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IN VIVO METABOLISM OF TOPICALLY APPLIED RETINOL AND ALL-TRANS RETINOIC ACID BY THE RABBIT CORNEA

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SUMMARY. Corneas of normal and vitamin A-deficient rabbits were treated topically with [11,12-^{3}H] retinol or [11,12-^{3}H] all-trans retinoic acid. Methanol extracts of these corneas were analyzed by high pressure liquid chromatography. Radiolabeled compounds were extracted from the corneas which co-migrated chromatographically with known retinoid standards. In agreement with studies on other tissues and organs, retinol was metabolized to retinoic acid and more polar compounds by corneas of normal and vitamin A-deficient rabbits. All-trans retinoic acid was isomerized to 13-cis retinoic acid in normal rabbit corneas; however, this trans-cis isomerization did not occur in vitamin A-deficient, xerophthalmic corneas.

INTRODUCTION. The effects of vitamin A deficiency on the cornea recently have been reviewed by Sommer (1) and several investigators have also reported on the use of topically applied retinoids for the treatment of xerophthalmia (2,3), corneal epithelial wounds (4), and dry-eye disorders (5). While the metabolism of retinoids by various tissues and organs has been studied extensively (6-11), similar studies have not been conducted on the cornea. In context of studies of the use of topical retinoids for treatment of corneal disease, we have now investigated the metabolism of retinol and retinoic acid by the cornea of normal and vitamin A-deficient rabbits.

MATERIALS AND METHODS

Unlabeled all-trans retinol and all-trans retinoic acid were purchased from Sigma Chemical (St. Louis, MO). [11,12-^{3}H] Retinol (40-50 Ci/mmole) was obtained from Amersham (Chicago, IL). Unlabeled 13-cis retinoic acid, 5,6-epoxy retinoic acid, and [11,12-^{3}H] all-trans retinoic acid (40 Ci/mmole) were provided by Drs. W.E. Scott and P.F. Sorter of Hoffman-LaRoche (Nutley, NJ). The retinoids were checked for purity by HPLC. All solvents used were of HPLC grade.

ABBREVIATIONS. HPLC = high performance liquid chromatography, RA = retinoic acid, RBG = retinoyl-β-glucuronide, UV = ultraviolet, BHT = butylated hydroxytoluene

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Vitamin A-deficient rabbits were prepared as previously described by Van Horn et al. (12). The rabbits were used after 14-18 weeks on the vitamin A-deficient diet when the corneas had reached stage 3-4 xerophthalmia with severe keratinization and plaque formation.

[11,12-^{3}H] Retinol or [11,12-^{3}H] all-trans retinoic acid was dissolved in corn oil at a concentration of 0.5 μCi/μl. In each experiment both eyes of three rabbits were treated with 30 μl of corn oil containing labeled retinoid followed by application of a second 30- l dose 15 minutes later. This dosage regimen was required to reach detectable levels of retinoid in the corneal tissue. The 30- l volume is the maximum which can be retained in the cul-de-sac of the rabbit's lower eyelid. After 2 hrs the rabbits were killed by a sodium pentobarbital overdose. This time period was chosen based on pharmacokinetic studies, which showed that peak retinoid levels are present in the cornea 1 to 4 hours after treatment, and also on the retinoid metabolism studies of other investigators (8,9). The corneas were removed, washed three times in saline solution, and extracted using the method of Zile et al. (8). Three corneas were pooled, homogenized in 1.5 ml of an aqueous solution of EDTA (0.5 mg/ml) and n-propyl gallate (0.5 mg/ml), and lyophilized. The remaining three corneas were treated in the same way. The dried samples were extracted with HPLC grade methanol containing BHT (20 μg/ml). The extracts were pooled, evaporated under nitrogen to a volume of 1 ml, and filtered through a Gelman Acrodisc 0.2-μm filter.

