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THE FORMATION OF ALL-*TRANS*-RETINOIC ACID FROM ALL-*TRANS*-RETINOL IN HAIRLESS MOUSE SKIN

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Abstract—All-*trans*-retinoic acid formation from topically applied retinol has been demonstrated in the skin of skh/hr1 (hairless) mice. The all-*trans*-retinoic acid was identified on the basis of its chromatographic properties on HPLC at various pH values, its photoisomerization to reaction products identical to those formed from authentic all-*trans*-retinoic acid, and its co-chromatography with methyl retinoate after methylation with diazomethane. Topically applied retinol is about 2-fold less potent at inducing epidermal hyperplasia and 7-fold less potent at inhibiting the induction of epidermal ornithine decarboxylase by phorbol esters than all-*trans*-retinoic acid in this strain of mice. To elucidate the possible role all-*trans*-retinoic acid formation from retinol may have in these pharmacological activities, the epidermal and dermal all-*trans*-retinoic acid levels were compared in mice treated topically with retinol or [11-^{3}H]-all-*trans*-retinoic acid. The levels of all-*trans*-retinoic acid found after retinol treatment were several orders of magnitude lower than those found after [11-^{3}H]-all-*trans*-retinoic acid treatment, and they were insufficient to account for the difference in potencies between all-*trans*-retinoic acid and retinol. Retinol was eliminated from the epidermis at a rate similar to that of all-*trans*-retinoic acid after topical administration, but the initial tissue levels achieved were lower. These results suggest that the lower potencies of retinol may simply reflect lower tissue uptake.

Vitamin A is required for normal epithelial growth and differentiation. These requirements can be fulfilled by all-*trans*-retinoic acid (vitamin A acid) in vitamin A deficient animals, and it may be a physiologically active form of vitamin A in these areas [1]. All-*trans*-retinoic acid is a retinoid metabolite found in some tissues [2, 3], and it is formed by the oxidation of retinol via the aldehyde retinal, or directly from retinal [1]. The latter oxidation step is irreversible and explains why all-*trans*-retinoic acid cannot completely replace retinol in the diet since it cannot be reduced back to retinal or retinol, which are required for normal vision and reproduction.

Retinol is the circulating form of vitamin A and is present in the plasma in a retinol/serum retinol-binding protein–transthyretin complex, specific receptors for which are present on epidermal cells [4]. Saponified extracts of mouse epidermis contain about 6 nmoles retinol/g [5], although this value presumably reflects mainly the levels of retinol esters. Mouse epidermis contains enzymes which can interconvert retinol and retinal and can oxidize retinal to retinoic acid [6]. Although retinoid metabolism in the epidermis has not been reported in depth, indirect evidence suggests that acid forms of vitamin A are physiologically important in this retinoid target tissue. The epidermis is apparently rich in cellular retinoic acid binding protein (CRABP) [7, 8], a molecule implicated in the mediation of retinoid action. The therapeutic use of retinoids in dermatology currently centers on acidic species such as all-*trans*-retinoic acid, 13-*cis*-retinoic acid, and the ester and free acid forms of etretinate, although retinol shows some clinical efficacy.

Topical retinoid treatment of hairless mouse skin stimulates epidermal proliferation and turnover [9], producing a dose-dependent thickening of the prickle and granular cell layers (epidermal hyperplasia) [10]. Retinol and retinal are about 50% as potent as all-*trans*-retinoic acid at inducing the hyperplasia [10]. A retinol dose of 1.7 nmoles has been reported to inhibit the induction of ornithine decarboxylase activity in phorbol ester treated mouse epidermis by about 50% compared to a 57% inhibition after 0.17 nmole all-*trans*-retinoic acid [11]. The reasons for the lower potencies of retinol are unclear but may reflect differences in tissue uptake and loss, or a requirement for metabolic activation (such as oxidation to the acid).

In the present study we measured the endogenous non-esterified retinol levels in hairless mouse skin, and examined the cutaneous metabolism of retinol after topical application, establishing that conversion of retinol to the acid form did occur in the skin. In an attempt to elucidate the relation, if any, of all-*trans*-retinoic acid formation to the mechanism of action of pharmacological doses of retinol, we compared the epidermal and dermal all-*trans*-retinoic acid levels found after topical retinol treatment to those found after topical all-*trans*-retinoic acid treatment.

