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13,14-Dihydroxy-retinol, a New Bioactive Retinol Metabolite*

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Deprivation of vitamin A (retinol) leads to reduced potential of B cell proliferation and nearly complete block of T cell activation *in vitro*. Retinol, which is thought to function as a pro-hormone, is enzymatically converted into intracellular messenger molecules. Thus, 14-hydroxy-*retro*-retinol (14-HRR) is an intracellular messenger molecule linked to activation and growth regulation of lymphocytes; whereas, anhydroretinol, another natural *retro*-retinoid, is an antagonist of 14-HRR effects. In this article, we describe the isolation, structure determination, synthesis, and biological properties of a new intracellular retinol derivative, 13,14-dihydroxy-retinol (DHR), which also supports the viability of retinol-deprived lymphocytes. DHR is found in numerous cell lines representing a large cross-section of tissues and animals from insects to mammals. In T lymphocytes the production of DHR and 14-HRR is up-regulated by phorbol ester. DHR is converted to 14-HRR by mild acid treatment, *but not by cells*; therefore DHR is not a biosynthetic intermediate in the conversion of retinol to 14-HRR. DHR is a distinct end point of retinol metabolism. Although it is linked to cell proliferation, its biological role remains to be determined.

When B or T lymphocytes encounter an antigen, they execute, when this event is appropriately amplified by co-receptor interactions, a preordained program of proliferation and differentiation. The receptor stimulation triggers a complex network of signals needed to induce changes in the cell biochemistry and architecture, and to provide commands for the requisite genomic changes. While this signal network is highly organized and includes many interwoven circuits, one mode of signal transduction is carried out by small diffusable messenger molecules, which comprise polar substances (*i.e.* inositol phosphates, cyclic nucleotides), and small lipophilic molecules (steroids, vitamin D, thyroid hormone, and retinoic acid). The latter class of compounds bind to and activate specific receptors which belong to the superfamily of steroid receptors, inducing transcriptional activation (1–4). Thus, all-*trans*-retinoic acid **1** (Fig. 1) and its 9-cis isomer bind to and activate the retinoic acid nuclear receptors, whereas, at higher concentrations, 9-cis-RA[1] activates also the retinoid X nuclear receptors (5, 6). In addition, 4-oxo-RA, considered so far to be an elimination catabolite product of RA, has been reported to be a potent ligand of the retinoic acid nuclear receptors.

On the other hand, retinol **2** itself, or metabolites distinct from the retinoic acid series, are essential for the regulation of spermatogenesis (8, 9) and the immune system (10–12). In particular, 14-hydroxy-4,14-*retro*-retinol (14-HRR) **3**, a new retinol metabolite, and the first bioactive *retro*-retinoid reported, is a potent intracellular mediator of B cell proliferation and T cell activation and growth (13, 14). In contrast, anhydroretinol (AR) **4**, another naturally occurring *retro*-retinoid (15, 16), long known as the inactive retinoid (17, 18), acts as a reversible inhibitor of retinol and 14-HRR effects (19–21). 14-HRR and AR have been identified in various mammalian and insect cell lines (19–22); they constitute the first naturally occurring agonist/antagonist pair of small lipophilic messenger molecules described.

In addition to these *retro*-retinoids, we have identified in lymphoblastoid 5/2 cells grown in the presence of [^{3}H]retinol, another retinol metabolite P1, more polar than 14-HRR; we have now characterized it as 13,14-dihydroxy-retinol (DHR) **5**. DHR is also active in the B cell proliferation and the T cell activation assays. Because of its structure and its shared biological properties with 14-HRR, P1 was thought first to be the precursor of 14-HRR. However, P1 is not converted to 14-HRR by the cells; this leads to the conclusion that both retinoids are independent end points of retinol metabolism.

MATERIALS AND METHODS

Cells and Culture Conditions

The human lymphoblastoid cell line 5/2 was obtained by Epstein-Barr virus transformation of peripheral blood lymphocytes. HL-60 cells were obtained from the American Type Culture Collection (Rockville, MD). 5/2 and HL-60 cells were grown in RPMI 1640 with 7% fetal bovine serum, and HeLa cells in minimal essential medium supplemented with 10% horse serum. All cells were tested for mycoplasma every 2 months. The defined serum-free medium (ITLB medium) consisted of RPMI 1640 with 0.12% delipidated bovine serum albumin, 5 μg/ml insulin, 5 μg/ml transferrin, 10^{-6} M linoleic acid, and 2 mM L-glutamine (Sigma). All-*trans*-retinol and 11,12-[^{3}H]retinol were purchased from Sigma and DuPont/NEN (Cambridge, MA), respectively. The anti-CD3 producing hybridoma 2C11 was obtained from Dr. Bluestone (23) and monoclonal antibody was purified by protein A-Sepharose. T cells from pooled lymphocytes and spleen of BALB/c mice were purged of B cells and monocytes by passage over anti-IgM-coated plastic dishes. They were >90% pure by immunofluorescence analysis.

Bioassays

5/2 cells were grown in RPMI 1640 with 7% fetal bovine serum, and on the day of assay washed twice with serum-free medium and transferred to multiwell plates at a density of 5×10^4 cells/ml in ITLB medium as described (12). Retinol, natural, or synthetic DHR were

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[1] The abbreviations used are: RA, retinoic acid; 14-HRR, 14-hydroxy-4,14-*retro*-retinol; AR, anhydroretinol; DHR, 13,14-dihydroxy-retinol; HPLC, high-pressure liquid chromatography; PMA, phorbol myristate acetate; EI/MS, electron impact/mass spectroscopy; CI, chemical ionization.

