Extraction of Human Epidermis Treated With Retinol Yields *Retro*-Retinoids in Addition to Free Retinol and Retinyl Esters

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Vitamin A, all-*trans*-retinol, is metabolized to retinoic acid *in vivo* by a tightly controlled two-step conversion. Retinoic acid then binds to nuclear receptors and modulates cellular proliferation and differentiation. Because only a small fraction of retinol applied topically can be metabolized to retinoic acid, alternative pathways of retinol metabolism in skin were investigated. Retinol (0.4%) was applied to adult human skin under occlusion for 6 h to 4 d. The conversion of retinol into various metabolites such as 14-hydroxy-4,14-*retro*-retinol, anhydroretinol, 4-oxo-retinol, retinyl esters, and retinyl glucuronides was investigated. The level of 14-hydroxy-*retro*-retinol was increased from undetectable at time 0 to 326 ng/g wet weight of tissue at 6 h (6% of the retinol level) and maintained approximately the same concentration at 24 h to 409 ng/g wet weight (1.9% of the retinol level); it decreased to 48 ng/g wet weight of tissue (12% of its maximum level) by 4 d. Anhydroretinol was undetectable at time 0, increased only slightly at 6 h, and remained at the same level. We did not detect 4-oxo-retinol. Because 14-hydroxy-*retro*-retinol was found in the retinol-treated areas, its effects on epidermis were compared with those of retinol. Topical application of *trans*-retinol (0.3%) significantly increased both epidermal thickness and cellular retinoic acid binding protein II mRNA, whereas 14-hydroxy-4,14-*retro*-retinol (0.3%) did not increase either of these well-characterized cutaneous retinoid responses. Retinol, when applied topically in pharmacologic doses to human epidermis, remained as free retinol, was metabolized primarily to retinol ester, and was metabolized to a lesser extent to *retro*-retinoids and didehydroretinol. *Key words: retinyl glucuronides/didehydroretinol/pharmacology/metabolism. J Invest Dermatol 107: 178–182, 1996*

Topical application of retinoic acid (RA) to adult human skin produced local erythema, increased epidermal thickness (Fisher *et al*, 1991), increased cellular retinoic acid binding protein II (CRABPII) mRNA (Elder *et al*, 1993), and induced RA 4-hydroxylase activity (Duell *et al*, 1992). These effects are thought to be mediated through RA binding to specific nuclear receptors, with subsequent transactivation of specific genes (Krust *et al*, 1989). Application of *trans*-retinol (*t*-ROL) to adult human skin produces many of these same effects, except that no detectable erythema or RA formation is observed (Kang *et al*, 1995). In keratinocyte cell cultures, *t*-ROL is converted mainly to retinyl esters, with a small but detectable amount metabolized to RA (Kurlandsky *et al*, 1994). The small amount of *t*-RA can be quantitated, because in contrast to induction of the enzyme observed *in vivo* after *t*-ROL application to skin, RA 4-hydroxylase is not inducible in cultured keratinocytes. In lymphocytes, other metabolites of *t*-ROL, such as 14-hydroxy-4,14-*retro*-retinol (14-HRR) (Buck *et al*, 1991; Eppinger *et al*, 1993; Derguini *et al*, 1994b), anhydroretinol (AR) (Buck *et al*, 1993; Eppinger *et al*, 1993; Derguini *et al*, 1994a), and 13,14-dihydroxy-retinol (Derguini *et al*, 1995), have been shown to regulate proliferation, but not differentiation. In this study, we investigated the formation and biologic activity of these compounds in human epidermis.

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Abbreviations: 14-HRR, 14-hydroxy-4,14-*retro*-retinol; AR, anhydroretinol; dd-ROL, didehydroretinol; MeCN, acetonitrile; RA, retinoic acid; ROL ester, retinyl ester; *t*-RA, *trans*-retinoic acid; *t*-ROL, *trans*-retinol.

MATERIALS AND METHODS

Materials Retinoid standards purchased from Sigma Chemical Co. (St. Louis, MO) were as follows: *t*-ROL, *trans*-retinaldehyde, *t*-RA, 13-*cis*-ROL, 13-*cis*-retinaldehyde, and 13-*cis*-RA. The following were gifts: 4-hydroxy-RA, 13-*cis*-4-oxo-RA, 4-oxo-RA, 5,6-epoxy-RA, and AR (from M. Rosenberger and P.F. Sorter, Hoffmann LaRoche, Nutley, NJ), and 9-*cis*-RA (from Drs. J. Grippo and A. Levin, Hoffmann LaRoche). We prepared 14-HRR according to Derguini *et al* (1994b); 4-oxo-retinol was obtained according to literature procedures (Boehm *et al*, 1990). The retinyl esters (ROL esters) were gifts from Dr. Anders Vahlquist (University of Linköping, Sweden) and Dr. Christine Huselton (Hoffmann LaRoche). Retinoyl glucuronide was a gift from Dr. Arun Barua (University of Iowa, Sioux City, IA), 9-*cis*-retinoyl glucuronide from Dr. Jörn Oliver Sass (Freie Universität, Berlin, Germany), and 13-*cis*-retinoyl glucuronide from Hoffmann LaRoche. All organic solvents for extractions and chromatography were high-performance liquid chromatography (HPLC) grade. HPLC was carried out using a Hewlett Packard 1090M Chem Work Station and a Spherisorb ODS1 column (4.6 mm × 250 mm) (Phase Separations, Norwalk, CT) to separate the retinoids (Duell *et al*, 1992). The HPLC

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effluent flowed directly into a Radiomatic flow through scintillation spectrometer Model 295A to quantitate recoveries.

