Characteristics of Retinol Accumulation from Serum Retinol-Binding Protein by Cultured Sertoli Cells†

Janet L. Shingleton,‡ Michael K. Skinner,§ and David E. Ong*,‡

Departments of Biochemistry and Pharmacology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

Received June 1, 1989; Revised Manuscript Received August 4, 1989

ABSTRACT: The uptake of retinol was examined in cultured Sertoli cells when retinol was provided as a complex with the transport protein retinol-binding protein (RBP). Sertoli cells accumulated [^{3}H]retinol in a time- and temperature-dependent manner. At 32 °C, the rate of retinol accumulation was biphasic. Accumulation was linear for approximately 1 h, but then accumulation continued at a linear but decreased rate for 23 h. The change in rate of retinol accumulation occurred when the cells had accumulated approximately 0.53 pmol of retinol/μg of cellular DNA. This amount of retinol was approximately equal to the cellular content of cellular retinol-binding protein (CRBP). Extraction and HPLC analysis of the cell-associated radioactivity yielded retinol and retinyl esters, indicating that a significant proportion of the accumulated retinol was esterified. Excess unlabeled retinol–RBP competed with [^{3}H]retinol–RBP for [^{3}H]retinol delivery to the cells, indicating that RBP delivery of retinol was a saturable and competable process. However, free [^{3}H]retinol associated with Sertoli cells in a noncompetable manner. The transport constant for specific retinol accumulation from RBP was 3.0 μM, suggesting that any change in the normal circulating retinol–RBP level (approximately 2 μM) would directly affect the rate of retinol accumulation. Neither iodinated nor reductively methylated RBP was accumulated by or tightly bound to Sertoli cells. In addition, energy inhibitors and lysosomal poisons had no effect on [^{3}H]retinol accumulation, indicating that RBP delivery of retinol to Sertoli cells did not occur by endocytosis of the retinol–RBP complex. Competition studies indicated, however, that protein recognition is important in the retinol uptake process. RBP, CRBP, and CRBP(II) competed with [^{3}H]retinol–RBP for [^{3}H]retinol accumulation, but free retinol, retinol–bovine serum albumin, and retinol–β-lactoglobulin did not. Transthyretin, bound to [^{3}H]retinol–RBP in the physiological 1:1 ratio, decreased [^{3}H]retinol accumulation by the cells by 25–30% compared to [^{3}H]retinol accumulation from [^{3}H]retinol–RBP. These studies indicated that Sertoli cell uptake of retinol involved recognition of the retinol–RBP complex at the cell surface with subsequent internalization of retinol, but not RBP. The fate of the internalized retinol may first have involved binding by CRBP, but eventually a significant portion of the accumulated retinol was esterified.

Vitamin A is essential for normal testicular structure and function. When animals are deprived of vitamin A, their germinal epithelium degenerates, and spermatogenesis ceases (Wolbach & Howe, 1925). These alterations are completely reversed when the vitamin A alcohol retinol is restored to the diet, but retinoic acid cannot prevent this degeneration (Howell et al., 1963). It does appear, however, that both of these retinoids play a role in vitamin A action in the testis. Retinol and retinoic acid, as well as their specific intracellular-binding proteins, cellular retinol-binding protein (CRBP) and cellular retinoic acid binding protein (CRABP), are present in high levels in the testis (Ito et al., 1974; Ong et al., 1982). Both retinol and retinoic acid have been shown to directly influence Sertoli cell function and differentiation (Skinner & Griswold, 1982). In addition, a nuclear retinoic acid receptor, which is in the superfamily of steroid hormone receptor proteins, has recently been described and is expressed in testis (Giguere et al., 1987).

Vitamin A circulates in the plasma as retinol bound to a specific carrier protein, retinol-binding protein (RBP), which, in turn, is bound by transthyretin (TTR). This transport complex serve to solubilize and stabilize the hydrophobic retinol molecule. Cells which require vitamin A are thought to have plasma membrane receptors which recognize RBP. Potential receptors have been demonstrated for the testis by binding studies using membrane fractions from whole testis (Bhat & Cama, 1979), and in vivo by localization of iodinated RBP on interstitial cells (McGuire et al., 1981). In addition, Sertoli cells in culture will internalize retinol from the retinol–RBP–TTR complex (Bishop & Griswold, 1987), indicating that these cells may also have RBP receptors. Since tight junctions between adjacent Sertoli cells form a blood–testis barrier, vitamin A destined for the late spermatocytes and spermatids of the adluminal compartment must pass through the Sertoli cell. Sertoli cells have been shown to contain considerable CRBP, which may participate in this process (Huggenvik & Griswold, 1981; Porter et al., 1985; Kato et al., 1985). We have therefore utilized Sertoli cells isolated from 20-day-old rats as a model cell type for further characterization of receptor-mediated retinol accumulation from the retinol–RBP complex.

Materials and Methods

Cell Preparation and Culture. Sertoli cells were isolated from the testis of 20-day-old rats by sequential digestion with trypsin, collagenase, and hyaluronidase (Dorrington & Fritz, 1975; Tung et al., 1984). Sertoli cells were then plated in

† This work was supported by National Institutes of Health Grants DK32642 and CA20850 (to D.E.O.) and HD20583 (to M.K.S.). M.K.S. is a PEW Scholar.

* Address correspondence to this author.
‡ Department of Biochemistry.
§ Department of Pharmacology.

0006-2960/89/0428-9641$01.50/0

24-well (1 mL per well) Linbro plates at approximately 5 × 10^5 cells per well or 150-mm dishes at a similar density. Cells were maintained at 32 °C in a 5% CO_2 atmosphere in Ham's F12 medium (Gibco). Sertoli cells isolated and maintained in this manner were shown by histochemical examination to have less than 2% contamination by other cell types (Anthony & Skinner, 1989). Sertoli cultures were treated at the time of plating and retreated after 48 h of culture with 0.1 mM dibutyryl-cAMP when the medium was replenished. Cells were harvested on day 5 or 6 of serum-free culture.

