Retinoid metabolism in spontaneously transformed mouse fibroblasts (Balb/c 3T12-3 cells): enzymatic conversion of retinol to anhydroretinol

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Abstract Spontaneously transformed mouse fibroblasts (Balb/c 3T12-3 cells) displayed an increased adhesion when cultured in the presence of 10^{-6} M all-*trans* retinol and acquired morphological characteristics of the normal phenotype. Thus it was of interest to investigate the metabolism of [15-^{14}C]retinol in this system. Within 24 hours of culture, approximately 4.25% of the [^{14}C]retinol was taken up by the cells. The hydrocarbon [^{14}C]anhydroretinol was a major metabolic product and was identified by gas–liquid chromatography and by its typical ultraviolet absorption spectrum with maxima at 386, 364, and 346 nm. At 24 and 40 hours anhydroretinol represented 27% and 55%, respectively, of the total nonpolar metabolites or approximately 16% and 30% of the total radioactive products. Formalin-fixed fibroblasts or cultured intestinal mucosal cells did not convert retinol into anhydroretinol. A more polar product with a UV absorption maximum at 310 nm was also found. The time course of the synthesis of this product by 3T12 cells suggested a precursor–product relationship with anhydroretinol. A microsomal preparation from 3T12 cells was also active in synthesizing [^{14}C]anhydroretinol and [^{14}C]metabolite-310 from [^{14}C]retinol. Moreover incubation of metabolite-310 with the 3T12 microsomes yielded anhydroretinol (40% conversion in 30 minutes), suggesting that metabolite-310 is an intermediate in the synthesis of anhydroretinol by these cells. Anhydroretinol appears to be an end product of the metabolism of retinol in 3T12-3 cells, as suggested by the finding that over 90% of [^{14}C]anhydroretinol incubated for 30 hours with 3T12-3 cells was recovered unaltered, without the formation of detectable retroretinol, retinol, or retinoic acid.—**Bhat, P. V., L. M. De Luca, S. Adamo, I. Akalovsky, C. S. Silverman-Jones, and G. L. Peck.** Retinoid metabolism in spontaneously transformed mouse fibroblasts (Balb/c 3T12-3 cells): enzymatic conversion of retinol to anhydroretinol. *J. Lipid Res.* 1979. **20:** 357–362.

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Recent studies (1) have shown that retinoids are active in restoring properties of normal growth to cultured, spontaneously transformed mouse fibroblasts (Balb/c 3T12-3 cells) (2). These cells display higher adhesive properties and lower saturation densities upon treatment with several retinoids. These acquired properties were lost upon culturing treated cells in unsupplemented medium (3).

Since retinol was very active in increasing adhesion of 3T12-3 cells, it was of interest to investigate its metabolism in this system.

MATERIALS AND METHODS

Culturing of Balb/c 3T12-3 mouse fibroblasts

Balb/3T12-3 mouse fibroblasts were obtained from the American Type Culture Collection (Rockville, MD) and cultured in T25 flasks (Falcon Plastics, Oxnard, CA) in 3 ml of medium. Dulbecco's Modified Eagle Medium (Grand Island Biological Co., Grand Island, NY) was supplemented with 10% calf serum (Flow Laboratories, Rockville, MD), 25 mM HEPES, pH 7.3, and 50 μg/ml gentamicin (Microbiological Associates, Walkersville, MD). The medium was changed every day. Trypsinizations (both for cell counting and passages), the adhesion assay, and growth curves are described elsewhere (1, 3). [Carbinol-^{14}C]retinol (12 mCi/mmol) (Amersham-Searle, Arlington Heights, IL) was dissolved in dimethylsulfoxide (DMSO) and added to the culture medium at a concentration of 10 μg/ml.

Culturing of mouse dermal primary cells

Epidermis-free skins were obtained from newborn Balb/c mice as described (4), and incubated in a trypsinization flask with 0.25% crude collagenase (Worth-

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ington) in phosphate-buffered saline (PBS) for 30 min at 37°C. The suspension was filtered and spun at 700 *g* for 3 min. The pellet was resuspended in complete medium and spun at 350 *g* for 3 min. The supernatant was spun again at 350 *g* for 3 min. The last supernatant was diluted with complete medium and the cells were plated at 20,000 cells/cm².