Human Subjects and Biopsy Procedure In adult volunteers, solutions of vehicle (95% ethanol:propylene glycol, 7:3, v/v), *t*-ROL, 14-HRR, or *t*-RA were applied to the buttock area with occlusion for various periods of time. Before removal of tissue with a keratome, the area was infiltrated with 1% lidocaine. Areas were tape stripped to remove stratum corneum and applied materials not absorbed into the viable layers. A 4-mm punch biopsy was taken to verify the completeness of the stripping procedure as well as for determination of epidermal thickness by routine light histology. The keratome biopsy specimens were immediately frozen in liquid nitrogen and stored at −80°C until extracted. All subjects gave written consent to the protocol, approved by the University of Michigan Institutional Review Board.

Extraction of Retinoids Extraction of tissue for the presence of retinoids was carried out with organic solvents butanol:acetonitrile (1:1) (McClean *et al*, 1982). The tissue was ground under liquid nitrogen in a mortar and pestle until a fine powder was obtained. The powder was transferred to a homogenizer containing trace amounts of [^{3}H]*t*-RA (to calculate recoveries) in organic solvents at a ratio of 10:1 (ml solvent per g wet weight tissue). The tissue was homogenized with six to eight strokes of the pestle and transferred to a screw-capped glass centrifuge tube. The samples were vortexed for 5 min and centrifuged at 500 rpm for 5 min. The organic phase was transferred to a fresh tube, and the remaining material was re-extracted using the same procedure. The combined organic phases were evaporated to dryness under nitrogen and resuspended in 200 μl of acetonitrile (MeCN). The samples were filtered into HPLC vials, followed by evaporation to dryness and resuspension in a final volume of 30 μl of MeCN. Retinoids were separated on a Spherisorb ODS1 reverse-phase column with a gradient of increasing MeCN concentration (Duell *et al*, 1992). Separation time was extended from 42 min to 110 min with a flow rate increase from 0.5 ml/min to 1 ml/min at 40 min to separate and elute retinyl esters from the column.

Extraction of 14-HRR From Epidermis Epidermal strips from six individuals were extracted with butanol:MeCN and prepared for HPLC separation as described earlier, except that tracer RA was not present. Twenty-four aliquots of the material were injected on the HPLC, and fractions were collected at 30-s intervals from 15 min to 25 min of the HPLC run. The test tubes containing the peak eluting at the elution time of authentic 14-HRR were pooled, concentrated, and analyzed by ultraviolet (UV) absorption and mass spectroscopy. The UV spectra were taken from the diode array contour plots; the low-resolution electron impact mass spectrum and the high-resolution electron impact mass spectrum (reference perfluorokerosine) were measured on a JEOL DX-303 HF spectrometer (JEOL USA, Inc., Peabody, MA).

Other Methods Punch biopsy specimens were processed for hematoxylin and eosin staining and were examined by light microscopy for epidermal thickness. The slides were read in a blinded fashion with respect to treatment group. The epidermal thickness in micrometers was the average of five measurements from the bottom of the stratum corneum to the epidermal basement membrane at different inter-rete ridge sites.

RNA isolation and quantitation of CRABPII mRNA were as described previously (Elder *et al*, 1993; Kang *et al*, 1995). Forty micrograms of total RNA was loaded per lane, as determined by OD_{260}.

Data were analyzed by either the Student's t test for paired data or the repeated measures analysis of variance. All p values are two-sided.

RESULTS

A 0.4% *t*-ROL solution in ethanol:propylene glycol (7:3) with 0.5 mg butylated hydroxytoluene (as an antioxidant) per ml was applied to adult volunteers for 24 h under occlusion. Compounds extracted from this site produced the chromatogram shown in **Fig 1**. The numbers indicate the point in the separation where standard compounds eluted from the column. As expected, the dominant peak was *t*-ROL. Because of isomerization during penetration from the surface to the viable layers of the skin, the next most prominent peak was 13-*cis* ROL. In addition, there was a prominent unknown peak that eluted at 22 min and exhibited a UV spectrum with an absorption maximum at 348 nm and a vibrational fine structure identical to that of 14-HRR (Buck *et al*, 1991; Derguini *et al*, 1994b).