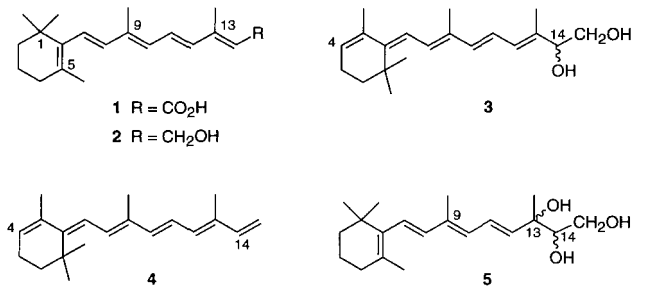

FIG. 1. **Retinoid structures.**

added at the onset of the culture, at indicated graded concentrations, and cultures grown in 5% CO_2 at 37 °C. Proliferation was measured on day 3 by use of [^{3}H]thymidine pulse (0.8 μCi/well, specific activity 6.7 Ci/mM) administered during the last day of culture.

To evaluate the effects of retinoids on T cell activation, multiwell plates were coated with anti-CD3 antibody at 10 μg/well in phosphate-buffered saline overnight, and washed. Retinoids were added to washed thymocytes of 4–6-week-old BALB/c mice, which were suspended in ITLB medium at 2×10^6 cells/ml. Cell cultures were pulsed on day 4 for 4 h with [^{3}H]thymidine (0.8 μCi/well).

To evaluate the production of DHR by normal T cells, pooled lymphocytes from spleens and lymph nodes of BALB/c mice were purged of adherent cells by culture in untreated Petri dishes for 1 h. Unattached cells were transferred to Petri dishes coated with anti-IgM antibody (10 μg/ml) at a rate of 10^8 cells/10-cm dish, and the plates were kept on ice for 3 h. 35% of input cells were recovered as unattached T cells and enriched to 85% purity as shown by immunofluorescence staining with anti-Thy-1.2 antibody. Aliquots of 4×10^7 cells in 1 ml of serum-free ITLB medium were pulsed with 10 μl of HPLC purified [^{3}H]retinol and placed in a tissue culture incubator. At the onset of culture, half of the aliquots were stimulated with phorbol myristate acetate (PMA) at 100 ng/ml. Stimulated and unstimulated cultures were each harvested at times 0, 1, 2, and 4 h, and the cells extracted by the method of McClean (24). The extracts were analyzed by reversed phase HPLC (analytical C_{18} column, Vydac, Hesperia, CA) using the elution gradient described in Fig. 3. The approximate amounts of DHR and 14-HRR formed were estimated by the integration of the respective radioactive peaks and the known specific radioactivity of the input retinol, assuming that dilution by endogenous retinoids did not occur.

Cell growth curves of HL-60 cells were obtained by taking aliquots from cultures in 12-well culture dishes (Costar, Cambridge, MA) and counting the cells in a Neubauer chamber. Cell numbers were kept between 1 and 5×10^6 cells/ml. Viability was assessed by trypan blue exclusion. Assays were done in duplicate. For detection of differentiation, HL-60 cells were tested for their ability to reduce nitro blue tetrazolium (Sigma). During a 30-min stimulation with 10^{-7} M PMA (Sigma), cells were provided with 0.61 mM nitro blue tetrazolium and analyzed for staining under the microscope (25, 26). The percentage of differentiated cells was determined in relation to the total number of viable trypan blue negative cells. Cells of positive controls were treated for 3 days with 10^{-6} M retinoic acid.

Bulk Extraction of Retinoids from HeLa Cells

10-Liter batches of HeLa cells at $5–8 \times 10^5$ cells/ml were incubated with all-*trans*-retinol at a concentration of 10^{-5} M for 18 h. The cells were collected by centrifugation, snap-frozen, and delipidated according to the method of McClean (24). To the thawed pellets (2.5 ml of packed cells) suspended in 7.5 ml of phosphate-buffered saline were added 4 ml of butanol/acetonitrile, 1/1 mixture (v/v). Addition of 3 ml of saturated K_2HPO_4 solution, followed by centrifugation at 10,000 rpm for 10 min, led to quantitative recovery of the organic phase. This extraction was repeated once. The combined organic extracts were purified by reversed-phase HPLC (preparative C_{18} and analytical C_4 columns; Vydac, Hesperia, CA) using a gradient water/methanol as eluent.

For quantitative assays of DHR and 14-HRR biosynthesis, cells (40×10^6) in ITLB medium were labeled with 10 μCi of [^{3}H]retinol and cultured for the indicated time periods. Cells were recovered by centrifugation in the cold and extracted as described above (24), using 1 ml of extraction buffer. The recovered butanol extract was analyzed by HPLC as described in the legend to Fig. 3. The eluate was monitored by an on-line scintillation counter (Radiomatic, Tampa, Fl) with quench correction.

To check whether DHR was a biosynthetic precursor of 14-HRR,

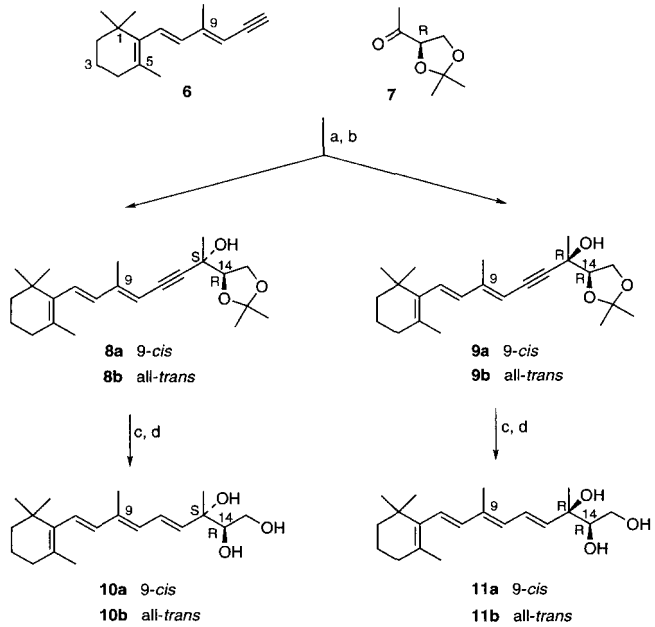

FIG. 2. **Synthetic steps for (13*S*,14*R*)-, and (13*R*,14*R*)-13,14-DHR: (*a*) CH_3MgBr, 6, THF, reflux, followed by addition of 7 in THF, reflux, 84%; (*b*) flash chromatography SiO_2, gradient hexane/ethyl acetate 95/5 to 90/10; (*c*) pTsOH, CH_3OH, RT, 65%; (*d*) lithium aluminum hydride (*LAH*), ether, reflux, 80%.**

