Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 4658–4662, July 1985
Biochemistry

Metabolism of retinoic acid and retinol during differentiation of F9 embryonal carcinoma cells

(retinoids/teratocarcinoma/laminin ELISA)

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Communicated by Ronald W. Estabrook, April 1, 1985

ABSTRACT **Retinol and retinoic acid dose–response curves were obtained for promotion of the differentiation of F9 murine embryonal carcinoma cells with an enzyme-linked immunoadsorbent assay for laminin, a product of differentiated F9 cells. Retinoic acid produced a half-maximum response at 1.3 nM and a maximum response at about 30 nM; retinol was 1/175th as potent. Maximum differentiation required 48 hr of continuous exposure to retinoic acid, whereas retinol required 72 hr of exposure. The half-time of retinoic acid conversion into polar metabolites was 3.5 hr; metabolism was accelerated by pretreating F9 cells with retinoic acid. An inhibitor of oxidative metabolism, ketoconazole, decreased the rate of retinoic acid metabolism and decreased the concentration of retinoic acid required to produce a half-maximum response. Unchanged retinoic acid was the sole compound isolated from nuclei of F9 cells incubated with retinoic acid. Retinol had a half-life approximately 5-fold longer than retinoic acid, attained greater cell concentrations, and was converted into retinoic acid by F9 cells. These data indicate that retinoic acid itself directs the differentiation of F9 cells and may mediate differentiation induced by retinol.**

Retinol, the major plasma retinoid, supports all known vitamin A functions *in vivo* (1, 2). Retinoic acid, a metabolite in the physiological pathway of retinol metabolism (3, 4), supports growth and epithelial differentiation but does not support vision or reproduction in mammals (5). Retinol and retinoic acid may function in part through two distinct cytoplasmic binding proteins (6, 7): cellular retinol-binding protein, which specifically binds retinol; and cellular retinoic acid-binding protein, which specifically binds retinoic acid. Retinoids appear to have both nuclear (8, 9) and extranuclear mechanisms of action (10, 11). The biochemical roles of retinoic acid relative to retinol are unclear, as is the function of retinoic acid metabolism.

Embryonal carcinoma (EC) cells are studied as models of differentiation (12). Naturally occurring retinoids are the only physiological substances that induce differentiation of the murine EC cell line F9 (13, 14). F9 cell responses to retinoic acid include decreased proliferation, loss of tumorigenicity, cell surface antigen changes, and alterations in protein synthesis. Retinoic acid-differentiated F9 cells secrete laminin, type IV collagen, and plasminogen activator, whereas these proteins are synthesized only at low levels, if at all, in undifferentiated cells (15, 16). Retinoic acid treatment stimulates adenylate cyclase activity (17) and cAMP-dependent protein kinase activity (18) in F9 cells. EC cells are sensitized by retinoic acid to cAMP, which promotes their differentiation into parietal endoderm (15). Thus, EC cells, with a homogeneous cell population and quantifiable biochemical markers of vitamin A action, provide a model system for investigating the structure/activity relationships of retinoids.

This report will demonstrate that retinoic acid is 175-fold more potent than retinol in inducing F9 cell differentiation and that retinoic acid itself, rather than its metabolites, mediates differentiation. The response to retinol may be mediated by retinoic acid.

MATERIALS AND METHODS

Retinoids. Retinol and retinoic acid were purchased from Sigma. 5,6-Epoxyretinoic acid was synthesized (19). [11,12-^{3}H]Retinoic acid (23–32 Ci/mmol, 1 Ci = 37 GBq), 13-*cis*-retinoic acid, and the all-*trans*-isomers of 4-oxo-16-hydroxy-, 4-oxo-, and 4-hydroxyretinoic acid were gifts of Hoffmann–La Roche (Nutley, NJ). [^{3}H]Retinoic acid was purified by elution through a reversed-phase HPLC column with 10 mM ammonium acetate in methanol/water (7:3, vol/vol; elution volume 56 ml). [11-^{3}H]Retinol (1.9 Ci/mmol) was prepared as described from [11-^{3}H]retinal (20), which was obtained from the Biological and Chemical Chemoprevention Program, Division of Cancer Cause and Prevention, National Cancer Institute. [11,12-^{3}H]Retinol (43 Ci/mmol) was purchased from Amersham. Retinol was purified by elution through a normal-phase HPLC column with acetone/hexane (7:93, vol/vol; elution volume 25 ml).