Preparation of Proteins. RBP and TTR were purified from outdated human plasma. DEAE-cellulose chromatography (Kopelman et al., 1976) and gel filtration in 1 mM Tris–acetate, pH 8.3, buffer (Said et al., 1981) separated RBP from TTR. RBP was then purified by TTR affinity chromatography (Vahlquist et al., 1971). TTR, recovered from the gel filtration step, was further purified by DEAE-cellulose chromatography using a 0.05 M Tris–acetate (pH 8.3)–0.33 M Tris–acetate/0.2 M NaCl gradient. TTR was identified by use of commercial radial immunodiffusion plates (Behring Diagnostics). CRBP was purified from rat liver (Ong & Chytil, 1978) and CRBP(II) from adult rat small intestine (Schaefer et al., 1989).

[^{3}H]Retinol was prepared by reduction of *all-trans*-retinaldehyde (Sigma) with NaB^3H_4 (20 Ci/mmol; Research Products International, Madison, WI) as described by Liau et al. (1981), and was purified by HPLC (Ong et al., 1987). [^{3}H]Retinol–RBP was prepared essentially as described by Futterman and Heller (1972). Pure retinol–RBP (3 mg) was lyophilized in a 30-mL Corex centrifuge tube and then stirred with 10 mL of ethanol for 5 min at room temperature. The protein was pelleted by centrifugation at 18000*g* for 15 min and 8 mL of the ethanol removed. The extraction was repeated until no retinol fluorescence was observed in the ethanol fraction. The pelleted protein was then dried under a stream of N_2 and was redissolved in 0.14 M sodium chloride/0.01 M sodium phosphate, pH 7.4 (PBS). [^{3}H]Retinol (1.1 mol/mol of RBP) in dimethyl sulfoxide (Me_2SO) was added immediately, with vortexing, and after 15 min at room temperature, the solution was applied to an affinity column of TTR immobilized on Sepharose 4B. [^{3}H]Retinol–RBP eluting from the column with distilled water was used in the uptake studies. The specific activity of these preparations was 3.5–5 Ci/mmol.

RBP was reductively methylated as described by Ong et al. (1982). The reaction mixture was submitted to gel filtration on a PD-10 column, and the reductively methylated protein obtained was then applied to an affinity column of TTR immobilized on Sepharose 4B. The specific activity of the modified RBP that eluted from the TTR column with distilled water was 25 Ci/mmol and had an A_{330}/A_{280} ratio of 0.97. RBP was labeled with ^{125}I by the lactoperoxidase method of Roth (1975) and by the chloramine T method of Greenwood et al. (1963). Both preparations were submitted to gel filtration on a PD-10 column and TTR affinity chromatography. Two peaks were obtained, one which retained affinity for TTR and one which did not. Both species were examined in binding studies.

CRBP Radioimmunoassay. Culture medium was removed from Sertoli cells in 24-well plates on the fifth day of culture, and 0.5 mL of 20 mM NaCl, 5 mM EDTA, 0.02% sodium azide, and 10 mM Tris-HCl (pH 7.8) was added to each well. The cells, still in the culture vessels, were sonicated for 10 s with a Branson sonifier fitted with a microtip at a power output of 7. Sonicated samples were then aliquoted for radioimmunoassay and DNA determination. CRBP radioimmunoassay was performed as described by Ong et al. (1982) using Pansorbin (Calbiochem) in place of the second antibody (Ong & Amédée-Manesme, 1987).

Retinol Accumulation Assay. Sertoli cells cultured for 5 or 6 days were released from 150-mm culture plates with dispase following the method of Pfeffer et al. (1986). Briefly, culture medium was removed by aspiration, and 10 mL of F-12 medium containing 2.4% dispase (Boehringer-Mannheim; Indianapolis, IN) and 100 mM sorbitol (Sigma) was added to the cells. After incubation at 32 °C for 1 h, cells were detached by gently pipeting the medium back onto the plates. The suspended cells were then pipeted into F-12 medium containing 10 μg/mL DNase I, 0.2 μg/mL aprotinin, and 0.1% BSA (all from Sigma). After centrifugation at 100*g* for 5 min, cells were resuspended in F-12 medium containing 0.1% BSA to give a final cell concentration of 100–200 μg of DNA/mL. DNA determinations were made by using the ethidium bromide fluorescence assay of Karstan and Wollenberger (1972) as modified by Skinner et al. (1988).

Cell suspensions were incubated with 1 μM [^{3}H]retinol–RBP at 32 °C with gentle shaking in F-12 medium containing 0.1% BSA in a final volume of 250 μL. Incubations were carried out in triplicate with one sample containing a 25-fold excess of unlabeled retinol–RBP. For competition experiments, retinol was complexed with BSA and β-lactoglobulin (Sigma) by adding a molar equivalent of *all-trans*-retinol (Sigma) in Me_2SO to the proteins in PBS. The retinol–protein complex was then submitted to gel filtration on a PD-10 column, and the concentration of the retinol–protein complex was determined by using an extinction coefficient of 50000 at 330 nm for bound retinol. Retinol accumulation studies performed in the presence of unlabeled retinol contained 0.1% ovalbumin (Sigma). When the effects of energy inhibitors and lysosomal poisons were investigated, the appropriate concentration of the inhibitor was preincubated with the cells in F-12 medium/0.1% BSA for 30 min at 32 °C prior to initiation of the assay. All experiments were terminated by the addition of 1 mL of ice-cold PBS containing 0.1% BSA to the incubation mixture; this was then quickly filtered through Millipore hydrophilic Durapore filters (GVWP) under vacuum. The filters were presoaked in F-12 medium containing 1% BSA. Each filter was washed twice with 5 mL of ice-cold PBS/0.1% BSA and was then allowed to soak overnight in 1 mL 0.5% SDS in scintillation vials. Ten milliliters of scintillation cocktail was added the next day; filters were removed, and the radioactivity in the vials was determined by scintillation counting. Efficiency was 20%, and quench was constant for all samples.