11,12-[^{3}H]DHR was isolated from extracts of murine spleen cells stimulated with PMA in the presence of 11,12-[^{3}H]retinol, and purified to homogeneity by reversed phase HPLC. Lymphoblastoid 5/2 cells were cultured for 4 h in serum-free medium in the presence of 6.4×10^{-9} M [^{3}H]DHR, and 10^{-6} M unlabeled retinol. The cell extract was then analyzed by reversed phase HPLC as described in the legend to Fig. 8.

Spectroscopic Methods

Absorption spectra were recorded on a Perkin-Elmer model Lambda 6B UV-vis spectrophotometer, and circular dichroism spectra (CD) on a JASCO J-700 spectropolarimeter. ^{1}H NMR spectra were measured on a Varian VXR-400 spectrometer in $CDCl_3$ or CD_3CN. The chemical shifts are given in ppm (δ reference peak: $CHCl_3$, 7.24; and CH_3CN, 1.93), and coupling constants (*J*) in Hertz. High-resolution electron impact mass spectra (EI/MS) were measured on a Jeol DX-303 HF spectrometer (matrix perfluorokerosine); low-resolution EI/MS and chemical ionization CI/MS (carrier gas methane) were recorded on a NERMAG R1010 spectrometer.

Synthesis of (13S,14R)- and (13R,14R)-13,14-DHR

As shown in Fig. 2, the synthesis involves condensation of the acetylenic Grignard reagent derived from **6** with ketone **7**, the latter being prepared from (*R*)-glyceraldehyde acetonide (27) after methylmagnesium bromide treatment and pyridinium chlorochromate oxidation of the resulting alcohol. Intermediate **6** was obtained according to literature procedure (28) as a cis/trans mixture at C-9. This procedure led to (13*S*,14*R*)-9-cis-**8a**, (13*S*,14*R*)-all-*trans*-**8b**, (13*R*,14*R*)-9-cis-**9a**, and (13*R*,14*R*)-all-*trans*-**9b** intermediates, which were separated in that order of elution by flash chromatography (E. Merck Silica Gel 60; solvent: gradient hexane/ethyl acetate 95/5 to 90/10). Deprotection of the terminal diol and lithium aluminum hydride reduction of the triple bond were carried out on each isomer, leading, respectively, to (13*S*,14*R*)-9-cis-DHR **10a**, (13*S*,14*R*)-all-*trans*-DHR **10b**, (13*R*,14*R*)-9-cis-DHR **11a**, and (13*R*,14*R*)-all-*trans*-DHR **11b**. Identification of the pair of geometrical isomers belonging to each diastereomer was achieved by ^{1}H NMR (comparison of the chemical shifts of the 13-Me) and HPLC analysis of photoconversion products resulting from irradiation of pure geometrical isomers.

The C-13 absolute configuration of synthetic DHR **10b** and **11b** was determined by the exciton chirality method (29) after lithium aluminum hydride reduction of the triple bond of the all-*trans*-14,15-acetonides **8b** and **9b**, respectively, and conversion of 13-OH to 13-*p*-methoxycinnamoyl ester (30).

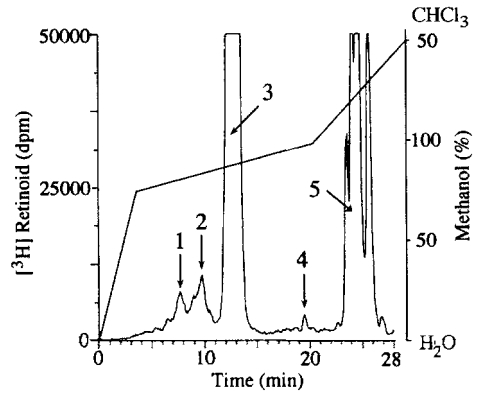

FIG. 3. **High-pressure liquid chromatography of retinoids from lymphoblastoid 5/2 cells.** Analytical C_{18} reversed-phase column; water/methanol/chloroform gradient; flow rate 1 ml/min; photodiode array detection. Disintegrations per min were determined with an on-line scintillation counter. The 5/2 cells (1×10^7 cells in 20 ml of RPMI, 10% fetal calf serum medium) were incubated with 40 μCi of [^{3}H]retinol; specific activity 49.3 Ci/mmol. After 16 h, retinoids were extracted from the washed cell pellet according to the procedure of McClean *et al.* (24). Unlabeled synthetic retinoids (*arrows*) were used as reference standards: 1) 13,14-dihydroxy-retinol; 2) 14-hydroxy-4,14-*retro*-retinol; 3) retinol; 4) anhydroretinol; 5) retinyl esters.