Cell Culture. F9 cells were obtained from Gail Martin (University of California, San Francisco) and were maintained in 2 ml of Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (GIBCO), sodium pyruvate (1 mM), sodium bicarbonate (44 mM), penicillin (100 units/ml), streptomycin (100 μg/ml), and Fungizone (25 mg/ml). Cells were grown in Corning dishes (35 mm, unless otherwise noted) in a humidified atmosphere of air/CO_2 (95:5) at 37°C with medium changes every 24 hr. Ketoconazole, obtained from Janssen Pharmaceutica (Piscataway, NJ), and retinoids were added in ethanol (2 μl). Cell number was determined with a hemocytometer in the presence of 0.1% trypan blue.

HPLC. HPLC was performed on Waters Associates equipment with reversed-phase columns purchased from Waters Associates (0.8 × 10 cm, radially compressed octadecylsilane bonded phase, 10 μm particles) or with normal-phase columns purchased from DuPont (0.46 × 25 cm, Zorbax-Sil). Flow rates were 2 ml/min, unless noted otherwise. Radioinert retinoids were detected by ultraviolet absorbance at 340 nm.

Analyses of Retinoid Metabolites. After incubating with [^{3}H]retinoic acid or [^{3}H]retinol, the medium was removed and the cells were harvested with 0.02% EDTA/phosphate-buffered saline. The cells were washed twice with phosphate-buffered saline, and the washes were combined with the

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medium. Water was removed from the samples under reduced pressure by azeotropic distillation with ethanol. The residues were extracted three times with methanol containing butylated hydroxytoluene (5 μg/ml). At least 95% of the radioactivity was recovered. Internal standards were added, the methanol solution was concentrated, and the metabolites were analyzed by HPLC. The data expressed in the text are the averages of two to three replicates. Controls consisted of incubating the [^{3}H]retinoid in medium alone.

Analysis of Nuclear Retinoic Acid Metabolites. Nuclei from four dishes (100 mm) of cells, incubated for 6 hr with 50 nM [^{3}H]retinoic acid (32 Ci/mmol), were isolated by established methods (8, 21). Briefly, cells were harvested by gentle scraping, washed three times with phosphate-buffered saline, and treated for 1 min with a buffer of 30 mM Hepes, 200 mM sucrose, 40 mM NaCl, 5 mM $MgCl_2$, and 0.5% Nonidet P-40 pH 8.0. Nuclei were pelleted at 7000 × *g* and were resuspended in a buffer of 50 mM Tris·HCl/300 mM sucrose/25 mM KCl/1 mM EDTA/4 mM $MnCl_2$/1 mM 2-mercaptoethanol, pH 7.5. The nuclei were recovered by centrifugation through 1.2 M sucrose at 7000 × *g* and were extracted with ethanol. Radioinert standards were added, and the retinoids were analyzed by HPLC.

Rates of Retinoic Acid Metabolism. [^{3}H]Retinoic acid was incubated with 10^6 cells for each determination, or with medium (2 ml) alone (control). Ketoconazole was added 30 min before the [^{3}H]retinoic acid. For cells pretreated with radioinert retinoic acid, the medium was removed; the cells were washed with phosphate-buffered saline; and fresh medium was added prior to adding [^{3}H]retinoic acid. The reactions were quenched by the addition of 0.2 M HCl in methanol (2 ml). Radioinert retinoic acid (5 μg) was added and each sample was extracted with hexane (2 ml). The hexane was removed under a stream of nitrogen and the residue was applied to a reversed-phase HPLC column. [^{3}H]Retinoic acid and 13-*cis*-[^{3}H]retinoic acid were eluted from the column with 10 mM ammonium acetate in methanol/water (75:25, vol/vol) in 33 and 27 ml, respectively. The amount of total [^{3}H]retinoic acid remaining was determined by measuring the radioactivity eluting with each isomer and correcting for recovery. Recovery was determined to be 80% from a standard curve relating the peak height of the radioinert retinoic acid to retinoic acid mass.

