Uptake and Metabolism of Retinol in Cultured Sertoli Cells: Evidence for a Kinetic Model†

Paul D. Bishop* and Michael D. Griswold
Department of Biochemistry/Biophysics, Washington State University, Pullman, Washington 99164-4660
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ABSTRACT: When cultured Sertoli cells derived from 20-day-old weanling rats were supplied [^{3}H]retinol bound to serum retinol binding protein–transthyretin complex, [^{3}H]retinol was rapidly incorporated and [^{3}H]retinyl esters were synthesized. Within 28 h after administration, 83% of the labeled retinoids were accounted for as retinyl esters (64% as retinyl palmitate). Sertoli cells derived from vitamin A deficient rats and supplied [^{3}H]retinol in culture under identical conditions likewise incorporated [^{3}H]retinol and synthesized retinyl esters. In contrast to normal Sertoli cells, vitamin A deficient Sertoli cells eventually metabolized virtually all of the cellular [^{3}H]retinol to retinyl esters. The primary metabolic fate of retinol administered to Sertoli cell cultures was the synthesis of retinyl esters under all conditions tested. However, administration of [^{3}H]retinol bound to serum retinol binding protein gave metabolic profiles having a higher proportion of retinyl esters and lower proportions of unresolved polar material than administration of [^{3}H]retinol alone. The kinetics of retinol uptake and intracellular retinyl ester synthesis in cultured Sertoli cells was complex. An initial, rapid phase of [^{3}H]retinol incorporation lasting 30 min was followed by a slower rate of incorporation and a concomitant decrease in the intracellular concentration of [^{3}H]retinol. During the time course the specific activity of [^{3}H]retinyl palmitate eventually exceeded that of intracellular [^{3}H]retinol. These observations suggest that two intracellular pools of retinol may exist in Sertoli cells.

Vitamin A is essential for the maintenance of male reproductive function (Thompson et al., 1964; Howell et al., 1963). In a vitamin A deficient (VAD)[1] animal the germinal epithelium degenerates and spermatogenesis ceases (Huang & Hembree, 1979; Huang et al., 1983). While administration of retinoic acid to VAD animals restores vital functions (Zile et al., 1979; Roberts & Frolik, 1979; Lotan, 1980), only retinol or its esters can restore testicular function and vision (Dowling & Wald, 1960; Thompson et al., 1964; Howell et al., 1963; Coward et al., 1969; Ahluwalia & Bieri, 1971). This phenomenon may be the result of a unique metabolic transformation that only retinol can undergo in these tissues. For example, only retinol and not retinoic acid can serve as a metabolic precursor for retinaldehyde and retinyl esters (Arens & van Dorp, 1946). In the testis, however, no absolute requirement for a metabolic transformation specific to retinol has yet been identified.

Sertoli cells are the somatic cells of the seminiferous tubules that interact with germinal cells and may regulate many germinal cell functions. Sertoli cells require vitamin A for the normal production of secreted proteins that are presumably very important in germinal cell development (Karl & Griswold, 1980; Skinner & Griswold, 1982; Griswold et al., 1984). Sertoli cells have been shown to contain high levels of cellular retinol binding protein (CRBP), and cellular retinoic acid binding protein (CRABP) has been localized in meiotic germinal cells (Huggenvik & Griswold, 1981; Porter et al., 1985; Kato et al., 1985a,b; Blaner et al., 1987). Thus both the support cells and the germinal elements of the testis may have important vitamin A requirements.

Sertoli cells form tight junctional complexes that create a blood–testis barrier which mediates the nutrient uptake of germinal cells (Fawcett, 1975). Thus the uptake and metabolism of the retinoids in Sertoli cells may play a critical role in the maintenance of germinal cells. It is possible that the absolute requirement for retinol by the testis may be related to the mechanism whereby retinol is taken up from the serum by Sertoli cells and transported to the germinal epithelium. One possible metabolic reaction that could be involved in this transport mechanism is the fatty acyl esterification of retinol.

Knowledge of testicular retinoid metabolism is limited to analytical surveys of whole organ extracts (Ito et al., 1974; Napoli & McCormick, 1981; Bhat & Lacroix, 1983) and metabolic studies utilizing extracts obtained from testis injected with [^{3}H]retinyl acetate (Chaudhary & Nelson, 1985). Evidence of the distribution of retinoids has been inferred from the histological examination of testicular tissue for labeled retinol and retinyl esters by autoradiography (Ahluwalia et al., 1975, 1980; Shakuntala et al., 1982) and by immunohistochemical analysis of CRBP and CRABP (Porter et al., 1985). Direct examination of metabolic events in individual testicular cell types is clearly required in order to elucidate the complex role the retinoids play in reproduction. This study describes the cellular kinetics of retinol metabolism in primary cultures of rat Sertoli cells and presents evidence for a kinetic model.

Experimental Procedures

Retinoids. Wherever possible, all operations involving retinoids were carried out in dim red light, under an Ar atmosphere at 0 °C using solvents thoroughly purged with Ar.

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* Address correspondence to this author.

[1] Abbreviations: BHT, butylated hydroxytoluene; CRABP, cellular retinoic acid binding protein; CRBP, cellular retinol binding protein; EtOH, ethanol; F-12 medium, Ham's F-12 medium; HBSS, Hank's basic salt solution; HPLC, high-performance liquid chromatography; NH_4O-Ac, ammonium acetate; RA, retinoic acid; RBP, serum retinol binding protein; RBP–TTR, the (apo)protein complex of RBP and TTR (transthyretin) without retinol bound to RBP; ROH, retinol; ROH:RBP–TTR, the (holo)protein complex of RBP and TTR with retinol bound to RBP; RP, retinyl palmitate; THF, tetrahydrofuran; VAD, vitamin A deficient. Asterisk superscripts denote specific radioactivity, e.g., *ROH.