MATERIALS AND METHODS

Materials. Retinol (all-*trans*-retinol), retinal, retinyl acetate and retinyl palmitate were obtained from the Sigma Chemical Co. (St. Louis, MO). All-*trans*- and 13-*cis*-methyl retinoates were synthesized from all-*trans*- and 13-*cis*-retinoic acids, respectively, using an excess of diazomethane generated by the method of Fales *et al.* [12]. [11-^{3}H]All-*trans*-retinoic acid

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(specific radioactivity 1.51 Ci/mmole) was provided by the National Cancer Institute. Other retinoids were gifts from Dr. Peter Sorter (Hoffmann-La Roche, Nutley, NJ). Purity of the retinoids was checked before use by HPLC and was greater than 98%. HPLC grade solvents were obtained from Fisher Scientific (Los Angeles, CA). Experiments were performed using female skh/hr1 (hairless) mice obtained from the Skin and Cancer Hospital, Temple University (Philadelphia, PA).

Treatments. To minimize chemical and photochemical degradation, all procedures were performed under reduced or dark-room lighting (Kodak No. 2 Safelight Filter), and retinoid solutions were prepared freshly. To investigate cutaneous retinol metabolism, 0.2 ml of acetone containing retinol (0, 50, 100, or 200 nmoles) was applied to the dorsal skins of groups of three to four mice using a micropipette with disposable tips. The mice were housed in the dark and were killed by cervical dislocation 0–24 hr after treatment. The treated areas of skin (15 cm^2) were removed by dissection, the skin was heated to 56° for 30 sec and then cooled in an ice/water bath, and the epidermis (80–140 mg) was scraped from the dermis (1.0 to 2.0 g) [13]. The tissues were weighed and the retinoids were extracted as described below. To study the handling of topically applied all-*trans*-retinoic acid, groups of three mice were dosed topically with 0.2 ml acetone containing 2, 8, 60, or 100 nmoles of all-*trans*-retinoic acid spiked with 2 μCi of [11-^{3}H]-all-*trans*-retinoic acid. At appropriate times the mice were killed, and the tissues were recovered and extracted as previously described [13].

Potencies for the inhibition of the induction of epidermal ornithine decarboxylase activity were determined by applying the retinoids (0.01, 0.1, 1.0, and 10 nmoles in 0.2 ml acetone) topically 1 hr prior to application of tetradecanoylphorbol-13-acetate (17 nmoles in 0.1 ml acetone). The mice were killed 4.5 hr later, and the epidermis was recovered, extracted, and assayed for ornithine decarboxylase activity as previously described [14].

Extraction procedures. The tissues from the retinol-treated mice were extracted as described in Ref. 13 with minor modification. The epidermis was homogenized in a Dounce type glass–glass tissue grinder in 20 vol. of chloroform–methanol (2:1) containing 50 mg/l butylated hydroxytoluene as an antioxidant. The homogenate was centrifuged at 1000 *g* for 5 min at room temperature, and the supernatant fraction was recovered. The pellet was resuspended in 2 ml of extraction solvent, and the mix was vortexed vigorously. The mixture was recentrifuged as above, and the supernatant fraction pooled with the first supernatant. Supernatant fractions were stored in amber vials at −70° until assayed. Dermis was freeze-dried prior to extraction and homogenized in a Brinkmann mechanical homogenizer, and otherwise treated as was the epidermis. In preliminary experiments the recovery of exogenous retinol and retinyl acetate added directly to the tissues prior to extraction was 90%. Extraction of all-*trans*-retinoic acid under these conditions was quantitative [13].