Quantification of Retinoic Acid Synthesis. Cells were harvested by gentle scraping and were suspended in 25-ml Erlenmeyer flasks in 2 ml of medium. [^{3}H]Retinol (43 Ci/mmol) was added; the flasks were purged with $O_2/CO_2/N_2$ (5:5:90, vol/vol), stoppered, and incubated in a shaking water bath at 37°C for 2 hr. KOH (25 mM) in methanol (2 ml) and radioinert retinoic acid (5 μg) were added. Each sample was extracted twice with 2 ml of hexane to remove neutral lipids. The aqueous phase was acidified with 4 M HCl (0.17 ml), and retinoic acid was extracted with 2 ml of hexane. The hexane was evaporated under a stream of N_2. Ethanol (0.2 ml) and diazomethane in diethyl ether (0.1 ml) were added to the residue. The solvents were evaporated; the residue was dissolved in column solvent and was applied to a normal-phase HPLC column. Methyl retinoate eluted in 27 ml with mobile phase 1 (toluene/hexane; 45:55, vol/vol); methyl 13-*cis*-retinoate eluted in 19 ml. Methyl retinoate was reapplied to the normal-phase HPLC column and was eluted with mobile phase 2 (methyl *tert*-butyl ether/hexane; 1:99, vol/vol) in 15 ml; methyl 13-*cis*-retinoate eluted in 11 ml; retinol, methyl 4-oxoretinoate, and methyl 5,6-epoxyretinoate eluted in greater than 25 ml. [^{3}H]Retinoic acid was quantified by measuring the radioactivity recovered in the fractions comigrating with radioinert methyl retinoate. Data were corrected for recovery by using a standard curve relating the peak height of radioinert methyl retinoate to the amount of retinoid.

Quantification of Laminin. Sixteen hours after cells were plated (3000/cm^2), the medium was changed, and dibutyryl cAMP (1 mM, Sigma) was added, as was either retinoid or vehicle alone (day 1). The medium and dibutyryl cAMP were replenished daily for a total of 4 days. The amount of laminin in the medium at the end of day 4 was measured by an enzyme-linked immunoadsorbent assay (ELISA) modified from the procedure of Rennard *et al.* (22). Briefly, 1:100 dilutions of medium aliquots or known amounts of purified laminin (standard curve) were incubated with rabbit antiserum to mouse laminin (E-Y Laboratories, San Mateo, CA) at 4°C for 16 hr. The samples were transferred to laminin-coated (100 ng/well) Nunc microtest plates. After 30 min at 25°C, the plates were washed, and a horseradish peroxidase-IgG conjugate was added to each well. After 90 min of incubation, unbound enzyme-IgG complexes were washed out, and *o*-phenylenediamine was added as substrate. The reaction was quenched after 30 min with 2 M sulfuric acid. Absorbance at 492 nm was read with a Titertek Multiscanner. Dose–response data were analyzed by computer, using a statistical method for fitting sigmoidal dose–response curves (23).

RESULTS

Dose–Response Curves. Complete dose-response curves quantifying the effectiveness of retinoic acid and retinol were obtained with an ELISA for laminin, a marker of F9 cell differentiation (15, 24). A half-maximum response was produced by either 1.3 nM retinoic acid or 226 nM retinol (Fig. 1*A*). Retinoic acid (30–50 nM) and retinol (3–5 μM) produced equivalent maximum responses, which resulted in laminin concentrations of 5 μg/ml, 10-fold greater than in control medium. Maximum laminin concentrations at the end of day 4 required 48 hr of continuous exposure to retinoic acid (on days 1 and 2). Exposure for 24 hr (day 1 only) to the same concentration of retinoic acid (30 nM) achieved only 80% of the maximum, whereas 12 hr of exposure (on the first 12 hr of day 1) achieved only 39% of the maximum. For retinol (3 μM), a maximum response required 72 hr of continuous exposure. Exposure for 24 hr achieved only 60% of the maximum laminin production. Ketoconazole, in the absence of retinoic acid, had no effect on laminin production, but 10