In studies of retinol metabolism, unlabeled retinol and all-trans retinoic acid were added to the tissue extracts as internal standards. In retinoic acid studies, unlabeled all-trans retinoic acid, 13-cis retinoic acid, and 5,6-epoxy retinoic acid were added to the extracts. Analysis by HPLC was performed using a Beckman 334 Gradient Liquid Chromatograph equipped with a Beckman 165 UV detector, a Du Pont Zorbax ODS reversed-phase column (0.46 cm x 25 cm), and a Rheodyne 7302 column inlet filter. Extract samples of 100 μl were applied to the column and eluted with a methanol:water mobile phase containing 10 mM ammonium acetate. Absorbance was monitored at 330 or 340 nm, and 1 ml fractions of elluent were collected for scintillation counting.

As controls for spontaneous breakdown or isomerization of retinoids during the treatment and corneal extraction procedure, during each experiment a sample of retinoid in oil was extracted with methanol containing BHT. A 100-μl sample of this extract was immediately analyzed by HPLC. The remainder was evaporated to dryness, 1 ml of the aqueous homogenization medium was added, and the preparation was lyophilized. The dried sample was dissolved in 1 ml of methanol-BHT, filtered, and analyzed by HPLC.

In this study all samples were kept on ice under nitrogen and stored at -80°C. All procedures were carried out in a room equipped with amber lights.

RESULTS

Retinol Metabolism. Experiments on retinol metabolism were repeated in three groups of normal rabbits and in one group of xerophthalmic, vitamin A-deficient rabbits. Figures 1A and 1B are representative chromatograms from these experiments. As shown in the chromatograms of control retinol samples (Figs. 1A,1B), no significant spontaneous degradation or isomerization of retinol occurred during the treatment and isolation procedures. Two major radioactive peaks in addition to retinol were extracted from the corneas of normal animals (Fig. 1A). The first, which accounted for 54% of the extracted

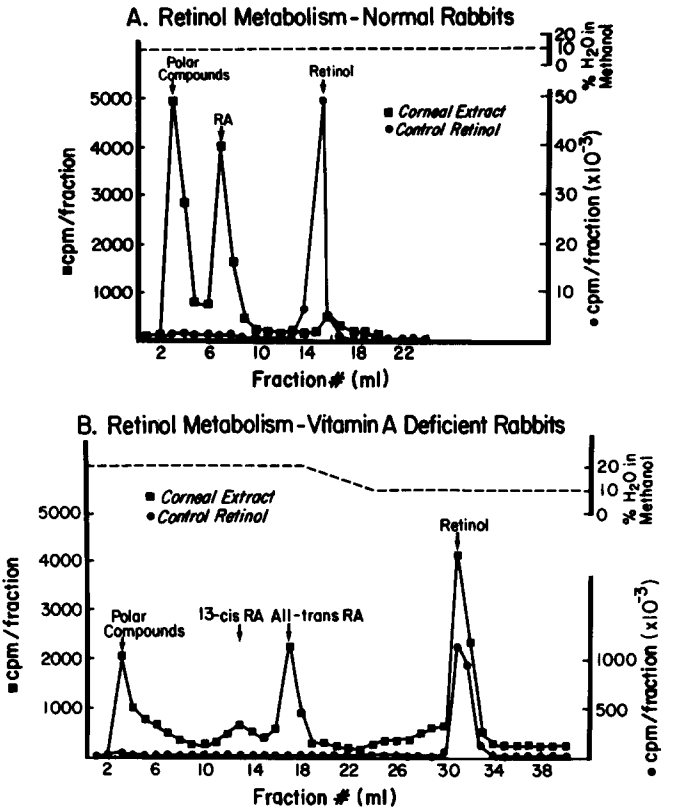

Figure 1. (A) Chromatographic profile of radioactivity in an extract of corneas of normal rabbits treated with [11,12-^{3}H] retinol (■). Also shown is a chromatogram of a control [11,12-^{3}H] retinol sample (●). (B) Chromatographic profile of radioactivity in an extract of vitamin A-deficient rabbit corneas treated with [11,12-^{3}H] retinol. Solvent conditions are shown in the upper portion of each graph. Labeled arrows show the elution positions of retinoid standards as detected by UV absorbance at 330 nm.

