setup using 3×10^6 cells per well in a volume of 3 mL. The percentage of [^{3}H]retinyl esters formed was determined by chromatography using 6% deactivated aluminum oxide columns (Saari & Bredberg, 1988). [^{3}H]Retinyl esters were eluted with 3% diethyl ether in petroleum ether (40–60 °C distillation fraction). Subsequently, [^{3}H]retinol was eluted with 50% diethyl ether in the same solvent. The [^{3}H]retinyl ester fraction and [^{3}H]retinol fraction were evaporated to dryness and dissolved in Ready Solve for liquid scintillation counting. [^{3}H]Retinyl ester formation was determined for [^{3}H]retinol–CRBP, [^{3}H]-retinol–lactoglobulin, and [^{3}H]retinol–RBP in three, two, and one separate experiments, respectively, at a concentration of 0.2 μM. Incubations were carried out in duplicate.

Harvesting of PC after Incubations with Retinol–^{125}I-Protein Complexes, and Determination of Degradation of Retinol–^{125}I-Protein Complexes. Both an excess of potassium iodide and BSA were added both to the combined culture medium and the rinsing fluid (600 μL in total) and to the harvested cells (300 μL) to reduce nonspecific binding and to maximize ^{125}I-protein precipitation. ^{125}I-Protein was denatured in trichloroacetic acid (TCA; final concentration 5% w/v) for 20 min at 4 °C and precipitated at 3000g for 3 min. Both the TCA-soluble fraction and the TCA-precipitable fraction were dissolved in 10 M urea, and radioactivity was determined by γ counting. The percentage of degraded ^{125}I-protein present in the culture medium was calculated by

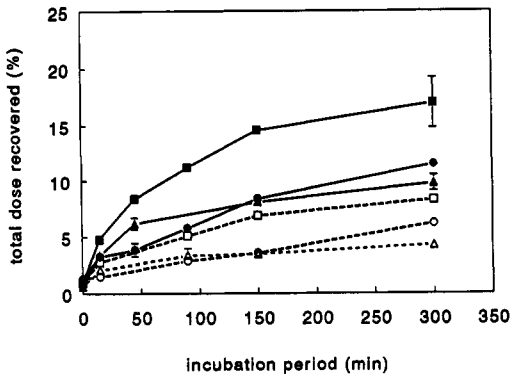

FIGURE 1: Effect of time and temperature on cell association of [^{3}H]retinol with PC. One typical experiment is shown using [^{3}H]-retinol–RBP, –CRBP, or –lactoglobulin. Rat liver PC were incubated at 37 °C (closed symbols) or 4 °C (open symbols) with [^{3}H]retinol–RBP (●, 18 000 cpm/well), [^{3}H]retinol–CRBP (■, 56 000 cpm/well), or [^{3}H]retinol–lactoglobulin (▲, 78 000 cpm/well) at a retinol concentration of 0.29 ± 0.05 μM, and the cell-associated radioactivity was determined. Values represent the mean of duplicates. Error bars represent the deviation from the mean. Only the values of [^{3}H]-retinol–CRBP incubations at 4 °C were from single determinations.

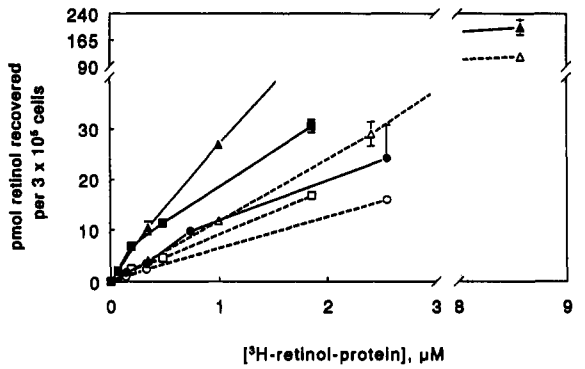

FIGURE 2: Effect of concentration and temperature on cell association of [^{3}H]retinol with PC. One typical experiment is shown using [^{3}H]-retinol–RBP, –CRBP, or –lactoglobulin. Rat liver PC were incubated for 90 min at 37 °C (closed symbols) or 4 °C (open symbols) with [^{3}H]retinol–RBP (●, 21 000 cpm/well), [^{3}H]retinol–CRBP (■, 69 000 cpm/well), or [^{3}H]retinol–lactoglobulin (▲, 81 000 cpm/well), and the cell-associated radioactivity was determined. Values represent the mean of duplicates. Error bars represent the deviation from the mean.

subtracting the radioactivity present in the TCA-soluble fraction at 0 min from that present at 300 min in three separate experiments.

