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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
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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

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

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substrate. However, the high level of CRBP in the testis, particularly in Sertoli cells (Blaner et al., 1987), suggests that retinol present in the testis will be bound by this protein. Retinol bound to CRBP has been shown to be a poor substrate for the ARAT of several tissues (Ong et al., 1987, 1988). A separate retinol esterifying activity, which can esterify retinol bound to CRBP, was identified in microsomal preparations from rat liver (Ong et al., 1988). This activity used an acyl donor that was present in the microsomal preparations and produced retinyl esters that were the same and in a similar ratio as those found in the liver. Similar CoA-independent retinyl ester synthases have been identified in rat small intestine (Ong et al., 1987; MacDonald & Ong, 1988a) and human liver (MacDonald & Ong, 1988b). The acyl donor in these esterifications appears to be phosphatidylcholine. The activity has been termed lecithin:retinol acyltransferase (LRAT) (MacDonald & Ong, 1988a).

In the present study, ARAT and LRAT esterifying activities were identified in microsomal preparations from Sertoli cell preparations from midpubertal and adult rats. The characteristics of retinol esterification in whole Sertoli cells suggest that, although both enzymes are present, LRAT is the activity responsible for retinol esterification in the rat Sertoli cell.

Experimental Procedures

Preparation of Proteins. CRBP was purified from rat liver (Ong & Chytil, 1978) and RBP from outdated human plasma (Shingleton et al., 1989). [^{3}H]Retinol was prepared by reducing *all-trans*-retinaldehyde with sodium boro[^{3}H]hydride (Liau et al., 1981). [^{3}H]Retinol–CRBP was prepared by adding [^{3}H]retinol to apo-CRBP as described (Ong et al., 1987), and [^{3}H]retinol–RBP was prepared by the method of Futterman and Heller (1972) as described (Shingleton et al., 1989).

Cell Preparation and Culture. Sertoli cells were isolated from the testis of 20-day-old rats by sequential digestion of decapsulated testis fragments with trypsin, collagenase, and hyaluronidase (Dorrington & Fritz, 1975; Tung et al., 1984). Sertoli cells were then plated in 150-mm dishes at a density of approximately 2×10^7 cells per plate. 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 culture. DNA determinations were made by using the ethidium bromide fluorescence assay of Karstan and Wollenberger (1972) as modified by Skinner et al. (1988).

Enriched cell types from adult rat testis were also prepared by the above method. Trypsin digestion of adult testis yielded tubule fragments that settled to the bottom of the incubation flask and an enriched preparation of Leydig cells that remained in the supernatant liquid. After the addition of soybean trypsin inhibitor, the supernatant liquid was collected and centrifuged at 100*g* for isolation of the Leydig cell fraction. The myoid cell fraction was obtained from the supernatant liquid after collagenase treatment of the tubules and centrifugation at 100*g* for 2 min for removal of Leydig and Sertoli cells. Myoid cells were then collected by centrifugation at 250*g* for 6 min. Sertoli and germ cell fractions were collected after the hyaluronidase digestion. Sertoli cells settled to the bottom of the incubation flask after the digestion and washes in Hank's medium. The supernatant liquid contained an enriched population of germ cells; these were collected by centrifugation at 250*g* for 5 min. Sertoli cells were pelleted by centrifugation at 100*g* for 5 min. Each enriched cell type was resuspended and washed in 0.14 M NaCl/0.01 M sodium phosphate, pH 7.4 (PBS), containing 1 mM dithiothreitol (DTT, Calbiochem).

Preparation of Microsomes. Cultured Sertoli cells were harvested on day 5 or 6. Culture medium was removed, and 10 mL of PBS containing 1 mM DTT was added to the plates. The cells were then scraped from the culture plates using a rubber policeman, and the cells were pelleted by centrifugation at 100*g*. Microsomes were prepared from cultured Sertoli cells, adult rat testis, and enriched cell fractions from adult rat testis after homogenization in 0.2 M KH_2PO_4, pH 7.2, by centrifugation of cell homogenates at 20000*g* for 15 min, followed by centrifugation of the resulting supernatant liquid at 113000*g* for 60 min (Ong et al., 1988). Protein concentrations were determined by a modification of the method of Lowry (Peterson, 1977).