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all-trans-[10,11-^{3}H]Retinol (specific activity 3.45 Ci/mmol) was synthesized from *all-trans*-[10,11-^{3}H]retinaldehyde (SRI, Menlo Park, CA) as follows: Toluene carrier was removed by evaporation in a stream of Ar, and the radioactive retinaldehyde residue was dissolved in 40 μL of EtOH and reduced with an aqueous solution of $NaBH_4$ (5 μL of 20 mg/mL). The [^{3}H]retinol product was then purified by HPLC as described under Chromatography. The fractions corresponding to retinol were pooled, evaporated to dryness, and redissolved in 100 μL of EtOH containing BHT (50 μg/mL) as an antioxidant and stored at −80 °C under Ar. In some experiments *all-trans*-[11,12-^{3}H]retinol (specific activity 43 Ci/mmol) was purchased from Amersham Corp. (Arlington Heights, IL) and repurified as described for *all-trans*-[10,11-^{3}H]retinol. Prior to use, [^{3}H]retinol was assayed for purity and specific activity by HPLC. The radiopurity of [^{3}H]retinol was greater than 95%, and the remaining label was accounted for as background. Unlabeled retinoids, *all-trans*-retinol, *all-trans*-retinaldehyde, *all-trans*-retinoic acid, retinyl acetate, and retinyl palmitate were purchased from Sigma Chemical Co. (St. Louis, MO).

Chromatography. HPLC analyses of retinoid standards (25 pmol each in 20 μL) or cellular extracts (20 μL) were performed on a C18, reverse-phase column (Spherisorb ODS, II, 5 μm, 25 × 0.46 cm, packed by Alltech Associates, Inc., Deerfield, IL) with a precolumn (Aquapore ODS, 10μm, 3 × 0.46 cm, Brownlee Labs Inc., Santa Rosa, CA). Base-line resolution of all of the standard retinoids was achieved with a programmed gradient elution using a Beckman Model 412 controller and two Model 112 pumps (Beckman Instruments, Inc., Berkeley, CA): solvent A was composed of 75 mM NH_4OAc (pH 6.8) in H_2O/CH_3CN (1:1); solvent B was CH_3CN/THF (5:3). The gradient consisted of the programmed sequence 35% B to 55% B in 20 min, 55% B to 92% B in 10 min, and 92% B to 100% B in 15 min. UV absorbance was monitored at the dual wavelengths of 325 nm (0.01 AFS) and 260 nm (0.05 AFS) with a Beckman Model 165 variable-wavelength detector. The eluent was collected in 0.5-mL fractions and the radioactivity determined by liquid scintillation counting by using a quench-corrected program for tritium.

Radiolabeled retinol preparations were purified with the same apparatus as described above using isocratic elution with CH_3CN/H_2O (5:1). Under these conditions retinoic acid was not retained, but retinol, retinaldehyde, and retinyl acetate were base-line resolved. The eluent was monitored for radioactivity as above but was not subjected to UV detection.

Extraction and Quantitation of Retinoids. Due to the deleterious effects of heat, light, pH extremes, and oxidative conditions on retinoids, great care was taken to minimize these effects as described under Retinoids. Control experiments were performed to assess potential deterioration of the retinoids by using dead cells as described under Cell Culture and Retinol Administration and by subjecting pure retinoid standards to the extraction procedure.

In all cases, Sertoli cells were collected, extracted, and assayed for retinoids as follows: Tissue culture medium was first removed from cell cultures by decantation, the cells were then gently washed twice with fresh F-12 medium, and the adhering cells were suspended in 1 mL of medium with a rubber policeman. The cells were then transferred to a 1.8-mL, conical centrifuge tube (West Coast Scientific, Inc., Emeryville, CA) with an additional 0.5 mL of medium. The cell suspension was then centrifuged at 8000*g* for 1 min in a Beckman Model 12 centrifuge. The supernatant was carefully removed from the pellet, and the resulting sample pellet was washed twice more by resuspension in F-12 followed by centrifugation, covered with Ar, and stored at −80 °C. When an entire set of experimental samples had been collected, they were placed in an ice bath and each was extracted by sonic disruption with 0.6 mL of ice-cold CH_3OH containing BHT (50 μg/mL) as antioxidant and 100 pmol of retinyl acetate added as internal standard. The resulting cell debris was then pelleted by centrifugation as before, and the CH_3OH extracts were transferred to clean centrifuge tubes. The remaining pellets were then extracted a second time with 0.5 mL of THF containing BHT (50 μg/mL), and the cell debris was removed by centrifugation. The combined extracts were then evaporated in a stream of Ar and redissolved in 30 μL of 2-propanol, and 20 μL was analyzed by HPLC as previously described under Chromatography.

Retinoids were quantified by using the absorbance at 325 nm for the peaks that corresponded to calibrated external standards. Peak heights and integrated peak areas were used as cross-checks for accuracy, and the ratios of the absorbances at 325 and 260 nm were used to assess peak purities. The radioactivity of individual retinoids was determined from the fractions that corresponded to the elution time of external standards. Unknown peaks that eluted in the region of the chromatogram corresponding to retinyl esters were saponified as described by Furr et al. (1984) to confirm their identity. Recoveries for all of the standard retinoids were greater than 95%. When freshly purified [^{3}H]retinol was subjected to this extraction procedure, greater than 90% of the radioactivity coeluted with added unlabeled retinol standard.

Preparation and Administration of [^{3}H]Retinol Bound to RBP–TTR. Rat RBP–TTR complex was partially purified (>95-fold) by using a modified procedure derived from McGuire and Chytil (1979). Briefly, citrated rat serum was dialyzed against 50 mM imidazole–acetate (pH 6.0) and then chromatographed on DE-52. The peak fractions yielding the highest fluorescence (excitation, 334 nm; emission, 465 nm) were pooled, dialyzed against water, lyophilized, and assayed for retinol binding activity as described below. [^{3}H]ROH:RBP–TTR was prepared as follows: A lyophilized preparation of RBP–TTR (5 mg) was extracted 3 times with 20-mL portions of EtOH at 2 °C over a 24-h period by tumble mixing followed by centrifugation, decantation, and resuspension in fresh EtOH. After the final centrifugation, the resulting pellet was dried under vacuum and dissolved in 4 mL of HBSS to which 25 nmol of [10,11-^{3}H]ROH dissolved in 100 μL of EtOH was added. This solution was allowed to equilibrate for 3 h at room temperature and then chromatographed in 1-mL aliquots to remove unbound [^{3}H]ROH on a 2 × 8 cm column of 200–400 mesh, Bio-Gel P-2 (Bio-Rad, Richmond, CA). The fractions corresponding to the void volume were then pooled and sterilized by filtration through a 0.22-μm Millex filtration unit (Millipore Corp., Bedford, MA). The concentration of [^{3}H]ROH:RBP–TTR was adjusted with sterile HBSS and directly administered to cultured Sertoli cells. The concentrations and specific activities of the resulting [^{3}H]ROH:RBP–TTR solutions were determined from lyophilized aliquots as described under Extraction and Quantitation of Retinoids. Nonspecific binding (<10%) and the apparent dissociation constant for the [^{3}H]ROH:RBP–TTR preparations (2×10^{-7} M) were determined by gel filtration as described above.