Plasma Clearance of ^{125}I-RBP, ^{131}I-RBP, ^{125}I-Tyramine Cellobiose (TC)–RBP, and ^{131}I-TC–RBP, and Tissue Uptake and Degradation of Radioiodinated TC–RBP. Purified rat plasma RBP was labeled either directly with ^{125}I or ^{131}I using the lactoperoxidase method or indirectly with ^{125}I-TC or ^{131}I-TC (Makover et al., 1988). The final specific activity of the radiolabeled complexes ranged from 25 to 45 nCi/μg of protein. Thirteen male Sprague-Dawley rats were catheterized via the external jugular veins. One catheter was used for injection of radiolabeled RBP and the other for blood sampling to determine plasma decay kinetics. Before the start of the experiments, rats were allowed to recover from the surgery for a period of at least 72 h. A mixture of ^{125}I-RBP and ^{131}I-TC–RBP or of ^{131}I-RBP and ^{125}I-TC–RBP (1–10 μCi of each) was injected, after which blood samples were collected in ethylenediaminetetraacetic acid-containing syringes after different time periods. Protein-associated radioactivity in plasma was determined after TCA precipitation (final TCA concentration 5% w/v). Uptake and degradation of ^{125}I-TC–RBP and ^{131}I-TC–RBP in various tissues, including liver, kidney, and muscle, were determined using the trapped ligand approach as described by Makover et al. (1988).

RESULTS

Cell Association of [^{3}H]Retinol to Rat Liver PC, Provided as a [^{3}H]Retinol–Protein Complex. (A) Time Dependence. Figure 1 gives the time-dependent cell association of [^{3}H]-retinol, provided as a complex with either RBP, CRBP, or lactoglobulin, using a retinol concentration of 0.29 μM. This concentration represents about 30% of the retinol concentration in rat blood (Wolf, 1980). Cell association of [^{3}H]retinol from RBP and lactoglobulin at 37 °C increased up to 8% of the administered dose by 150 min (Figure 1). Thereafter, the increase in cell-associated radioactivity was slower, reaching a value of about 11% by 300 min. The time-dependent cell-association characteristics of [^{3}H]retinol from [^{3}H]retinol–RBP observed in this study are in agreement with those reported previously using rat liver PC (Blomhoff et al., 1985) or rat Sertoli cells (Shingleton et al., 1989). When provided as a complex with CRBP, the cell-associated [^{3}H]retinol increased up to 15% by 150 min and increased less rapidly thereafter, reaching a value of 17% by 300 min. These data indicate that uptake of retinol from a retinol–protein complex does not specifically depend on the binding to RBP. In a preliminary experiment, addition of free [^{3}H]retinol to the culture medium resulted in cell association which increased up to 25% of the dose administered by 150 min and stabilized thereafter (data not shown). These data are in accordance with those reported previously using keratinocytes (Creek et al., 1989; Hodam et al., 1991) or Sertoli cells (Shingleton et al., 1989).

The time-dependent cell association of [^{3}H]retinol was also studied at 4 °C and was about 50% of that observed at 37 °C at all time points tested (Figure 1). This was also the case when free [^{3}H]retinol was added to the cultured cells (data not shown). These data indicate that even at low temperatures, at which cell-surface receptor internalization does not occur, cell association of retinol still continues.