Microsome Esterification Assay. All incubations were carried out under subdued yellow light. Sertoli cell microsomal preparations (250 μg of protein) from 20-day-old rats were incubated with 3 μM retinol and 40 μM lauroyl-CoA (for the ARAT assay) or 3 μM [^{3}H]retinol–CRBP (for the LRAT assay) in 0.2 M KH_2PO_4, pH 7.2. The incubation also contained 40 μM BSA and 1 mM DTT. The final volume was 0.5 mL. After a 30-min incubation at 37 °C in a shaking water bath, 2 mL of ice-cold 0.2 M KH_2PO_4/0.1% BSA was added, and the tubes were placed on ice. Microsomes were pelleted by centrifugation at 113000*g* for 60 min. Microsomal pellets were then immediately extracted, and the extracts were subjected to HPLC as described below.

When esterification assays were performed using microsomal preparations from adult rat testis or enriched cell fractions from adult rat testis, conditions were the same as described above except 100 μg of protein was incubated with 3 μM retinol–CRBP or unbound retinol. When designated, 40 μM lauroyl-CoA or 40 μM dilaurylphosphatidylcholine (DLPC, Sigma) was added. Phenylmethanesulfonyl fluoride (PMSF, Sigma), an inhibitor of LRAT activity, was added to the incubation when indicated. The inhibitor, dissolved in dimethyl sulfoxide (Me_2SO, Aldrich), was added to the microsomes 10 min prior to the addition of retinol, retinol–CRBP, and acyl donors.

Retinol Accumulation and Esterification in Cultured Sertoli Cells. Cells were released from culture plates and were used in the retinol accumulation assay essentially as described (Shingleton et al., 1989). Cells (10 μg of DNA) were incubated with 1 μM [^{3}H]retinol–RBP for various times at 32 °C. The incubation medium was PBS containing 0.1% BSA, and the final volume was 0.25 mL. PMSF, when present, was added to cells in Me_2SO 10 min prior to the addition of [^{3}H]retinol–RBP. Each time point was carried out in triplicate, with the third tube containing a 25-fold excess of unlabeled retinol–RBP for the determination of nonspecific cell-associated radioactivity. 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 of 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. Specific cell-associated radioactivity was determined by subtracting the nonspecific counts from the counts obtained in the absence of competitor.

[^{3}H]Retinyl esters formed by Sertoli cells were characterized as follows. Cell suspensions (100 μg of DNA) were incubated with 1 μM [^{3}H]retinol–RBP in PBS containing 0.1% BSA. PMSF, when present, was added to cells in Me_2SO 10 min prior to the addition of [^{3}H]retinol–RBP. The final volume was 2.5 mL. After incubation at 32 °C for 60 min, 5 mL of ice-cold PBS/0.1% BSA was added, and cell membranes were pelleted by centrifugation at 113000*g* for 60 min. These pellets were then extracted, and the extracts were subjected to HPLC.

Extraction of Lipids from Membrane Pellets and Analysis of Retinyl Esters by HPLC. Two milliliters of 100% ethanol containing 100 μg/mL butylated hydroxytoluene (BHT) was added to the cell membrane pellets or microsomal pellets, and the pellets were dispersed in the ethanol using a Pasteur pipet. Eight milliliters of hexane containing 100 μg/mL BHT and 2 mL of distilled water were then added. Tubes were vortexed for 30 s. The upper phase was removed and taken to dryness under nitrogen and then redissolved in 100 μL of methanol. The samples were subjected to HPLC analysis on a Waters Associates 10-μm reverse-phase μBondapak column. The mobile phase was 100% ethanol containing 24 mM $AgNO_3$ (J. T. Baker) which allows the resolution of retinyl oleate from retinyl palmitate (deRuyter & de Leenheer, 1979). If [^{3}H]retinol–RBP or [^{3}H]retinol–CRBP were used in the esterification assay, the HPLC column eluent was collected in 1-mL fractions in scintillation vials. After evaporation of the solvent, radioactivity in the vials was determined by scintillation counting.

Results

Demonstration of ARAT and LRAT Activity in Cultured Sertoli Cells. ARAT activity in cultured Sertoli cells was demonstrated by incubating microsomes with retinol and lauroyl-CoA. After incubation for 30 min at 37 °C, the microsomes were pelleted and retinoids extracted for HPLC analysis (Figure 1A). After elution of retinol and several peaks unrelated to the presence of retinol, a peak was observed that corresponded to the elution position previously determined for retinyl laurate. This peak was not observed in the absence of microsomes, lauroyl-CoA, or retinol (data not shown). The ARAT activity in these preparations, determined by quantitation of the retinyl laurate peak, was 4.4 pmol of retinyl ester (mg of protein)$^{-1}$ min^{-1} (Table I).