radioactivity, consisted of unidentified polar compounds which eluted with the solvent front. The second, which co-eluted with the all-trans retinoic acid standard, accounted for 40% of the extracted radioactivity. The third peak, retinol, accounted for only 6% of the radioactivity. The retinoic acid:retinol ratio in the extract was approximately 6.67. The extract from corneas of vitamin A-deficient animals was chromatographed using the gradient shown in Figure 1B which gave better separation than the isocratic 90:10 methanol:water ratio used in the experiment shown in Figure 1A. In addition to the peaks seen in extracts of normal corneas, a small peak co-migrating with 13-cis retinoic acid was resolved. The all-trans retinoic acid:retinol ratio in these corneas was approximately 0.50.

Retinoic Acid Metabolism. Experiments on retinoic acid metabolism were repeated on five groups of normal rabbits and two groups of vitamin A-deficient animals. Representative chromatograms are shown in Figures 2 and 3. The control retinoic acid shown in Figure 3A consisted of 80% all-trans isomer and 20% 13-cis isomer with a minor peak corresponding to 5,6-epoxy retinoic acid. This did not differ from the material with which the animals were treated. The majority of the radioactivity extracted from the corneas in the experiment shown in Figure 2A consisted of unidentified polar compounds and degradation products. In contrast to the control sample, the peaks