Synthetic DHR Spectroscopic Data

(13S,14R)-9-cis-DHR ***10a***—^{1}H NMR ($CDCl_3$) δ 1.00 [s, 6H, 1-$(CH_3)_2$], 1.37 (s, 3H, 13-CH_3), 1.45 (m, 2H, 2-H_2), 1.58 (m, 2H, 3-H_2), 1.70 (s, 3H, 5-CH_3), 1.92 (s, 3H, 9-CH_3), 2.00 (t, J = 7 Hz, 4-H_2), 3.47 (m, 1H, 14-H), 3.75 (m, 2H, 15-H_2), 5.65 (d, J = 15 Hz, 1H, 12-H), 5.92 (d, J = 11 Hz, 1H, 10-H), 6.16 (d, J = 16 Hz, 1H, 7-H), 6.58 (d, J = 16 Hz, 1H, 8-H), 6.80 (dd, J = 11 Hz, 15); CI/MS m/z 320 (M^+), 303 [(M-18)$^+$ + 1]; UV (CH_3CN) 288 nm.

(13S,14R)-All-trans-DHR ***10b***—^{1}H NMR ($CDCl_3$): δ 1.01 [s, 6H, 1-$(CH_3)_2$], 1.37 (s, 3H, 13-CH_3), 1.45 (m, 2H, 2-H_2), 1.59 (m, 2H, 3-H_2), 1.68 (s, 3H, 5-CH_3), 1.92 (s, 3H, 9-CH_3), 1.98 (t, J = 6 Hz, 2H, 4-H_2), 3.49 (m, 1H, 14-H), 3.75 (m, 2H, 15-H_2), 5.72 (d, J = 15 Hz, 1H, 12-H), 6.02 (d, J = 11 Hz, 1H, 10-H), 6.04 (d, J = 16 Hz, 1H, 8-H), 6.15 (d, J = 16 Hz, 1H, 7-H), 6.74 (dd, J = 11 Hz, 15 Hz, 1H, 11-H); CI/MS m/z 320 (M^+), 303 [(M-18)$^+$ + 1]; UV (CH_3CN) 292 nm.

(13R,14R)-9-cis-DHR ***11a***—^{1}H NMR ($CDCl_3$) δ 1.00 [s, 6H, 1-$(CH_3)_2$], 1.31 (s, 3H, 13-CH_3), 1.45 (m, 2H, 2-H_2), 1.58 (m, 2H, 3-H_2), 1.70 (s, 3H, 5-CH_3), 1.92 (s, 3H, 9-CH_3), 2.00 (t, J = 7 Hz, 4-H_2), 3.57 (m, 1H, 14-H), 3.75 (m, 2H, 15-H_2), 5.66 (d, J = 15 Hz, 1H, 12-H), 5.92 (d, J = 11 Hz, 1H, 10-H), 6.16 (d, J = 16 Hz, 1H, 7-H), 6.58 (d, J = 16 Hz, 1H, 8-H), 6.80 (dd, J = 11 Hz, 15 Hz); CI/MS m/z 320 (M^+), 303 [(M-18)$^+$ + 1]; UV (CH_3CN) 288 nm.

(13R,14R)-All-trans-DHR ***11b***—^{1}H NMR ($CDCl_3$): δ 1.00 [s, 6H, 1-$(CH_3)_2$], 1.32 (s, 3H, 13-CH_3), 1.44 (m, 2H, 2-H_2), 1.59 (m, 2H, 3-H_2), 1.67 (s, 3H, 5-CH_3), 1.92 (s, 3H, 9-CH_3), 1.98 (t, J = 6 Hz, 2H, 4-H_2), 3.56 (m, 1H, 14-H), 3.75 (m, 2H, 15-H_2), 5.75 (d, J = 15 Hz, 1H, 12-H), 6.02 (d, J = 11 Hz, 1H, 10-H), 6.04 (d, J = 16 Hz, 1H, 8-H), 6.15 (d, J = 16 Hz, 1H, 7-H), 6.75 (dd, J = 11 Hz, 15 Hz, 1H, 11-H); CI/MS m/z 320 (M^+), 303 [(M-18)$^+$ + 1]; UV (CH_3CN) 292 nm.

RESULTS AND DISCUSSION

Retinol Metabolites Constitutively Produced by 5/2 Cells—The HPLC profile of an extract of lymphocytes grown in the presence of [^{3}H]retinol shows a mixture of new radioactive compounds (Fig. 3), some of which were characterized by coelution with known standards using different HPLC gradients. Peaks 2, 4, and 5 correspond to 14-HRR, anhydroretinol (AR), and retinyl esters, respectively. The fraction eluting between 7 and 8 min (peak 1 or P1) is frequently observed in 14-HRR producing cells. Purification of a similar fraction, extracted from HeLa cells (grown for 18 h in the presence of 10^{-5} M retinol), using a series of reversed phase HPLC columns, led to homogeneous P1 (about 20 absorbance units at 292 nm).

Structure Determination of Natural DHR (P1)—The UV absorption maximum at 292 nm suggests that P1 has one double bond less than its precursor retinol (λmax 326 nm). P1 obtained from lymphocytes cultured in the presence of 1.5×10^{-6} M retinol exhibits a CD spectrum with a negative Cotton effect band at 294 nm (Fig. 4*A*). However, P1 extracted from HeLa cells, cultured in the presence of unphysiological concentrations of retinol (10^{-5} M), lost its optical activity.

The high resolution EI/MS[2] gave an observed value of 320.2354 (calculated for $C_{20}H_{32}O_3$ = 320.2351), indicating that P1 has two hydrogen atoms and two oxygen atoms (which could be accounted for by two hydroxyl groups) more than retinol, $C_{20}H_{30}O$.