To detect LRAT activity, an increase in the sensitivity of detection of newly synthesized retinyl esters was necessary. Thus, microsomes were next incubated with [^{3}H]retinol–CRBP in the absence of added acyl-CoA. Radioactivity eluted from the HPLC column as shown in Figure 1B. Peaks were observed at positions previously established for retinyl oleate and retinyl palmitate. A small peak corresponding with the elution position for retinyl stearate was also consistently observed. The production of retinyl esters by the microsomal preparations using an endogenous acyl donor and retinol bound to CRBP indicated that LRAT activity was present. In addition, the pattern of esters produced was similar to the ester pattern produced by LRAT in other tissues (Ong et al., 1987, 1988; MacDonald & Ong, 1988a,b). Quantitation of the retinyl esters produced indicated that the LRAT activity [0.22 pmol of retinyl esters (mg of protein)$^{-1}$ min^{-1}] was very low compared to LRAT activity in rat intestine and liver [44 and 52 pmol (mg of protein)$^{-1}$ min^{-1}] (Ong et al., 1987; MacDonald & Ong, 1988). The specific activity of LRAT was also only 5% of the observed ARAT activity, as shown in Table I.

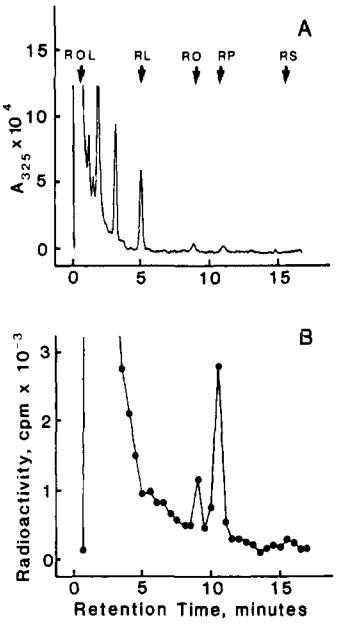

FIGURE 1: ARAT and LRAT activity in microsomal preparations from cultured Sertoli cells. Sertoli microsomes (250 μg of microsomal protein) were incubated with (A) 3 μM [^{3}H]retinol (5.0 Ci/mmol) and 40 μM lauroyl-CoA or (B) 3 μM [^{3}H]retinol–CRBP (5.0 Ci/mmol), in 0.2 M KH_2PO_4, pH 7.2, containing 40 μM BSA and 1 mM DTT. The reaction volume was 0.5 mL. Following a 30-min incubation at 37 °C, the microsomes were pelleted and extracted, and the extracts were subjected to HPLC. In (B), fractions collected from the HPLC column were analyzed for radioactivity. ROL, retinol; RL, retinyl laurate; RO, retinyl oleate; RP, retinyl palmitate; RS, retinyl stearate. The data shown are representative of three different experiments.

Table I: Comparison of LRAT and ARAT Activities in Microsomal Preparations from Midpubertal and Adult Rat Testis and Isolated Cell Types[a]

age	source of microsomes	act. (pmol of retinyl ester min^{-1} mg^{-1}) LRAT	ARAT
midpubertal (20 days old)	whole testis	0.3[b]	nd[c]
	Sertoli cells, freshly isolated	0.20[b]	nd
	Sertoli cells, cultured	0.22[b]	4.4[d]
adult (8 months old)	whole testis	19.5[e]	4.3[d]
	Sertoli cell fraction	15.4[e]	4.5[d]
	germ cell fraction	8.0[e]	nd
	interstitial cell fraction	8.9[e]	nd
	peritubular cell fraction	2.9[e]	nd

[a] Each value given is the average of at least two determinations and is representative of two experiments. SEM was less than 15%. [b] Microsomes (250 μg of microsomal protein) were incubated with 3 μM [^{3}H]retinol–CRBP (5.0 Ci/mmol) for 30 min at 37 °C. The final volume was 0.5 mL. After the incubation, the microsomes were pelleted and extracted, and the extracts were analyzed by HPLC. Fractions collected from the HPLC column were analyzed for radioactivity, and the radioactivities associated with the retinyl ester peaks were summed for determination of LRAT activity. [c] nd, not determined. [d] Microsomal protein (250 and 100 μg from midpubertal and adult rats, respectively) was incubated with 3 μM retinol, 40 μM lauroyl-CoA, and 2 mM PMSF. PMSF was added to inhibit esterification of retinol by LRAT. Incubation conditions and HPLC analysis were the same as in *b*. Quantitation of retinyl esters eluting as retinyl laurate was determined for estimation of ARAT activity. [e] Microsomes (100 μg of microsomal protein) were incubated with 3 μM retinol–CRBP. Incubation conditions and HPLC analysis were the same as in *b*. Peaks eluting as retinyl esters were summed for determination of LRAT activity.

