Gangull, P. K., & Theophanides, T. (1979) *Eur. J. Biochem. 101*, 377.
Harder, H. (1975) *Chem.-Biol. Interact. 10*, 27.
Kelman, A. D., & Buchbinder, M. (1978) *Biochimie 60*, 893.
Lee, C. C., & Harder, H. C. (1982) *Proc. Am. Assoc. Cancer Res.*, Abstr. No. 19.
Marcelis, A. T. M., Den Hartog, J. H. L., & Reedjik, J. (1982) *J. Am. Chem. Soc. 104*, 2664.
Pera, M. F., Rawlings, C. J., Shackleton, J., & Roberts, J. J. (1981) *Biochim. Biophys. Acta 655*, 152.
Roberts, J. J., & Friedlos, F. (1981) *Biochim. Biophys. Acta 655*, 146.
Robins, A. B. (1973) *Chem.-Biol. Interact. 6*, 35.
Royer-Pokora, B., Gordon, L. K., & Haseltine, W. A. (1981) *Nucleic Acids Res. 9*, 4595.
Strandberg, M. C., Bresnick, E., & Eastman, A. (1982) *Chem.-Biol. Interact. 39*, 169.
Tullius, T. D., & Lippard, S. J. (1981) *J. Am. Chem. Soc. 103*, 4620.
Ushay, H. M., Tullius, T. D., & Lippard, S. J. (1981) *Biochemistry 20*, 3744.
Zwelling, L. A., Anderson, T., & Kohn, K. W. (1979a) *Cancer Res. 39*, 365.
Zwelling, L. A., Filipski, J., & Kohn, K. W. (1979b) *Cancer Res. 39*, 4989.

13-*cis*-Retinoic Acid Metabolism in Vivo. The Major Tissue Metabolites in the Rat Have the All-Trans Configuration†

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ABSTRACT: The liver and intestinal metabolites of orally dosed 13-*cis*-[11-^{3}H]retinoic acid were analyzed in normal and 13-*cis*-retinoic acid treated rats 3 h after administration of the radiolabeled retinoid. *all-trans*-Retinoic acid was identified as a liver and intestinal mucosa metabolite in normal rats given physiological doses of 13-*cis*-[^{3}H]retinoic acid. *all-trans*-Retinoyl glucuronide was identified as the most abundant radiolabeled metabolite in mucosa and a prominent liver metabolite under the same conditions. Thus, the major 13-*cis*-retinoic acid metabolites retained in liver and mucosa, two retinoid target tissues, had the all-trans configuration. These data indicate that the isomerization of 13-*cis*-retinoic acid to *all-trans*-retinoic acid and the subsequent conversion to *all-trans*-retinoyl glucuronide are central events in the in vivo metabolism of 13-*cis*-retinoic acid in the rat. Moreover, the *all-trans*-retinoic acid detected in vivo could account for a significant fraction of the physiological activity of 13-*cis*-retinoic acid. The tissue disposition and metabolism of orally dosed 13-*cis*-[^{3}H]retinoic acid are modulated by retinoid treatment. Chronic 13-*cis*-retinoic acid treatment apparently increased the intestinal accumulation of *all-trans*-retinoic acid, *all-trans*-retinoyl glucuronide, and 13-*cis*-retinoyl glucuronide. The liver concentrations of tritiated *all-trans*-retinoic acid and *all-trans*-retinoyl glucuronide were also elevated in 13-*cis*-retinoic acid treated rats.