Identification of 14-HRR as a Component in Epidermal Extract To confirm the structure of this metabolite, fractions were collected from numerous HPLC separations of extracts

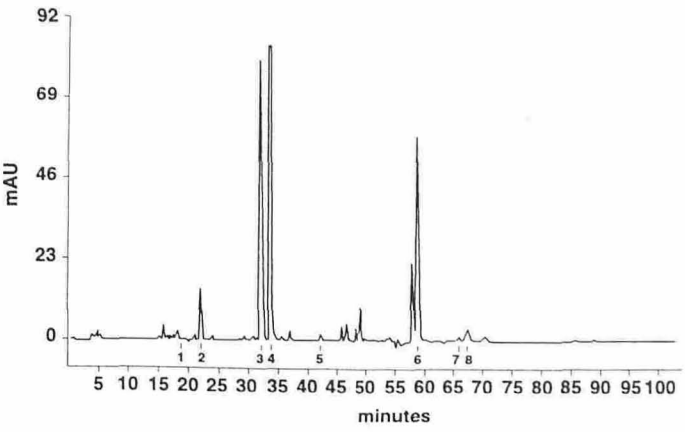

Figure 1. Chromatogram of epidermal extract obtained after exposure of epidermis to 0.4% *trans*-retinol under occlusion for 24 h. Epidermal biopsy specimen was extracted with butanol:acetonitrile (1:1), as described in *Materials and Methods*. Compounds were separated by HPLC on a Spherisorb ODS1 column with increasing MeCN. The numbers indicate the retention times of the following standards: 1, 4-oxo-retinol; 2, 14-hydroxy-*retro*-retinol; 3, 13-*cis*-retinol; 4, *t*-retinol; 5, anhydroretinol; 6, retinyl linoleate; 7, retinyl oleate; 8, retinyl palmitate.

obtained from *t*-ROL–treated epidermis. **Figure 2** shows the retention time of a standard solution of 14-HRR, and the insert shows the superimposed UV spectra of the standard and the pooled sample. Co-injection of the standard and extracted material gave a single sharp peak on the HPLC.

Mass spectral analysis of the pooled sample is given in **Fig 3**. Both the high-resolution **(Fig 3*A*)** and low-resolution **(Fig 3*B*)** mass spectra are in agreement with the literature (Buck *et al*, 1991); i.e., the observed mass of 302.2244 (calculated for $C_{20}H_{30}O_2$ = 302.2246) confirms that the new metabolite was indeed 14-HRR as suggested by the UV spectrum and the HPLC retention time. The circular dichroism spectrum (data not shown) did not show optical activity, indicating that 14-HRR produced under pharmacologic conditions is a racemic mixture (Derguini *et al*, 1994b).

Data showing the amount of 14-HRR extracted from tissue at time 0 and after a single application of 0.4% *t*-ROL with specimens

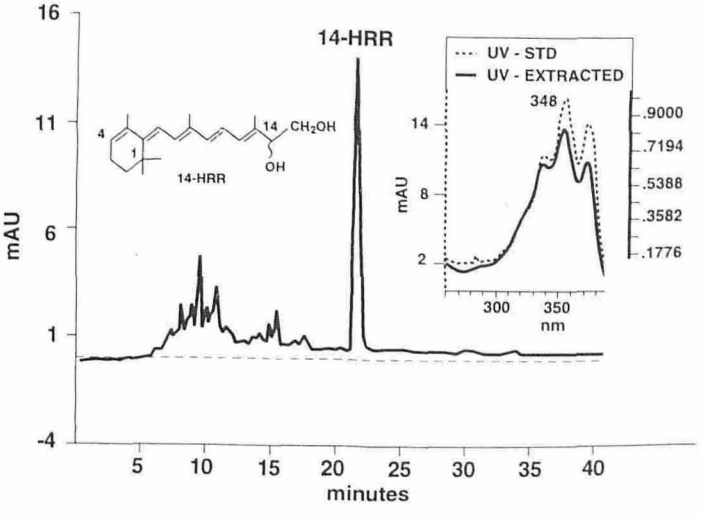

Figure 2. Chromatogram obtained by injection of a standard solution of 14-hydroxy-4,14-*retro*-retinol (14-HRR) onto the HPLC. Retention time of 14-HRR was 22 min, with HPLC conditions given in **Fig 1**. The *insert* shows the UV spectrum of the standard (----) and the UV spectrum of the pooled peak material extracted from the epidermis (—).