Retinoid analysis. Tissue extracts were evaporated to dryness under a stream of nitrogen and resuspended in the appropriate chromatography solvent. Samples were analyzed for retinol and retinoic acid by reverse phase HPLC on a 5 μm Spherisorb ODS column (BioRad Laboratories) fitted with a guard column, using an LKB HPLC system equipped with a variable wavelength detector set to monitor column eluant absorbance at 340 nm, and a Hewlett–Packard Integrator. Samples (0.04 to 0.2 ml) were injected onto the column through a Rheodyne injector fitted with a 2-ml sample loop. Column eluant was collected in an LKB Redirac fraction collector when required. The solvent system was 70% methanol, 30% 0.01 M ammonium acetate switching to 80% methanol, 20% 0.01 M ammonium acetate at 30 min, delivered at a flow rate of 1 ml/min, except where stated. The column was washed periodically with pure methanol to elute any accumulating non-polar materials. In studies of the effect of the pH of the eluting solvent on retention times, the samples were eluted with methanol–0.1 M potassium acetate mixtures titrated to the appropriate pH with acetic acid. Fluorescence and ultraviolet spectra were obtained with a Turner model 430 spectrofluorometer and a Beckman model 35 spectrophotometer respectively. Radioactivity was measured in a Beckman liquid scintillation counter.

Methods of calculation. Retinoid concentrations in the skin extracts from the retinol-treated mice were calculated from the integrated HPLC peak areas with reference to standard curves obtained by injecting known amounts of the authentic retinoids. The standard curves were linear over the range used, with a minimum measurable detection limit of 10 pmoles for both all-*trans*-retinoic acid and retinol. Tissue retinol levels were corrected for incomplete recovery (90%) during extraction. All-*trans*-retinoic acid levels in tissues from [^{3}H]-all-*trans*-retinoic acid treated mice were determined by counting the radioactivity in the appropriate radioactive peaks. The elimination half-lives were derived by regression analysis using the logarithm of the tissue retinoid concentrations found 2–24 hr after treatment [13], with allowance made for endogenous retinol, and are expressed as the value ± the standard error of the estimate. The retinoid concentrations in the dosing and standard solutions were determined from their absorbance (in methanol) using $E_{325\ nm}$ = 52,000 for retinol and $E_{351\ nm}$ = 45,000 for all-*trans*-retinoic acid.

RESULTS

*Induction of epidermal hyperplasia and the inhibition of ornithine decarboxylase induction by retinol and all-*trans*-retinoic acid.* The dose inhibiting the induction of ornithine decarboxylase activity by 50% in this strain of mice was 1.8 nmoles of retinol compared to 0.23 nmole of all-*trans*-retinoic acid. These values are similar to the published values for CD1 mice [11]. Retinol had a relative potency of 0.47 compared to all-*trans*-retinoic acid at inducing epidermal hyperplasia in skh/hr1 mice [10].

*Assignment of the identity of all-*trans*-retinoic acid.* The tissue extracts from mice treated with topical retinol contained variable amounts of a metabolite which absorbed at 340 nm and co-eluted