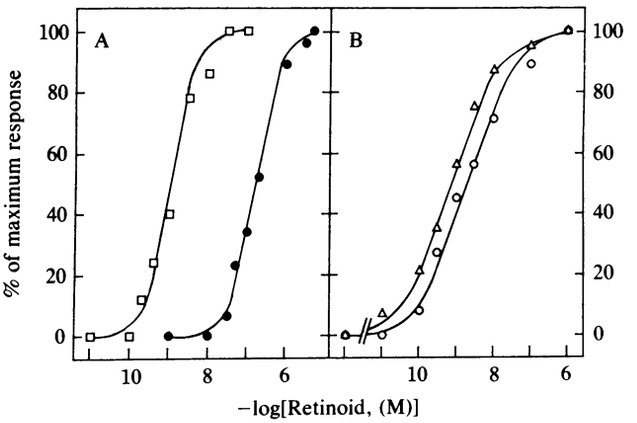

FIG. 1. Retinoid-induced differentiation of F9 cells. Cells were incubated with retinoids and dibutyryl cAMP (1 mM) for 96 hr (*A*) or 24 hr (*B*). □, Retinoic acid; ●, retinol; △, retinoic acid and 10 μM ketoconazole; and ○, retinoic acid in the absence of ketoconazole. Data are expressed as percent of maximum laminin concentration in the medium at the end of the 4th day. Each point is the mean of data from three culture dishes; laminin was assayed in triplicate. Variation among the nine values was less than 15%. Both experiments were repeated; the same results were obtained.

μM ketoconazole increased the potency of retinoic acid 3-fold (Fig. 1*B*).

Retinoic Acid Metabolism. During incubation of F9 cells with 50 nM [^{3}H]retinoic acid, the amount of cell-associated retinoic acid steadily decreased from 1 pmol per 10^6 cells at 2 hr to about 0.013 pmol per 10^6 cells at 24 hr (Fig. 2). Qualitatively similar metabolites were observed in both cells and media after 2, 4, 6, 12, and 24 hr. Reversed-phase HPLC profiles of metabolites obtained at 6 hr were representative (Fig. 3). Medium and cells each contained a group of polar metabolites (fractions 40–60), and a group of very polar metabolites (fractions 3–15), which were absent from the control. After 6 hr the medium contained 99% of the retinoids, but only 44% was retinoic acid, whereas 76% of the retinoids in the cells was retinoic acid. In a separate experiment, retinoic acid accounted for 94% of the retinoids in the nuclei after 6 hr. After 24 hr, the medium still contained at least 99% of the retinoids, but only 0.5% was retinoic acid; in contrast, 60% of the retinoids in the cells was retinoic acid. Quantitatively significant steady-state metabolites comigrating with 4-oxoretinoic acid or retinoyl glucuronide were not observed; the presence of 13-*cis*-retinoic acid and 5,6-epoxyretinoic acid in the control indicates that these retinoids were not metabolic products.

The rates of retinoic acid metabolism were measured in the presence of 10 μM ketoconazole, an inhibitor of oxidative metabolism (25), and in cells pretreated with 100 nM retinoic acid for 10 hr. A plot of the natural logarithm of the percent remaining retinoic acid versus time was linear in each case (correlation coefficients > 0.95) and revealed an apparent first-order clearance (Fig. 4). The half-life of retinoic acid in F9 cells was calculated ($t_{1/2}$ = 0.693/slope) to be 3.5 hr; 10 μM ketoconazole increased the half-life approximately 3-fold to 11.6 hr; retinoic acid pretreatment decreased it approximately 1.5-fold to 2.3 hr. Analysis of the metabolites in the media after 12 hr of incubating retinoic acid with the cells in the presence of ketoconazole revealed a 4-fold decrease in the metabolites in fractions 3–15, a 4-fold increase in 4-hydroxyretinoic acid, a catabolite, and a 5-fold increase in retinoic acid.

Retinol Metabolism. During incubation, [^{3}H]retinol (1 μM) had a half-life of 16 hr (data not shown). Intracellular retinol reached a concentration of 147 pmol per 10^6 cells at 4 hr and