The ^{1}H NMR spectrum (Fig. 4*B*) displays four olefinic protons which establish P1 as a 13,14-DHR; thus P1 has two asymmetric centers at C-13 and C-14. The two singlets at 1.22 and 1.23 ppm (Fig. 4*B*)[3] are characteristic of a C(OH)CH_3 group and correspond to 13-Me: P1 very likely exists as a mixture of diastereomers. That P1 exists as a mixture of diastereomers is supported by two doublets at 5.79 and 5.81 ppm (12-H; J 16) integrating for 1H. This was also confirmed by synthesis. Namely, the ^{1}H NMR spectra of minor natural P1 (δ 13-Me 1.23 ppm), and major natural P1 (δ 13-Me 1.22 ppm) are identical to those of the synthetic diastereomers all-*trans*-DHR **10b** and **11b**. The absolute configurations at C-13 and C-14, however, remain to be established. Therefore, we synthesized one set of the optically active diastereomers: (13*S*,14*R*)- and (13*R*,14*R*)-DHR.

Fig. 5*A* shows the CD spectra of (13*S*,14*R*)- and (13*R*,14*R*)-all-*trans*-DHR **10b** (first positive CD) and **11b** (first negative CD), respectively, and their absorption spectra. The (13*S*,14*R*)- and (13*R*,14*R*)-9-cis isomers exhibit opposite CD signs to that of their corresponding all-*trans* diastereomer (curves not shown). This inversion of the CD signs has been observed also with mono-cis carotenoids as compared to the all-*trans* isomer of the same carotenoid (31).

Fig. 5*B* (*solid line*) shows that the CD spectrum of the 13,14-acetonide, corresponding to (13*R*,14*R*)-all-*trans*-DHR **11b,** also exhibits a first negative CD band. However, the corresponding 13-*p*-methoxycinnamoyl-13,14-acetonide exhibits a prominent split CD centered at 294 nm due to exciton coupling between the polyene chain and the cinnamate chromophores, λmax = 292 and 311 nm, respectively (29). The positive sign of the first couplet (32) is in full agreement with the 13*R* absolute configuration in **11b**. As expected, the 13-*p*-methoxycinnamoyl-13,14-acetonide, corresponding to the (13*S*,14*R*)-diastereomer **10b**, exhibits a split CD with a first negative couplet (curve not shown) which is consistent with the 13*S* absolute configuration in **10b**.

Natural DHR exists as a mixture of isomers, and we know from the NMR data that at least two diastereomers are present in a 5/4 ratio. Indeed, the major diastereomer (δ 13-Me 1.22 ppm; Fig. 4*B*) has a ^{1}H NMR spectrum identical to that of (13*R*,14*R*)-all-*trans*-DHR **11b** (or its antipode); whereas the minor P1 (δ 13-Me 1.23 ppm; Fig. 4*B*), has the same ^{1}H NMR spectrum as (13*S*,14*R*)-all-*trans*-DHR **10b** (or its antipode). Its CD spectrum (Fig. 4*A*), however, does not look like a simple summation of the CD curves of two diastereomers. In addition,

[2] The low-resolution EI/MS shows the following peaks: mass-to-charge ratio m/z (%) 320 (50; M^+), 302 (22; M − H_2O), 284 (12; M − 2 × H_2O), 272 (22; M − H_2O − 2 × Me), 259 (100; M − CH[OH]CH_2OH).

[3] We have chosen to show in Fig. 2 the natural DHR ^{1}H NMR in CD_3CN, because DHR decomposes slowly in $CDCl_3$. However, the ^{1}H NMR in $CDCl_3$ is better resolved, and shows clearly that P1 is a mixture of diastereomers: δ 1.00 [s, 6H, 1-(CH3)$_2$], **1.32/1.37 (2 s, 3H, 13-CH_3)**, 1.44 (m, 2H, 2-H_2), 1.59 (m, 2H, 3-H_2), 1.67 (s, 3H, 5-CH_3), 1.92 (s, 3H, 9-CH_3), 1.98 (t, J = 6 Hz, 2H, 4-H_2), **3.49/3.56 (2 m, 1H, 14-H)**, 3.75 (m, 2H, 15-H_2), **5.72/5.75 (2d, J = 15 Hz, 1H, 12-H)**, 6.02 (d, J = 11 Hz, 1H, 10-H), 6.04 (d, J = 16 Hz, 1H, 8-H), 6.15 (d, J = 16 Hz, 1H, 7-H), **6.74/6.75 (2dd, J = 11 Hz, 15 Hz, 1H, 11-H)**.