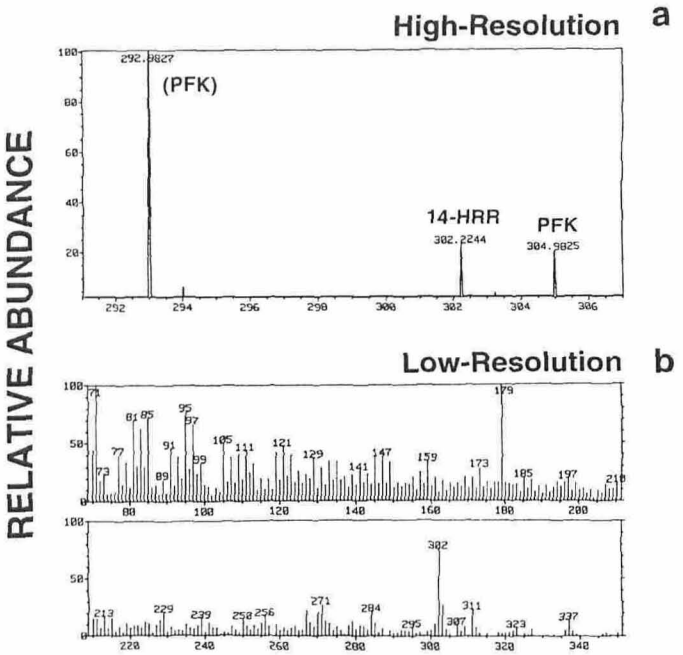

Figure 3. Mass spectra of pooled peak material extracted from retinol-treated epidermis. *a*) High-resolution electron impact mass spectrum (m/e 302.2244). *b*) Low-resolution electron impact mass spectrum (M^+ 302) of the same extracted 14-hydroxy-4,14-*retro*-retinol (14-HRR). Retinoid extraction and HPLC separation as in **Fig 1**. The pooled peak material had a retention time and UV spectrum similar to those of 14-HRR shown in **Fig 2**. PFK, perfluorokerosine.

at 6, 24, and 96 h are given in **Fig 4**. We did not detect 14-HRR at time 0. The concentration of 14-HRR in nanograms per gram wet weight was similar at 6 h (326 ± 73) and 24 h (409 ± 107), but was markedly reduced by 96 h (48 ± 3) (mean ± SEM). In contrast, *t*-ROL concentrations reached a maximum at 24 h and returned to 6-h levels at 96 h (Kang *et al*, 1995).

Topical 14-HRR Does Not Produce the Usual Retinoid Cutaneous Response The effects of 14-HRR on epidermal proliferation and differentiation in comparison with the effects of *t*-ROL and *t*-RA were investigated using the 4-d patch test for clinical and histologic evaluation. In addition, tape-stripped keratome biopsy specimens were extracted to determine penetration of the applied compounds into the viable layers and to determine

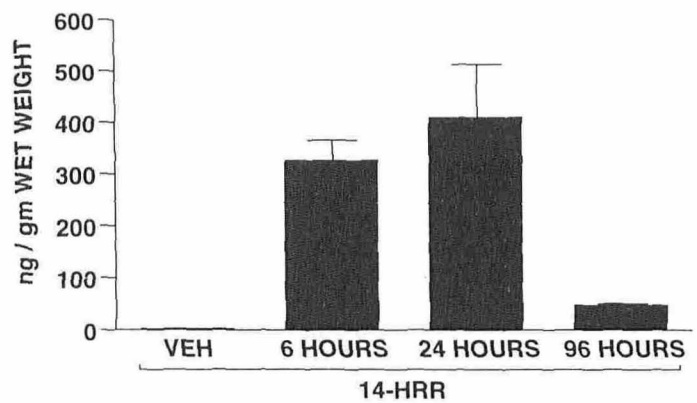

Figure 4. Content of 14-hydroxy-4,14-*retro*-retinol (14-HRR) in epidermal extracts from epidermis treated with *trans*-retinol. Epidermal biopsy specimens were extracted and retinoids were separated as in **Fig 1**. Changes in the amounts of 14-HRR extracted from the epidermis at each time are given as mean ± SEM; n = 5. VEH, vehicle.

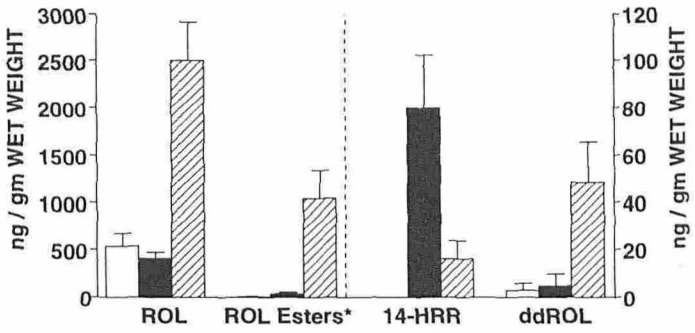

Figure 5. Retinoids extracted from viable epidermis 4 d after application of 14-hydroxy-4,14-*retro*-retinol (14-HRR) and *trans*-retinol (*t*-ROL). Either 0.3% 14-HRR, 0.3% *t*-ROL, or vehicle was applied to the epidermis under occlusion. After tape stripping to remove the stratum corneum and unabsorbed materials, tissue was extracted with butanol:MeCN. Retinoids were separated as in **Fig 1**. Data are mean ± SEM; n = 5. *Open bars*, vehicle; *closed bars*, 0.3% 14-HRR; *hatched bars*, 0.3% *t*-ROL. ddROL, didehydroretinol; ROL esters, retinyl esters.