Extraction of cells

All operations were conducted in red or yellow light. At the end of each incubation with [carbinol-^{14}C]retinol, the medium was removed and the cells were rinsed three times with 5 ml of cold PBS, which was discarded. The rinsed cells were immediately lyophilized for 2 hr and the metabolites were extracted two times with 50 ml of 99% methanol containing 0.05% butylated hydroxytoluene (BHT). The methanolic extract was used for chromatography on plates of silica gel in toluene–methanol–chloroform 4:1:1 (solvent A), in which retinylphosphate, retinol, and anhydroretinol have respective R_f values of 0.05, 0.6, and 0.82–0.86. The least polar fraction (R_f 0.82–0.86) was eluted from the scraped gel by methanol and used for chromatography on alumina.

Alumina column chromatography

Brockman Grade III Alumina was prepared as described (5) and a (1 × 7.5 cm) column was equilibrated with hexane. Anhydroretinol was eluted from this column with hexane, retinyl esters with 2% acetone in hexane, and retinol with 10% acetone in hexane (5).

Gas–liquid chromatography

Samples were analyzed on a Hewlett-Packard 5700 A gas chromatograph with flame ionization detector. A 42-cm glass column, internal diameter 4 mm made by Hewlett-Packard, was used for the chromatography. The column was packed with SE 30, 3%, 100/120 mesh, on Gas Chrom Q from Applied Science Laboratories, Inc.

The column temperature was 180°C, and the auxiliary temperature was 300°C. The carrier gas flow was 62 ml/min. The retention time for anhydroretinol was 5.6 min (338 sec) and for retinol was 11.2 min (670 sec).

A 10:1 splitter permitted the collection of the radioactive material into 2-in long capillary tubes with an internal diameter of 1 mm at the collection end and 2 mm at the exit end. Condensation of the eluted material was facilitated by cooling the collection tubes in dry ice. The capillary tubes were either counted directly in Econofluor or the condensed material was eluted with hexane. Efficiency of counting was 93% for radiocarbon. A Hewlett-Packard integrator 3370B was used for detection of peak areas.

Efficiency of collection was determined with standard anhydroretinol. Known quantities were injected into the gas chromatograph and peak areas were collected into capillary tubes, which were washed with hexane to dissolve the eluates. These were then scanned to record their UV spectra. The efficiency of collection was 52%.

The hexane-eluted fraction (5,300 dpm) from the alumina column was dried under nitrogen. This was then resuspended in 5 μl of hexane containing standard anhydroretinol (15 μg) and retinol (25 μg) and then injected into the gas chromatograph. Nine continuous areas were monitored with capillary tubes and counted directly in Econofluor. The eluate corresponding to anhydroretinol had 71% (2635 dpm) of the total eluted counts (3.6–6.4 min). The eluate before anhydroretinol had a total of 12.4% (467 dpm) of the counts. The area eluted immediately after anhydroretinol had 3.6% (135 dpm) of the counts (6.4–8.8 min), and 0.6% (23 dpm) were eluted in the retinol area (10.4–12.4 min). Two minutes after the retinol peak had been eluted the temperature of the column was raised to 250°C. Twelve percent (452 dpm) of the counts were collected in this area (15.2–22.4 min).

Microsomal incubation

Microsomes were prepared from 3T12-3 cells and incubated with 1 μg/ml of [carbinol-^{14}C]retinol dissolved in 10 μl of DMSO in the presence of 0.3 M Tris-HCl buffer (pH 7.6), 0.1 M $MnCl_2$, and 1.2 mg of microsomal protein in a final incubation volume of 0.2 ml for different time intervals. The reaction was stopped by the addition of 5 ml of 99% methanol containing 0.05% butylated hydroxytoluene. The methanolic extracts were evaporated under vacuum and anhydroretinol, retinol, and metabolite-310 were separated by alumina column chromatography.