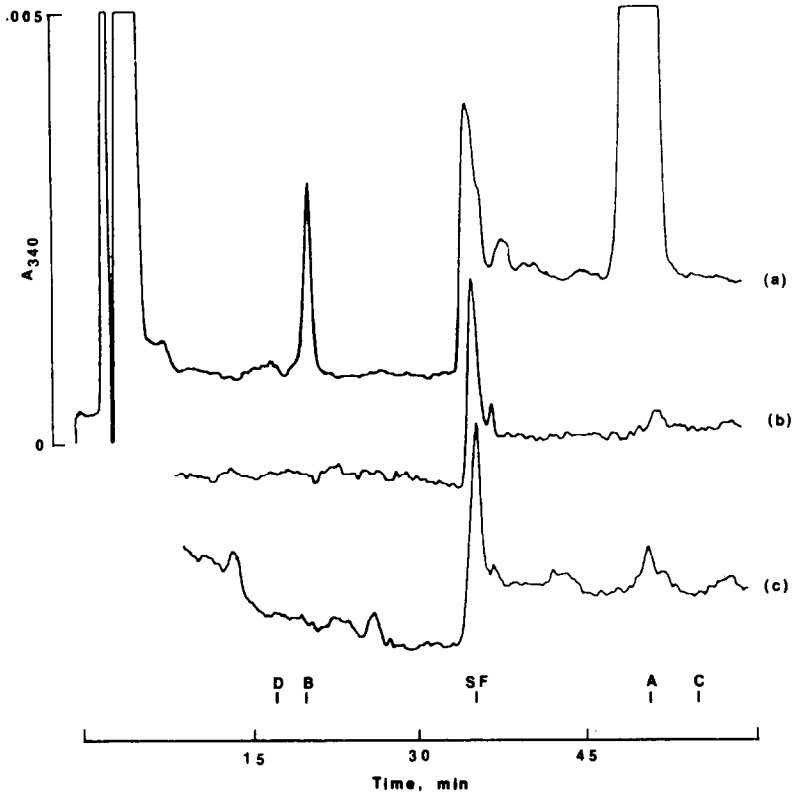

Fig. 1. Cutaneous retinol and all-*trans*-retinoic acid levels in control and retinol-treated mice. HPLC chromatography (conditions as described in the text) of extracts from (a) 20 mg epidermis recovered 4 hr after 100 nmoles retinol; (b) comparable amount (20 mg) control epidermis; and (c) 60 mg control epidermis showing endogenous retinol peak. The letters mark the elution positions of (A) retinol, (B) all-*trans*-retinoic acid, (C) retinal, (D) 13-*cis*-retinoic acid, and (SF) solvent front.

with all-*trans*-retinoic acid on HPLC (Fig. 1). The metabolite peak was recovered for further characterization. When the metabolite was irradiated with visible light from a fluorescent lamp for 1 hr and then re-chromatographed, the irradiated metabolite contained several new chromophores (Fig. 2), corresponding to the photoisomerization products obtained from authentic all-*trans*-retinoic acid. Samples of the metabolites were methylated, after evaporating the metabolite peak to dryness under a stream of nitrogen, by passing an excess of diazomethane into the metabolite resuspended in diethyl-ether–methanol (9:1) for 15 min. The ethereal metabolite solution was evaporated to dryness, resuspended in methanol–water (90:10), and analyzed by HPLC. The methylated metabolite co-migrated with all-*trans*-methyl retinoate (Fig. 3) obtained by methylation of authentic all-*trans*-retinoic acid. In keeping with an acidic retinoid structure, the retention time of the metabolite was markedly pH dependent [15]. The retention time of the metabolite on reverse phase columns decreased as the pH of the eluting solvent increased (21.2 min at pH 5.45, 19.6 min at pH 5.86, 12.7 min at pH 6.65, 8.6 min at pH 6.95; determined using a Resolvex C18 column eluted with methanol–0.1 M potassium acetate (80:20) at a flow rate of 3 ml/min), and it co-chromatographed with authentic all-*trans*-retinoic acid at each pH. In contrast, the elution position of retinol was unchanged. Residual 340 nm absorbing chromophores were absent from the original site of elution of the putative all-*trans*-retinoic acid peak on chromatography of representative tissue extracts on the Spherisorb ODS column when the pH of the eluting solvent was shifted. Thus, the all-*trans*-retinoic acid values were not overestimated due to the presence of co-eluting non-acidic retinol metabolites. All-*trans*-retinoic acid in the extracts from [^{3}H]-all-*trans*-retinoic acid treated mice was further identified by its co-migration with authentic all-*trans*-retinoic acid on thin-layer chromatography [13] and from its ultraviolet spectrum.

Cutaneous retinol levels, and the elimination and metabolism of exogenous retinol. Retinol was identified in epidermal and dermal extracts from untreated mice by its co-elution on HPLC with authentic retinol, by its fluorescence spectrum (excitation maximum 350 nm at an emission wavelength of 480 nm in hexane), by its absorbance spectrum, by its destruction on irradiation with ultraviolet light, and by the appearance of small amounts of a chromophore with the chromatographic properties of 13-*cis*-retinol when the recovered (putative) retinol peak was rechromatographed. The endogenous retinol concentration was 8-fold higher in the epidermis than in the dermis (Table 1). Tissue extracts from control animals prepared in the absence or presence of exogenous retinyl acetate or retinyl palmitate