all-trans-Retinoic acid (*all-trans*-RA),[1] a normal intermediate in retinol metabolism (McCormick & Napoli, 1982), is at least an order of magnitude more potent than retinol in the maintenance of epithelial differentiation in cultured trachea (Sporn et al., 1976) and prostate (Lasnitzki & Goodman, 1974), and in the induction of embryonal carcinoma cell (Strickland & Mahdavi, 1978; Strickland, 1978; Jetten et al., 1979) and S91 melanoma cell (Lotan et al., 1980) differentiation in culture. These data argue that *all-trans*-RA, or a further metabolite, is the form of retinol active in directing differentiation. 13-*cis*-Retinoic acid (13-*cis*-RA) has been reported as an in vivo metabolite of *all-trans*-RA by several laboratories (Napoli & McCormick, 1981; Sundaresan & Bhat, 1982; Zile et al., 1982a) and is a physiological retinol metabolite in the rat.[2] 13-*cis*-RA is equipotent to *trans*-RA in promoting growth (Zile & DeLuca, 1968), maintaining differentiation in tracheal organ cultures (Frolik et al., 1980), and inducing embryonal carcinoma cell differentiation (Strickland, 1978). Moreover, 13-*cis*-RA has potent chemopreventive activity in urinary bladder (Becci et al., 1978, 1981; Thompson et al., 1981), respiratory tract (Port et al., 1975), and skin (Mayer et al., 1978; Verma et al., 1979). Thus, 13-*cis*-RA may be a physiologically important metabolite of retinol.

Limited information is available concerning the in vivo metabolism of 13-*cis*-RA. The cis isomer is rapidly metabolized to 13-*cis*-4-hydroxyretinoic acid and 13-*cis*-4-ketoretinoic acid in vivo (Frolik et al., 1979; Vane & Buggé, 1981) and in vitro (Frolik et al., 1979; Vane et al., 1982). These C(4) oxidized metabolites are less active than the parent retinoid in maintaining epithelial differentiation in cultured trachea (Frolik et al., 1979). Further metabolism of 13-*cis*-4-keto-RA in vitro produced the 2-hydroxy and 3-hydroxy derivatives

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[1] Abbreviations: HPLC, high-performance liquid chromatography; RP-HPLC, reverse-phase high-performance liquid chromatography; NP-HPLC, normal-phase HPLC; *trans*-RA, *all-trans*-retinoic acid; 13-*cis*-RA, 13-*cis*-retinoic acid; 5,6-epoxy-RA, 5,6-epoxyretinoic acid; 4-hydroxy-RA, 4-hydroxyretinoic acid; 4-keto-RA, 4-ketoretinoic acid; 13-*cis*-5,6-epoxy-RA, 13-*cis*-5,6-epoxyretinoic acid; 13-*cis*-4-keto-RA, 13-*cis*-4-ketoretinoic acid; UDPGA, uridine-5′-diphosphoglucuronic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

[2] J. L. Napoli, unpublished results.

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(Vane et al., 1982). 13-*cis*-Retinoyl glucuronide and 13-*cis*-4-ketoretinoyl glucuronide have been identified as biliary 13-*cis*-RA metabolites (Frolik et al., 1981). Little data are available on the target-tissue metabolites of 13-*cis*-RA. Nor has it been established whether 13-*cis*-RA possesses inherent biological activity or if it must be converted to an active metabolite in vivo.

The present study was undertaken to characterize the target-tissue metabolites of 13-*cis*-RA in the rat. This paper will demonstrate that the major liver and intestine metabolites have the all-trans configuration. In addition, this work will show that a major route of 13-*cis*-RA metabolism in vivo is isomerization to *all-trans*-RA and the subsequent synthesis of *all-trans*-retinoyl glucuronide.

Materials and Methods

Chemicals. HPLC grade, glass-distilled, methanol, hexane, and toluene were purchased from MCB. AR grade hexane for retinoid extraction was obtained from MCB. Ethanol (95%) was obtained from U.S. Industrial Chemicals. DEAE-Sephadex A-25 was purchased from Pharmacia Fine Chemicals, Piscataway, NJ. The resin was converted to the hydroxyl form by treatment with 20% methanolic ammonium hydroxide. Excess reagent was removed by washing the derivatized resin with methanol.