Retinol Esterification Assay. Cells suspended by dispase treatment (50–100 μg of DNA) were incubated with 1 μM [^{3}H]retinol–RBP in F-12 medium containing 0.1% BSA in a final volume of 2.5 mL. The incubations were carried out in duplicate at 32 or 4 °C for 3 h with gentle shaking. Incubation mixtures were then transferred to conical centrifugation tubes, and cells were pelleted by centrifugation at 100*g*. Cell pellets were resuspended in PBS containing 0.1% BSA and repelleted twice. The buffer was removed from the cells by aspiration, and 2.5 mL of 80% ethanol containing 80 μg/mL butylated hydroxytoluene (BHT) was added to the cell pellet. The pellet was sonicated for 10 s with a Branson sonifier at a power output of 7. Eight milliliters of hexane and 2 mL of distilled water were added, and the solution was vortexed for 30 s. The upper phase was removed, dried under a stream of N_2, and redissolved in 100 μL of hexane/dioxane (95:5). Ninety microliters was injected onto a 5-μm Whatman Partisil 5 (4.6 mm × 25 cm) silica column coupled to a 5-μm Supelcosil (2

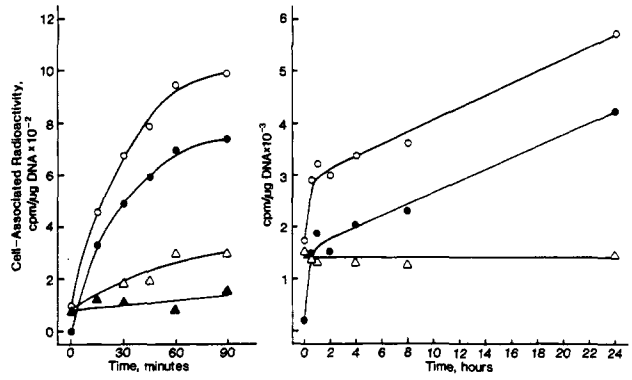

FIGURE 1: Effect of time and temperature on [^{3}H]retinol accumulation in cultured Sertoli cells. Cells (5 μg of DNA in left panel, 9 μg of DNA in right panel) were incubated with 1 μM [^{3}H]retinol–RBP (3.7 and 4.7 Ci/mmol in left and right panels, respectively) in 250 μL of F-12 medium containing 0.1% BSA in the absence (○) or presence (△) of a 25-fold excess of unlabeled retinol–RBP for various times at 32 °C or at 4 °C in the absence of unlabeled retinol–RBP (▲). Specific accumulation is also shown (●). The data shown in each panel are representative of four separate experiments.

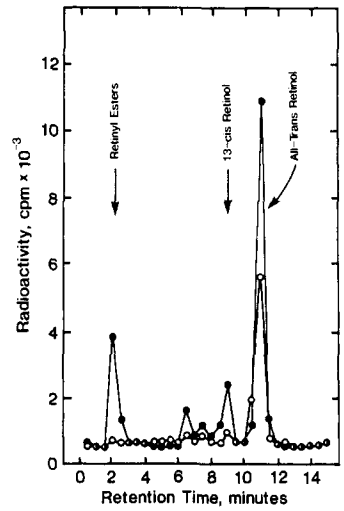

FIGURE 2: HPLC analysis of radioactivity accumulated by Sertoli cells. Cells (60 μg of DNA) were incubated for 3 h with 1 μM [^{3}H]retinol–RBP (5.0 Ci/mmol) at 32 °C (●) or 4 °C (○). Incubation volume was 2.5 mL. Cell extracts were subjected to HPLC, and fractions collected from the column were analyzed for radioactivity. The data shown are representative of four separate experiments.

cm) silica guard column. The mobile phase was hexane/dioxane (95:5); otherwise, conditions were the same as described by MacDonald and Ong (1987). One-milliliter fractions were collected from the column. Radioactivity in the fractions was determined by scintillation counting after solvent evaporation.

Results

Demonstration of Uptake of Retinol from RBP by Sertoli Cells. Sertoli cells from 20-day-old weanling rats were cultured for 5 days in retinol-free medium and then enzymatically detached for incubation in suspension with 1 μM [^{3}H]-retinol–RBP. The cells were able to accumulate [^{3}H]retinol over a 24-h period, with a transition in rate of accumulation occurring at approximately 1 h (as shown in Figure 1). The presence of excess unlabeled retinol–RBP reduced [^{3}H]retinol uptake considerably. Specific cell-associated radioactivity was calculated as the difference between accumulation in the presence and absence of excess unlabeled retinol–RBP. The amount of retinol specifically accumulated by Sertoli cells at 1 h (the time when the rate of uptake changed) was 0.53 ± 0.05 pmol of retinol/μg of DNA (mean ± SEM for five experiments), which was essentially identical with the CRBP content of these cells (0.54 ± 0.06 pmol/μg of DNA by radioimmunoassay). Whether CRBP was initially present in the cells as the holo- or apoprotein was not determined, but the cells had been cultured for 5 days in the absence of retinol prior to use. Incubation at 4 °C diminished [^{3}H]retinol accumulation to values somewhat lower than observed in the presence of excess unlabeled retinol–RBP (Figure 1, left panel). Cell-associated radioactivity observed at 4 °C was not reduced by the presence of excess unlabeled retinol–RBP (data not shown), indicating that the accumulation of radioactivity by Sertoli cells at 4 °C was not specific.

Retinol accumulation by cells attached to culture plates was the same as that observed with cells released by enzymic treatment (results not shown), suggesting that enzymic treatment did not perturb cell-surface components involved in uptake. However, it also indicated that cell-surface receptors were equally accessible to retinol–RBP whether the cells were attached or free in suspension. Microscopic examination of dispase-treated cells revealed single cells and cell clumps which were greater than 98% viable, as judged by their ability to exclude trypan blue. Retinol accumulation was linear with cell number up to 15 μg of DNA per assay (results not shown).