LRAT activity in freshly isolated Sertoli cells was found to be comparable to the LRAT activity observed from cells cultured for 5 days (used for these studies), indicating the level

was not altered by cell culturing. However, LRAT activity in microsomal preparations from adult whole rat testis was much greater than that observed for either 20-day-old testis or isolated Sertoli cells of those testes (Table I).

Demonstration of LRAT Activity in Enriched Sertoli Cell Preparations from Adult Rat Testis. Since testicular LRAT activity was much higher in the adult compared to the mid-pubertal rat, we examined the cellular distribution of the activity in the adult testis. Cell types were separated by enzymatic digestion according to the method of Dorrington and Fritz (1975), as modified by Tung et al. (1984). Microsomes were prepared from the enriched cell types, and LRAT activity was assayed by incubating the microsomes with retinol–CRBP and monitoring retinyl ester formation by HPLC. The results are shown in Table I. All of the cell fractions contained LRAT, but the highest activity was found in the Sertoli cell fraction. LRAT activity in Sertoli cells from 20-day-old rats was 1.4% of the adult Sertoli cell LRAT activity. This was comparable to the difference in the LRAT activity in whole rat testis, where 20-day-old testis LRAT activity was 1.9% of the activity observed in adult testis. In contrast to the developmental increase observed for LRAT in the Sertoli cell fractions, ARAT activity remained constant (Table I).

Characterization of LRAT and ARAT in the Enriched Sertoli Cell Fraction from Adult Rat Testis. Because LRAT activity was considerably greater in Sertoli cells isolated from adult rat testis than in the cells isolated from 20-day-old rats, enriched Sertoli cell fractions from the adult were used for further characterization of LRAT and ARAT activity.

Microsomal preparations from adult Sertoli cells synthesized considerable retinyl ester from either retinol–CRBP or unbound retinol and an endogenous acyl donor (Figure 2A,B). Retinyl palmitate was the predominate ester synthesized; retinyl oleate and retinyl stearate were produced in lesser amounts. The ester pattern was the same whether microsomes were incubated with free or bound retinol, and was similar to that observed with microsomes from 20-day-old Sertoli cells. When dilaurylphosphatidylcholine (DLPC) was added, retinyl laurate was synthesized in addition to the retinyl esters previously observed (Figure 2C,D, upper tracings). Preincubating the microsomes with 2 mM PMSF, an inhibitor of LRAT but not ARAT (Ong et al., 1988), inhibited both the esterification using the exogenously added acyl donor DLPC and the esterification using the endogenous acyl donor (Figure 2C,D, lower tracings). The ability to use DLPC as an acyl donor and either free retinol or retinol–CRBP as substrate as well as the sensitivity of the esterification to PMSF established that the LRAT activity of Sertoli cells was similar to that previously described for rat small intestine and liver (Ong et al., 1988; MacDonald & Ong, 1988). The endogenous acyl donor was presumed to be phosphatidylcholine.

Compared to unbound retinol, retinol–CRBP was a poor substrate for the ARAT activity, with little retinyl laurate synthesized when lauroyl-CoA was provided (Figure 2E, upper tracing). Esters attributable to the LRAT reaction were the major esters observed and were much reduced by pretreatment of the microsomes with PMSF (Figure 2E, lower tracing). The inability to utilize retinol–CRBP as substrate has been previously observed with ARAT activities in other tissues (Ong et al., 1988). ARAT activity could be demonstrated by incubating microsomes with unbound retinol and lauroyl-CoA, as shown in Figure 2F, upper tracing. Now, considerable retinyl laurate was synthesized, as well as the retinyl esters synthesized by LRAT using the endogenous acyl donor. However, LRAT activity (determined by subtracting retinyl