Preparation of anhydroretinol

The procedure of Dunagin and Olson (6) was followed. The ultraviolet spectrum of the product in hexane showed maxima at 346, 366, and 386 nm, as reported by Dunagin and Olson (6). The yield of the reaction was about 39%.

Metabolism of [15-^{14}C]anhydroretinol by 3T12-3 cells

3T12-3 cells were seeded at 10,000 cells/cm² in six (10 cm) dishes and allowed to grow for 4 days. At day 5, 5 μg/ml of chemically prepared [15-^{14}C]anhydroretinol (sp act 5 μCi/μmol) was added to the dishes and two dishes were fixed with formalin just before adding the hydrocarbon.

Incubation proceeded for 30 hr as usual and the

methanolic extracts were processed for the isolation of anhydroretinol by chromatography on thin layers of silica gel (E. Merck A.G., Darmstadt, Germany) in solvent system A.

RESULTS

Formation of [15-^{14}C]anhydroretinol from [carbinol-^{14}C]retinol in 3T12-3 cells

Spontaneously transformed mouse fibroblasts (3T12-3 cells) incubated with [carbinol-^{14}C]retinol at 34×10^{-6} M for 24 hr take up approximately 4.25% of the radioactive precursor. Chromatography of the methanolic extract on thin layers of silica gel in solvent A showed three major radioactive peaks at R_f 0.05, 0.59, and 0.82 (**Fig. 1**). The compound at R_f 0.05 constituted 3.2% of the total radioactivity at 24 hr of incubation (**Table 1**). This peak did not show characteristics of retinylphosphate (7) by a variety of chromatographic techniques. It was also resistant to mild alkali under conditions that yield anhydroretinol from standard retinylphosphate. No [^{14}C]retinylphosphate was detected in any of six experiments conducted with 3T12-3 fibroblasts.

The compound at R_f 0.59 was shown to be intact [^{14}C]retinol, which represented 78% of the total cell-associated radioactivity at 1 hr and 33% at 24 hr (Table 1). Finally the least polar fraction at R_f 0.82–0.86 (Fig. 1) represented about 6.9% of the cell-associated radioactivity at 1hr and 53% at 24 hr of incubation (Table 1). Thus, as the amount of retinol

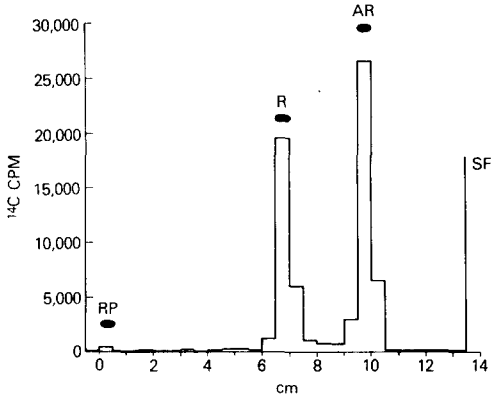


Fig. 1. Formation of metabolites of [15-^{14}C]retinol in 3T12-3 cells. Cells were incubated with [15-^{14}C]retinol, as described in Materials and Methods. An aliquot of the methanolic extracts was applied on thin layers of silica gel, which was developed in solvent system A (toluene–chloroform–methanol 4:1:1). Bands of 0.5 cm were scraped with an automatic zonal scraper, suspended in 250 μl of 99% methanol and counted. The positions of standard retinylphosphate (R_f 0.05), retinol (R_f 0.59), and anhydroretinol (R_f 0.82–0.86) are shown.

TABLE 1. Time-course study of the cellular uptake of [15-^{14}C]retinol and its conversion into compounds at R_f 0.82–0.86 by 3T12-3 cells

Incubation Time (hr)	Cellular Uptake % [^{14}C]Retinol	Percent of Total Cellular Radioactivity at		
		R_f 0.05 (RP)	R_f 0.59 (Free Retinol)	R_f 0.82 (Hydrocarbons, Retinyl Esters)
1	2.25	1.4	78	6.9
3	2.13	0.8	72	15.0
5	2.50	2.7	56	19.0
10	3.00	1.7	52	23.0
24	4.25	3.2	33	53.0
40	7.10	2.6	39	55.0

decreased, the amount of the least polar fraction increased.