(B) Concentration Dependence. The concentration dependence of cell association of [^{3}H]retinol was studied using 90-min incubations at both 37 and 4 °C. The highest concentrations used for [^{3}H]retinol–RBP and [^{3}H]retinol–CRBP were in the range of 2–3 μM, representing about twice the retinol–RBP concentration in blood. Concentrations up to about 9 μM were used in the incubations with [^{3}H]retinol–lactoglobulin. At 37 °C, cell association of [^{3}H]retinol from [^{3}H]retinol–RBP increased linearly with the ligand concentration to 25 pmol/3 × 10^5 cells at about 3 μM (Figure 2). For [^{3}H]retinol–CRBP, cell association of [^{3}H]retinol increased linearly to 30 pmol/3 × 10^5 cells at 2 μM. For [^{3}H]-retinol–lactoglobulin, a linear increase of cell-associated [^{3}H]retinol to 200 pmol/3 × 10^5 cells at about 9 μM was observed. The percentages of the dose recovered as cell-associated were respectively 3%, 6%, and 8% for [^{3}H]retinol–RBP, [^{3}H]retinol–CRBP, and [^{3}H]retinol–lactoglobulin, at all concentrations tested. In a preliminary experiment, addition of free [^{3}H]retinol to the culture medium resulted in cell association which increased to 110 pmol/3 × 10^5 cells at 2 μM, representing 17% of the administered dose (data not shown). Cell association of [^{3}H]retinol from [^{3}H]retinol–RBP, [^{3}H]retinol–CRBP, [^{3}H]retinol–lactoglobulin, or [^{3}H]-retinol free in solution also increased linearly with retinol concentration at 4 °C. The values were about 50% of those at 37 °C (Figure 2), similar to the data obtained in the time-dependence experiments.

(*C*) *Competition.* To further investigate the specificity of the cell association of [^{3}H]retinol from RBP, competition of [^{3}H]retinol–RBP with an excess of an unlabeled retinol–protein complex or free retinol was investigated using a 90-min incubation at 37 °C. Cell association of [^{3}H]retinol delivered by 1 μM [^{3}H]retinol–RBP (280 000 cpm/well) was not inhibited by a 2-, 5-, or 10-fold molar excess of unlabeled retinol–RBP, retinol–lactoglobulin, or free retinol (data not shown).

These data suggest that uptake of retinol by PC, irrespective of its carrier, is not saturable even at concentrations which are severalfold higher than physiological. Furthermore, uptake of retinol is not inhibited by excess unlabeled retinol–protein complex.

Metabolism of [^{3}H]Retinol in Rat Liver PC, Provided either as a [^{3}H]Retinol–Protein Complex or Free in Solution. The esterification of [^{3}H]retinol into [^{3}H]retinyl esters was studied to establish whether the carrier used to deliver the retinol to the PC influences the metabolic processing of cell-associated [^{3}H]retinol. The percentage of [^{3}H]retinyl esters formed was determined for incubations with [^{3}H]retinol–CRBP and [^{3}H]-retinol–lactoglobulin at 150 and 300 min. The percentage of [^{3}H]retinyl ester formed increased from about 20% by 150 min to about 40% by 300 min for incubations with [^{3}H]retinol–CRBP and [^{3}H]retinol–lactoglobulin (data not shown). [^{3}H]-Retinyl esters are also formed, in similar percentages, when rat liver PC are incubated with [^{3}H]retinol–RBP or free [^{3}H]-retinol (data not shown). These data show that the PC-associated [^{3}H]retinol is converted to [^{3}H]retinyl esters, irrespective of its carrier. Similar data were reported by Creek et al. (1989) in their studies on keratinocytes and by Shingleton et al. (1989) using Sertoli cells.

Cell Association of Retinol–^{125}I-Protein Complexes to Rat Liver PC. (*A*) *Time Dependence.* Cell association of the ^{125}I-binding protein moieties of retinol–protein complexes was studied to investigate whether retinol uptake occurs as a retinol–protein complex. Cell association of the protein moieties of retinol–^{125}I-RBP, retinol–^{125}I-CRBP, and retinol–^{125}I-lactoglobulin was studied using a retinol concentration of 0.47 ± 0.20 μM. Cell association of retinol–^{125}I-RBP, retinol–^{125}I-CRBP, and retinol–^{125}I-lactoglobulin (all 110 000 cpm/well) at 37 °C was low at both 90 and 300 min and never exceeded 4% of the administered dose (data not shown). In contrast to the incubations with [^{3}H]retinol–protein complexes, no time-dependent increase of cell-associated radioactivity was observed with any of the retinol–^{125}I-protein complexes.

(*B*) *Concentration Dependence.* In order to expand these observations, the concentration-dependent cell association of retinol–^{125}I-protein complexes was studied at 90 min (Figure 3) using the same concentration range as in the [^{3}H]retinol cell-association studies described above. Similar to the time-dependence studies, concentration-dependent cell association of ^{125}I-RBP, ^{125}I-CRBP, and ^{125}I-lactoglobulin at 37 °C was low and did not exceed 2% at any of the concentrations tested. Furthermore, the radioactivity recovered increased linearly and was not observed to saturate up to concentrations of 3, 4, and 7 μM, respectively, for ^{125}I-RBP, ^{125}I-CRBP, and ^{125}I-lactoglobulin (Figure 3). These data indicate that the association of the ^{125}I-RBP moiety of the retinol–RBP complex with cells is not saturable even at concentrations exceeding the retinol–RBP concentration found in blood by more than a factor of 2. These findings are consistent with our observations obtained in the [^{3}H]retinol cell-association studies (see above).