Retinoids. *all-trans*-Retinoic acid (*trans*-RA) was purchased from Sigma. 13-*cis*-Retinoic acid (13-*cis*-RA), 13-*cis*-4-ketoretinoic acid (13-*cis*-4-keto-RA), 4-ketoretinoic acid (4-keto-RA), and 5,6-epoxyretinoic acid (5,6-epoxy-RA) were a gift from Dr. Beverly Pawson, Hoffmann-La Roche Inc., Nutley, NJ. 13-*cis*-5,6-Epoxyretinoic acid (13-*cis*-5,6-epoxy-RA) was synthesized by epoxidation of 13-*cis*-RA with monoperoxyphthalic acid as described previously (McCormick et al., 1978). 13-*cis*-[11-^{3}H]Retinoic acid (1.75 Ci/mmol) was obtained from the Chemopreventive Program, Division of Cancer Cause and Prevention, National Cancer Institute. The purity of the [^{3}H]-13-*cis*-RA, determined by RP-HPLC analysis, was greater than 99%. All retinoids were stored under N_2 at −85 °C and handled under yellow fluorescent lighting.

Animals. Male Crl:CD (SD)Br Sprague-Dawley rats (75–100 g) were obtained from Charles River Breeding Laboratories, Wilmington, MA. The animals were maintained on stock chow throughout the experimental period. After a 7-day acclimation period, rats were dosed with 13-*cis*-RA (1 mg/kg body weight) dissolved in Wesson oil for 3 days (experiment II) or for 14 days (experiment III). Control rats (experiment I) received an equal volume of corn oil vehicle.

General. High-performance liquid chromatography (HPLC) was performed with a Waters Associates ALC/GPC 204 liquid chromatograph equipped with an additional M45 solvent delivery system and a Model 660 solvent programmer for gradient elution. A Waters Associates RCM-100 fitted with a C_{18} Radial-Pak cartridge (8 mm × 10 cm, 10-μm spherical particles) was used for reverse-phase HPLC, and an analytical Du Pont Zorbax-SIL column (0.46 × 25 cm) was used for normal-phase HPLC. A Whatman Magnum 9 ODS-2 column was used for semipreparative reverse-phase HPLC. Radioactivity was measured with a Beckman LS-330 liquid scintillation counter equipped with automatic external standardization. Samples were counted in 6 mL of Hydrocount (J.T. Baker Co.) with a tritium counting efficiency of 25–35%.

Gas chromatography/mass spectroscopy (GC/MS) was performed with a Finnigan 4021 mass spectrometer served by an INCOS data system. A glass column (0.25 in. × 6 ft) packed with 3% OV-1 on S-port (60/80) supplied by Finnigan, Palo Alto, CA, was used for gas chromatography. The column temperature was 245 °C, and the helium flow rate was 30 mL/min. The injector, transfer line, and jet separator were held at 250 °C. Mass spectra were obtained at 70 eV in the electron impact mode.

In Vivo Metabolism Experiments. Three experimental protocols were used to examine 13-*cis*-RA metabolism in vivo. Rats not treated with exogenous retinoids (experiment I) and 13-*cis*-RA (1.3×10^7 dpm μg) pretreated rats (experiment II) were administered 1 μg of [11-^{3}H]-13-*cis*-RA orally to investigate its physiological metabolism and also to determine the effects of 13-*cis*-RA treatment on its metabolism in vivo. A third group of rats were chronically treated with 13-*cis*-RA (1 mg kg^{-1} day^{-1}) for 14 days (experiment III) to generate sufficient quantities of the major metabolites in intestinal mucosa and liver for isolation and structural identification. The last dose was 200 μg of [^{3}H]-13-*cis*-RA (1×10^5 dpm/μg). In each of the three experiments, the tritiated retinoid was given 24 h after the last 13-*cis*-RA treatment to allow for clearance of most of the unlabeled compound (Frolik et al., 1981). All rats were sacrificed 3 h after the tritiated 13-*cis*-RA was dosed. Liver, small intestine, kidney, and serum were collected. Intestinal tissue was washed with ice-cold 0.15 M NaCl to remove the contents, and the mucosa was collected by scraping the entire length of the small intestine with a microscope slide. Tissues were homogenized in one volume of an *n*-propyl gallate solution (50 mg/L) and the homogenates were lyophilized. Retinoids were extracted from the tissue residues with distilled methanol (3 × 150 mL) fortified with butylated hydroxytoluene (50 mg/L). The methanol extracts were concentrated to 50 mL. Triplicate aliquots were measured for radioactivity. This method yields virtually quantitative extraction of tissue retinoids (Napoli & McCormick, 1981).