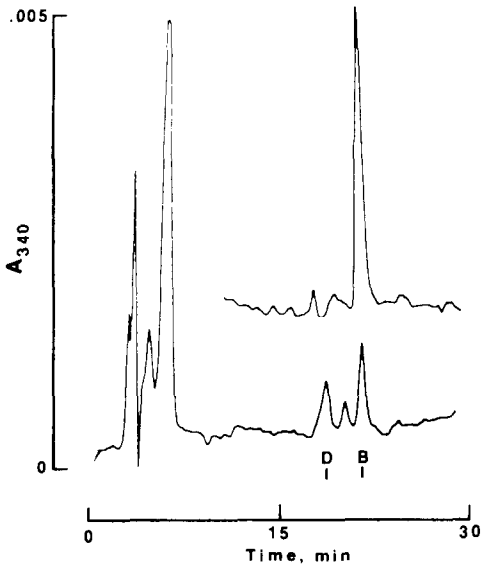

Fig. 2. Effect of light exposure on the putative all-*trans*-retinoic acid peak. Epidermal extracts from retinol-treated mice were pooled and chromatographed on HPLC (30% 0.01 M ammonium acetate–70% methanol, 1 ml/min). The metabolite peak (upper trace) was recovered, irradiated with visible light for 1 hr, and re-chromatographed (lower trace). The letters mark the elution positions of (B) all-*trans*-retinoic acid, and (D) 13-*cis*-retinoic acid.

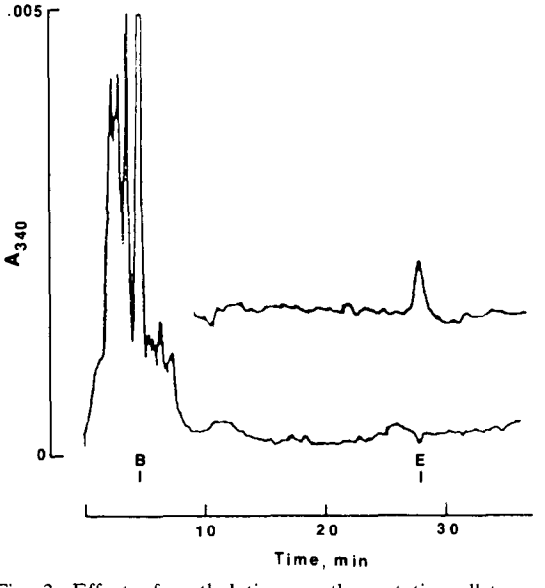

Fig. 3. Effect of methylation on the putative all-*trans*-retinoic acid peak. The putative all-*trans*-retinoic acid peaks were pooled and methylated with diazomethane. On chromatography (15% 0.01 M ammonium acetate–75% methanol, at a flow rate of 1 ml/min) the methylated metabolite (upper trace) migrated with the same retention time as (E) all-*trans*-methyl retinoate. The lower trace shows the unmethylated metabolite in the same solvent system.

contained similar levels of retinol, demonstrating that the free retinol recovered from the tissues was not an artifact due to hydrolysis of retinyl esters during extraction. All-*trans*-retinoic acid was not detected in extracts of epidermis or dermis from control mice.

Although unchanged retinol was by far the most dominant chromophore in extracts from retinol-treated mice, the extracts contained several 340 nm absorbing materials which were absent from extracts from controls (Fig. 1). One of these eluted from the column with the same retention time as all-*trans*-retinoic acid (Fig. 1), and this identity was confirmed as described above. The other metabolite peaks were not identified; however, retinal and 13-*cis*-retinoic acid were not detected in any of the extracts. The HPLC elution conditions used in the metabolism studies were not harsh enough to elute the endogenous retinyl esters.

Table 1 gives the retinol and all-*trans*-retinoic acid levels found in epidermis and dermis at various times after treatment with 100 nmoles retinol. The all-*trans*-retinoic acid levels tended to decrease as the tissue retinol levels fell, and by 24 hr all-*trans*-retinoic acid could not be detected in epidermal or dermal extracts. Figures 4 and 5 show the retinol and all-*trans*-retinoic acid levels found in the epidermis and dermis 4 hr after treatment with various doses of retinol. The epidermal retinol level appeared to saturate after a dose of 100 nmoles (Fig. 4), and a similar though less marked trend was evident in the dermis (Fig. 5). These trends were echoed in the retinol-derived all-*trans*-retinoic acid levels, although they were 1.5 to 2 orders of magnitude lower than the retinol levels at all doses. Figures 4 and 5 also show the all-*trans*-retinoic acid levels determined after treatment of mice with various doses of tritium-labeled all-*trans*-retinoic acid. It is evident that the epidermal and dermal all-*trans*-retinoic acid levels found after retinol treatment were at least 2 orders of magnitude less than after an equivalent treatment with all-*trans*-retinoic acid.