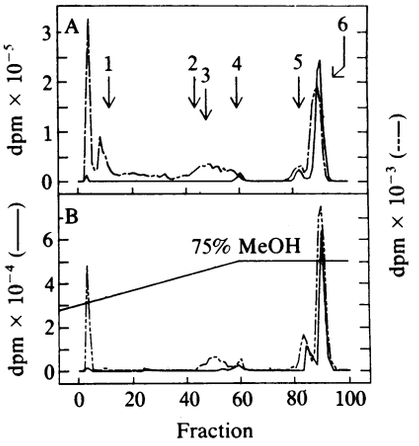

FIG. 3. HPLC analysis of retinoic acid metabolism. [11,12-^{3}H]Retinoic acid (32 Ci/mmol, 50 nM) was incubated with F9 cells or in medium alone (control) for 6 hr. (*A*) —, Control medium; - - -, medium of cells. (*B*) —, Nuclei; - - -, cells. The elution positions of radioinert internal retinoic acid standards are indicated: 1, 4-oxo-16-hydroxy-; 2, 4-oxo-; 3, 4-hydroxy-; 4, 5,6-epoxy-; 5, 13-*cis*-retinoic acid; and 6, retinoic acid. Extracts were eluted from a reversed-phase HPLC column over a 30-min period by using a linear gradient of methanol/water (45:55 to 75:25, vol/vol) containing 10 mM ammonium acetate, at a flow rate of 2.0 ml/min. Data are plotted as total radioactivity per fraction; fractions were 1 ml each.

then declined to 48 pmol per 10^6 cells by 24 hr (Fig. 2). Retinyl esters, found exclusively in the cells, accumulated to an apparent steady-state concentration of 17 pmol per 10^6 cells by 10 hr. The metabolites of retinol present at 2, 4, 10, and 24 hr were qualitatively similar. HPLC profiles obtained at 24 hr (Fig. 5) were representative. After 24 hr, 68% of the retinoids in the cells was retinol. The major metabolite in the cells comigrated with retinyl palmitate (17% of the total). The remaining metabolites, 14% of the total after correcting for the amount observed in the control, were polar metabolites (fractions 39–53; Fig. 5). In the medium, 35% of the retinoids was retinol. Three groups of polar metabolites were observed in the medium (fractions 2–10, 39–45, and 46–53; Fig. 5) representing 14, 17, and 4%, of the total, respectively. The

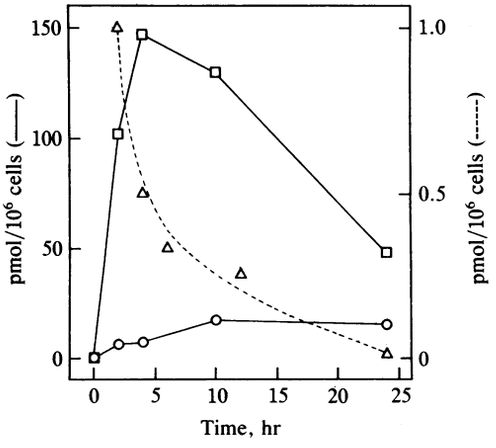

FIG. 2. Concentrations of retinoic acid, retinol, and retinyl esters in F9 cells as a function of time. F9 cells were incubated with either 50 nM [11,12-^{3}H]retinoic acid (32 Ci/mmol) or 1 μM [11-^{3}H]retinol (1.9 Ci/mmol). △, Retinoic acid; □, retinol; ○, retinyl esters. Each point is the mean of two or three replicates. Variations were less than 15%. The amounts of retinoids present were measured by HPLC as described in the legend of Fig. 3 (retinoic acid) or Fig. 5 (retinol and retinyl esters).

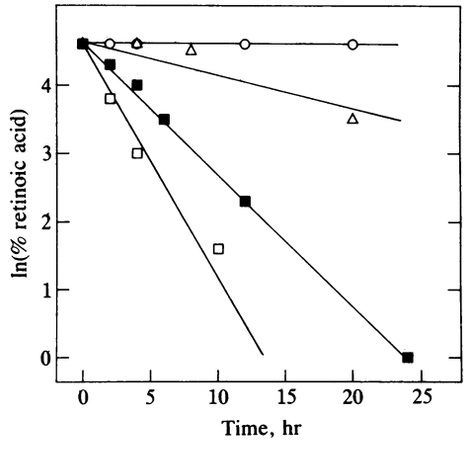

FIG. 4. Rates of F9 cell retinoic acid metabolism. The amount of [11,12-^{3}H]retinoic acid (23 Ci/mmol, 50 nM) remaining as a function of time was determined in medium alone (○), in the presence of F9 cells (■), in the presence of cells and 10 μM ketoconazole (△), and in the presence of cells pretreated with 100 nM radioinert retinoic acid (□). Measurements were made on two dishes per time point by reversed-phase HPLC. The duplicate values for each point differed by less than 7%.