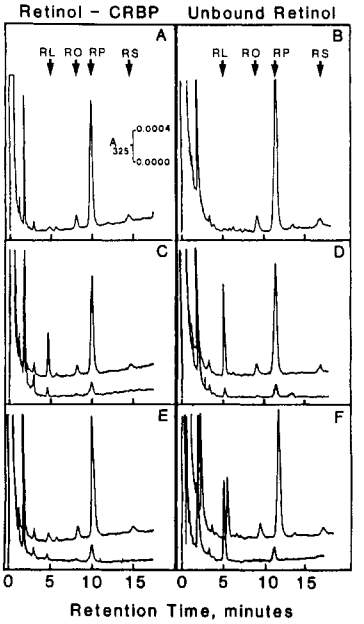

FIGURE 2: Characterization of LRAT and ARAT activity in the Sertoli cell fraction from adult rat testis. Sertoli microsomes (50 μg of microsomal protein) were preincubated in the absence (panels A and B and upper tracing in panels C–F) or presence (lower tracing in panels C–F) of 2.0 mM PMSF for 10 min at 37 °C. Following the addition of 40 μM DLPC (panels C and D) or 40 μM lauroyl-CoA (panels E and F), esterification reactions were initiated by the addition of either 3 μM retinol–CRBP (panels A, C, and E) or 3 μM retinol (panels B, D, and F). The final volume was 0.5 mL. Reactions were incubated for 30 min at 37 °C, extracted into hexane, and analyzed by HPLC. Ester labels are the same as in Figure 1. The upper tracing in panel F was offset to allow visualization of the retinyl laurate peak in the lower tracing. Data shown are representative of two experiments.

laurate from total ester) was responsible for 82% of the total esters synthesized. Pretreatment with PMSF greatly reduced the LRAT reaction (i.e., the esterification using the endogenous acyl donor) but had no effect on the ARAT reaction with lauroyl-CoA as acyl donor (Figure 2F, lower tracing). The upper tracing was offset to allow visualization of the retinyl laurate peak in the lower tracing.

An increased recovery of retinyl laurate was observed for the ARAT reaction (Figure 2F) in the presence of PMSF (6.75 pmol) compared to the recovery of retinyl laurate in the absence of PMSF (4.95 pmol). This may indicate the presence of a PMSF-sensitive esterase activity which reduced the yield of newly synthesized retinyl esters. Microsomal preparations from adult Sertoli cells were indeed able to hydrolyze added retinyl esters, indicating that such an esterase activity was present (results not shown). This activity was also identified in microsomes from 20-day-old Sertoli cells.

Effects of PMSF on Retinol Accumulation and Esterification by Cultured Sertoli Cells. Because LRAT activity was considerably lower than ARAT activity in microsomal preparations from 20-day-old Sertoli cells (Table I), it appeared unlikely that it would be the dominant reaction in the whole cell. Since LRAT and ARAT exhibited a differential sensitivity to PMSF, we treated cells with PMSF to see if we might be able to establish which of the activities was responsible for Sertoli cell esterification of retinol accumulated from retinol–RBP. Cells incubated in the presence of PMSF were determined to be greater than 95% viable by trypan blue exclusion. In addition, PMSF treatment did not inhibit retinol accumulation by the cells (Figure 3). In fact, the rate of specific retinol accumulation was unexpectedly enhanced in the presence of PMSF.

In the absence of PMSF, cultured Sertoli cells synthesized

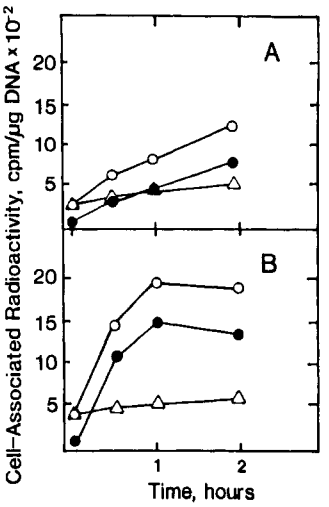

FIGURE 3: Effect of 5 mM PMSF on retinol accumulation in Sertoli cells. Cells (10 μg of cellular DNA) were preincubated in the absence (panel A) or presence (panel B) of 5 mM PMSF for 10 min at 32 °C. The cells were then incubated for the indicated periods of time with 1 μM [^{3}H]retinol–RBP (5.0 Ci/mmol) in the absence (○) (total cell associated radioactivity), or presence (△) (nonspecific cell-associated radioactivity), of a 25-fold molar excess of unlabeled retinol–RBP. The final volume was 250 μL. Specific cell-associated radioactivity (●) was the difference between the total and the nonspecific counts. The data shown are representative of three different experiments.

Table II: Comparison of the Relative Proportion of Retinyl Esters Synthesized

retinyl ester formed	cells + [^{3}H]retinol–RBP[b,d] (% of total)	cells + [^{3}H]retinol[b,e] (% of total)	microsomes[a] + [^{3}H]retinol–CRBP[c,d] (% of total)
retinyl palmitate	64	65	76
retinyl stearate	2	4	6
retinyl oleate	25	20	16
retinyl linoleate	9	11	<3

[a] Prepared from cultured Sertoli cells from midpubertal rats. [b] Experimental conditions as described in Figure 4. [c] Experimental conditions as described in Figure 1. [d] Average of four determinations. [e] Average of two determinations.