The elimination half-lives for retinol following topical administration of 100 nmoles retinol were 3.0 ± 0.2 and 8.6 ± 1.5 hr from the epidermis and dermis, respectively, and were similar to the corresponding values for an equivalent dose of all-*trans*-retinoic acid (3.2 ± 0.1 and 5.43 ± 0.35 hr respectively). However, the derived maximum tissue contents, calculated by extrapolating back to zero time, were lower after retinol administration (124.5 ± 24.4 nmoles/g epidermis and 1.81 ± 0.33 nmoles/g dermis) than after all-*trans*-retinoic acid administration (547 ± 59 nmoles/g epidermis and 4.56 ± 0.43 nmoles/g dermis). That comparatively lower tissue levels were achieved after retinol administration at other doses is evident from the trends in Figs. 4 and 5.

DISCUSSION

These experiments demonstrate that all-*trans*-retinoic acid is formed in mouse skin after treatment with pharmacological doses of retinol. Retinal reducing and oxidizing enzymes have been demonstrated previously in extracts of mouse epidermis [6]. Retinal is a presumed intermediate in the oxidation of retinol to the acid, and is the active form of vitamin A in vision. We did not find retinal in the tissue extracts even after the highest doses of retinol (200 nmoles),

Table 1. Epidermal and dermal retinol and all-*trans*-retinoic acid levels at various times after treatment with retinol

	Time (hr)	All-*trans*-retinoic acid (pmoles/g)	Retinol (pmoles/g)
Epidermis			
Treated	2	2910 ± 1170	110,000 ± 24,600
	4	1830 ± 780	50,800 ± 6710
	8	899 ± 275	13,900 ± 2630
	24	ND*	1280 ± 717
Untreated (control)		ND	1160 ± 633
Dermis			
Treated	2	187 ± 123	2700 ± 551
	4	38.3 ± 9.6	1250 ± 354
	8	18.2 ± 4	861 ± 178
	24	ND	437 ± 26
Untreated (control		ND	164 ± 46

Mice were treated topically with 100 nmoles retinol, and the epidermal and dermal retinol and all-*trans*-retinoic acid levels were determined by HPLC. Values are means ± SD; N = 3 except for the untreated controls where N = 4.
* Not detected.

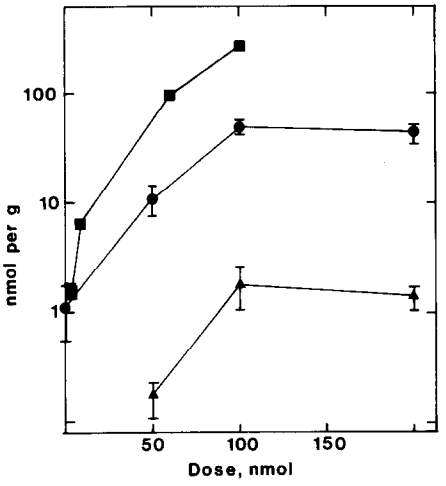

Fig. 4. Epidermal retinol and all-*trans*-retinoic acid levels. Mice were treated with retinol and the epidermis was examined 4 hr later for retinol (—●—●—) and all-*trans*-retinoic acid (—▲—▲—), or they were treated with [11-^{3}H]-all-*trans*-retinoic acid and the tissues were examined for all-*trans*-retinoic acid (—■—■—). Error bars denote the standard deviations (N = 3–4); in some cases these are obscured by the points.