Total radioactivity associated with Sertoli cells after incubation with 1 μM [^{3}H]retinol–RBP for 30 min was less than 1% of the radioactivity added to the incubation. In contrast, when cells were incubated with 1 μM [^{3}H]retinol not bound to RBP, 30% of the added radioactivity was recovered with the cells, and inclusion of a 25-fold excess of unlabeled retinol had no effect on that association. Thus, the association of retinol with Sertoli cells in the absence of RBP was a nonspecific and noncompetable process.

The radioactivity associated with Sertoli cells after incubation with [^{3}H]retinol–RBP was characterized by HPLC analysis of cell extracts. The majority of the cell-associated radioactivity was found to elute at positions previously determined for *all-trans*-retinol and retinyl esters, with 20% and 33% of the total radioactivity eluting as retinyl ester after a 3-h and a 6-h incubation, respectively (Figure 2). Minor amounts of cis isomers of retinol were also observed, which were probably generated during the extraction procedure. It should be noted that possible metabolism of retinol to retinoic acid would not be detected in this system, since the [^{3}H]retinol used was labeled in the C-15 position. Cells incubated with [^{3}H]retinol–RBP at 4 °C did not synthesize [^{3}H]retinyl esters (Figure 2).

Effect of RBP Concentration on Retinol Delivery. When Sertoli cells were incubated with 1 μM [^{3}H]retinol–RBP in the presence of increasing concentrations of unlabeled retinol–RBP, accumulation was progressively decreased, with a 25-fold or greater excess reducing cell-associated radioactivity to 20% of that observed in the absence of competition (Figure 3, left panel). These data were transformed to total cell-associated retinol, producing the biphasic curve in Figure 3, right panel. This curve, like the competition curve that generated it, has two components: one which is competable and saturable and one which is noncompetable and nonsaturable. The nonsaturable component was determined by the graphical method of Neame and Richards (1972) and was subtracted from total cell-associated retinol to generate the curve shown for specific and saturable retinol accumulation by Sertoli cells. Eadie–Hofstee analysis of the specific retinol accumulation data (inset, Figure 3, right panel) allowed calculation of the K_t for retinol transport into Sertoli cells. The value obtained (average for three experiments) was 3.0 ± 1.1 μM (mean ±

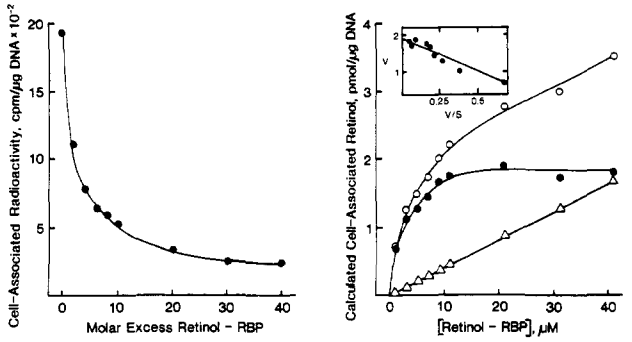

FIGURE 3: Concentration dependence of retinol accumulation in cultured Sertoli cells. (Left panel) Competition for [^{3}H]retinol accumulation (from [^{3}H]retinol–RBP) by increasing concentrations of unlabeled retinol–RBP. Cells (7.5 μg of DNA) were incubated with 1 μM [^{3}H]retinol–RBP (4.7 Ci/mmol) in the absence or presence of increasing concentrations of unlabeled retinol–RBP for 1.5 h at 32 °C. (Right panel) Determination of the concentration dependence of specific retinol accumulation by transformation of the data displayed in the left panel. (○) Calculated cell-associated retinol (determined by correcting for the dilution of the specific activity in the left panel); (△) nonspecific component of total cell-associated retinol curve (determined by the graphical method of Neame and Richards); and (●) calculated specific retinol accumulation (obtained by subtraction of the nonspecific component from the total accumulated retinol curve). The *inset* represents a linear transformation of the saturation curve for specific retinol accumulation. The data shown are representative of three separate experiments.

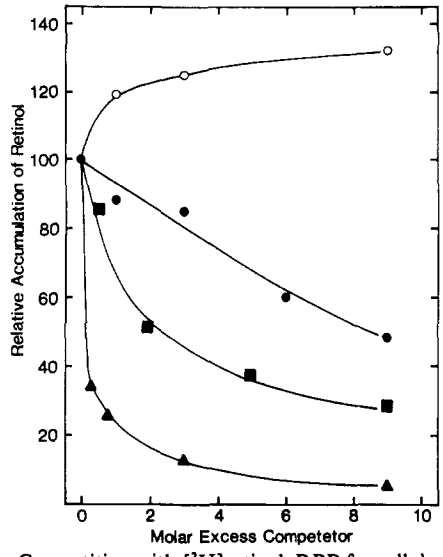

FIGURE 4: Competition with [^{3}H]retinol–RBP for cellular [^{3}H]retinol accumulation by free retinol and several retinol–protein complexes. Cells (7.5 μg of DNA) were incubated with 1 μM [^{3}H]retinol–RBP (4.6 Ci/mmol) in the absence or presence of increasing amounts of unlabeled retinol–RBP (●), retinol–CRBP(II) (■), retinol–CRBP (▲), or free retinol (○). Incubations were for 30 min at 32 °C in F-12 medium containing either 0.1% ovalbumin (for free retinol points) or 0.1% BSA (for all other points). The data shown are representative of three separate experiments.

SD). This was close to the normal circulating level of retinol–RBP in the blood of approximately 2 μM.