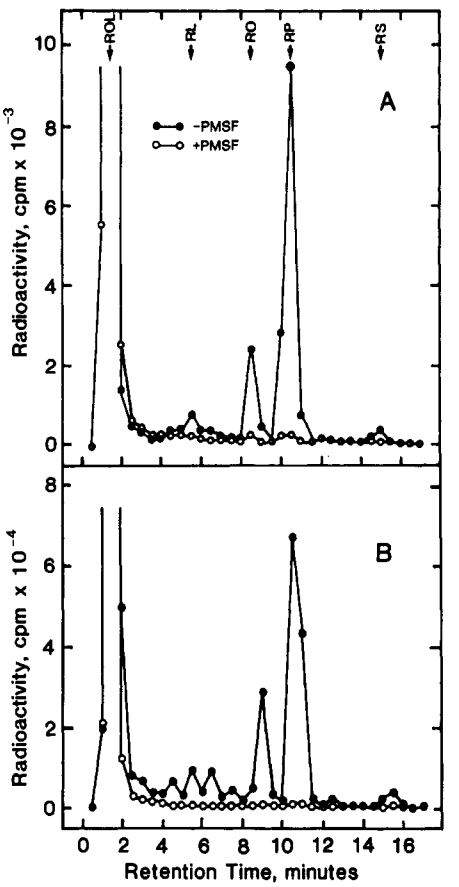

FIGURE 4: Effect of 5 mM PMSF on Sertoli cell esterification of retinol. Cells (100 μg of cellular DNA) were preincubated in the absence (●) or presence (○) of 5 mM PMSF for 10 min at 32 °C. One micromolar [^{3}H]retinol–RBP (5.0 Ci/mmol) (panel A) or 1 μM [^{3}H]retinol (5.0 Ci/mmol) (panel B) was added to a final volume of 2.5 mL. After 1 h at 32 °C, the cell membranes were isolated and extracted, and the extracts were analyzed by HPLC. Fractions collected from the HPLC column were analyzed for radioactivity. ROL, retinol; RL, retinyl linoleate; RO, retinyl oleate; RP, retinyl palmitate; RS, retinyl stearate. The data shown are the average of two determinations.

[^{3}H]retinyl linoleate, [^{3}H]retinyl oleate, [^{3}H]retinyl palmitate, and [^{3}H]retinyl stearate from [^{3}H]retinol–RBP (Figure 4A). However, this esterification was completely inhibited when the cells were pretreated with PMSF. Incubating the cells with free [^{3}H]retinol in the absence of PMSF also resulted in retinyl ester production (Figure 4B), and the pattern of esters synthesized was similar, although total ester recovery was 9-fold greater. However, treatment with PMSF also blocked this higher level of ester synthesis.

A further point was that the pattern of retinyl esters synthesized by the Sertoli cells, from both free and bound retinol, was the same and in similar proportions to those produced by LRAT in microsomal preparations (Table II). The major esters produced by either whole cells or microsomal preparations were retinyl palmitate and retinyl oleate; minor amounts of retinyl linoleate and retinyl stearate were also synthesized. Cells incubated with free [^{3}H]retinol also synthesized minor amounts of several unidentified esters (Figure 4B).

Discussion

The studies presented here indicate that retinol esterification in cultured Sertoli cells is catalyzed by lecithin:retinol acyltransferase (LRAT). Cells incubated with retinol–RBP or free retinol synthesized retinyl esters that were the same and in a similar ratio as those produced by LRAT in vitro in Sertoli cell microsomes. In addition, cellular retinol esterification was completely inhibited in the presence of PMSF, a selective inhibitor of LRAT esterification. Microsomal preparations from Sertoli cells also contained an ARAT activity that was 20-fold greater than the observed LRAT activity, but since esterification by testicular ARAT was insensitive to PMSF, the complete inhibition of cellular retinol esterification by PMSF implied that ARAT was not contributing to the esterification of retinol in the whole cell.

Cultured Sertoli cells accumulate retinol from retinol–RBP in a specific and saturable process (Shingleton et al., 1989). PMSF did not inhibit the specific accumulation of retinol by Sertoli cells, but rather was found to enhance it. Specific accumulation in PMSF-treated cells was 4-fold greater than the accumulation observed in untreated cells after 30 min. Interestingly, this increased rate of retinol accumulation ended at about 1 h, when the cellular retinol concentration was 0.58 pmol/μg of DNA. This is approximately the same value that we obtained for CRBP levels in these cells (Shingleton et al., 1989). In that study, we found that the rate of retinol accumulation in the absence of PMSF also leveled off when the cells had accumulated retinol about equivalent to the cellular CRBP concentration. Although PMSF-treated Sertoli cells accumulated retinol, the ARAT activity present was unable to esterify this retinol. Perhaps this was because internalized

retinol is bound to CRBP. In vitro experiments here showed that retinol–CRBP was a poor substrate for testicular ARAT.