The fraction at R_f 0.82–0.86 was eluted from the silica gel and rechromatographed on columns of alumina to yield two components, one eluted as standard anhydroretinol with hexane and the other eluted in 2% acetone in hexane. The UV absorption spectrum of the initial material obtained from the silica gel at R_f 0.82–0.86 is shown in **Fig. 2*A*** and has several maxima; the UV absorption spectrum of the compound eluted from alumina with hexane is identical to that of authentic anhydroretinol (Fig. 2*B*). Moreover calculations of specific radioactivity of the starting [carbinol-^{14}C]retinol and the known molar extinction coefficient of anhydroretinol at 346 nm revealed that 97% of the radiocarbon was present in this fraction as [^{14}C]-anhydroretinol. Gas–liquid chromatography of the hydrocarbon fraction eluted from alumina was performed as described in Materials and Methods on an SE-30 column. Of the material eluted from the column, 71% coeluted with external standard anhydroretinol.

The UV absorption spectrum of the more polar compound eluted in the 2% acetone in hexane fraction is shown in Fig. 2*C*, and displays a maximum at 310 nm. Neither compound was synthesized by formalin-fixed cells or by cultured intestinal cells from the hamster. The relationship between the [^{14}C]-anhydroretinol and ^{14}C-labeled metabolite-310 was investigated further in a time-course study, as shown in **Table 2.** The metabolite-310 represented 93% of the material that migrated at R_f 0.82–0.86 in solvent A at 1 hr of incubation. It had decreased to 42% at 40 hr. Conversely, at the same time, [^{14}C]anhydroretinol had increased from 7.3% to 55% (Table 2). This finding supported the concept that metabolite-310 is an intermediate in the conversion of retinol to anhydroretinol.

The conversion of retinol into metabolite-310 and anhydroretinol was also studied in cultured Balb/c 3T3 mouse fibroblasts as well as in primary mouse

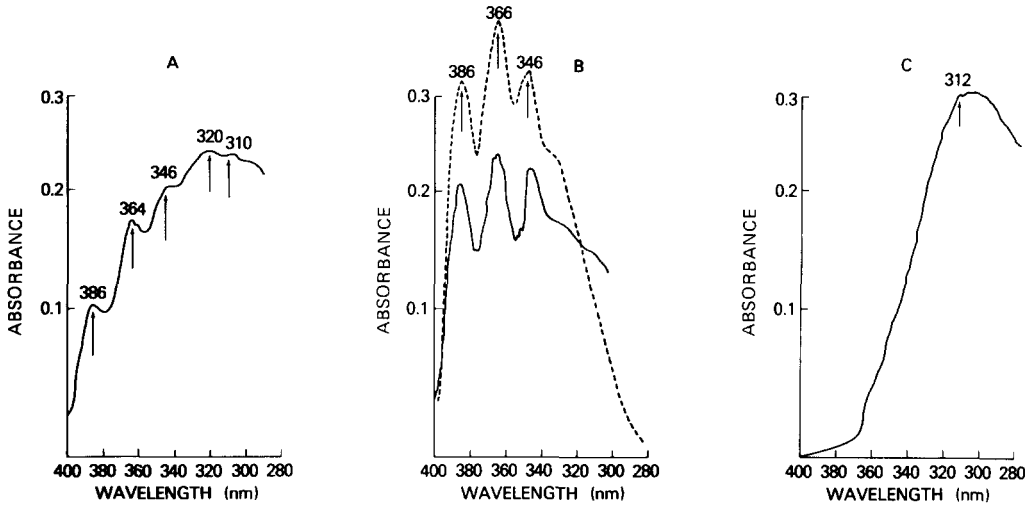


Fig. 2. *A.* Absorption spectra of the eluate obtained from thin-layer chromatography in solvent A. The compound at R_f 0.82–0.86 was obtained as described in Fig. 1. *B.* Absorption spectrum of the fraction obtained by alumina column chromatography by elution with hexane. The mixture of compounds at R_f 0.82–0.86 obtained by thin-layer chromatography was subjected to alumina column chromatography, as described in Table 2, and the hexane fraction was collected. An aliquot of the hexane eluate was taken for UV absorption spectroscopy. The dashed line represents the absorption spectrum of chemically prepared anhydroretinol (1.5 μg/ml in hexane). *C.* Absorption spectrum of the 2% acetone in hexane eluate. Alumina column chromatography of the mixture of compounds obtained at R_f 0.82–0.86 from thin-layer chromatography was carried out as described in Materials and Methods.