Competition for RBP Delivery of Retinol by Other Retinol–Protein Complexes. Competition studies were also conducted by incubating cells with 1 μM [^{3}H]retinol–RBP and increasing concentrations of free retinol, or retinol bound to several other proteins (Figure 4). As previously mentioned, [^{3}H]retinol accumulation was diminished in the presence of increasing concentrations of retinol–RBP. However, free retinol showed no ability to compete. Interestingly, retinol–CRBP and retinol–CRBP(II), two intracellular retinol-binding proteins, also reduced [^{3}H]retinol accumulation and were

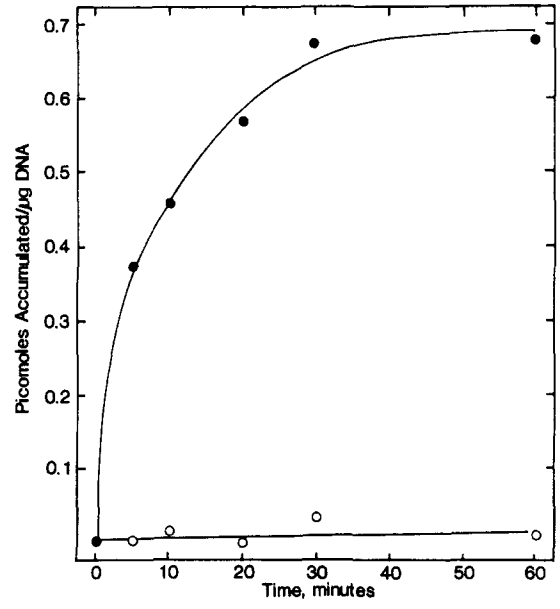

FIGURE 5: Determination of RBP and retinol accumulation by Sertoli cells. Cells (7 μg of DNA) were incubated with 1 μM [^{3}H]-retinol–RBP (4.7 Ci/mmol) (●) or 1 μM retinol–[^{3}H]RBP (25 Ci/mmol) (○) for various times at 32 °C. Specific accumulation of retinol or RBP (picomoles per microgram of DNA) was determined by subtracting the radioactivity observed in the presence of a 25-fold excess of unlabeled retinol–RBP. The data shown are representative of three separate experiments.

Table I: Effect of Energy Inhibitors and Lysosomal Poisons on Specific Accumulation of Retinol by Sertoli Cells[a]

addition	[^{3}H]retinol accumulated (pmol/μg of DNA)
none	0.240 ± 0.01
sodium azide (5 mM), 2-deoxyglucose (50 mM)	0.280 ± 0.01
chloroquine (0.1 mM)	0.290 ± 0.03
ammonium chloride (10 mM)	0.320 ± 0.04

[a] Cells (9 μg of DNA) were preincubated with the indicated compounds for 30 min in F-12 medium containing 0.1% BSA at 32 °C. [^{3}H]Retinol–RBP (1 μM, 5.0 Ci/mmol) ± retinol–RBP (25 μM) were added to appropriate tubes, and the incubation was continued for 30 min more at 32 °C. The data shown are the average of two determinations and are representative of two experiments.

considerably more effective than retinol–RBP. In contrast, no competition was observed for retinol bound to either BSA or bovine β-lactoglobulin (data not shown).

Lack of RBP Accumulation by Sertoli Cells. The fate of RBP, radioactively labeled by either iodination or reductive methylation, was followed to determine if RBP internalized during the process of retinol delivery. When Sertoli cells were incubated with iodinated holo-RBP at 4 or 32 °C, neither specific binding nor uptake of the labeled protein was observed (data not shown). Further studies used RBP labeled by reductive methylation of lysine residues. Reductively methylated RBP still bound retinol (A_{330}/A_{280} = 0.97) and was recognized by TTR as indicated by retention on a TTR affinity column (as described under Materials and Methods). In addition, methylated RBP was identical with unmodified RBP in its ability to compete with [^{3}H]retinol–RBP for [^{3}H]retinol accumulation (data not shown). Therefore, this modification did not adversely affect three important interactions of the protein. No accumulation of reductively methylated RBP by Sertoli cells was observed at any point of a 60-min incubation (Figure 5). Over the same time period, the Sertoli cells would accumulate a considerable amount of [^{3}H]retinol from un-

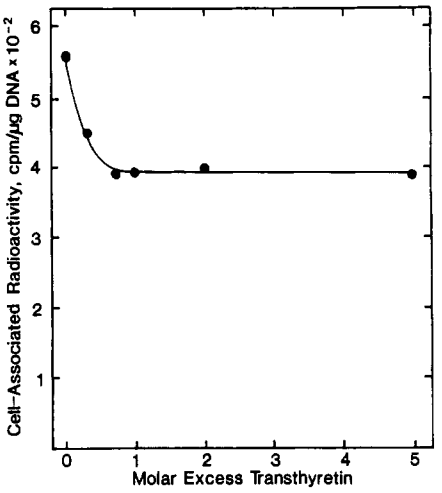

FIGURE 6: Effect of transthyretin on Sertoli cell accumulation of [^{3}H]retinol from [^{3}H]retinol–RBP. Cells (8.5 μg of DNA) were incubated with 1 μM [^{3}H]retinol–RBP (4.0 Ci/mmol) for 1 h at 32 °C in the absence or presence of increasing concentrations of transthyretin. The data shown are representative of two separate experiments.

modified RBP. The lack of RBP accumulation could be due to a rapid uptake and recycling of the protein. However, retinol accumulation was not affected by the energy inhibitors sodium azide and 2-deoxyglucose (Table I). Thus, retinol uptake did not involve endocytosis, a process which is energy dependent. In addition, the lysosomal poisons chloroquine and ammonium chloride did not inhibit retinol accumulation, suggesting that receptor recycling was not involved in the uptake process.

Effect of TTR on RBP Delivery of Retinol to Sertoli Cells. In plasma, retinol–RBP is bound to TTR in a 1:1 molar ratio. The effect of TTR on RBP delivery of retinol to Sertoli cells was determined by monitoring [^{3}H]retinol accumulation from RBP in the presence of increasing concentrations of TTR. [^{3}H]Retinol accumulation was somewhat decreased until a 1:1 molar ratio of RBP to TTR was reached (Figure 6), but higher concentrations of TTR had no additional effect. This suggested that uptake from the [^{3}H]retinol–RBP–TTR complex was slower than from retinol–RBP. Examination of [^{3}H]-retinol accumulation over time from preformed [^{3}H]retinol–RBP–TTR complex confirmed that the rate of accumulation was 25–30% slower compared to accumulation from [^{3}H]-retinol–RBP (results not shown).