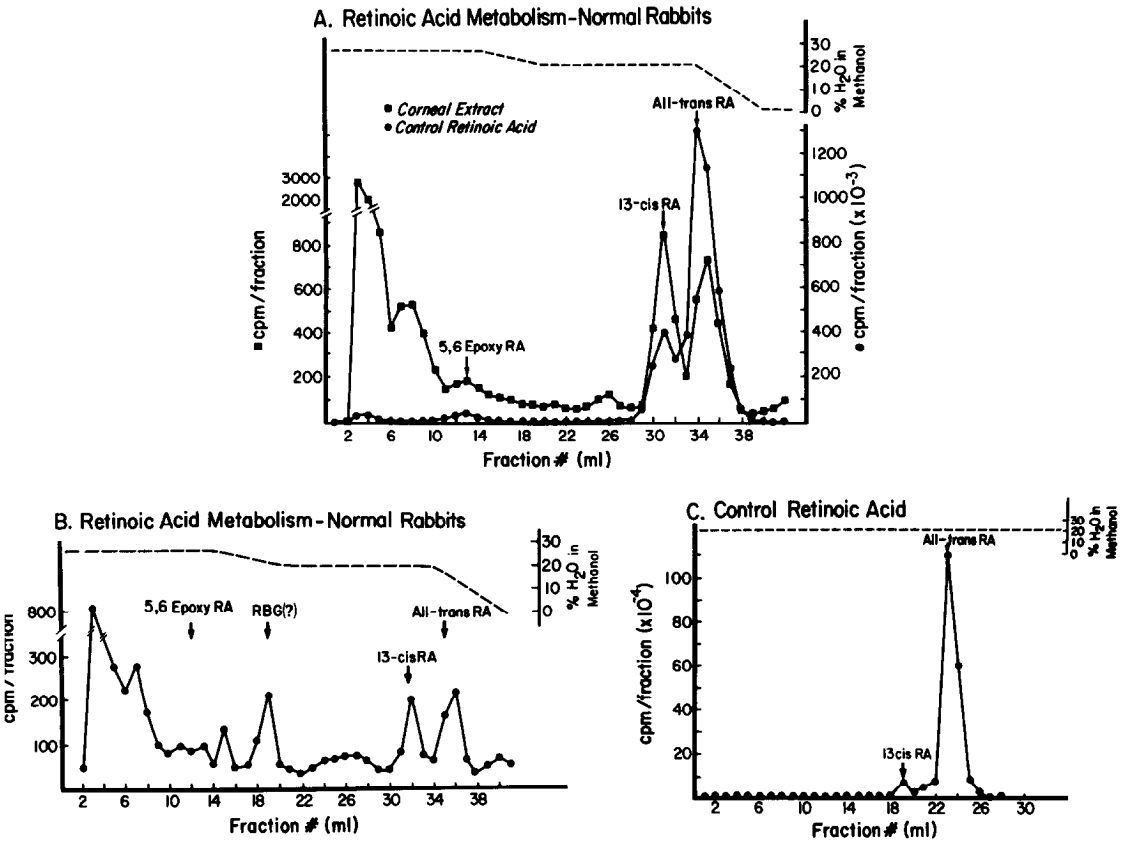

Figure 2. (A) Chromatographic profile of radioactivity in an extract of corneas of normal rabbits treated with [11,12-^{3}H] retinoic acid (■). Also shown is chromatogram of a control [11,12-^{3}H] retinoic acid sample (●). (B) Chromatogram of an extract of normal rabbit corneas treated with [11,12-^{3}H] retinoic acid. A peak is present which may correspond to retinoyl-β-glucuronide. (C) Chromatogram of control retinoic acid used in (B). Solvent conditions are shown in the upper portion of each graph. Labeled arrows show elution positions of retinoid standards as detected by UV absorbance at 340 nm.

attributable to retinoic acid contained 50% all-trans retinoic acid and 50% 13-cis retinoic acid. Corneas in the experiment shown in Figure 2B were treated with a preparation containing 94% all-trans retinoic acid and 6% 13-cis retinoic acid (Fig. 2C). Extracts of these corneas showed the same pattern of polar compounds, 13-cis retinoic acid, and all-trans retinoic acid shown in Figure 2A; however, an additional peak of radioactivity was eluted which may correspond to retinoyl-β-glucuronide (8). This could not be confirmed for lack of a retinoyl-β-glucuronide standard. The extracts from corneas of vitamin A-deficient rabbits treated with retinoic acid (Fig. 3A) contained a lower level of polar compounds than those from normal rabbits, and there was no evidence of isomerization of all-trans retinoic acid to 13-cis retinoic acid in vitamin A-deficient rabbit corneas (Fig. 3B).

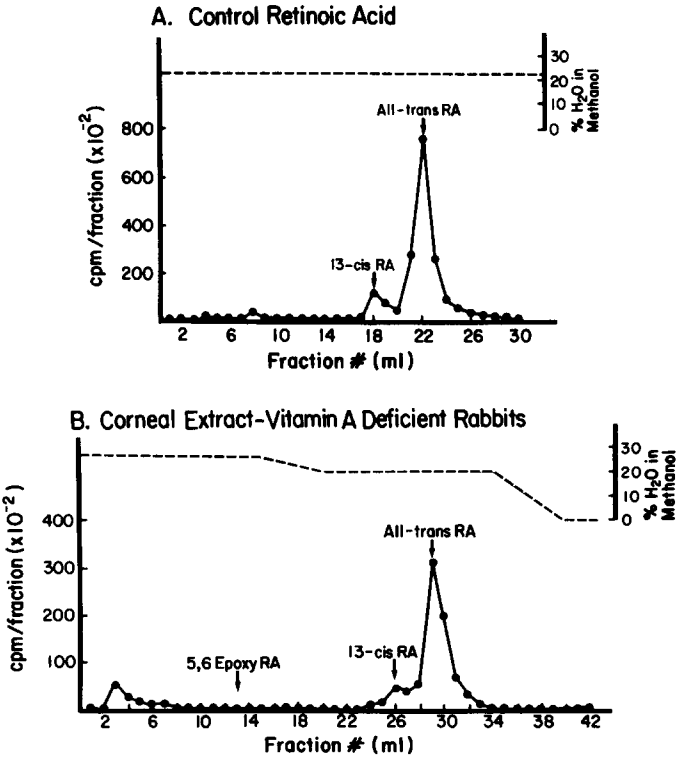

Figure 3. (A) Control [11,12-^{3}H] retinoic acid. (B) Chromatographic profile of radioactivity in an extract of vitamin A-deficient rabbit corneas treated with [11,12-^{3}H] retinoic acid. Solvent conditions are shown in the upper portion of each graph. Labeled arrows show elution positions of retinoid standards as detected by UV absorbance at 340 nm.