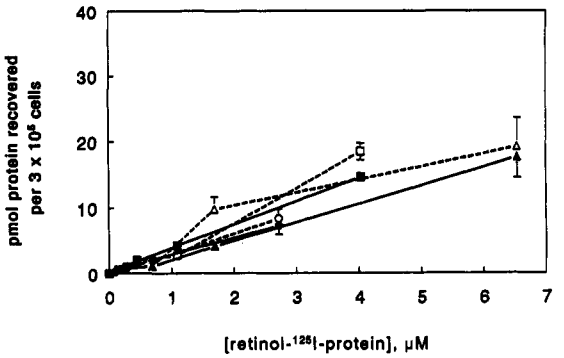

FIGURE 3: Effect of concentration and temperature on cell association of ^{125}I-binding proteins with PC. One typical experiment is shown using retinol–^{125}I-RBP, –^{125}I-CRBP, or –^{125}I-lactoglobulin. Rat liver PC were incubated for 90 min at 37 °C (closed symbols) or 4 °C (open symbols) with retinol–^{125}I-RBP (●, 110 000 cpm/well), retinol–^{125}I-CRBP) (■, 100 000 cpm/well), or retinol–^{125}I-lactoglobulin (▲, 100 000 cpm/well), and the cell-associated radioactivity was determined. Values represent the mean of duplicates. Error bars represent the deviation from the mean.

(*C*) *Temperature Dependence.* To further characterize the association process with PC of the ^{125}I-protein moieties, the concentration dependence and time dependence of cell association were also studied at 4 °C. Cell associations of ^{125}I-RBP, ^{125}I-CRBP, and ^{125}I-lactoglobulin were similar, both at 4 °C and at 37 °C (Figure 3; data for time dependence not shown), suggesting that cell association of these binding proteins represents nonspecific binding. Low or undetectable cell association of ^{125}I-RBP was also reported in other studies using Sertoli cells (Shingleton et al., 1989), keratinocytes (Creek et al., 1989; Hodam et al., 1991), and mucosa cells (Rask & Peterson, 1976).

TCA Precipitation. Both for the time-dependence and for the concentration-dependence studies, cell association of ^{125}I-protein for the three retinol–protein complexes was about a factor of 10 lower than that observed for [^{3}H]retinol. To examine the possibility that ^{125}I-binding proteins are taken up and degraded, and that degradation products are resecreted into the culture medium, the percentage of TCA-soluble ^{125}I-binding protein present in the culture medium was determined by TCA precipitation. The TCA-soluble radioactivity present in the PC culture medium was always 0.2% or less after a 300-min incubation at 37 °C with about 0.5 μM retinol–^{125}I-RBP, retinol–^{125}I-CRBP, or retinol–^{125}I-lactoglobulin using 100 000–500 000 cpm/well (data not shown). These data indicate that the difference between cell association of retinol and its binding protein is not due to uptake and subsequent degradation of the binding protein, and resecretion of the degradation products into the culture medium.

Plasma Clearance of ^{125}I-RBP, ^{131}I-RBP, ^{125}I-TC–RBP, and ^{131}I-TC–RBP. Gjoen et al. (1987) have reported that rat parenchymal cells and fat-storing cells take up radiolabeled TC–RBP injected intravenously into rats. Since in their study RBP iodinated either by the sodium hypochlorite method or by covalent attachment of iodinated tyramine cellobiose showed the same plasma decay characteristics, a significant uptake of ^{125}I-RBP by PC was predicted in our study. However, only little uptake of ^{125}I-RBP iodinated by the lactoperoxidase method was observed in vitro (see above). Therefore, plasma decays of ^{125}I-lactoperoxidase-iodinated RBP and ^{131}I-TC–RBP were compared by injecting into rats a mixture of the two radiolabeled proteins. In a separate experiment, a mixture of ^{131}I-RBP and ^{125}I-TC–RBP was injected to exclude the effect of the radioisotope applied. Both ^{125}I-RBP and ^{131}I-RBP were cleared more rapidly as compared