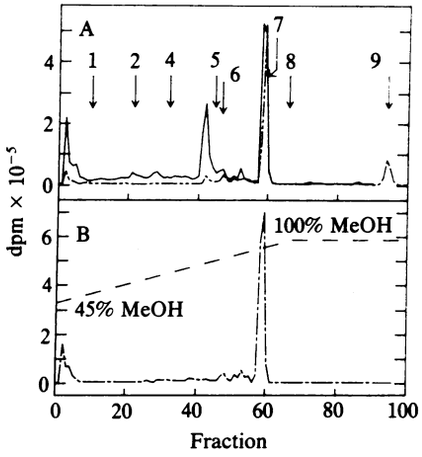

FIG. 5. HPLC analysis of retinol metabolites. [^{3}H]Retinol (1 μM, 1.9 Ci/mmol) was incubated with F9 cells or in medium alone (control) for 24 hr. (*A*) - - -, Cells; —, medium of cells. (*B*) Control medium. The elution positions of radioinert internal standards are indicated: 1–6 are identified in the legend of Fig. 3; 7, retinol; 8, methyl all-*trans*-retinoate; 9, retinyl palmitate. Retinoids were quantified by reversed-phase HPLC using a linear gradient of methanol/water (45:55) to methanol, containing 10 mM ammonium acetate, over a 45-min period at a flow rate of 3 ml/min. Fractions were 2 ml each.

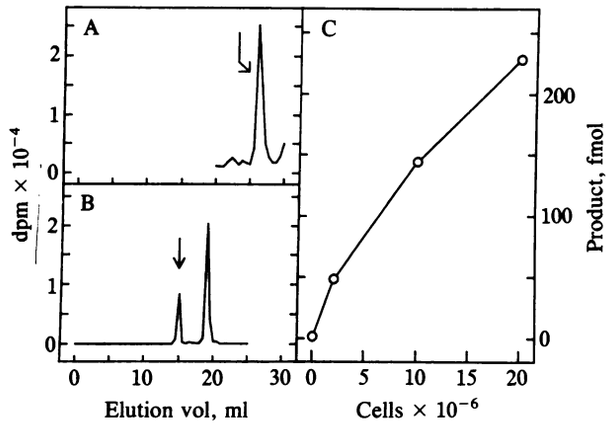

FIG. 6. Conversion of retinol into retinoic acid by F9 cells. [11,12-^{3}H]Retinol (150 nM, 43 Ci/mmol) was incubated with increasing numbers of F9 cells for 2 hr. (*A*) The extracts were first analyzed on HPLC with mobile phase 1; 0.5-ml fractions were collected from 20 to 31 ml. (*B*) Material recovered from HPLC mobile phase 1 was reanalyzed with mobile phase 2; 0.5-ml fractions were collected. The arrows mark the elution positions of methyl all-*trans*-retinoate. (*C*) Relationhip between cell number and amount of products migrating with methyl retinoate. Each point is the mean of duplicate determinations; differences were less than 15%.

formation of the polar materials in the control was dependent upon the length of incubation and was not caused by the analytical methods because the peaks were not observed after 2 hr of incubation. No peak that was greater than the peaks in the controls was detected migrating with retinoic acid in cells or medium. Treating the predominant medium peak (fractions 39–45) with β-glucuronidase (19) did not affect its elution position from HPLC—*i.e.*, did not release retinoic acid.

The limit of detection was no lower than 1 pmol with the low specific activity retinol and with the methods used to examine the spectrum of retinol metabolites. An experimental approach with greater sensitivity was applied to determine whether F9 cells produced small amounts of retinoic acid. The procedure relied upon [^{3}H]retinol of 23-fold higher specific activity and methylation of the extract, followed by sequential analysis on two normal-phase HPLC systems to reduce background and to ensure specificity. A radioactive peak migrating with authentic methyl retinoate was observed during the first HPLC analysis (Fig. 6*A*); its production was cell dependent (Fig. 6*C*). Reanalysis on the second HPLC system (Fig. 6*B*) showed that the peak consisted of retinoic acid (33%) and an unidentified metabolite (66%). The rate of retinoic acid synthesis was determined with the data from the second HPLC column to be 54 fmol/2 hr per 10^7 cells. Radioactive material in the controls, which eluted with retinoic acid from the first HPLC column and would include the unidentified metabolite, was below the limit of detection (<2.5 fmol; <250 dpm). The unidentified metabolite did not elute with any known retinoid.