The methanol extracts were individually applied to DEAE-Sephadex A-25 columns (2.5 × 15 cm), equilibrated in methanol. Neutral lipids were eluted in 150 mL of methanol. The acidic retinoids were eluted with 150 mL of 0.25 M ammonium bicarbonate in 10% aqueous methanol. Triplicate aliquots of the neutral and acidic fractions were measured for radioactivity. The solvent and ammonium bicarbonate were removed from the acidic retinoid fraction under reduced pressure. Residues were dissolved in 15 mL of distilled methanol, and the samples were stored under N_2 at −85 °C.

HPLC Profiles of Tissue Retinoids. Aliquots of the tissue retinoid extracts were evaporated, dissolved in 200 μL of methanol, and filtered through 0.5-μm Millex-SR filters. Each chromatographic sample was fortified with 5 μg each of 13-*cis*-4-keto-RA, 4-keto-RA, 13-*cis*-5,6-epoxy-RA, 5,6-epoxy-RA, 13-*cis*-RA, and *trans*-RA. 13-*cis*-RA metabolites were examined by reverse-phase HPLC by using either a 30-min linear gradient of 45–80% methanol in water with a 10 min hold at initial conditions (experiments I and II) or a 30-min linear gradient of 40–80% methanol in water (experiment III). Both chromatographic solvents used to prepare the gradients contained 10 mM ammonium acetate. In all experiments, chromatography at final conditions (80% methanol) was continued until the 13-*cis*-RA and *all-trans*-RA internal standards eluted. One-milliliter fractions were collected and counted in 6 mL of Hydrocount. The chromatographic data were corrected for recovery (93–97%) and were then normalized to dpm per gram of tissue (dpm/mL of serum).

Control experiments were conducted for each experiment by adding 10 μg of [^{3}H]-13-*cis*-RA (1×10^6 dpm) to each tissue homogenate at 0 °C. Tissue samples were lyophilized and analyzed as described above. Recovery of added radio-

activity in the acidic retinoid fraction was 96–99%. A total of 96–98% of the added radioactivity was recovered as unchanged 13-*cis*-RA. The remainder of the radioactivity in the control tissues comigrated with *all-trans*-RA. The artifactually produced *all-trans*-RA in each control sample was subtracted from the amount observed in the corresponding experimental sample before the data were converted to dpm per gram of tissue.

β-Glucuronidase Treatment. Retinoyl glucuronides were treated with β-glucuronidase (500 units) in 1 mL of 50 mM Mops, pH 7.0, for 1.5 h at 37 °C (Napoli et al., 1982). Control incubations contained 1 mL of heat-inactivated enzyme. Reactions were quenched with 5 mL of ice-cold ethanol, and the precipitated protein was removed by centrifugation. The recovered solvent was dried under a stream of N_2. The resulting residues were dissolved in methanol (200 μL), filtered through 0.5-μm filters, and immediately analyzed by RP-HPLC.

In Vitro Synthesis of 13-cis-Retinoyl Glucuronide and all-trans-Retinoyl Glucuronide. Livers of 80-g normal male Sprague-Dawley rats were used to prepare microsomes. A 20% (w/v) liver homogenate was prepared in 50 mM Mops, 250 mM sucrose, and 2.5 mM EGTA, pH 7.4. The homogenate was centrifuged at 500*g* for 15 min. The supernatant fraction was centrifuged at 18000*g* for 15 min to obtain the postmitochondrial supernatant. Microsomes were obtained by centrifugation of the postmitochondrial supernatant at 105000*g* for 1 h in a Beckman type 30 rotor. The microsomal pellet was resuspended in 50 mM Mops, 5 mM EGTA, and 10 mM $MgCl_2$, pH 7.0, with a glass Dounce homogenizer to a final protein concentration of 2 mg/mL as determined by the Bradford protein assay (Bradford, 1976). Retinoyl glucuronides were synthesized by incubating 10 μg of 13-*cis*-RA or *all-trans*-RA, 1 mg of microsomal protein, and 2.5 mg of UDPGA in a total volume of 0.55 mL at 37 °C for 1 h. Reactions were quenched as described for the β-glucuronidase reactions. The retinoyl glucuronides were purified by RP-HPLC with a solvent of methanol–water (7:3) containing 10 mM ammonium acetate. The purified glucuronide standards were stable for a 1-week period if stored in ethanol at −85 °C.