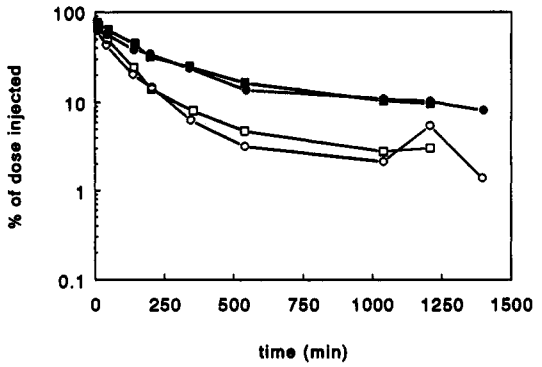

FIGURE 4: Plasma clearance of $^{125/131}$I-labeled and $^{125/131}$I-TC-labeled RBP. One typical experiment is shown in which a mixture of ^{125}I-RBP (○) and ^{131}I-TC–RBP (●) or of ^{131}I-RBP (□) and ^{125}I-TC–RBP (■) (1–10 μCi of each) was injected into two rats, respectively. Blood samples were collected after different periods of time, and TCA-precipitable radioactivity was determined.

to both ^{125}I-TC–RBP and ^{131}I-TC–RBP (Figure 4). No significant differences in ^{125}I- and ^{131}I-RBP plasma decay were observed, indicating that the radioisotope used did not affect plasma decay. The plasma half-life was about 90 min for the directly iodinated RBP and about 180 min for the TC-labeled RBP (Figure 4). These data suggest that the uptake and metabolism of lactoperoxidase-iodinated RBP are different from those of TC-iodinated RBP. The shorter half-life of ^{125}I-RBP as compared to ^{131}I-TC–RBP is most likely not due to differences in TTR binding. This was tested in two ways. First, a mixture of ^{125}I-RBP and ^{131}I-TC–RBP was injected into a rat and circulated for 30 min in order to allow binding to TTR. Plasma containing ^{125}I-RBP and ^{131}I-TC–RBP was taken from this animal and injected into other rats. The same difference in plasma half-life was observed for the two labeled RBP complexes (data not shown). Second, TTR affinity chromatography was used to purify ^{125}I-RBP and ^{131}I-TC–RBP through TTR binding capacity. The plasma decay of the two purified forms of RBP, both of which bind TTR, remained different (data not shown). Therefore, the difference in plasma decay of the two labeled RBP complexes is likely not caused by differences in TTR binding.

Of the 13 experimental animals tested, approximately 18–27% of the injected ^{125}I-TC–RBP or ^{131}I-TC–RBP was taken up by the liver and 11–12% by the kidney after 24 h. These findings are in agreement with those described by Gjoen et al. (1987). In our study, the muscle was the site of greatest uptake of radioiodinated TC–RBP, accounting for between 24 and 34% of the injected dose.

DISCUSSION

Both the mechanism of retinol delivery to cells and the role of RBP in this process have been extensively studied in a variety of cells and isolated cell membranes [see Blaner (1989) for a review]. At present, it is still controversial as to whether RBP receptors exist on cell membranes and what role such receptors would play in the delivery of retinol to the cell (Blaner, 1989). The evidence for the presence of a cell-surface RBP receptor is not conclusive, and no such cell-surface receptor has been unequivocally purified and characterized, although much literature supporting the possible existence of the RBP cell-surface receptor has been reported in the last 15 years (Heller, 1975; Rask & Peterson, 1976; Forsum et al., 1977; Sege & Peterson, 1978; Bhat & Cama, 1979; McGuire et al., 1981; Blomhoff et al., 1985; Törmä & Vahlquist, 1984, 1986; Eriksson et al., 1986; Pfeffer et al., 1986; Gjoen et al., 1987; Ottonello et al., 1987; Sivaprasadarao & Findlay, 1988a,b; Shingleton et al., 1989; Senoo et al., 1990). However, more recently other mechanisms for the uptake of retinol from RBP by cells, which do not invoke the need for an RBP receptor, have been proposed. A recent study (Noy & Xu, 1990a) indicated that the dissociation of retinol from RBP is spontaneous and rapid, with a dissociation constant of 0.1 12^{-1}. This process is much more rapid than the observed uptake rate of retinol by several cell types, and therefore is not rate limiting for uptake (Noy & Xu, 1990a).