[^{3}H]ROH:RBP–TTR was administered to Sertoli cells on the fifth day of cell culture. Spent culture medium was removed by aspiration, the cells were washed twice with fresh HBSS (the residual HBSS being carefully aspirated off), and

Table I: Relative Distribution of [^{3}H]Retinoids in Cultured Sertoli Cells Administered [^{3}H]Retinol[a,b]

	normal	control[c]
retinol	16 ± 2	47 ± 3
retinyl palmitate	28 ± 3	0[f]
∑ retinyl esters[d]	45 ± 7	0[f]
∑ polar[e]	39 ± 6	46 ± 1

[a] Data were obtained from cultured Sertoli cells administered [11,12-^{3}H]retinol (94.6 dpm/fmol) for 32 h as described for Figure 1A. Data are given as the mean ± SEM of five separate determinations (cultures). See text for experimental details. [b] Expressed as percent of total extractable label. [c] Cultured Sertoli cells killed by exposure to microwave radiation. [d] Sum of the saponifiable label corresponding to fractions 55–90 of the HPLC profile (see Experimental Procedures). [e] Sum of the label eluting prior to retinol in the HPLC chromatograms. [f] No saponifiable peaks were observed.

0.5 mL of the [^{3}H]ROH:RBP–TTR solution (preequilibrated in the incubator atmosphere) was added. The period of administration varied as stated under Results. At the end of the administration period the [^{3}H]ROH:RBP–TTR solution was removed by aspiration and frozen at −80 °C for subsequent analysis. The cultured Sertoli cells were then washed twice with fresh F-12 medium and either harvested as previously described or allowed to continue in culture in fresh, unsupplemented medium for a specified time period.

Cell Culture. Sertoli cells obtained from the testes of 20-day-old rats were prepared and cultured as previously described (Kissinger et al., 1982; Dorrington & Fritz, 1975). Rat Sertoli cells prepared from adult and VAD rats were done as described by Wilson and Griswold (1979). These cultures were judged to be greater than 90% Sertoli cells by light microscopy and were shown to synthesize transferrin and transferrin mRNA (Hugly & Griswold, 1987). Cells were plated in 100-mm Falcon dishes in 10 mL of F-12 medium and cultured for 4 days prior to addition of radiolabeled retinoids.

Controls were obtained from 5-day cultures killed by brief exposure to microwave radiation (425 W for 15 s) prior to addition of radiolabeled retinoids.

Animals. Male Sprague-Dawley rats were obtained from the Laboratory Animal Resource Center at Washington State University. In vitamin A deficient studies, weanling rats were maintained on a vitamin A free diet (United State Biochemical Corp.) for 10 weeks.

Results

Dependence of Retinol Uptake and Metabolism on the Mode of Administration. When primary cultures of 20-day-old rat Sertoli cells were administered [10,11-^{3}H]ROH, a number of labeled retinoids were found to accumulate. In Table I the relative distribution of label found in Sertoli cells 32 h after administration of [^{3}H]ROH is given for both live cultures and for cultures killed by microwave irradiation (controls). The most quantitatively significant metabolite found over controls were the retinyl esters (45 ± 7%), with retinyl palmitate (RP) being the single most abundant metabolite (28 ± 3%). The relatively large amounts of polar material (i.e., material that eluted prior to retinol during HPLC analysis) found in these cells did not appear to significantly differ from that found in control cells.

During the course of these experiments it was found that when equivalent doses of [^{3}H]ROH were administered to Sertoli cell cultures incubated in F-12 medium alone or in F-12 medium containing 2% calf serum, the presence of serum limited both cellular accumulation of [^{3}H]ROH and the rate of RP synthesis by greater than a factor of 10 (data not shown). The possibility that this effect was due to serum

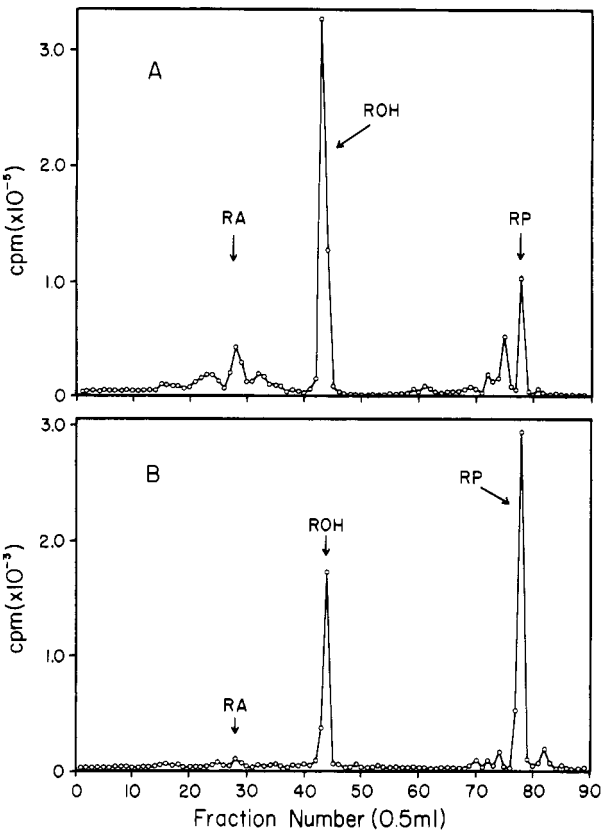


FIGURE 1: HPLC analysis of retinoid metabolites obtained from Sertoli cells administered [^{3}H]retinol. Chromatograms indicate the profile of radioactivity eluted from cellular extracts of (A) cultured Sertoli cells administered [11,12-^{3}H]retinol (94.6 dpm/fmol) at a final concentration of 240 nM in 0.5 mL of F-12 medium for 4 h and (B) cultured Sertoli cells obtained from a parallel experiment except that [11,12-^{3}H]retinol (4.33 dpm/fmol) was administered bound to RBP–TTR at a final concentration of 450 nM.

factors was investigated by administering [^{3}H]ROH bound to the rat serum retinol binding protein–transthyretin complex ([^{3}H]ROH:RBP–TTR). A comparison of representitive HPLC profiles of cultured Sertoli cells given either [^{3}H]ROH or [^{3}H]ROH:RBP–TTR is shown in Figure 1. Differences in the relative distribution of [^{3}H]ROH metabolites are evident; in cells given [^{3}H]ROH (Figure 1A) the relative distribution of polar material (35%) is approximately 3-fold greather than the proportion found in cells given [^{3}H]-ROH:RBP–TTR (Figure 1B). Further important differences included a lower proportion of retinyl esters (11% as retinyl palmitate) found in cells given [^{3}H]ROH compared to that found in cells given [^{3}H]ROH:RBP–TTR (51% as retinyl palmitate).