Isolation of Tissue Metabolites. The acidic retinoid fraction from intestinal mucosa obtained in experiment III was applied to a semipreparative RP-HPLC column in 400–500-μL portions. The peak E metabolites were eluted with methanol–water (65:35) containing 10 mM ammonium acetate. The peak E metabolites were resolved into two distinct peaks, E1 (74–84 mL) and E2 (90–100 mL). Tissue retinoic acids were eluted from this column with methanol and saved for future analysis. E1 and E2 were each treated with β-glucuronidase, and the liberated retinoids were collected for further purification. The putative 13-*cis*-RA and *all-trans*-RA were each purified by RP-HPLC by using a C_{18} Radial-Pak cartridge and a chromatographic solvent of methanol–water (65:35) plus 10 mM ammonium acetate. The 13-*cisRA* (62–70 mL) and *all-trans*-RA (76–84 mL) peaks obtained were each evaporated to dryness, dissolved in 100 μL of distilled methanol, and methylated with diazomethane (McCormick et al., 1978). The methylated derivatives were purified by RP-HPLC with a solvent of methanol–water (85:15). The putative methyl 13-*cis*-retinoate eluted at 61 mL as did synthetic methyl 13-*cis*-retinoate used to standardize the column immediately prior to metabolite purification. Putative methyl retinoate eluted at 78 mL as did the synthetic standard. The final purification of each methylated retinoid was accomplished by normal-phase HPLC with a hexane–toluene (70:30) solvent system. In this system, the biological samples of methyl 13-*cis*-retinoate and methyl retinoate eluted at 15 and 21 mL, respectively, as did the corresponding synthetic compounds. The methyl 13-*cis*-retinoate (14–16 mL) and methyl retinoate (20–22 mL) peaks were each evaporated to dryness, and the residues were dissolved in HPLC-grade hexane for GC/MS analysis and HPLC comigration experiments.

The acidic retinoid fraction from the livers of chronic 13-*cis*-RA-treated rats was prepared as previously described. The peak E metabolite(s) was (were) isolated by RP-HPLC by using a 40–80% linear methanol–water gradient. Material eluting between 58 and 66 mL was pooled, the solvent was evaporated, and the residue was dissolved in methanol (2 mL) and stored at −85 °C under a N_2 atmosphere.

As mentioned above, the 13-*cis*- and *all-trans*-RA isomers were obtained in a single fraction and reserved during the purification of intestinal peaks E1 and E2. The entire fraction was concentrated to 300 μL of methanol, and the *all-trans*-RA isomer was obtained by RP-HPLC by using a solvent system of methanol–water (7:3) containing ammonium acetate (10 mM). The putative *all-trans*-RA peak (80 mL) eluted in the same position as authentic *all-trans*-RA, well separated from the 13-*cis*-RA peak (66 mL). The recovered *all-trans*-RA was methylated with diazomethane, and the resulting methyl ester was purified by RP-HPLC with methanol–water (85:15). The putative methyl retinoate eluted at 75 mL as did the synthetic standard chromatographed immediately after the methylated metabolite. In this system, methyl 13-*cis*-retinoate eluted at 61 mL (see Figure 6).