Other studies have demonstrated that retinol transfer from RBP to phospholipid bilayers and across the lipid bilayer is very rapid and spontaneous (Fex & Johannesson, 1987, 1988; Noy & Xu, 1990a,b). Recently, Noy and Blaner (1991) have reported that the association of retinol with apo-CRBP will not be a rate-limiting step for retinol uptake by cells and that the thermodynamic need to maintain equilibrium will cause retinol to be drawn into the cell as long as apo-CRBP is available within the cell. Thus, these authors have suggested that the level of apo-CRBP within cells controls retinol flux into cells and that, therefore, the presence of a cell-surface receptor for RBP will not be necessary to draw retinol into cells. The studies reported here were designed to explore retinol internalization by hepatic parenchymal cells.

Several authors (Gjoen et al., 1987; Senoo et al., 1990) have proposed the existence of a specific RBP receptor on at least two hepatic cell types, the hepatic PC and FSC. It has been suggested that the RBP receptor on PC and FSC is important for the recycling of the retinol–RBP complex from peripheral tissues back to the liver (Blomhoff et al., 1985; Gjoen et al., 1987; Senoo et al., 1990), thus providing an alternative for renal RBP catabolism (Goodman, 1984). This RBP may originate from the liver itself or may be synthesized in extrahepatic tissues expressing RBP–mRNA (Soprano et al., 1986). In vivo studies, using ^{125}I-TC-labeled RBP, have indicated that a significant amount of RBP is taken up by hepatic PC (Gjoen et al., 1987). Other studies, employing electron microscopic immunocytochemical techniques, reported that when very large quantities of human RBP were injected intravenously into rats, some of the RBP was taken up through a receptor-mediated endocytic process by both PC and FSC (Senoo et al., 1990). Our initial studies were designed to characterize such a receptor on isolated rat liver PC. Our studies included an examination of the concentration, time, and temperature dependence of the association of both [^{3}H]retinol and ^{125}I-RBP with PC as well as the competition of unlabeled retinol–RBP and [^{3}H]retinol–RBP for cell association. The concentration dependence of cell association of [^{3}H]retinol–RBP with the parenchymal cells was linear for up to at least twice the retinol–RBP concentration in blood (>2 μM). Furthermore, the uptake of physiological amounts of [^{3}H]retinol from RBP was not inhibited by a 10-fold molar excess of unlabeled retinol–RBP. Incubation at 4 °C diminished [^{3}H]retinol uptake by only 50%. The cell association of ^{125}I-RBP was not found to saturate even at concentrations up to 3 μM. The amount of cell-associated ^{125}I-RBP was very low and showed no time dependence. At 4 °C, the association of ^{125}I-RBP to PC was similar to that at 37 °C. Furthermore, in our studies, cultures of hepatic PC were found to take up and degrade only 0.2% or less of the ^{125}I-RBP, in a period of 300 min. These observations can be taken to suggest that an RBP receptor is not present on the surfaces of rat liver PC. A time-dependent increase of cell-associated [^{3}H]retinol was observed for all three [^{3}H]retinol–protein complexes. This time dependence leveled off after prolonged incubation periods. Time dependence is expected

both in non-receptor-mediated and in receptor-mediated uptake processes. Recently, Hodam et al. (1991) showed that re-addition of retinol after a prolonged incubation period resulted in the same time-dependent increase and leveling off of retinol accumulation. The latter data indicate that the time dependence of retinol uptake may mainly depend on culture conditions. Therefore, we do not consider this time-dependent uptake of [^{3}H]retinol to be supportive of a particular (non-receptor-mediated or receptor-mediated) uptake process.

We observed that little (<0.2%) of ^{125}I-RBP provided to parenchymal cells was taken up and degraded by these cells. This finding was in apparent contradiction with the results described by Gjoen et al. (1987), which showed that considerable percentages of ^{125}I-TC–rat RBP were recovered in rat liver PC isolated at 1, 5, and 24 h after injection. Uptake of ^{125}I-TC–RBP was considered to be representative for uptake of sodium hypochlorite-labeled ^{125}I-RBP since no difference in plasma decay of the two differently radiolabeled RBP molecules was observed. In our study, however, lactoperoxidase-labeled ^{125}I-rat RBP was cleared more rapidly from the circulation than ^{125}I-TC–rat RBP. Moreover, the percentage of TC-labeled RBP recovered in the kidney was considerably lower than expected on the basis of data in the old literature using ^{125}I-RBP which showed that the kidney plays a major role in RBP catabolism (Vahlquist et al., 1973). Therefore, the uptake of TC-labeled RBP may not be representative for the tissue site of uptake of lactoperoxidase-iodinated RBP.