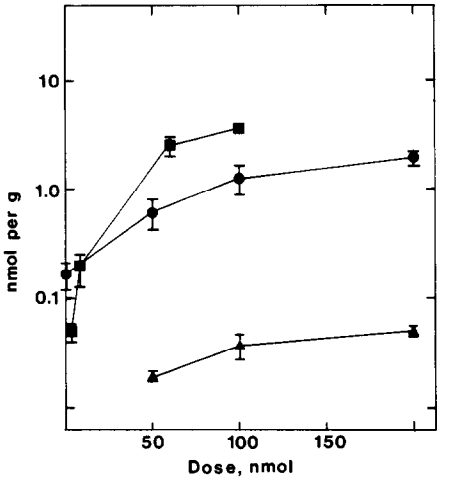

Fig. 5. Dermal retinol and all-*trans*-retinoic acid levels. Mice were treated with retinol and the dermis was examined 4 hr later for retinol (—●—●—) and all-*trans*-retinoic acid (—▲—▲—), or they were treated with [11-^{3}H]-all-*trans*-retinoic acid and the tissues were examined for all-*trans*-retinoic acid (—■—■—). Error bars denote the standard deviations (N = 3–4); in some cases these are obscured by the points.

although the sensitivity of detection for retinal was comparable to that of all-*trans*-retinoic acid, and we did not observe any undue sensitivity of retinal to degradation during handling. In a study of the fate of physiological doses of radioactive retinyl acetate, Cullum and Zile [3] found that retinoic acid but not retinal is formed in the intestinal mucosa and liver. Thus, in common with other retinoid-metabolizing tissues, the reduction and oxidation of retinal are not rate-limiting steps in retinol metabolism in the skin. All-*trans*-retinoic acid was not detected in extracts of epidermis or dermis from untreated mice. Simple extrapolation of the data presented in Figs. 4 and 5 indicates that, if present at all, the endogenous level of the acid is several orders of magnitude below that of free retinol (less than 10 pmoles/g), and is below the current limits of detection. This observation has interesting implications in view of the postulated role of cytosolic binding proteins in mediating retinoid action in target tissues, such as the epidermis. The CRABP levels in the epidermis are relatively high [7, 8], in hairless mouse epidermis being of the order of 500 pmoles/g (Connor and Smit, unpublished observations), and in view of the low all-*trans*-retinoic acid level must be present largely in the apo (i.e. ligand free) form; thus, CRABP-mediated retinoid activities will be determined by all-*trans*-retinoic acid availability, and all-*trans*-retinoic acid formation from retinol could act to regulate CRABP-mediated retinoid activities under physiological conditions in the epidermis.

We did not find 13-*cis*-retinoic acid in extracts from

the retinol-treated mice. There is some evidence to suggest that 13-*cis*-retinoic acid is a naturally occurring metabolite formed from retinol *in vivo* [3]. However, 13-*cis*-retinoic acid is readily formed by light or chemical isomerization of all-*trans*-retinoic acid as an artifact during extraction, and similar isomerizations occurred in retinoid-treated skin when irradiated with visible light *in situ* (Connor and Smit, unpublished observations). The absence of 13-*cis*-retinoic acid may suggest, therefore, that it is not formed enzymically in the skin in significant amounts.

The aims of the present study were to observe if retinol was metabolized to all-*trans*-retinoic acid in the skin, and to investigate the possible role of this in epidermal responses to retinol. Compared to all-*trans*-retinoic acid, topically applied retinol has a relative potency at inducing epidermal hyperplasia of 0.47 [10], and at inhibiting the induction of epidermal ornithine decarboxylase activity by tetradecanoylphorbol-13-acetate of 0.13. Since the amount of all-*trans*-retinoic acid generated in the epidermis from retinol was 2–3 orders of magnitude lower than that found after equivalent doses of all-*trans*-retinoic acid, this would not be sufficient to account for the smaller differences in potencies. These studies thus suggest that metabolism to all-*trans*-retinoic acid is not an obligatory step in the mechanism by which topically applied retinol induces epidermal hyperplasia or inhibits the induction of epidermal ornithine decarboxylase. That the elimination half-lives of retinol and all-*trans*-retinoic acid from the epidermis were the same, but that lower retinol levels were achieved, indicates that the lower potency of retinol in these pharmacological assays may be due to lower tissue uptake.

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