When Sertoli cells are incubated with free retinol, the cells take up retinol by a nonsaturable and nonspecific process (Shingleton et al., 1989). Retinol transfers rapidly across bilayer membranes and from one membrane to another, with half-lives of less than 30 s and 10 min, respectively (Fex & Johannesson, 1988). Thus, retinol taken up by the cells in this manner was probably distributed throughout the cell membrane system. In the absence of PMSF, this retinol was available for esterification. In fact, ester synthesis was almost 10-fold greater when the cells were incubated with free retinol than when the retinol was bound to RBP. Yet the pattern of the esters synthesized and the sensitivity of this esterification to PMSF again suggested that the retinol esterification was catalyzed only by LRAT. Microsomal ARAT esterification still did not occur even though the cellular retinol concentration was greatly increased compared to retinol accumulation from retinol–RBP.

Although the results from whole cell studies indicated that LRAT was responsible for retinol esterification in Sertoli cells, the LRAT activity found in microsomal preparations from these cells was very low. The LRAT activity in microsomal preparations from adult rat testis was ninety-fold greater than the activity found in Sertoli cell microsomes (Table I). The low LRAT activity was not explained by cell culturing since an equally low LRAT activity was found in microsomal preparations from freshly isolated 20-day-old Sertoli cells. An esterase activity was identified in the immature Sertoli cell microsomes which would decrease the recovery of synthesized retinyl esters, but a comparable esterase activity was also identified in microsomes from enriched preparations of adult Sertoli cells. Therefore, this activity could not explain the 70-fold difference in retinyl ester recovery in the 20-day-old and the adult Sertoli cell microsomes. Instead, the difference in the LRAT activity appeared to be due to a developmental regulation of LRAT in the rat testis. LRAT activity in the 20-day-old testis was less than 2% of the activity observed in the adult rat, and a corresponding difference was observed in the 20-day-old and adult Sertoli cells. In contrast, the ARAT activity showed little change. The increased LRAT activity in the adult testis in comparison to the testis from immature rats is similar to developmental increases in other factors important in sperm production, such as FSH receptors and androgen-binding protein secretion (Ritzén et al., 1977; Sanborn et al., 1977). Therefore, it appears that LRAT activity increases as the Sertoli cell differentiates during puberty and is maintained at high levels in the adult testis to support the vitamin A dependence of adult testis function.

It should be stressed that the cell types isolated from sequential digestion of the adult rat testis were not completely pure. The peritubular cell fraction can be contaminated with interstitial cells and Sertoli cells, the Sertoli cell fraction can be contaminated with peritubular cells and germ cells, and the germ cell fraction can contain Sertoli cells. The degree of contamination was not quantitatively assessed here. However, since the highest LRAT activity was observed in the enriched Sertoli cell fraction, it seems clear that LRAT was indeed present in the adult Sertoli cells.

LRAT of rat liver and small intestine utilize an endogenous acyl donor to generate retinyl esters with a fatty acid composition similar to the retinyl ester composition observed in vivo. Thus, LRAT was suggested to represent the physiologically important retinyl ester synthase in these tissues. Characterization of retinol esterification by microsomes from the adult Sertoli cell fraction determined that LRAT activity in these cells was similar to the LRAT activity in microsomal preparations from rat liver and small intestine. Either free or bound retinol was esterified by using an endogenous acyl donor, producing retinyl esters in proportions similar to those produced by the whole cell. Phosphatidylcholine, the proposed acyl donor, served as a substrate for this esterification. In contrast, microsomal ARAT activity synthesized retinyl esters from free retinol only, and was dependent on exogenously added acyl-CoA.

Ottonello et al. (1987) recently characterized retinol accumulation in a plasma membrane enriched fraction from bovine retinal pigment epithelium. They concluded retinol uptake at the plasma membrane is coupled to esterification of retinol, with subsequent hydrolysis of plasma membrane associated retinyl esters stimulated by apo-CRBP. If this process occurred in Sertoli cells, we would not expect to observe the plasma membrane associated retinyl esters under normal conditions since apo-CRBP would stimulate their hydrolysis, but the results presented in the current study suggest that a coupled retinol uptake, esterification, and deesterification do not occur at the Sertoli cell plasma membrane. If retinol esterification at the plasma membrane was catalyzed by an LRAT activity, then PMSF should inhibit both retinol uptake and esterification. We saw inhibition of retinol esterification, but retinol accumulation was enhanced in the presence of PMSF. If the plasma membrane esterifying activity was PMSF insensitive (i.e., an ARAT activity), then retinol uptake and esterification at the plasma membrane should occur in the presence of PMSF. PMSF would, however, very likely inhibit any plasma membrane esterase activity, resulting in an accumulation of plasma membrane retinyl esters. Since no retinyl esters were synthesized in cultured Sertoli cells in the presence of PMSF, this would not appear to fit the mechanism of plasma membrane retinol uptake, esterification, and ester hydrolysis suggested by Ottonello et al.