In other experiments, we tested whether it is necessary for [^{3}H]retinol to be specifically bound to RBP in order for PC to take up retinol. Our data show that [^{3}H]retinol uptake by PC cultures from CRBP and lactoglobulin was similar to that for [^{3}H]retinol uptake from RBP. Furthermore, the data indicate that the type of binding protein also did not affect subsequent retinyl ester formation. These data and the low level of association of ^{125}I-RBP, ^{125}I-CRBP, or ^{125}I-lactoglobulin with isolated parenchymal cells (see Results) suggest that retinol is dissociated from its binding protein before it associates with the cells. Similar conclusions were reached in other studies exploring retinol uptake in rat Sertoli cells (Shingleton et al., 1989) and human keratinocytes (Creek et al., 1989; Hodam et al., 1991). Thus, the cellular uptake of retinol by hepatic parenchymal cells may not require a specific interaction between RBP and the cell surface. These experiments also indicate that retinol delivery to the cell will direct retinol toward esterification, independent of its carrier.

The rate of [^{3}H]retinol uptake from RBP observed in our study (2 pmol/min at 90 min using 99 pmol of holo-RBP per 3×10^5 PC: also see Figure 1) was much less than the rate of dissociation of retinol from RBP ($v = k$[RBP] = 0.112×99 pmol/min = 11 pmol/min). Therefore, the release of [^{3}H]-retinol from RBP was not the rate-limiting step for [^{3}H]retinol uptake in our study. The rate-limiting step in [^{3}H]retinol uptake by parenchymal cells may be in the metabolic processing or transport of [^{3}H]retinol after it has passed to the inner leaflet of the parenchymal cell plasma membrane. Hepatic parenchymal cells have been shown to possess high levels of CRBP (Blaner et al., 1985), and in addition, it has been reported that most of the retinol present in the liver is bound to CRBP (Harrison et al., 1987). Thus, the binding of retinol in the parenchymal cell by apo-CRBP may play a central role in the internalization of retinol by the cell. The retinol–CRBP complex is the substrate for the esterification reaction catalyzed by lecithin:retinol acyltransferase (LRAT) (Blaner et al., 1990), and retinyl esters do not bind to CRBP (Chytil & Ong, 1984). Therefore, as retinol enters the parenchymal cells, its esterification catalyzed by LRAT (Blaner et al., 1990) could serve as one of the possible mechanisms to stimulate retinol uptake by the parenchymal cells.