possible changes in the content of endogenous retinoids in the epidermis as a result of the applied compounds. The concentrations of *t*-ROL, 14-HRR, and ROL esters extracted from the tissue after application of 0.3% of each compound in ethanol:propylene glycol vehicle are shown in **Fig 5**. The results showed that 14-HRR penetrated into the viable layers of the epidermis (80 ± 23 ng/g wet weight; n = 5). The 14-HRR–treated epidermis, in comparison with vehicle-treated tissue, contained the same amount of *t*-ROL, an increased amount (4.7-fold) of ROL esters (40 ± 20 ng/g wet weight; n = 5), and an increased amount (67%) of dd-ROL. In contrast, the application of 0.3% *t*-ROL increased *t*-ROL by 3.7-fold (2499 ± 428 ng/g wet weight; n = 5), increased ROL esters 150-fold (1042 ± 304 ng retinyl component per g wet weight; n = 5), and increased dd-ROL 15-fold (48 ± 18 ng/g wet weight; n = 5). There was a small amount of 14-HRR extracted at 4 d (16 ± 8 ng/g wet weight; n = 5). This is in agreement with the time-course data given previously **(Fig 4)**. Application of 0.025% *t*-ROL, 14-HRR, or *t*-RA did not yield detectable amounts of 14-HRR in the viable layers, and the *t*-ROL–treated sites showed only a 26% increase in *t*-ROL content above vehicle levels (data not shown).

Epidermal thickness was evaluated in histologic sections from punch biopsy specimens taken just before keratome biopsies. Neither concentration (0.3% or 0.025%) of 14-HRR altered epidermal thickness significantly ($p > 0.05$), and only 0.3% *t*-ROL produced a significant ($p < 0.05$) increase in epidermal thickness, similar to that observed with 0.025% *t*-RA, as is shown in **Fig 6**.

Increased CRABPII mRNA in epidermis has been associated with increased retinoic acid content of the epidermis, which also alters epidermal proliferation and differentiation (Elder *et al*, 1993). Significant increases in CRABPII mRNA in comparison with vehicle were noted in response to topical application of 0.025% RA, 0.025% *t*-ROL, or 0.3% *t*-ROL ($p < 0.05$), whereas neither concentration of 14-HRR increased CRABPII mRNA content in the epidermis, as is shown in **Fig 7**. In data not shown, 14-HRR did not displace tritiated ligands from cellular retinol binding protein, CRABPI or II, or from nuclear retinoid receptors (personal communication, Subhash Datta and Gary Fisher, Department of Dermatology, University of Michigan).

Other Retinol Metabolites The formation of AR, a reversible inhibitor of 14-HRR (Buck *et al*, 1993; Derguini *et al*, 1994a), after application of *t*-ROL was also investigated. As shown in **Fig 8**, the retention time of a standard solution of AR was 43 min. The insert shows the UV spectrum of standard AR and the UV spectrum obtained by diode array detector of the compound that eluted at 42.9 min. The amount of AR present in the extracts from the 0.4% *t*-ROL–treated tissue was approximately 1% of the 14-HRR levels

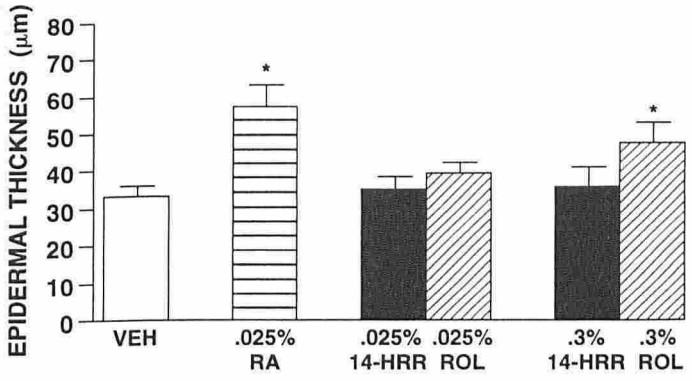

Figure 6. An increase in epidermal thickness was observed 4 d after application of 0.3% *trans*-retinol (ROL) but not 0.3% 14-hydroxy-4,14-*retro*-retinol (14-HRR). Each of the retinoids was applied in ethanol: propylene glycol (7:3) vehicle and occluded for 4 d. Data are mean ± SEM; n = 8. *Statistically significant difference ($p < 0.05$) between retinoic acid (RA) or retinol (ROL) and vehicle (VEH)-treated tissue.

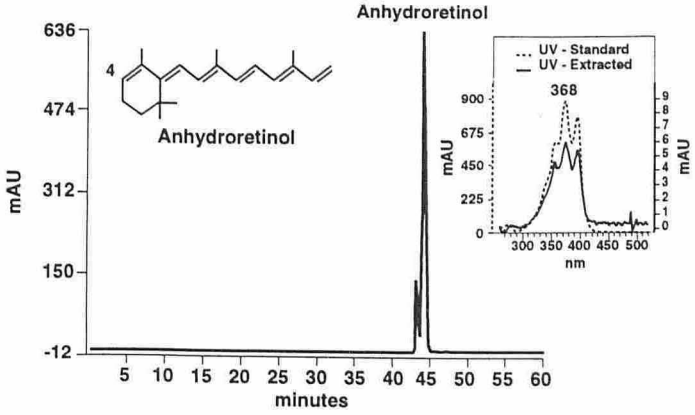

Figure 8. HPLC chromatogram of standard solution of anhydroretinol. Retention time of AR was determined by reverse phase chromatography as in **Fig 1**. The *insert* shows the UV spectrum of the standard (····) and the peak obtained in the epidermal extract (—), captured with the diode array detector during elution of the standard and epidermal extract from the column.