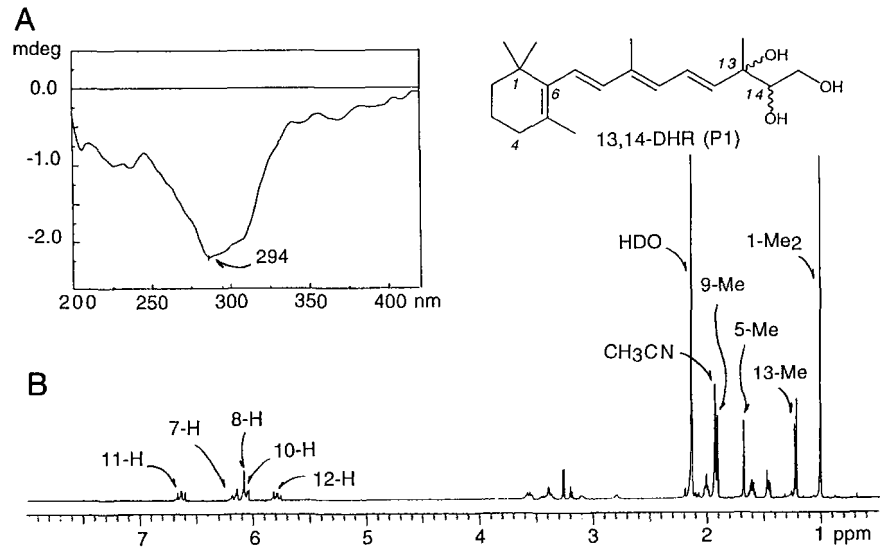

FIG. 4. *A*, CD spectrum in CH_3OH; and *B*, ^1H NMR in CD_3CN of natural DHR: δ 1.06 (s, 6H, 1-$(CH_3)_2$), 1.22, 1.23 (2 s, 3H, 13-Me), 1.46 (m, 2H, 2-CH_2), 1.61 (m, 2H, 3-CH_2), 1.68 (s, 3H, 5-CH_3), 1.91 (s, 3H, 9-CH_3), 2.02 (t, J = 8 Hz, 2H, 4-CH_2), 3.32–3.62 (m, 3H, 14-H and 15-H_2), 5.79, 5.81 (2 d, J = 16 Hz, 1H, 12-H), 6.07 (d, J = 16 Hz, 1H, 8-H), 6.08 (d, J = 12 Hz, 1H, 10-H), 6.17 (d, J = 16 Hz, 1H, 7-H), 6.64 (dd, J = 12 Hz, 16 Hz, 1H, 11-H).

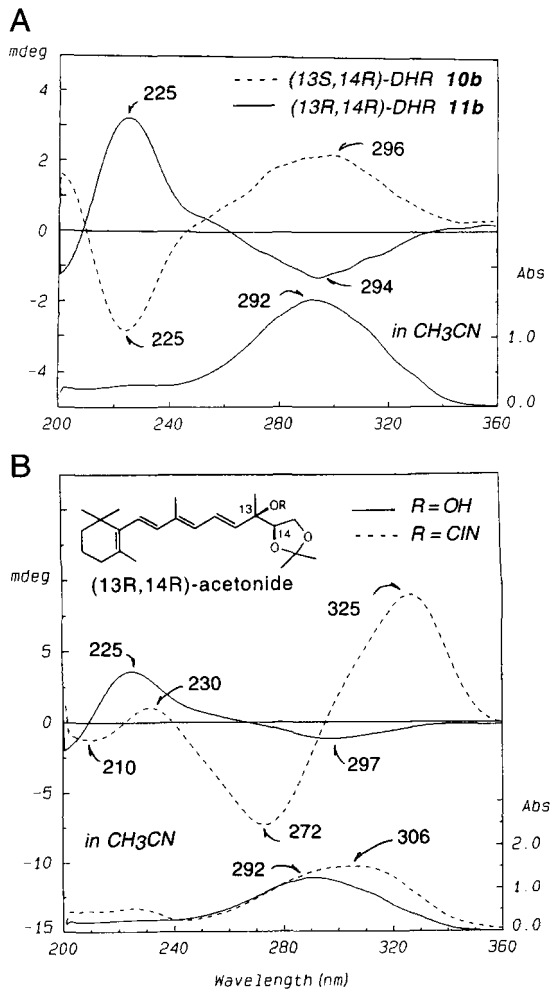

FIG. 5. *A*, CD spectra of synthetic (13*S*,14*R*)-, and (13*R*,14*R*)-all-*trans*-DHR **10b** and **11b**, respectively, and their corresponding absorption spectrum. *B*, CD spectrum of 14,15-acetonide of (13*R*,14*R*)-all-*trans*-DHR, its 13-*p*-methoxycinnamoyl ester showing a first positive Cotton effect characteristic of a 13*R* configuration, and their respective UV spectra.

the optical activity of "natural" P1 varies with the concentrations of retinol added to the cell cultures: under unphysiological conditions (10^{-5} M retinol) P1 was even racemic! Just like 14-hydroxy-*retro*-retinol (14), optically active natural P1 may result from the opening of optically active 13,14-epoxyretinol, which can be produced via stereospecific 13,14-ene epoxidation of retinol bound to cellular retinol-binding protein (1–3 × 10^{-7} M). Under unphysiological conditions, most retinol within the cells is free or nonspecifically bound to membranes and nonspecific cellular proteins, leading to random epoxidation and ring opening. Thus, it is possible that as in the case of 14-HRR (14), the stereochemistry of P1 is dependent on subtle biosynthetic conditions and varies from case to case (Fig. 6). The minute amount of P1 extracted under physiological conditions (*i.e.* cells cultured in medium containing 10% serum) precluded the determination of its absolute configuration.

Biological Activity and Distribution of DHR—Growth of cells, notably those of hemopoietic origin, frequently depends on retinol (10–12, 21). At 1 × 10^{-7} to 5 × 10^{-9} M concentrations, 14-HRR reverses the growth stagnation of retinol starved lymphocytes and HL-60 cells. DHR displays the same ability. Indeed, the purified material from lymphocytes or HeLa cells was able to replace retinol in the B cell (data not shown) and T cell assays, as well as in the HL-60 proliferation assay at 1.2 × 10^{-6} to 1 × 10^{-7} M concentrations (Fig. 7). In the T cell assay (Fig. 7*A*), retinol is active at a 2–4-fold lower concentration than DHR, and 14-HRR shows 2-fold less activity. However, these differences may be attributable to the shorter half-life in culture of DHR and 14-HRR (3 h) as compared to retinol (24 h). Attempts to compensate for the shorter half-life by periodically replenishing the retinoids during the assay led to a 30-fold increase in activity for 14-HRR (13); a similar experiment carried out with DHR did not shift down its dose-response curve. All four pure synthetic isomers, (13*S*,14*R*)-9-cis-DHR, (13*S*,14*R*)-all-*trans*-DHR, (13*R*,14*R*)-9-cis-DHR, and (13*R*,14*R*)-all-*trans*-DHR, showed the same supportive bioactivities as the natural all-*trans* racemic DHR. Because the assays last several days, it is difficult to conclude whether isomerization did not occur during the course of the experiment.

Contrary to retinoic acid (≥10^{-8} M) and retinol (≥2 × 10^{-7} M), the *retro*-retinoids 14-HRR and AR do not induce differentiation of HL-60 cells to granulocytes (21). Similarly, DHR (2 × 10^{-6} or 1 × 10^{-8} M) does not induce HL-60 cell differentiation as measured by the nitro blue tetrazolium reduction assay.