Kinetics of Retinol Metabolism in Cultured Sertoli Cells Derived from 20-Day-Old Weanling Rats. Sertoli cells obtained from 20-day-old weanling rats were cultured in the presence of ROH for 5 days, at which time the endogenous cellular concentrations of ROH and retinyl palmitate were 75 ± 13 and 309 ± 53 fmol/μg, respectively (n = 19). The cell cultures were then administered [^{3}H]ROH:RBP–TTR for up to 4 h (pulsed), at which time the medium was removed and replaced with fresh, ROH-free medium for the remainder of the time course (washed out). A representative time course for cellular ROH accumulation and RP synthesis is shown in Figure 2. [^{3}H]ROH accumulation was initially rapid, with the cellular concentration of [^{3}H]ROH reaching a maximum within 30 min. However, within 1 h of administration, [^{3}H]ROH accumulation declined sharply and approached a steady-state rate (medium [^{3}H]ROH:RBP–TTR remained

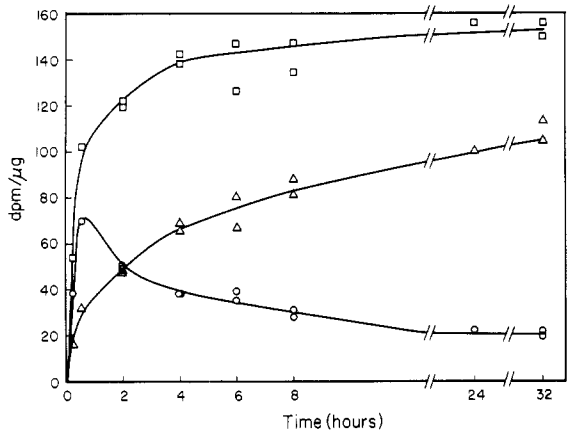


FIGURE 2: Time course for the concentrations of [^{3}H]retinol metabolites in cultured Sertoli cells from 20-day-old rats. Cultured Sertoli cells were administered [10,11-^{3}H]retinol (4.33 dpm/fmol) bound to RBP–TTR at a final concentration of 450 nM for 4 h (pulse), at which time the incubation medium containing the label was removed and fresh medium was added (wash out). At the times indicated the cells were collected, washed, and analyzed by HPLC as under Experimental Procedures. The cellular concentrations of label are given for the retinoids: (○) retinol; (△) retinyl palmitate; (□) total label that was extractable from the cells.

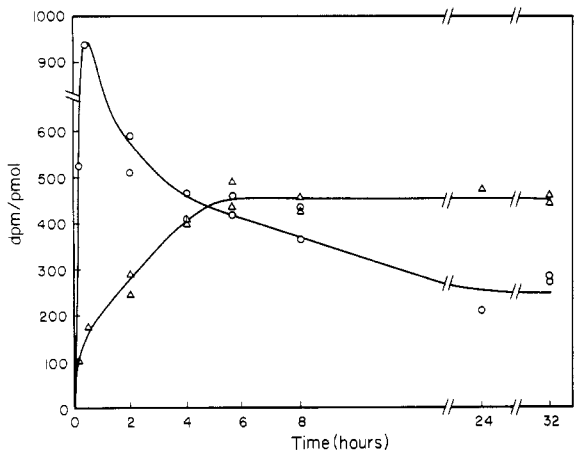


FIGURE 3: Time course for the specific activities of [^{3}H]retinol metabolites in cultured normal Sertoli cells from 20-day-old rats. Experimental conditions are as described in Figure 2. The cellular specific activities are given for the retinoids: (○) retinol; (△) retinyl palmitate.

virtually constant during the 4-h course of administration). By 8 h the concentration of cellular [^{3}H]ROH was essentially constant. The time course for the specific radioactivities of the cellular ROH and RP is given in Figure 3. Initially, the rise in the specific activity of ROH was extremely sharp—much faster than that for RP. Following this initial 30-min period the specific activity of ROH declined while that of RP continued to rise, with the specific activity of RP eventually exceeding that for ROH.

In Tables II and III the cellular concentrations and specific activities, respectively, of [^{3}H]ROH and [^{3}H]RP are given for cultured Sertoli cells administered [^{3}H]ROH:RBP–TTR for 4 h (end of pulse) and 28 h after removal of exogenous label (end of wash out). The experimental conditions were identical with those described for the experiment shown in Figure 3. The data are presented as pooled averages of six to seven independent determinations (i.e., from separate cultures) obtained from three separate Sertoli cell preparations.

The data in Table II indicate the general pattern of ROH uptake and RP synthesis seen in Sertoli cell cultures given [^{3}H]ROH:RBP–TTR. These data indicate the following: (1)

Table II: Concentration of [^{3}H]Retinol and [^{3}H]Retinyl Palmitate in Cultured Sertoli Cells Administered [^{3}H]Retinol Bound to RBP–TTR[a,b]

	4 h	32 h
retinol[c]	19 ± 6 (6)	16 ± 4 (7)
retinol control[d]	98 ± 15 (6)	2.4 ± 0.6 (6)
retinyl palmitate	53 ± 4 (6)	94 ± 11 (7)
retinyl palmitate control	0 (6)	0 (6)
total extracted label	125 ± 24 (6)	146 ± 13 (7)
total extracted label control	128 ± 17 (6)	37 ± 14 (6)

[a] The cellular concentrations of labeled retinol and retinyl palmitate are given for Sertoli cell cultures administered [10,11-^{3}H]retinol bound to RBP–TTR (4.33 dpm/fmol) after 4 h (end of pulse) and at 32 h (end of wash-out period). Data are given as the mean ± SEM for the number of independent determinations (cultures) indicated in parentheses. The cultures were obtained from three separate Sertoli cell preparations. See text for experimental details. [b] Values expressed as dpm/μg of cell protein. [c] For cultured Sertoli cells. [d] For cultured Sertoli cells killed by exposure to microwave radiation.