The final purification of the methylated intestinal metabolite was performed by NP-HPLC with a hexane–toluene (7:3) solvent system. In this system, the putative methyl retinoate eluted at 21 mL as did a synthetic methyl retinoate standard. Methyl 13-*cis*-retinoate eluted at 15 mL.

Results

Tissue 13-cis-RA Metabolites. The tissue distribution of the dosed [^{3}H]-13-*cis*-RA in the three experimental groups is shown in Table I. In rats not treated with exogenous retinoid, the liver contained the highest percentage of a physiological dose. Intestinal mucosa, kidney, and serum each had an order of magnitude less [^{3}H]retinoid. 13-*cis*-RA pretreatment resulted in a 2-fold increase in total radioactivity in liver and mucosa but had no effect on total radioactivity in kidney or serum. In the chronically treated rats, given a 200-μg dose of [^{3}H]-13-*cis*-RA, the tissue distribution of radioactivity was markedly different. The highest percentage of dosed radioactivity was recovered in the intestinal mucosa, whereas liver contained about 5-fold less. Kidney and serum each had 20-fold less radioactivity than intestine.

Reverse-phase HPLC analyses of intestinal mucosal 13-*cis*-RA metabolites after a physiological dose of tritiated retinoid are shown in Figure 1. Five major radioactive peaks were detected in the acidic retinoid fraction of mucosa from untreated animals (Figure 1A). Two of the metabolite peaks comigrated with the 13-*cis*-RA (80 mL) and *trans*-RA (85 mL) internal standards. The largest peak, designated E, was noted at 70 mL. Two additional polar peaks, designated C (43 mL) and D (53 mL), were also observed. Several polar peaks, not detected in untreated rat mucosa, were seen in the mucosa of the 13-*cis*-RA-pretreated rats (Figure 1B). The two novel peaks were designated A (34 mL) and B (39 mL). Notably, peaks C and D were absent after 13-*cis*-RA treatment. The total radioactivity in the 13-*cis*-RA peak, the putative *all-trans*-RA peak, and peak E in mucosa was not affected by 13-*cis*-RA pretreatment.

Table I: Tissue Distribution of Radioactivity 3 h after Oral Administration of 13-*cis*-[11-^{3}H]Retinoic Acid

tissue	treatment	[^{3}H]-13-*cis*-RA dose[c] (μg)	no. of animals	% dose	% dose/g	ng of [^{3}H]-retinoids/g
liver	none (I)[a]	1	4	27	2.3	22.3
	1 mg/kg; 3 days (II)	1	4	58	4.8	47.6
	1 mg/kg; 14 days (III)	200	14	1.6	0.1	280
intestine (mucosa)	none (I)	1	4	3.0	0.6	5.6
	1 mg/kg; 3 days (II)	1	4	7.3	1.3	13.1
	1 mg/kg; 14 days (III)	200	14	8.9	1.6	3272
kidney	none (I)	1	4	1.7	0.7	6.8
	1 mg/kg; 3 days (II)	1	4	1.5	0.6	5.9
	1 mg/kg; 14 days (III)	200	14	0.5	0.2	358
serum[b]	none (I)	1	4	2.1	0.7	7.2
	1 mg/kg; 3 days (II)	1	4	2.3	0.8	7.8
	1 mg/kg; 14 days (III)	200	14	0.4	0.1	240

[a] The experiment number is shown in parentheses. [b] Nanograms of [^{3}H]retinoids per milliliter. [c] The specific radioactivity of the dosed [^{3}H]-13-*cis*-RA in experiments I and II was 1.3×10^7 dpm/μg and 1.0×10^5 dpm/μg in experiment III.