(Fig 1) and did not seem to vary during the 6-h to 4-d period. AR was not detected in vehicle-treated areas.

Recently, 4-oxo-ROL (a further metabolite of 4-OH-ROL) has been identified in extracts obtained from F9 cells treated first with *t*-RA, followed by addition of *t*-ROL as the cells are differentiating (Achkar *et al*, 1996). The retention time and UV absorbance spectrum of a 4-oxo-ROL standard obtained by the diode array detector of the HPLC are shown in **Fig 9**. Because the HPLC instrument captures and stores the spectra of all compounds detected as peaks with absorbance in the range of 320 to 380 nm, the chromatograms obtained during this investigation were studied for the presence of measurable quantities of 4-oxo-ROL, as assessed by its characteristic spectrum. Although peaks occurred on the chromatograms near the retention time of 4-oxo-ROL, the UV spectra were not compatible with that of 4-oxo-ROL. Either 4-oxo-ROL was not formed *in vivo*, was formed in too small a quantity to be detected, or was not stable to extraction conditions.

Figure 10 summarizes the possible routes of ROL metabolism. Possible pathways of conversion other than those described in this study are conversion of *t*-ROL to *t*-RA and formation of retinyl esters or glucuronides. From previous work, little or no detectable *t*-RA was extracted from tissue treated *in vivo* with *t*-ROL, but formation of retinyl esters was greatly increased (Kang *et al*, 1995). To examine possible glucuronidation of *t*-ROL, six extracts from *t*-ROL–treated tissues were divided in half. Half was treated with β-glucuronidase before separation by HPLC. There were no detectable changes in the elution patterns with β-glucuronidase treatment, i.e., no change in peak size or retention times, between treated and untreated samples.

DISCUSSION

In normal physiologic conditions, ROL is transported to tissue bound to retinol binding protein (Soprano and Blaner, 1994) and sequestered in the cell by cellular retinol binding protein (Ong *et al*, 1994). Application of pharmacologic doses of retinol to human skin circumvents this normal control and resulted in increased levels of free ROL and increased formation of retinyl esters in the epidermis (Kang *et al*, 1995). Additional studies of ROL-treated skin have identified an unknown compound as 14-HRR based on chromatographic retention time, UV spectrum, mass spectrum, and co-elution with standard 14-HRR; 14-HRR was first identified in extracts from lymphocytes treated with ROL (Buck *et al*, 1991). Addition of pharmacologic amounts of ROL to lymphocytes in

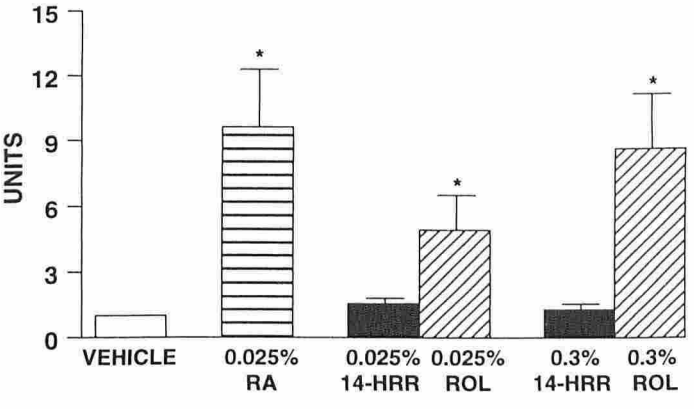

Figure 7. CRABPII mRNA content of epidermis is increased 4 d after application of *trans*-retinol (ROL) but not after application of 14-hydroxy-4,14-*retro*-retinol (14-HRR). Retinoids were applied as in **Fig 6**. Isolation and identification of mRNA are given in *Materials and Methods*. Data are mean ± SEM; n = 8. *Statistically significant difference ($p < 0.05$) between retinoic acid (RA), 0.025% retinol (ROL), or 0.3% ROL and vehicle-treated tissue.

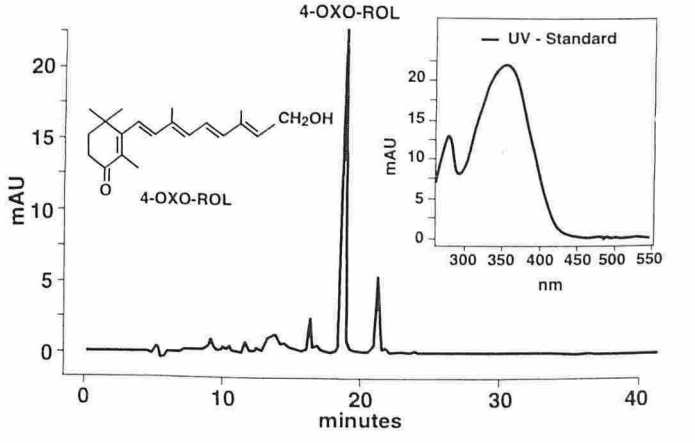

Figure 9. HPLC chromatogram of standard solution of 4-oxo-retinol. Retention time of 4-oxo-ROL was determined with chromatographic conditions as in **Fig 1**. The *insert* contains the UV spectrum captured with the diode array detector during elution of the standard. These data were used to scan previous chromatograms obtained with extracts from treated skin for the presence of 4-oxo-ROL.