dermal fibroblasts. Both cell types were active in synthesizing anhydroretinol. However the primary cells only converted 10–15% of the retinol in 24 hr, whereas 3T3 cells yielded 40% anhydroretinol, which is comparable to 3T12 cells. Cell viability was not altered by retinol treatment. Cells from hamster intestinal mucosa did not convert retinol either to metabolite-310 or to anhydroretinol.

Formation of [15-^{14}C]anhydroretinol by microsomes

A microsomal system obtained from 3T12-3 cells was also active in converting [^{14}C]retinol to metabolite-310 and to [^{14}C]anhydroretinol as shown in **Fig. 3.** The formation of the metabolite-310 appeared to precede the synthesis of [^{14}C]anhydroretinol. Neither compound was synthesized by boiled microsomes (Fig. 3).

Microsomes also converted metabolite-310 into

TABLE 2. Kinetics of the formation of anhydroretinol and metabolite-310 by 3T12-3 cells incubated with [15-^{14}C]retinol

Incubation Time (hr)	% Radioactivity in Hexane	% Radioactivity Eluted in 2% Acetone in Hexane
1	7.3	93
3	11	89
5	20	80
10	18	78
24	27	73
40	55	42

The methanolic extracts from cells incubated with [15-^{14}C]retinol were applied to thin layers of silica gel and then developed by solvent system A (toluene–methanol–chloroform 4:1:1). The radioactive peak corresponding to anhydroretinol was scraped from the plate and eluted with 99% methanol. This fraction was further separated by chromatography on alumina (1 × 7.5 cm) into a hexane fraction that contained the hydrocarbon anhydroretinol and a 2% acetone in hexane fraction that contained metabolite-310.

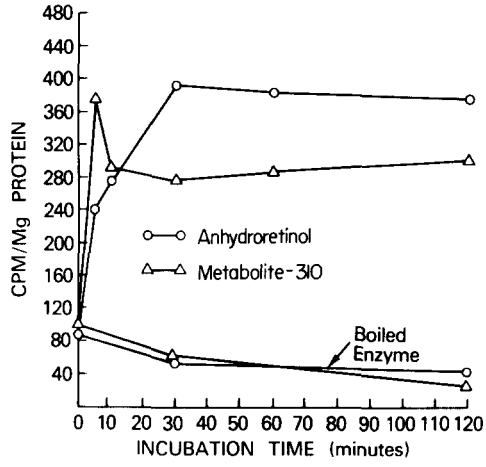


Fig. 3. Formation of anhydroretinol and metabolite-310 by microsomes prepared from 3T12-3 cells. Microsomes from 3T12-3 cells were incubated with 1 μg/ml of [15-^{14}C]retinol at pH 7.6 for different time intervals. A boiled enzyme control was also carried out for three time points. The incubation was stopped by the addition of 5 ml of 99% methanol. The methanolic extracts were evaporated and compounds with polarity of anhydroretinol, retinol, and retinyl esters were separated by alumina column chromatography as described in Materials and Methods. ○—○ Anhydroretinol; △—△ metabolite-310.

anhydroretinol as shown in **Fig. 4.** About 30–40% of the metabolite was converted to anhydroretinol in 5 min and 50–55% in 30 min. After this time, there was no conversion of metabolite-310 into the hydrocarbon, probably because some required factors may have been exhausted after 30 min of incubation.