DISCUSSION

Fundamental to understanding the mechanisms of vitamin A action is elucidating which retinoids elicit specific responses. The complex array of retinol and retinoic acid metabolites produced *in vivo*, the influence of vitamin A status on their production, and the rapid rate of retinoic acid turnover (19, 26) indicate that obtaining such knowledge could be problematic. In this regard, tissue homogenates or tissue explants suffer the disadvantages of heterogeneous cell populations, the inability to relate appearance of metabolites with the expression of specific responses, or both. F9 EC cells are a homogeneous population of undifferentiated cells and retinoids are the only naturally occurring substances that promote their differentiation (13–17). Thus, these cells afford a propitious model system in which to examine the relationship between metabolism and differentiation induced by vitamin A.

The extensive production of metabolites during differentiation of F9 cells may allow accumulation of an active metabolite(s), decrease the effectiveness of retinoic acid, or both. No metabolite was observed in the cells in greater abundance than was observed in the medium; no differences were noted between the cell and medium metabolites; and exposing cells to retinoic acid accelerated its metabolism. These observations indicate that retinoic acid metabolism most likely represents conversion into hydrophilic excretion products and is similar to its disposition *in vivo* (19, 26, 27). Although the cellular concentration of retinoic acid decreased with time, retinoic acid remained the most abundant retinoid in the cells. Ketoconazole, which inhibited the appearance of polar metabolites and produced a 3-fold increase in the half-life of retinoic acid, yielded a corresponding 3-fold increase in the potency of retinoic acid. Our results are consistent with the conclusion that retinoic acid acts directly to induce differentiation and modulates its own activity by accelerating the rate of its degradation.

Retinol has been estimated to be inactive in F9 cells (14), two orders of magnitude less active than retinoic acid (28) and approximately 10% as active as retinoic acid (29). These various conclusions were based on qualitative assessments of plasminogen activator activity and limited data. For example, the highest estimate of activity was based on the assessment of a single retinol concentration. In contrast, in this study complete dose–response curves were obtained by direct quantification of a product of differentiated cells (laminin), and retinol that was purified by normal-phase HPLC prior to assay was used. Commercially available retinol is impure, and retinol is transformed into polar materials on storage. The standard method of purification of retinol by reversed-phase HPLC is much less effective (30) than normal-phase.

The most likely explanation for the lesser potency of retinol compared to that of retinoic acid is that its activity requires metabolism, perhaps to retinoic acid. Several ob-

servations support the requirement of metabolic conversion for activity. Retinoic acid is produced from retinol by F9 cells, albeit at a low rate. The amount of cellular retinoic acid that commits cells to differentiate is also low. In medium containing 50 nM retinoic acid, the average amount of cellular retinoic acid found was less than 0.1 pmol per 10^6 cells between 12 and 24 hr—a period in which the number of cells committed to differentiate doubled (Fig. 2). At 1 μM retinol, which is virtually equipotent to 50 nM retinoic acid, cellular retinol levels increased rapidly and remained high (Fig. 2), providing ample retinol to support continuous production of retinoic acid. Moreover, the induction of maximum F9 cell differentiation required considerably longer exposures to retinol (72 hr) than to retinoic acid (48 hr). This finding and the low rate of conversion of retinol into retinoic acid are consistent with a requirement for accumulation of sufficient product as a prerequisite to differentiation induced by retinol. The alternate explanation, that retinol and retinoic acid act independently, is not excluded by these data. Its demonstration, however, would require an ability to quantify femtomole amounts of retinoic acid during F9 cell differentiation and an inhibitor of retinoic acid synthesis.

Our observation of retinoic acid as the sole retinoid in F9 cell nuclei after incubation of intact cells with retinoic acid complements the recent report that cellular retinoic acid-binding protein mediates specific uptake of retinoic acid by isolated F9 cell nuclei (31). These data are consistent with and expand current concepts that alteration of gene expression is one mechanism of retinoid action (32). It would now be interesting to determine why a need exists for 24 to 48 hr of exposure of cells to retinoic acid to effect a maximum response.

We are grateful for the expert technical assistance of Mr. Charles O. Shields, for helpful conversations with Dr. A. M. McCormick, and for the computer graphics programs of Dr. Julian A. Peterson and Dr. Peter W. Roome. This research was supported by Grant AM26535 from the National Institutes of Health and by National Institutes of Health Service Award GM07062.

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