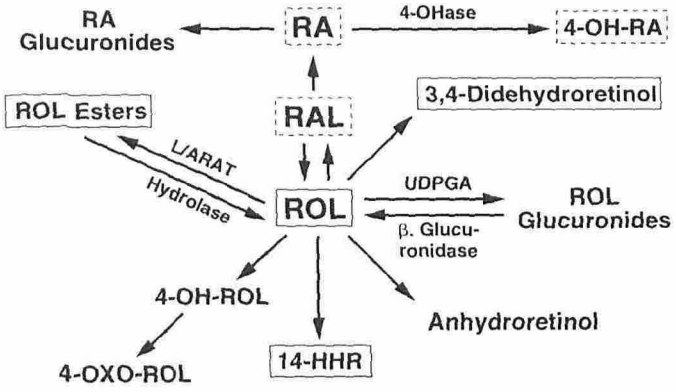

Figure 10. Possible routes of retinol metabolism in skin. Compounds enclosed in *solid lines* were extracted from epidermis treated with *t*-ROL; compounds enclosed in *dashed lines* were not detected, but their formation was inferred from other data generated previously from human skin or keratinocytes. No evidence for the formation of the other compounds (unenclosed) was found. 4-OH-RA, 4-OH-retinoic acid; L/ARAT, lecithin/acyl:retinol acyltransferase; RAL, retinaldehyde; UDPGA, uridine diphosphoglucuronic acid.

culture also produced a racemic mixture of 14-HRR (Derguini *et al*, 1994b), and this was observed in skin as well. Derguini *et al* (1994b) speculated that epoxidation of free ROL might occur in a nonstereospecific manner, whereas epoxidation of ROL bound to its specific binding protein might occur in a stereospecific manner.

Originally, 14-HRR was identified as an inducer of lymphocyte proliferation (Buck *et al*, 1991), and AR as a reversible inhibitor of 14-HRR effects (Buck *et al*, 1993). A similar role for 14-HRR on keratinocytes should lead to increased epidermal thickness. In the 4-d patch test, 0.3% 14-HRR did not increase epidermal thickness or erythema, which argues against 14-HRR as an inducer of proliferation in human epidermis *in vivo*. Because AR is a competitive inhibitor of 14-HRR (Buck *et al*, 1993), the small amount of AR present in the epidermis after ROL application would not be sufficient to antagonize 14-HRR–induced proliferation. The lack of induction of CRABPII mRNA also indicates that 14-HRR is not functioning as do other retinoids, such as *t*-RA and *t*-ROL, as both retinoids induce mRNA for CRABPII in human skin *in vivo*. Thus, the function(s) of *retro*-retinoids present in epidermis remains unknown.

Other known pathways of *t*-ROL metabolism are *t*-RA formation in keratinocytes (Kurlandsky *et al*, 1994), retinyl esters (Törmä and Vahlquist, 1990; Kang *et al*, 1995), and dd-ROL (Törmä and Vahlquist, 1991). Measurable quantities of *t*-RA are rarely found when *t*-ROL is applied to human skin *in vivo*, even with subsequent extraction of the tissue for retinoid content with organic solvents chloroform:methanol (2:1) and methanol:water at pH 3.2 (85:15). This is probably due to the induction of *t*-RA 4-hydroxylase (Duell *et al*, 1992), which prevents *t*-RA accumulation. Addition of *t*-RA to keratinocytes in culture does not induce *t*-RA 4-hydroxylase activity. Both *t*-RAL and *t*-RA were isolated from such cultures after the addition of *t*-ROL. The metabolism of *t*-ROL to *t*-RA was required to demonstrate biologic activity (Kurlandsky *et al*, 1994).

An increasing gradient of ROL ester content in normal epidermis from basal layer to stratum corneum has been reported by other investigators (Törmä and Vahlquist, 1990). The ROL ester content of epidermis in the experiments reported here was somewhat low because of loss of the upper layers of epidermis by tape stripping just before biopsy of the site. Ester formation, however, remains the main route by which epidermal cells handle excess *t*-ROL (Kang *et al*, 1995). Some conversion of *t*-ROL to dd-ROL was detected in the *t*-ROL–treated tissues, which agrees with previous reports of dd-ROL levels of 5–18% of *t*-ROL levels (Törmä and Vahlquist, 1991). The *t*-ROL or dd-ROL levels were unchanged or marginally changed after treatment with 14-HRR.

The formation of glucuronides is a possible mechanism for removing excess *t*-ROL in the epidermis, but β-glucuronidase treatment produced no changes in the chromatograms. It is nevertheless possible that glucuronides formed in epidermis *in vivo* would be exported into the dermis and then into the circulatory system, thereby rendering them undetectable in epidermal samples.

In summary, although numerous pathways are available for *t*-ROL metabolism in skin, retinyl ester formation is the primary route for disposition of pharmacologic doses of *t*-ROL. The final metabolite that is the important retinoid for biologic activity in keratinocytes, however, is *t*-RA.