Table III: Specific Activity of [^{3}H]Retinol and [^{3}H]Retinyl Palmitate in Cultured Sertoli Cells Administered [^{3}H]Retinol Bound to RBP–TTR[a,b]

	cellular sp act.[b]	
	4 h	32 h
retinol[c]	428 ± 24 (6)	132 ± 45 (7)
retinyl palmitate	196 ± 42 (6)	281 ± 68 (7)
difference (*ROH − *RP)[d]	232[e] ± 55 (6)	−149[e] ± 34 (7)

[a] The cellular specific activities of labeled retinol and retinyl palmitate are given for Sertoli cell cultures administered [10,11-^{3}H]retinol bound to RBP–TTR (4.33 dpm/fmol) for 4 h (end to pulse) and at 32 h (end of wash-out period). Data are given as the mean ± SEM for the number of independent determinations (cultures) indicated in parentheses. The cultures were obtained from three separate Sertoli cell preparations. See text for experimental details. [b] Values expressed as dpm/pmol. [c] For cultured Sertoli cells. [d] The average of the paired differences between the specific activities of retinol and retinyl palmitate determined in each separate culture. [e] $p < 0.01$.

The synthesis of RP was greatest during the period of [^{3}H]-ROH:RBP–TTR administration. (2) The change in the concentration of [^{3}H]ROH associated with the Sertoli cells between 4 and 32 h did not fully account for the increase in the concentration of [^{3}H]RP observed during the same time period. (3) RP synthesis was not detected in controls consisting of killed Sertoli cell cultures administered [^{3}H]ROH:RBP–TTR. (4) A significant amount of [^{3}H]ROH was associated with killed Sertoli cell controls at the end of the period of [^{3}H]ROH:RBP–TTR administration (4 h); however, at the end of the subsequent 28-h wash-out period (32 h) most of this [^{3}H]ROH was lost.

The data for the specific activities of ROH and RP given in Table III indicate that by the end of the wash-out period the specific activity of [^{3}H]RP exceeded that of its precursor [^{3}H]ROH. Since the experiment has a random-block design, it is appropriate to employ a paired-difference test for comparing the specific activities of the metabolites (i.e., to calculate the average of the differences between the specific activities of retinol and retinyl palmitate determined in each separate culture). Thus, by use of Student's t statistic the observation that the specific activity of RP exceeded that of ROH at the end of the wash-out period was found to be significant at $p < 0.01$.

The relative distribution of labeled retinoid metabolites present in Sertoli cells administered [^{3}H]ROH:RBP–TTR as described above is given in Table IV. The table shows that under the conditions of these experiments (1) retinyl esters account for the largest proportion of labeled ROH metabolites, (2) RP is apparently the most abundant retinyl ester syn-

Table IV: Relative Distribution of [^{3}H]Retinoids in Cultured Sertoli Cells Compared to Control (Killed) Cells and Medium Alone[a,b]

	4 h	32 h
retinol[c]	16 ± 4 (6)	11 ± 2 (7)
retinol control[d]	76 ± 3 (6)	7 ± 0.6 (6)
retinol blank[e]	81 ± 2 (10)	NA[i]
retinyl palmitate	50 ± 3 (6)	64 ± 2 (7)
retinyl palmitate control	0 (6)	0 (6)
retinyl palmitate blank	0 (10)	NA
$\sum$ retinyl esters[f]	65 ± 4 (6)	83 ± 2 (7)
$\sum\sum$ retinyl esters control[g]	10 ± 3 (6)	28 ± 2 (6)
$\sum\sum$ retinyl esters blank[g]	3 ± 1 (10)	NA
$\sum\sum\sum$ polar[h]	19 ± 5 (6)	8 ± 3 (7)
$\sum\sum\sum$ polar control	14 ± 4 (6)	65 ± 3 (6)
$\sum\sum\sum$ polar bank	16 ± 2 (10)	NA

[a] Data compiled from the time course of retinol metabolism as described in Table II are compared on a percent basis to parallel time course incubations of [10,11-^{3}H]retinol bound to RBP–TTR (4.33 dpm/fmol) with control cell cultures and in incubation medium alone. Data are given as the mean ± SEM for the number of independent determinations (cultures) indicated in parentheses. The cultures were obtained from three separate Sertoli cell preparations. [b] Expressed as percent of total extractable label. [c] Cultured Sertoli cells. [d] Cultured Sertoli cells killed by exposure to microwave radiation. [e] F-12 medium containing [^{3}H]ROH:RBP–TTR only. [f] Sum of the saponifible label corresponding to fractions 55–99 of the HPLC analysis (see Experimental Procedures). [g] Label above background—no resolvable peaks were saponifible. [h] Sum of the label eluting prior to retinol in the HPLC chromatograms. [i] Nonapplicable.

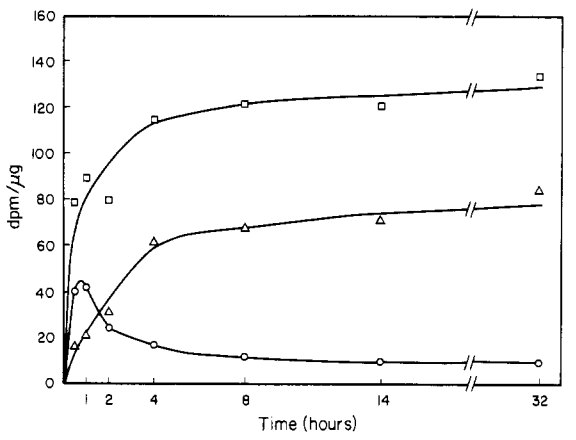


FIGURE 4: Time course for [^{3}H]retinol metabolism in normal adult Sertoli cells depleted of vitamin A in tissue culture. Experimental conditions are as described under Figure 2 except that Sertoli cells were derived from normal, 60-day-old, adult rats and were depleted of vitamin A for 5 days in culture prior to administration of [10,11-^{3}H]retinol (specific activity 4.33 dpm/fmol) bound to RBP–TTR at a final concentration of 450 nM. The cellular concentrations of label are given for the retinoids: (○) retinol; (△) retinyl palmitate; (□) total label that was extractable from the cells.

thesized, and (3) few differences were found between the proportions of the polar material associated with Sertoli cell cultures, killed Sertoli cell cultures (controls), and incubation medium alone (blank) at the end of 4 h. It may be noted that the increase in the proportion of polar material in the control cells was due to the loss of [^{3}H]ROH (and therefore total label) and not to a net synthesis of polar material. Note also that for the control cells the label which corresponded to retinyl esters was not found to be saponifible and was therefore apparently background.