The cell growth promoting effect of 14-HRR is reversibly antagonized by AR (19, 20), suggesting that 14-HRR and AR bind to the same receptor. Surprisingly, assays carried out with the T cell line ERLD show clear evidence for reversible inhibition of AR cytotoxicity by 13,14-DHR (data not shown). Al-

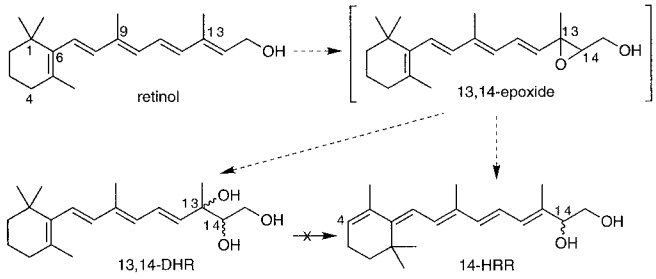

FIG. 6. **Plausible routes leading to 14-HRR and DHR from the retinol precursor; the epoxide is a putative intermediate.**

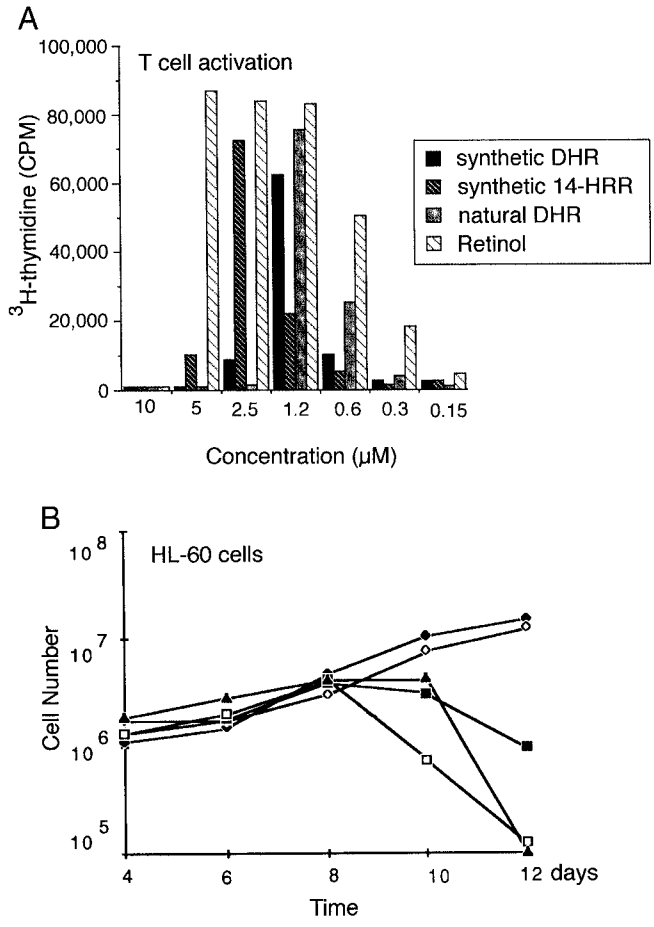

FIG. 7. **Effect of DHR on T cell activation and growth of promyelocytic HL-60 cells.** *A*, comparative dose responses of DHR, 14-HRR, and retinol in T cell activation. Thymic T cells of BALB/c mice were activated with immobilized anti-CD3ε antibody and graded doses of retinoids added at the onset of culture. Proliferation was measured by incorporation of tritiated thymidine into DNA on day 3. Means of triplicate measurements that were within 15% of each other are plotted. Natural racemic DHR, a mixture of synthetic 9E/9Z diastereomeric DHR, and synthetic racemic 14-HRR were used. *B*, HL-60 cells from stock cultures in 10% fetal calf serum were reseeded in serum- and retinol-free ITLB medium and natural racemic DHR added daily to the final concentrations shown. Cell numbers and viability were determined by trypan blue exclusion and microscopic counting in cultures with different retinoid concentrations. The cell density was kept between 3×10^5 and 2×10^6 cells/ml by providing additional culture medium accordingly. Shown is the total cell number from days 4 through 12 of cultures. ◆, 0.3 μM DHR; ◇, 0.1 μM DHR; ■, 0.03 μM DHR; □, 0.01 μM DHR; ▲, none.

though structural differences suggest different receptors for 14-HRR and DHR, binding to the same receptor could be also rationalized by the fact that DHR, just like retinol bound to cellular retinol-binding protein (33, 34), can adopt a 6-s-*trans* conformation leading to ring/chain coplanarity, which in 14-HRR is imposed by the *retro* structure.

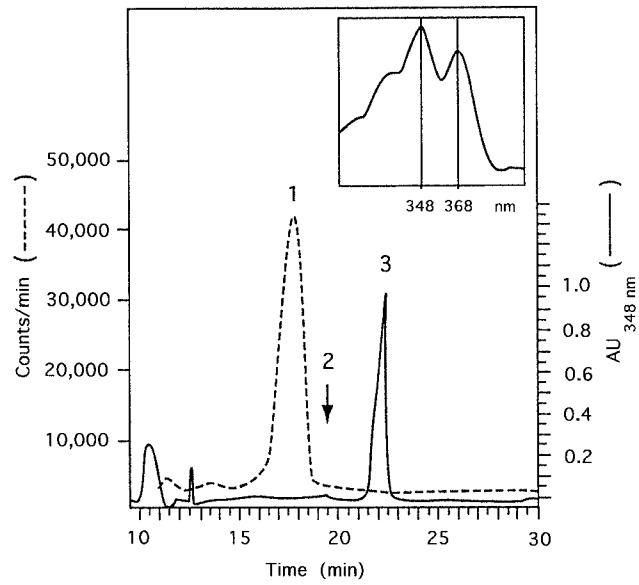

FIG. 8. **Lymphoblastoid 5/2 cells do not convert 13,14-DHR to 14-HRR.** Lymphoblastoid 5/2 cells were cultured for 4 h in serum-free medium in the presence of [^{3}H]DHR and 10^{-6} M unlabeled retinol. The cell extract was analyzed using the conditions described in the legend to Fig. 2. The radioactive peaks were monitored by an on-line liquid scintillation counter (*dotted line*); the *solid line* corresponds to the chromatogram monitored at 348 nm, the λmax of 14-HRR. The positions of DHR (*1*), 14-HRR (*2*), and retinol (*3*) were determined in calibration runs.