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REFERENCES

Båvik, C. O., Eriksson, U., Allen, R. A., & Peterson, P. A. (1991) *J. Biol. Chem. 266*, 14978–14985.
Bhat, M. K., & Cama, H. R. (1979) *Biochim. Biophys. Acta 587*, 273–281.
Blaner, W. S. (1989) *Endocr. Rev. 10*, 308–316.
Blaner, W. S., & Goodman, D. S. (1990) *Methods Enzymol. 189*, 193–206.
Blaner, W. S., Hendriks, H. F. J., Brouwer, A., De Leeuw, A. M., Knook, D. L., & Goodman, D. S. (1985) *J. Lipid Res. 26*, 1241–1251.
Blaner, W. S., Van Bennekum, A. M., Brouwer, A., & Hendriks, H. F. J. (1990) *FEBS Lett. 274*, 89–92.
Blomhoff, R., Norum, K. R., & Berg, T. (1985) *J. Biol. Chem. 260*, 13571–13575.
Chytil, F., & Ong, D. E. (1984) in *The Retinoids* (Sporn, M. B., Roberts, A. B., & Goodman, D. S., Eds.) Vol. 2, pp 89–123, Academic Press, Inc., New York.
Creek, K. E., Silverman-Jones, C. S., & De Luca, L. M. (1989) *J. Invest. Dermatol. 92*, 283–289.
Eriksson, U., Hansson, E., Nilsson, M., Jönsson, K.-H., Sundelin, J., & Peterson, P. A. (1986) *Cancer Res. 46*, 717–722.
Fex, G., & Johannesson, G. (1987) *Biochim. Biophys. Acta 901*, 255–264.
Fex, G., & Johannesson, G. (1988) *Biochim. Biophys. Acta 944*, 249–255.
Forsum, U., Rask, L., Tjernlund, U. M., & Peterson, P. A. (1977) *Arch. Dermatol. Res. 258*, 85–88.
Gjoen, T., Bjerkelund, T., Blomhoff, H. K., Norum, K. R., Berg, T., & Blomhoff, R. (1987) *J. Biol. Chem. 262*, 10926–10930.
Goodman, D. S. (1984) in *The Retinoids* (Sporn, M. B., Roberts, A. B., & Goodman, D. S., Eds.) Vol. 2, pp 41–88, Academic Press, Inc., New York.
Harrison, E. H., Blaner, W. S., Goodman, D. S., & Ross, A. C. (1987) *J. Lipid Res. 28*, 973–981.
Heller, J. (1975) *J. Biol. Chem. 250*, 3613–3619.
Hendriks, H. F. J., Brouwer, A., & Knook, D. L. (1987) *Hepatology 7*, 1368–1371.
Hendriks, H. F. J., Brouwer, A., & Knook, D. L. (1990) *Methods Enzymol. 190*, 49–58.
Hodam, J. R., St. Hilaire, P., & Creek, K. E. (1991) *J. Invest. Dermatol. 97*, 298–304.
Kato, M., Kato, K., & Goodman, D. S. (1984) *J. Cell Biol. 98*, 1696–1704.
Makover, A., Moriwaki, H., Ramakrishnan, R., Saraiva, M. J. M., Blaner, W. S., & Goodman, D. S. (1988) *J. Biol. Chem. 263*, 8598–8603.
McGuire, B. W., Orgebin-Crist, M.-C., & Chytil, F. (1981) *Endocrinology 108*, 658–667.
Noy, N., & Xu, Z.-J. (1990a) *Biochemistry 29*, 3878–3883.
Noy, N., & Xu, Z.-J. (1990b) *Biochemistry 29*, 3883–3888.
Noy, N., & Blaner, W. S. (1991) *Biochemistry 30*, 6380–6386.
Ottonello, S., Petrucce, S., & Maraini, G. (1987) *J. Biol. Chem. 262*, 3975–3981.
Pfeffer, B. A., Clark, V. M., Flannery, J. G., & Bok, D. (1986) *Invest. Ophthalmol. Visual Sci. 27*, 1031–1040.
Randolph, R. K., & Ross, A. C. (1991) *J. Lipid Res. 32*, 809–820.
Rask, L., & Peterson, P. A. (1976) *J. Biol. Chem. 251*, 6360–6366.

Roth, J. (1975) *Methods Enzymol. 37*, 223–233.

Saari, J. C., & Bredberg, D. L. (1988) *J. Biol. Chem. 263*, 8084–8090.

Sege, K., & Peterson, P. A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2443–2447.

Senoo, H., Stang, E., Nilsson, A., Kindberg, G. M., Berg, T., Roos, N., Norum, K. R., & Blomhoff, R. (1990) *J. Lipid Res. 31*, 1229–1239.

Shingleton, J. L., Skinner, M. K., & Ong, D. E. (1989) *Biochemistry 28*, 9641–9647.

Sivaprasadarao, A., & Findlay, J. B. C. (1988a) *Biochem. J. 255*, 561–569.

Sivaprasadarao, A., & Findlay, J. B. C. (1988b) *Biochem. J. 255*, 571–579.

Soprano, D. R., Soprano, K. J., & Goodman, D. S. (1986) *J. Lipid Res. 27*, 166–171.

Törmä, H., & Vahlquist, A. (1984) *Arch. Dermatol. Res. 276*, 390–395.

Törmä, H., & Vahlquist, A. (1986) *Placenta 7*, 295–305.

Tsutsumi, C., Okuno, M., Tannous, L., Piantedosi, R., Allan, M., Goodman, D. S., & Blaner, W. S. (1992) *J. Biol. Chem. 267*, 1805–1810.

Vahlquist, A., Peterson, P. A., & Wibell, L. (1973) *Eur. J. Clin. Invest. 3*, 352–362.

Wolf, G. (1980) in *Nutrition and the adult-Micronutrients* (Alfin-Slater, R. B., & Kritchevsky, D., Eds.) pp 97–203, Plenum Press, New York and London.