Kinetics of Retinol Metabolism in Cultured Sertoli Cells Derived from Normal Adult and Vitamin A Deficient Adult Rats. Sertoli cells were obtained from normal adult rats and were cultured in the absence of ROH for 5 days (to deplete intracellular retinoid stores), at which time the intracellular concentrations of ROH and RP were 11 ± 8 and 15 ± 2

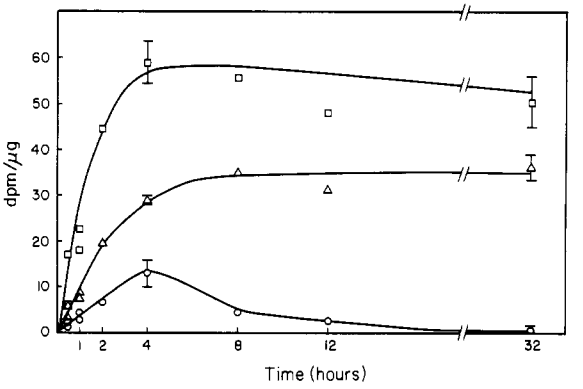


FIGURE 5: Time course for [^{3}H]retinol metabolism in vitamin A deficient cultured Sertoli cells. Experimental conditions are as described under Figure 2 except that Sertoli cells, derived from VAD rats, were maintained deficient in culture until the time of administration of [10,11-^{3}H]retinol bound to RBP–TTR. The cellular concentrations of label are given for the retinoids: (○) retinol; (△) retinyl palmitate; (□) total label that was extractable from the cells.

Table V: Concentration of [^{3}H]Retinol and [^{3}H]Retinyl Palmitate in Cultured VAD Sertoli Cells Administered [^{3}H]Retinol Bound to RBP–TTR[a,b]

	4 h	32 h
retinol[c]	13 ± 3 (4)	0.5 ± 0.3 (5)
retinol control[d]	39 (2)	1 (2)
retinyl palmitate	28 ± 1 (4)	36 ± 3 (5)
retinyl palmitate control	0	0
total extracted label	59 ± 9 (4)	51 ± 6 (5)
total extracted label control	48 (2)	2 (2)

[a] The cellular concentrations of labeled retinol and retinyl palmitate are given for Sertoli cell cultures administered [10,11-^{3}H]retinol (4.33 dpm/fmol) bound to RBP–TTR at 4 h (end of pulse) and at 32 h (end of wash-out period). Data are given as the mean ± SEM for the number of independent determinations (cultures) indicated in parentheses. The cultures were obtained from two separate VAD Sertoli cell preparations. See text for experimental details. [b] Values expressed as dpm/μg of cell protein. [c] For cultured VAD Sertoli cells. [d] For cultured VAD Sertoli cells killed by exposure to microwave radiation.

fmol/μg (n = 7), respectively. The cell cultures were then incubated with [^{3}H]ROH:RBP–TTR under experimental conditions identical with those described above for the Sertoli cell cultures from 20-day-old rats. The rate of cellular ROH accumulation and RP synthesis (Figure 4) was qualitatively similar to that seen in the time course for cultured Sertoli cells derived from 20-day-old rats (Figure 2). By the end of the wash-out period, cellular [^{3}H]ROH and [^{3}H]RP accounted for 8% and 70% of the total cellular label, respectively. The primary purpose of these cultures was to serve as a normal, adult Sertoli cell control for the VAD Sertoli cell studies described below.

Sertoli cells derived from VAD adult rats were cultured as described for normal adult rats; no endogenous ROH or RP was detected in VAD Sertoli cells. The time course of ROH metabolism in cultured Sertoli cells derived from VAD rats is shown in Figure 5. The experimental conditions were identical with those just described. The general pattern of ROH accumulation and RP synthesis differs in several important respects from that seen in Sertoli cells derived from either weanling rats (Figure 2) or normal adult rats (Figure 4): (1) Cellular [^{3}H]ROH accumulation was linear during the entire 4-h period of [^{3}H]ROH:RBP–TTR administration. (2) The change in the concentration of cellular [^{3}H]ROH between 4 and 32 h (Table V) appeared to account for the increased concentration of [^{3}H]RP. (3) After removal of [^{3}H]ROH:RBP–TTR from the medium (wash-out period), cellular [^{3}H]ROH eventually accounted for only 0.9 ± 0.6%

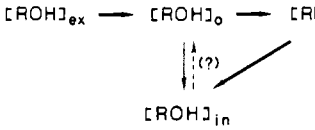
($n = 5$) of the total label incorporated. (4) RP synthesis was significantly lower ($p < 0.001$) in Sertoli cells from VAD rats than in Sertoli cells from 20-day-old rats.

Discussion

In the present study, the synthesis of retinyl esters has been demonstrated to occur in well-characterized primary cultures of Sertoli cells obtained from weanling (20-day-old) rats. The extent of esterification was striking; 32 h after administration of [^{3}H]ROH:RBP–TTR to cultured Sertoli cells, retinyl esters constituted 83 ± 2% of the total cellular label (64 ± 2% of the total label as RP). This distribution of labeled retinoids differs from that reported by Chaudhary and Nelson (1985) for whole testes. These workers found that 24 h after injection of normal testes with [^{3}H]retinyl acetate 52% of the label was present as ROH and only 15% was accounted for as RP. In spite of these differences (which are likely to result from the variety of cell types present in whole testes and from the modes of administration) it is clear from both of these studies that retinyl esters can be rapidly synthesized in testicular cells. The concentration of RP found in the present investigation for cultured Sertoli cells (309 ± 53 fmol/μg) was intermediate to that determined for isolated liver parenchymal cells (112 ± 36 fmol/μg; Blomhoff et al., 1985) and for isolated hepatocytes (520 μ 60 fmol/μg; Drevon et al., 1984) but significantly higher than that reported for whole rat testis (20 fmol/μg; Bhat & Lacroix, 1983). On the basis of these comparisons, it is likely that Sertoli cells are capable of ROH esterification at physiologically relevant concentrations.