DISCUSSION

The results of this study indicate that retinoic acid is a major corneal metabolite of retinol and that all-trans retinoic acid undergoes isomerization to 13-cis retinoic acid in the cornea. It must be noted that these data represent metabolism in the whole cornea which is not a homogeneous tissue but rather a complex organ consisting of epithelium, stroma, and endothelium. Each layer is made up of a unique cell type containing intracellular retinoid receptors (13).

Retinoic acid has previously been identified as a retinol metabolite in liver, intestinal mucosa, and kidney (6,7). Our findings in cornea are in agreement with previous studies although the percentage of retinoid extracted from the tissue as retinoic acid is much higher than in other tissues. The conversion of retinol to retinoic acid may, therefore, be a particularly important step in the rabbit cornea although it is not known whether metabolism of retinol to retinoic acid is a necesary step for the biological activity of vitamin A (14).

Retinoic acid metabolism has been studied in several tissues. 5,6,-epoxy retinoic acid, a physiologic (11) but non-essential metabolite of retinoic acid (16), has been identified in small amounts in intestinal mucosa and kidney. No evidence for expoxidation of retinoic acid was found in corneal extracts. Retinoyl-β-glucuronide, once thought only to be a biliary metabolite of retinoic acid, is now known to be a major metabolite of all-trans retinoic acid in the intestinal mucosa (8). Our data do not allow us to make a conclusion concerning glucuronidation of retinoic acid in rabbit cornea; however, this reaction certainly does not appear to occur in cornea to the extent reported for intestinal mucosa. Isomerization of all-trans retinoic acid to 13-cis retinoic acid has been reported in many tissues of normal, vitamin A-deficient, and retinoic acid supplemented vitamin A-deficient rats (8,9,16). The reverse reaction from 13-cis to all-trans also occurs when animals are treated with 13-cis retinoic acid and an equilibrium

exists between the two isomers (9,10). Our study agrees with these findings in that approximately equal amounts of both isomers were extracted from corneas of normal animals treated with a preparation containing at least 80% all-trans retinoic acid. In dealing with isomers in this type of study, it is important to confirm that isomerization is a physiologic event rather than an artifact of the experimental procedure. In addition to our control samples, we have evidence in our study that trans-cis isomerization was a physiological event in normal rabbits since the isomerization did not occur in vitamin A-deficient rabbit corneas. Normal and vitamin A-deficient rabbit corneas have been shown to take up equal amounts of retinoic acid (16). In comparison with other metabolism studies, however, the lack of a trans-cis isomerization in vitamin A-deficient corneas is a unique finding. This may be due to a tissue or species difference. Alternately, it may be related to the stage of vitmain A deficiency in the animals. The rabbits were used for experiments three to four weeks after the weight plateau when the corneas were severely keratinized. At this stage retinoic acid is clearly taken up by the cornea; however, the enzymes of retinoid metabolism may be down-regulated so that the isomerization step and the formation of polar metabolites do not occur. All-trans retinoic acid is, however, very effective in reversing keratinization at this stage of vitamin A deficiency when applied topically to the cornea (3). If all-trans retinoic acid is the active retinoid in this process, the equilibrium of reactions would be shifted in the direction of the all-trans isomer. This would explain the lack of isomerization of all-trans retinoic acid by the vitamin A-deficient rabbit cornea observed in our study.

Retinol and retinoic acid are thought to be involved in control of keratin expression and glycoprotein synthesis in the cornea (18-20). The direct mechanism of action of these compounds and the importance of their metabolites in these activities remains to be elucidated.

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