Discussion

Vitamin A is a nutrient essential for normal growth and differentiation of germ and Sertoli cells in the testis (Wolbach & Howe, 1925; Skinner & Griswold, 1982). However, because of the blood–testis barrier that is formed by Sertoli cells at the periphery of seminiferous tubules, vitamin A destined for late spermatocytes and spermatids must first pass through the Sertoli cell. Sertoli cells have access to the retinol–RBP–TTR complex that circulates in plasma, and it seemed likely that these cells would have the ability to obtain retinol from that complex. Indeed, uptake and esterification of retinol from the retinol–RBP–TTR complex by cultured Sertoli cells were recently reported by Bishop and Griswold (1987). The results described here extend those observations and strongly suggest that specific receptors for RBP are present on Sertoli cells. RBP delivered retinol to Sertoli cells in primary culture in a time- and temperature-dependent manner; this uptake was competable and saturable in contrast to what was observed when free retinol was incubated with Sertoli cells. Although RBP was not internalized in the delivery process, and RBP binding at the cell surface could not be directly demonstrated, competition studies with free retinol or retinol bound to other proteins indicated that protein recognition was an essential step for specific uptake of retinol. Mouse keratinocytes in primary culture have recently been found to accumulate retinol by a similar process (Creek et al., 1989).

Retinol accumulation in Sertoli cells was saturable at increasing concentrations of retinol–RBP; a transport constant of 3.0 μM was obtained. Since the normal plasma level of RBP-bound retinol is about 2.2 μM (Goodman, 1984), these results suggest that any decrease in the normal level of circulating retinol–RBP would lead to a decrease in the rate of retinol accumulation by Sertoli cells. To our knowledge, no studies have examined the effect of chronically depressed retinol–RBP levels on sperm production, but relatively small decreases in the circulating retinol–RBP level do appear to have deleterious effects on other vitamin A responsive tissues. Vahlquist et al. (1978a,b) reported that plasma RBP levels below 1.1 μM in patients with intestinal and liver disease were correlated with a proportional decrease in dark adaptation ability. Russell et al. (1978) observed that poor dark vision was associated with low plasma RBP levels (mean ± SEM for 14 patients was 1.5 ± 0.2 μM) and that vitamin A therapy which increased RBP levels led to improvement. Severe acne also has been associated with low plasma RBP levels (Michaëlsson et al., 1977; Vahlquist et al., 1978a,b). The level of retinol–RBP circulating in serum is homeostatically maintained when vitamin A is sufficient. Results here suggest why relatively modest decreases in circulating retinol–RBP levels caused by inadequate vitamin A intake or processing can have deleterious effects for vitamin A requiring organs.

Although initial rates of accumulation varied between different cell preparations, we consistently observed a change in rate of accumulation of retinol by Sertoli cells when accumulation approached the level of CRBP in the cells. It is possible that retinol uptake by the cells required a transfer of retinol to CRBP. Retinol uptake after saturation of available CRBP might then be diminished to the rate of generation of apo-CRBP, perhaps by esterification of the CRBP–retinol. Additional work will be required to test this suggestion.

A significant portion of accumulated retinol was esterified (20 and 33% after a 3- and 6-h incubation, respectively), but these values were lower than those reported by Bishop and Griswold (1987). Their experiments were terminated by centrifuging the cells at 8000*g*, which might have led to some cell disruption and subsequent loss of retinol bound to CRBP. Esters are not soluble and would remain with the disrupted cell, leading to an overestimate of ester percentage.

Bishop and Griswold observed retinol uptake in Sertoli cells from the retinol–RBP–TTR complex. This suggests that TTR does not prevent RBP delivery of retinol to target cells even though TTR has been suggested to bind to RBP at the entrance to the β-barrel that contains retinol (Sandblom et al., 1986). Our studies also indicate that transfer of retinol to Sertoli cells from the retinol–RBP–TTR complex can occur, but at a reduced rate compared to transfer from RBP alone.

RBP delivery of retinol to pigment epithelial cells (Heller, 1975; Bok & Heller, 1976) and intestinal mucosal cells (Rask & Peterson, 1976) occurs without uptake of RBP, in contrast to the accumulation of RBP that has been observed in parenchymal and stellate cells from the liver (Gjøen et al., 1987). Here, neither iodinated RBP nor a well-characterized preparation of reductively methylated RBP was taken up by Sertoli cells. In addition, metabolic inhibitors and lysosomal poisons

did not interfere with retinol accumulation. Therefore, RBP delivery of retinol to Sertoli cells, like pigment epithelial cells and intestinal mucosal cells, does not appear to involve uptake or recycling of RBP.

However, recognition of the protein moiety does appear to be involved in Sertoli cell uptake of retinol. Retinol bound to RBP, CRBP, and CRBP(II) competed with [^{3}H]retinol–RBP for [^{3}H]retinol uptake, but free retinol, and retinol bound to BSA or β-lactoglobulin, did not. CRBP and CRBP(II) are intracellular proteins, and the ability of these proteins to compete with RBP was unexpected. While these intracellular proteins have significant sequence homology to each other (56%), they do not have significant homology to RBP (Li et al., 1986; Demmer et al., 1987). Demmer et al. (1987) have noted a limited sequence homology between CRBP(II) and RBP in the retinol-binding region. It may be that these proteins, although very different in sequence, have tertiary structure similarities that are recognized by the membrane receptor. The observed competition by retinol–CRBP might also suggest a role for CRBP in the extracellular transport of retinol. Alternatively, the plasma membrane receptor, which recognizes RBP extracellularly, might have a similar or identical intracellular binding site that recognizes CRBP. Recently, Ottonello et al. (1987) have observed that retinol–CRBP diminished RBP delivery of retinol to bovine pigment epithelium plasma membrane preparations. Bovine β-lactoglobulin, on the other hand, does have limited sequence homology and a similar tertiary structure to RBP (Pervaiz & Brew, 1985; Papiz et al., 1986). This protein binds retinol and has been proposed to deliver retinol to receptors on mucosal cells in the small intestine in the process of vitamin A absorption from mother's milk. Uptake from retinol–β-lactoglobulin by rat small intestine is competed for by RBP (Said et al., 1989), but retinol–β-lactoglobulin did not compete with [^{3}H]-retinol–RBP here. Retinol bound to BSA, a protein which binds many lipophilic molecules, also did not reduce uptake. The lack of competition by these two retinol–protein complexes, in contrast to the competition observed when retinol was bound to RBP, CRBP, and CRBP(II), suggests that specific retinol accumulation by these cells is dependent on recognition of certain ligand–protein structural features. While the retinol molecule may be a part of this structure, it by itself does not appear to be sufficient for specific retinol accumulation, since free retinol could not compete for uptake from the retinol–RBP complex.