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REFERENCES

Achkar CC, Derguini F, Blumberg B, Langston A, Levin AA, Speck J, Evans RM, Balado J Jr, Nakanishi K, Buck J, Gudas L: 4-Oxoretinol, a new natural ligand and transactivator of the retinoic acid receptors. *Proc Natl Acad Sci USA* 93:4879–4884, 1996

Boehm MF, Gawinowicz MA, Foucault A, Derguini F, Nakanishi K: Photoaffinity labeling studies of bacteriorhodopsin with (15-^{3}H)-3-diazo-4-keto-all-*trans*-retinal. *J Am Chem Soc* 112:7779–7782, 1990

Buck J, Derguini F, Levi E, Nakanishi K, Hämmerling U: Intracellular signaling by 14-hydroxy-4,14-*retro*-retinol. *Science* 254:1654–1656, 1991

Buck J, Grün F, Derguini F, Chen Y, Kimura S, Noy N, Hämmerling U: Anhydroretinol: a naturally occurring inhibitor of lymphocyte physiology. *J Exp Med* 178:675–680, 1993

Derguini F, Nakanishi K, Buck J, Hämmerling U, Grün F: Spectroscopic studies of anhydroretinol, an endogenous mammalian and insect *retro*-retinoid. *Angew Chem Int Ed Engl* 33:1837–1839, 1994a

Derguini F, Nakanishi K, Hämmerling U, Buck J: Intracellular signaling activity of synthetic (14R)-, (14S)-, and (14RS)-14-hydroxy-4, 14-*retro*-retinol. *Biochemistry* 33:623–628, 1994b

Derguini F, Nakanishi K, Hämmerling U, Chua R, Eppinger T, Levi E, Buck J: 13,14-Dihydroxy-retinol, a new bioactive retinol metabolite. *J Biol Chem* 270: 18875–18880, 1995

Duell EA, Åström A, Griffiths CEM, Chambon P, Voorhees JJ: Human skin levels of retinoic acid and cytochrome P-450-derived 4-hydroxyretinoic acid after topical application of retinoic acid *in vivo* compared to concentrations required to stimulate retinoic acid receptor-mediated transcription *in vitro*. *J Clin Invest* 90:1269–1274, 1992

Elder JT, Cromie MA, Griffiths CEM, Chambon P, Voorhees JJ: Stimulus-selective induction of CRABP-II mRNA: a marker for retinoic acid action in human skin. *J Invest Dermatol* 100:356–359, 1993

Eppinger TM, Buck J, Hämmerling U: Growth control or terminal differentiation: endogenous production and differential activities of vitamin A metabolites in HL-60 cells. *J Exp Med* 178:1995–2005, 1993

Fisher GJ, Esmann J, Griffiths CEM, Talwar HS, Duell EA, Hammerberg C, Elder JT, Finkel LJ, Karabin GD, Nickoloff BJ, Cooper KD, Voorhees JJ: Cellular, immunologic and biochemical characterization of topical retinoic acid-treated human skin. *J Invest Dermatol* 96:699–707, 1991

Kang S, Duell EA, Fisher GJ, Datta SC, Wang ZQ, Reddy AP, Tavakkol A, Yi JY, Griffiths CEM, Elder JT, Voorhees JJ: Application of retinol to human skin *in vivo* induces epidermal hyperplasia and cellular retinoid binding proteins characteristic of retinoic acid but without measurable retinoic acid levels or irritation. *J Invest Dermatol* 105:549–556, 1995

Krust A, Kastner PH, Petkovich M, Zelent A, Chambon P: A third human retinoic acid receptor, hRAR-γ. *Proc Natl Acad Sci USA* 86:5310–5314, 1989

Kurlandsky SB, Xiao JH, Duell EA, Voorhees JJ, Fisher GJ: Biological activity of all-*trans* retinol requires metabolic conversion to all-*trans* retinoic acid and is mediated through activation of nuclear retinoid receptors in human keratinocytes. *J Biol Chem* 269:32821–32827, 1994

McClean SW, Ruddel ME, Gross EG, DeGiovanna JJ, Peck GL: Liquid-chromatographic assay for retinol (vitamin A) and retinol analogs in therapeutic trials. *Clin Chem* 28:693–696, 1982

Ong DF, Newcomer ME, Chytil F: Cellular retinoid binding proteins. In: Sporn MB, Roberts AB, Goodman D (eds.). *The Retinoids: Biology, Chemistry and Medicine*. Raven Press, New York, 1994, pp 283–317

Soprano DR, Blaner WS: Plasma retinol-binding protein. In: Sporn MB, Roberts AB, Goodman D (eds.). *The Retinoids: Biology, Chemistry and Medicine*. Raven Press, New York, 1994, pp 257–281

Törmä H, Vahlquist A: Retinol uptake and metabolism to 3,4-didehydroretinol in human keratinocytes at various stages of differentiation. *Skin Pharmacol* 4:154–157, 1991

Törmä H, Vahlquist A: Vitamin A esterification in human epidermis: a relation to keratinocyte differentiation. *J Invest Dermatol* 94:132–138, 1990