The functional similarity of DHR and 14-HRR and their common existence in cells suggest that DHR could be the biosynthetic intermediate in the pathway to 14-HRR. Indeed, DHR is easily converted to 14-HRR upon mild acid treatment. However, studies with high doses (10^{-6} M) of synthetic DHR, or [^{3}H]DHR (obtained biosynthetically from [^{3}H]retinol) did not lead to detectable amounts of DHR in 5/2 lymphoblastoid cells. Indeed, the chromatogram of extract from lymphoblastoid 5/2 cells cultured in the presence of [^{3}H]DHR and unlabeled retinol (Fig. 8) does not exhibit radioactivity above background at 19.5 min, the retention time of 14-HRR. However, a small amount of 14-HRR is synthesized from unlabeled retinol (see absorption spectrum in Fig. 8, *inset*), indicating that the 14-HRR biosynthetic pathway was active at the time of the experiment. The estimated limit of detection is about 1–5% of the input radioactivity, and thus a small amount of 14-HRR could have gone undetected. However, because steady-state concentrations of 14-HRR and DHR in cells approximate a 1/1 ratio, we would have expected conversion of half the input DHR to 14-HRR. Thus, DHR is not a metabolic intermediate in the biosynthesis of 14-HRR from retinol, but rather a distinct end point of retinol metabolism alongside 14-HRR. In other experiments carried out with HeLa and Jurkat T cells, synthetic unlabeled DHR was not metabolized to 14-HRR (data not shown).

The production of 14-HRR and DHR is not limited to transformed cell lines. They are produced in normal T cells where their synthesis is up-regulated by phorbol ester, as shown in Fig. 9. Moreover, a limited trace labeling study with [^{3}H]retinol shows a radioactive peak coeluting with DHR in myeloid cells and tumor cell lines derived from lung, colon, prostate, breast, and nervous tissues. The DHR peak is also present in tissue culture cells of various insects (*Aedes*, *Manduca*, *Drosophila*). In fact, virtually all dividing cells seem to produce simultaneously DHR and 14-HRR. DHR is also found in amphibians. Thus, extraction of 7 ml of untreated *Xenopus laevis* oocytes, followed by HPLC purification using water/methanol and

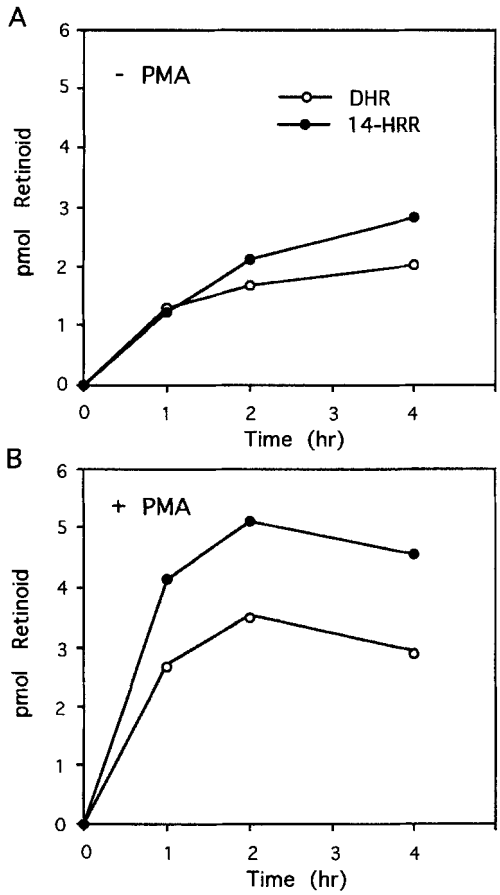

FIG. 9. **The production of DHR and 14-HRR in T lymphocytes is up-regulated by phorbol ester.** Purified T cells of BALB/c mice were cultured in serum-free medium in the presence of [^{3}H]retinol. *A*, control cultures produced both DHR and 14-HRR, reaching steady state levels 60 min after the initiation of cultures. *B*, however, PMA treated cultures led to 2–4 times more DHR and 14-HRR.

water/acetonitrile gradients, successively, led to 4×10^{-9} M DHR and 2×10^{-8} M 14-HRR. These data suggest a broad evolutionary conservation of retinol metabolic pathways, ranging from invertebrates, to amphibians, to mammals.

Our data support the hypothesis that metabolism of retinol leads to several molecules with different biological functions involving either control of cell growth or cell differentiation. The latter is exemplified by the acid derivatives of retinol, *i.e.* all-*trans*-RA, 9-cis-RA, and all-*trans*-4-oxo-RA, which induce differentiation accompanied by growth arrest via activation of nuclear receptors. On the other hand, 14-HRR and DHR serve as growth-promoting factors for retinol-dependent cells, but do not induce differentiation. AR is a functional antagonist of 14-HRR and DHR with growth-inhibiting activity. Whether 14-HRR, DHR, and AR function through nuclear, cytoplasmic, or membrane receptor is under investigation.

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