These studies indicate that Sertoli cell uptake of retinol involves recognition of the retinol–RBP complex at the cell surface with subsequent internalization of retinol, but not RBP. The internalized retinol may first be bound by CRBP, but eventually a significant portion of the accumulated retinol is esterified. In the intact testis, germ cells would eventually acquire the vitamin A accumulated by the Sertoli cell by mechanisms still to be discovered.

Acknowledgments

We thank Byron Glenn for technical assistance and acknowledge the assistance of the Vanderbilt University Reproductive Biology Research Center Tissue Culture Core Laboratory.

Registry No. Retinol, 68-26-8.

References

Anthony, C. T., & Skinner, M. K. (1989) *Biol. Reprod. 40*, 811.

Bhat, M. K., & Cama, H. R. (1979) *Biochim. Biophys. Acta 587*, 273.

Bishop, P. D., & Griswold, M. D. (1987) *Biochemistry 26*, 7511.

Bok, D., & Heller, J. (1976) *Exp. Eye Res. 22*, 395.

Creek, K. E., Silverman-Jones, C. S., & DeLuca, L. M. (1989) *J. Invest. Dermatol. 92*, 283.

Demmer, L. A., Birkenmeier, E. H., Sweetser, D. A., Levin, M. S., Zollman, S., Sparkes, R. S., Mohandas, T., Lusis, A. F., & Gordon, J. I. (1987) *J. Biol. Chem. 262*, 2458.

Dorrington, J. H., & Fritz, I. B. (1975) *Endocrinology 96*, 879.

Futterman, S., & Heller, J. (1972) *J. Biol. Chem. 247*, 5168.

Giguere, V., Ong, E. S., Segui, P., & Evans, R. M. (1987) *Nature 330*, 624.

Goodman, D. S. (1984) in *The Retinoids* (Sporn, M. B., Roberts, A. B., & Goodman, D. S., Eds.) Vol. 2, p 42, Academic Press, Orlando, FL.

Gjøen, T., Bjerkelund, T., Blomhoff, H. K., Berg, T., & Blomhoff, R. (1987) *J. Biol. Chem. 262*, 10926.

Greenwood, F. C., Hunter, W. M., & Glover, J. S. (1963) *Biochem. J. 89*, 114.

Heller, J. (1975) *J. Biol. Chem. 250*, 3613.

Howell, J. McC., Thompson, J. N., & Pitt, G. A. J. (1963) *J. Reprod. Fertil. 5*, 159.

Huggenvik, J., & Griswold, M. D. (1981) *J. Reprod. Fertil. 61*, 403.

Ito, Y. L., Zile, M., Ahrens, H., & DeLuca, H. F. (1974) *J. Lipid Res. 15*, 517.

Karsten, U., & Wollenberger, A. (1972) *Anal. Biochem. 46*, 135.

Kato, M., Sung, W. K., Kato, K., & Goodman, D. S. (1985) *Biol. Reprod. 32*, 173.

Kopelman, M., Mokady, S., & Cogan, U. (1976) *Biochim. Biophys. Acta 439*, 442.

Li, E., Demmer, L. A., Sweetser, D. A., Ong, D. E., & Gordon, J. I. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 5779.

Liau, G., Ong, D. E., & Chytil, F. (1981) *J. Cell Biol. 91*, 63.

MacDonald, P. N., & Ong, D. E. (1987) *J. Biol. Chem. 262*, 10550.

McGuire, B. W., Orgebin-Crist, M.-C., & Chytil, F. (1981) *Endocrinology 108*, 658.

Michaëlsson, G., Vahlquist, A., & Juhlin, L. (1977) *Br. J. Dermatol. 96*, 283.

Neame, K. D., & Richards, T. G. (1972) in *Elementary Kinetics of Membrane Carrier Transport*, p 53, Blackwell Scientific Publications, Oxford.

Ong, D. E., & Chytil, F. (1978) *J. Biol. Chem. 253*, 828.

Ong, D. E., & Amédée-Manesme, O. (1987) *Hepatology 7*, 253.

Ong, D. E., Crow, J. A., & Chytil, F. (1982) *J. Biol. Chem. 257*, 13385.

Ong, D. E., Kakkad, B., & MacDonald, P. N. (1987) *J. Biol. Chem. 262*, 2729.

Ottonello, S., Petruccc, S., & Maraini, G. (1987) *J. Biol. Chem. 262*, 3975.

Papiz, M. Z., Sawyer, L., Eliopoulos, E. E., North, A. C. T., Finddlay, J. B. C., Sivaprasadarao, R., Jones, T. A., Newcomer, M. E., & Kraulis, P. J. (1986) *Nature 324*, 383.

Pervaiz, S., & Brew, K. (1985) *Science 228*, 335.

Pfeffer, B. A., Clark, V. M., Flannery, J. G., & Bok, D. (1986) *Invest. Ophthalmol. Visual Sci. 27*, 1031.

Porter, S. B., Ong, D. E., Chytil, F., & Orgebin-Crist, M.-C. (1985) *J. Androl. 6*, 197.

Rask, L., & Peterson, P. A. (1976) *J. Biol. Chem. 251*, 6360.