The mode of ROH delivery markedly affected the incorporation and metabolism of ROH in cultured Sertoli cells. Initial studies on Sertoli cells showed that the presence of serum in the culture medium dramatically altered the rate and pattern of ROH metabolism. In studies where labeled ROH was administered to cultured Sertoli cells directly (Figure 1A), it was found that cells contained a complex array of labeled retinyl esters with a large portion of total label present as unidentified polar compounds. In contrast, cells given [^{3}H]ROH:RBP–TTR (Figure 1B) showed a simpler pattern of ROH metabolism with RP accounting for >85% of the total retinyl esters and <15% of the total label present as unidentified polar compounds (also compare Tables I and IV). These findings indicate that the presence of serum factors (RBP–TTR) may affect the specificity of ROH esterification and the degree to which ROH is oxidized. In dilute aqueous solution ROH is labile to oxidation, whereas solutions of ROH:RBP–TTR are more stable (Goodman, 1984); it is possible that the increased amount of oxidized (polar) retinoids found in the Sertoli cell cultures administered ROH in the absence of serum factors was the result of nonspecific oxidation. The greater specificity of ROH esterification seen in Sertoli cells administered [^{3}H]ROH:RBP–TTR may be due to the lower ROH incorporation observed. This could have effectively reduced the concentration of the intracellular ROH available for esterification reactions and possibly restricted ROH esterification to a specific retinyl ester synthetase with a higher affinity for specific fatty acyl donors (e.g., palmitoyl CoA). Administration of [^{3}H]ROH:RBP–TTR resulted in clearer metabolite profiles than [^{3}H]ROH alone (i.e., a lower percent of unresolved polar material, a higher percent of retinyl esters, and a simpler pattern of esterification). In addition, ROH:RBP–TTP is believed to be the physiological form that is actually involved in the delivery of ROH to target tissues in vivo (Goodman, 1984). Therefore, [^{3}H]ROH:RBP–TTR was administered to Sertoli cell cultures in all of the metabolic time courses discussed below.

The metabolism of [^{3}H]ROH in cultured Sertoli cells exhibited interesting kinetics. Initially, when [^{3}H]ROH:RBP–TTR was administered to cultured Sertoli cells obtained from either normal weanling or adult rats (Figures 2 and 4), the rate of [^{3}H]ROH incorporation exceeded the rate of [^{3}H]RP synthesis by greater than 2-fold. Following an initial 30-min period, the concentration of cellular [^{3}H]ROH declined rapidly and approached a constant level as [^{3}H]ROH incorporation and [^{3}H]RP synthesis approached steady-state rates of nearly equal magnitude. When exogenous [^{3}H]ROH:RBP–TTR was removed during this steady-state period, the cellular concentrations of both [^{3}H]ROH and [^{3}H]RP became constant, with [^{3}H]ROH and [^{3}H]RP accounting for approximately 12% and 70% of the total label, respectively. This kinetic behavior might be accounted for if [^{3}H]ROH, taken up by the cell, enters an endogenous pool of [^{3}H]ROH that is in dynamic equilibrium with a pool of cellular retinyl esters. However, this simple model would predict that following removal of exogenous [^{3}H]ROH the specific activities of cellular ROH and RP should approach equilibrium (i.e., *ROH ≥ *RP). Careful determinations of the specific activities for cellular ROH and RP (Figure 3 and Table III) indicate that following removal of exogenous [^{3}H]ROH the specific activity of RP exceeded that of the cellular ROH (i.e., *RP > *ROH; $p < 0.01$). This observation is a classic indication that at least two pools of cellular ROH may exist with one pool serving as a privileged precursor to RP synthesis. A more simple equilibrium model also fails to adequately account for the kinetic behavior of Sertoli cells obtained from VAD rats. In contrast to normal Sertoli cells, VAD Sertoli cells (which lack endogenous ROH) metabolized virtually all newly incorporated [^{3}H]ROH primarily to [^{3}H]RP esters (Figure 5 and Table V), indicating that a dynamic equilibrium between cellular ROH and retinyl esters does not occur. A possible model that accounts for all of these observations is given in the reaction pathway



In this model, incorporation of extracellular retinol ($[ROH]_{ex}$) gives rise to a cellular pool of retinol ($[ROH]_0$) that can both serve as a privileged precursor for retinyl palmitate ([RP]) synthesis and deliver ROH to another more stable intracellular pool of retinol ($[ROH]_{in}$). These intracellular retinoid pools may exist in a state of quasi-equilibrium wherein RP could be hydrolyzed to replenish ROH losses possibly caused by oxidations, intercellular transport, or other metabolic processes.

While this model does not directly address the process of ROH uptake (i.e., the model does not distinguish ROH binding from the overall process of ROH uptake), several lines of evidence suggest that a specific binding event for ROH does occur. When preparations of [^{3}H]ROH:RBP–TTR were used to determine the binding of ROH to 20-day-old Sertoli cells (performed at 2 °C to prevent uptake and metabolism), Scatchard analysis gave a linear plot ($r = 0.99$, $n = 5$) that yielded an apparent dissociation constant for ROH of 6.8×10^{-7} M and a putative receptor density of 37 fmol/μg (unpublished data). Thus, under the conditions of [^{3}H]ROH:RBP–TTR administration in the present study, 20-day-old Sertoli cells would be expected to initially bind approximately 15 fmol/μg [^{3}H]ROH. In Figure 2 the maximum concentration of [^{3}H]ROH ($t = 30$ min) was 70 dpm/μg, which corresponds to 17 fmol/μg [^{3}H]ROH; in good agreement with the expected value. Further evidence for a specific binding

process comes from the observation that 4 h after administration of [^{3}H]ROH:RBP–TTR to killed Sertoli cell controls (Table II) the concentration of [^{3}H]ROH was 98 ± 15 dpm/μg, which is equivalent to 23 ± 3 fmol/μg [^{3}H]ROH and also in reasonable agreement with the value predicted from the Scatchard analysis. These control cells, which appeared morphologically intact by phase-contrast microscopy, apparently did not take up [^{3}H]ROH since 28 h following removal of medium [^{3}H]ROH:RBP–TTR the cellular concentration of [^{3}H]ROH fell to 2.4 ± 0.6 dpm/μg (0.6 ± 0.1 fmol/μg), suggesting that the binding process was reversible.

The implications of a two-pool model may prove to be important in assessing the role of retinyl esters in Sertoli cells and in understanding the function of the CRBP. The presence of CRBP in Sertoli cells has been clearly demonstrated in Sertoli cell cultures in our laboratory (Huggenvik & Griswold, 1981) and immunohistochemically by Porter et al. (1985). There also appears to be a quantitative correlation between the intracellular retinol pool ([ROH]$_{in}$) postulated in our model and CRBP. Using a sensitive radioimmunoassay, Blaner et al. (1987) have determined the concentration of CRBP in primary Sertoli cell cultures derived from normal 22-day-old rats to be 1.11 ± 0.2 μg/mg (75 ± 14 fmol/μg), in excellent agreement with the concentration of endogenous ROH (75 ± 13 fmol/μg; n = 19) found in the present study. Furthermore, the observations that the concentration of CRBP undergoes a significant decrease in the testis of VAD rats (Chytil & Ong 1984; Kato et al., 1985a,b) could provide an explanation for the very low concentration of cellular [^{3}H]-ROH that eventually resulted following [^{3}H]ROH:RBP–TTR administration to VAD Sertoli cells (compare Figure 5 with Figures 2 and 4 and Table II with Table V).