It is possible that the retinol esterification observed in plasma membrane enriched fractions from retinal pigment epithelium was due to microsomal contamination. Saari and Bredberg (1988) have found that the retinol esterifying activity of bovine retinal pigment epithelial microsomes is extremely high (103 nmol of retinyl ester mg^{-1} min^{-1}). This microsomal activity is high enough to account for the activity observed by Ottonello et al. in their plasma membrane preparations which they estimated had a 2% or greater microsomal content. Here, no retinol esterifying activity was found in plasma membrane enriched fractions from cultured Sertoli cells (results not shown). Since LRAT activity was low in cultured Sertoli cell microsomes, a microsomal retinol esterifying activity would not mask or contaminate a plasma membrane retinol esterifying activity in this system.

The role that retinyl esters play in vitamin A function in the testis is unclear. Perhaps retinol is esterified by Sertoli cells for local storage of the vitamin. Alternatively, this may be the form of the vitamin that is transported to germ cells. Further investigation is needed to clarify this and other aspects of vitamin A metabolism and action in the testis.

In conclusion, the studies presented here indicate that although microsomal preparations from cultured Sertoli cells contain two retinol esterifying activities, LRAT and ARAT, only one of these activities (LRAT) functions in the whole cell to esterify retinol. These results, and other recent studies (Ong et al., 1988; MacDonald & Ong, 1988a,b) suggest that LRAT, and not ARAT, is the physiologically significant retinol esterifying activity.

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Registry No. LRAT, 117444-03-8; ARAT, 81295-48-9; retinol, 68-26-8; retinyl palmitate, 79-81-2; retinyl stearate, 631-87-8; retinyl oleate, 631-88-9; retinyl linoleate, 631-89-0; retinyl laurate, 1259-24-1; dilaurylphosphatidylcholine, 18285-71-7.

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A Differential Scanning Calorimetric Study of the Bovine Lens Crystallins†

Bryan L. Steadman,‡ Philip A. Trautman,‡ Erlinda Q. Lawson,‡ Matthew J. Raymond,‡ Deborah A. Mood,‡ John A. Thomson,‡ and C. Russell Middaugh*,§

Department of Molecular Biology, University of Wyoming, Box 3944, University Station, Laramie, Wyoming 82071, and Merck Sharp & Dohme Research Laboratories, WP26-331, West Point, Pennsylvania 19486

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ABSTRACT: Differential scanning calorimetry was performed on the five major lens crystallin fractions [HM-α, α, β_H, β_L, and (β_s + γ)] of the bovine lens as well as on more purified forms of α- and γ-crystallins. All were found to be relatively thermally stable although the α-crystallin fractions were found to at least partially unfold at an approximately 10 °C lower temperature than the β and γ fractions. Increasing protein concentration had little effect on γ-crystallin thermograms but had marked effects on those of the α- and β-crystallins. Increases in the thermal stability with increasing protein concentration for the β-crystallins can be explained most simply by the known β_L/β_H equilibrium, but, in the case of the α-crystallins, excluded volume effects may be an important factor. In both cases, the increased stability at high concentrations could be of physiological relevance. As well as the expected endothermic unfolding transitions, all of the lens crystallins revealed exothermic peaks that correlate with protein precipitation. Interestingly, this phenomenon occurs only after extensive structural alteration in the case of the α-crystallins but is present very early in the initial stages of structural perturbation of the β- and γ-crystallins.

Alterations in the conformational integrity of the lens crystallins have often been postulated to play a central role in cataract formation (Harding & Crabbe, 1984). Thus, since there is virtually no protein turnover during the life of a mammalian lens, structural stability would seem an essential functional prerequisite for lens crystallins. The structural stability of a protein is often inferred from spectroscopic measurements, with the protein usually at a relatively low concentration and in the presence of a chemical denaturant.

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* To whom correspondence should be addressed.
‡ University of Wyoming.
§ Merck Sharp & Dohme Research Laboratories.

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