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

Russell, R. M., Morrison, S. A., Smith, F. R., Smith, F. R., Oaks, E. V., & Carney, E. A. (1978) *Ann. Int. Med. 88*, 622.
Said, H. M., Ong, D. E., & Shingleton, J. L. (1989) *Am. J. Clin. Nutr. 49*, 690.
Sandblom, P., Åqvist, J., Jones, T. A., Newcomer, M. E., van Gunsteren, W. F., & Tapia, O. (1986) *Biochem. Biophys. Res. Commun. 139*, 564.
Schaefer, W. F., Kakkad, B., Crow, J. A., Blair, I. A., & Ong, D. E. (1989) *J. Biol. Chem. 264*, 4212.
Skinner, M. K., & Griswold, M. D. (1982) *Biol. Reprod. 27*, 211.
Skinner, M. K., Fetterhoff, P. M., & Anthony, C. T. (1988) *J. Biol. Chem. 263*, 2884.
Tung, P. S., Skinner, M. K., & Fritz, I. B. (1984) *Biol. Reprod. 30*, 199.
Vahlquist, A., Nilsson, S. F., & Peterson, P. A. (1971) *Eur. J. Biochem. 20*, 160.
Vahlquist, A., Michaëlsson, G., & Juhlin, L. (1978a) *Acta Derm-Venereol. 58*, 437.
Vahlquist, A., Sjölund, K., Nordén, A., Peterson, P. A., Stigmar, G., & Johansson, B. (1978b) *Scand. J. Clin. Lab. Invest. 38*, 301.
Wolbach, S. B., & Howe, P. R. (1925) *J. Exp. Med. 43*, 753.

Retinol Esterification in Sertoli Cells by Lecithin–Retinol Acyltransferase†

Janet L. Shingleton,‡ Michael K. Skinner,§ and David E. Ong*,‡
Departments of Biochemistry and Pharmacology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232
Received June 1, 1989; Revised Manuscript Received August 4, 1989

ABSTRACT: Esterification of retinol occurs during the metabolism of vitamin A in the testis. An acyl-CoA:retinol acyltransferase (ARAT) activity has been described for microsomes isolated from testis homogenates. That activity was also observed here in microsomal preparations obtained from cultured Sertoli cells from 20-day-old (midpubertal) rats. ARAT catalyzed the synthesis of retinyl laurate when free retinol and lauroyl-CoA were provided as substrates. However, in the absence of exogenous acyl-CoA, retinol was esterified by a different activity in a manner similar to the lecithin:retinol acyltransferase (LRAT) activity described recently for liver and intestine. Microsomal preparations obtained from enriched Sertoli cell fractions from the adult rat testis had 75-fold higher levels of LRAT than the preparations from midpubertal animals, but ARAT activity was the same in both these preparations. LRAT utilized an endogenous acyl donor and either unbound retinol or retinol complexed with cellular retinol-binding protein (CRBP) to catalyze the synthesis of retinyl linoleate, retinyl oleate, retinyl palmitate, and retinyl stearate. The addition of exogenous dilaurylphosphatidylcholine (DLPC) resulted in the synthesis of retinyl laurate. The esterification from both exogenous DLPC and endogenous acyl donor was inhibited by 2 mM phenylmethanesulfonyl fluoride (PMSF). ARAT activity was not affected by similar concentrations of PMSF. Furthermore, retinol bound to CRBP, a protein known to be present in Sertoli cells, was not an effective substrate for testicular ARAT. When retinol uptake and metabolism were examined in cultured Sertoli cells from 20-day-old rats, the cells synthesized the same retinyl esters that were produced by microsomal LRAT in vitro. Pretreating the cells with PMSF did not prevent specific retinol accumulation but did inhibit retinol esterification. Consequently, the LRAT-like retinyl esters produced by cultured Sertoli cells and the sensitivity of this esterification to PMSF suggest that LRAT, and not ARAT, is the physiologically important retinyl ester synthase in the Sertoli cell.

Sertoli cells, the somatic cells of the seminiferous tubules that provide structural and nutritional support for the developing germ cells, require vitamin A for the normal secretion of proteins that are postulated to be for germ cell development (Skinner & Griswold, 1982; Karl & Griswold, 1980). Retinoids have been speculated to directly affect Sertoli cell uridine nucleotide metabolism (Carson & Lennarz, 1983). Furthermore, Sertoli cells have been shown to contain high levels of cellular retinol-binding protein (CRBP) (Porter et al., 1985; Blaner et al., 1987). The presence of cellular retinoic acid binding protein in meiotic germinal cells suggests that these cells also have important vitamin A requirements (Porter et al., 1985; Blaner et al., 1987). However, since late spermatocytes and spermatids are sequestered behind the blood–testis barrier created by tight junctions between adjacent Sertoli cells, these developing germ cells are dependent on Sertoli cells for delivery of vitamin A as well as for all other nutritional requirements (Fawcett, 1975; Ritzén et al., 1981). Thus, the uptake and metabolism of vitamin A in Sertoli cells are important components of vitamin A action in the testis.

One step in Sertoli cell metabolism of vitamin A is the esterification of retinol with fatty acids. Ahluwalia and Gambhir (1976) studied [^{3}H]retinol esterification in rat seminiferous tubules both in vivo and in vitro and concluded that Sertoli cells were responsible for the esterification. In addition, Sertoli cells in culture esterify retinol that is delivered to the cells as retinol bound to plasma retinol-binding protein (RBP) or as retinol–RBP that is complexed with transthyretin (Shingleton et al., 1989; Bishop & Griswold, 1987).

An acyl-CoA-dependent retinol esterifying activity has been described in microsomal preparations from rat testis (Chaudhary & Nelson, 1987). This activity, designated acyl-CoA:retinol acyl transferase (ARAT) was characterized in vitro by using retinol dispersed from organic solution as the

† This work was supported by National Institutes of Health Grants DK32642 (to D.E.O.) and HD20583 (to M.K.S.). M.K.S. is a PEW Scholar.
* Address correspondence to this author.
‡ Department of Biochemistry.
§ Department of Pharmacology.

0006-2960/89/0428-9647$01.50/0