Study of the metabolism of [15-^{14}C]anhydroretinol by 3T12-3 cells

[15-^{14}C]Anhydroretinol of known specific radioactivity was prepared and incubated for 30 hr with live and formalin-fixed cells, as mentioned in Materials and Methods. The methanolic extract was separated by thin-layer chromatography in solvent A. Live cells took up 4.3% of the label whereas 0.5% was taken up by the fixed cells. Thin-layer chromatography in solvent system A showed that over 90% of the radioactive material comigrated with anhydroretinol. This compound was identified from its typical absorption spectrum after purification through alumina. The rehydration products of anhydroretinol, retroretinol, or retinol (R_f 0.75) were not detected. Over 90% of the radioactivity taken up by fixed cells was recovered as anhydroretinol.

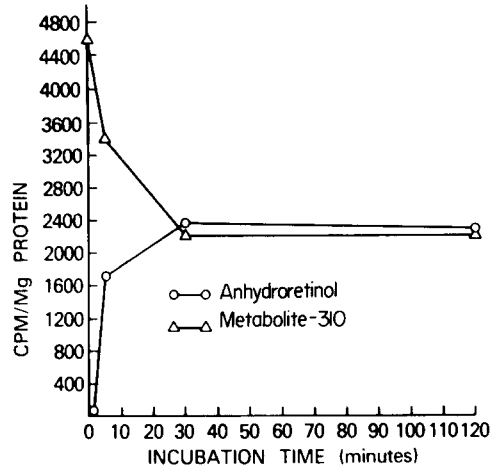


Fig. 4. Formation of anhydroretinol from metabolite-310 by microsomes prepared from 3T12-3 cells. The [15-^{14}C]metabolite-310 was obtained by incubation of 3T12-3 cells with [15-^{14}C]retinol as described in Table 2. Microsomes were incubated with 1 μg/ml of [15-^{14}C]metabolite-310 for different time intervals. A boiled enzyme control was also incubated for different time intervals. The incubation was stopped by the addition of 5.0 ml of 99% methanol. The metabolite-310 and anhydroretinol were separated by alumina column chromatography as described in Materials and Methods. ○ —— ○ Anhydroretinol; △ —— △ metabolite-310.

DISCUSSION

Retinol increases the adhesion of spontaneously transformed mouse fibroblasts (Balb/c 3T12-3 cells) to each other and to the culture dish surface and modifies their morphology to resemble a more normal phenotype (1–3). For this reason a study of its metabolism was undertaken. We found that retinol was readily converted to a metabolite with an absorption maximum at about 310 nm, and to anhydroretinol. This compound accounted for as much as 30% of the initial radioactivity after 40 hr of culture with live cells but was not synthesized by fixed cells or by cultured cells from hamster intestine. Anhydroretinol was also formed by 3T3 cells and by primary mouse fibroblasts obtained from the dermis.

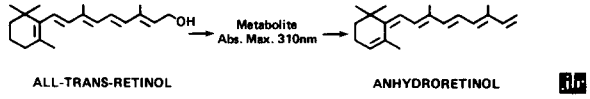
[15-^{14}C]Anhydroretinol incubated with 3T12-3 cells was recovered unaltered without the formation of retroretinol. Thus it appears that anhydroretinol is an end product of the metabolism of retinol in this cellular system.

Crude microsomal membranes also contain the dehydratase activity and convert [^{14}C]retinol into ^{14}C-labeled metabolite-310 and into [^{14}C]anhydroretinol. This microsomal system is also active in converting ^{14}C-labeled metabolite-310 into [^{14}C]anhydroretinol, suggesting metabolite-310 as an intermediate in this dehydration reaction. The described in vitro systems are, so far, the only ones in which the enzymic conversion of retinol into anhydroretinol has been found; thus the significance of this reaction in vivo remains to be established.

Contrary to our findings in epithelial cell systems (8), the three cultured fibroblast systems were apparently inactive in synthesizing retinylphosphate or its mannosyl derivative from [^{14}C]retinol, or at least these compounds could not be detected.

Retinoic acid, on the other hand, appears to be metabolized by 3T12 fibroblasts into products which are chromatographically identical with retinylphosphate (9) and its mannosyl derivative (3). Whether such products function in place of retinylphosphate and its derivatives in this mesenchymal system cannot be excluded at present.

In conclusion, the following pathway for retinol has been demonstrated for the first time in this work:



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