The function of retinol esters in Sertoli cells is not known; possibly they are primarily a storage reserve for cellular ROH, or possibly they are also involved in intercellular transport of ROH. Since it is well established that ROH, but not retinoic acid, can serve as a precursor for retinyl esters (Arens & van Dorp, 1946) and that only ROH and retinyl esters can support spermatogenesis (Thompson et al., 1964; Howell et al., 1964), it is possible that a direct link between retinyl esters and spermatogenesis may exist. In the testis, Sertoli cells form intercellular tight-junctional complexes (a blood–testis barrier) that effectively block direct transfer of serum constituents to germinal cells (Fawcett, 1975). It is possible that ROH taken up by Sertoli cells from serum is first esterified before being transferred to germinal cells. Ottonello et al. (1987) have recently shown that in a cell-free system from bovine pigment epithelial cells ROH uptake from RBP underwent a 4-fold reduction under conditions where retinol esterification was specifically inhibited. These same studies presented evidence that ROH bound to a plasma membrane receptor was the actual substrate for esterification and also that apo-CRBP could activate the hydrolysis of retinyl esters, resulting in the formation of ROH bound to CRBP. Thus it would appear from these studies that ROH uptake and retinyl esterification are functionally coupled—a conclusion consistent with the model presented in the present study. Although more work is clearly needed to establish the mechanism of transport of retinol from Sertoli cells to germinal cells, it is likely that retinyl esters function as an intermediate species during this process.

Acknowledgments

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Registry No. ROH, 68-26-8; [10,11-$^3H_2$]ROH, 110773-30-3; retinyl palmitate, 79-81-2; vitamin A, 11103-57-4; [10,11-$^3H_2$]-retinaldehyde, 110773-31-4.

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Kinetics and Concentration Dependency of cAMP-Induced Desensitization of a Subpopulation of Surface cAMP Receptors in *Dictyostelium discoideum*†

Peter J. M. Van Haastert
Cell Biology and Genetics Unit, Zoological Laboratory, University of Leiden, 2300 RA Leiden, The Netherlands
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ABSTRACT: Extracellular cAMP induces the rapid activation of guanylate cyclase, which adapts within 10 s to constant cAMP concentrations. A new response can be induced either by a higher cAMP concentration or by the same cAMP concentration at some time ($t_{1/2}$ = 90 s) after removal of the previous stimulus. Stimulation of guanylate cyclase is supposed to be mediated by a subpopulation of cell surface cAMP receptors (B-sites). These sites can exist in three states, B^F, B^S, and B^{SS}, which interconvert in a cAMP and guanine nucleotide dependent manner. It has been proposed that the transition of B^S to B^{SS} represents the activation of a guanine nucleotide regulatory protein [Van Haastert, P. J. M., De Wit, R. J. W., Janssens, P. M. W., Kesbeke, F., & DeGoede, J. (1986) *J. Biol. Chem. 261*, 9604-9611]. Binding of [^{3}H]cAMP to these sites was measured after a short preincubation with an identical concentration of nonradioactive cAMP. [^{3}H]cAMP could still bind to B^F and B^S, but not to B^{SS}, indicating that the transition of B^S to B^{SS} is blocked by the preincubation with cAMP. This blockade was rapid and showed first-order kinetics with $t_{1/2}$ = 4 s. A half-maximal blockade was induced by 0.7 nM cAMP; at this concentration only 5% of the B-sites are occupied with cAMP. The blockade of the transition of B^S to B^{SS} was released by two conditions: (i) When the concentration of cAMP was increased, the blockade was released within a few seconds. (ii) When cAMP was removed, the blockade was released slowly with $t_{1/2}$ = 90 s. Finally, cAMP did not induce the blockade under conditions where guanylate cyclase did not adapt, i.e., at 0 °C and in cells starved for 2 h or less. These results suggest that the interaction of cAMP with the B-sites induces a rapid and reversible blockade of the terminal step in the generation of an active G-protein and that this blockade could be the molecular basis of adaptation of guanylate cyclase.

The eukaryotic microorganism *Dictyostelium discoideum* is a suitable organism to study desensitization of signal transduction pathways. In this organism the hormone-like substance is cAMP, which is detected by cell surface receptors. Extracellular cAMP induces the rapid activation of guanylate cyclase (Mato & Malchow, 1978) and the slower activation of adenylate cyclase (Roos & Gerisch, 1976). Intracellular cGMP reaches a peak at 10 s and declines to prestimulated levels within about 30 s (Mato et al., 1977). Intracellular cAMP reaches maximal levels after 60-120 s and is secreted, thus acting as an autocatalytic feedback loop (Gerisch & Wick, 1975).

Prolonged stimulation of *D. discoideum* cells with constant cAMP concentrations induces desensitization by at least two mechanisms: (i) One is down-regulation of cAMP-binding activity after a long incubation (5 min) with high cAMP concentrations (above 0.1 μM). After removal of cAMP, cells resensitize with a half-life of about 60 min (Klein & Juliani, 1977; Klein, 1979). (ii) Another is a rapid desensitization of the cAMP-mediated activation of adenylate and guanylate cyclases by nanomolar cAMP concentrations with the characteristics of adaptation; i.e., the activity of the cyclases fades in the presence of a constant cAMP concentration but can be reactivated by increasing the cAMP concentration (Devreotes & Steck, 1979; Van Haastert & Van der Heijden, 1983). After removal of cAMP, cells deadapt with a half-life of 1-2 min for the guanylate cyclase and 3-4 min for the adenylate cyclase. Adaptation of the cAMP-mediated cGMP accumulation is completed within 10 s, while adaptation of the cAMP accumulation is completed after about 5 min (Dinauer et al., 1980a,b; Van Haastert & Van der Heijden., 1983).

Adaptation of adenylate cyclase stimulation has been correlated with a covalent modification, presumably phosphorylation, of the receptor (Devreotes & Sherring, 1985; Klein, C., et al., 1985; Klein, P., et al., 1985, 1987). This receptor modification is probably not the molecular mechanism for adaptation of guanylate cyclase stimulation, since adaptation

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