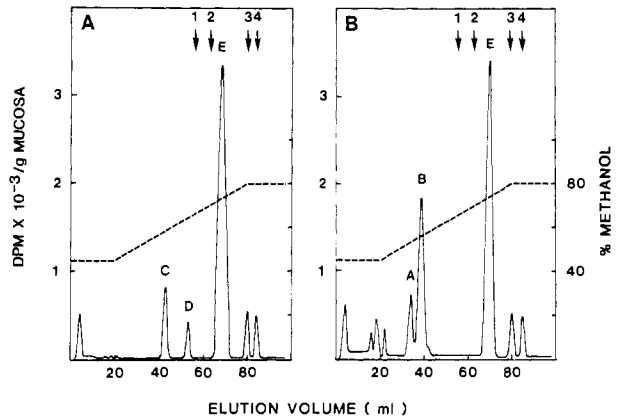

FIGURE 1: Reverse-phase HPLC profiles of intestinal mucosa 13-*cis*-RA metabolites after oral administration of 1 μg of [^{3}H]-13-*cis*-RA. The metabolites were resolved with a linear methanol–water gradient from 45 to 80% methanol with a 10-min hold at initial and final conditions. Both solvents contained ammonium acetate (10 mM). The flow rate was 2 mL/min. Fractions of 1 mL were collected and measured for tritium. The elution positions of the internal standards, monitored at 340 nm, are marked by the arrows: 1, 4-keto-RA; 2, 5,6-epoxy-RA; 3, 13-*cis*-RA; 4, *trans*-RA. (B) Metabolite profile from intestinal mucosa of rats pretreated with 13-*cis*-RA (1 mg kg^{-1} day^{-1} for 3 days) before administration of 1 μg of [^{3}H]-13-*cis*-RA. The radiolabeled 13-*cis*-RA was given 24 h after the third dose of unlabeled retinoid.

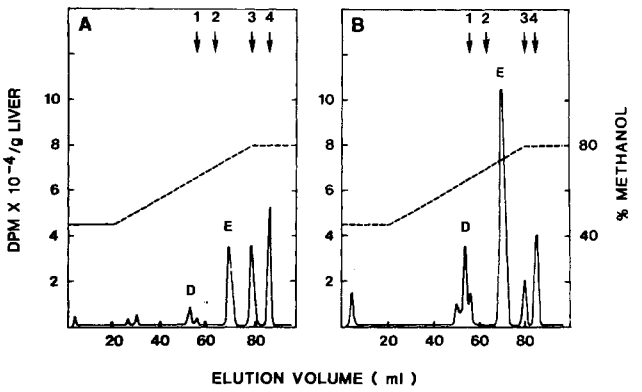

FIGURE 2: Reverse-phase HPLC profiles of liver 13-*cis*-RA metabolites after oral administration of 1 μg of [^{3}H]-13-*cis*-RA. Chromatographic conditions were as described in Figure 1. (A) Metabolite profile from liver of normal rats given 1 μg of tritiated 13-*cis*-RA. (B) Metabolite profile from liver of rats pretreated with 13-*cis*-RA (1 mg kg^{-1} day^{-1} for 3 days) before administration of 1 μg of tritiated 13-*cis*-RA. The radiolabeled 13-*cis*-RA was given 24 h after the third dose of unlabeled retinoid.

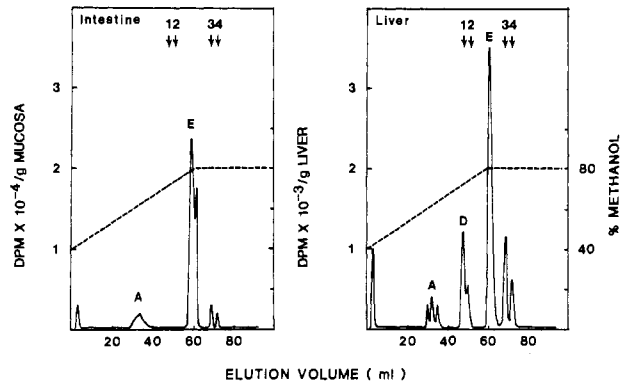

FIGURE 3: Reverse-phase HPLC profiles of intestinal mucosa and liver 13-*cis*-RA metabolites in rats receiving chronic, pharmacological doses of 13-*cis*-RA (1 mg kg^{-1} day^{-1} × 15 days). The metabolites were resolved with a 40–80% linear methanol–water gradient with both solvents containing 10 mM ammonium acetate. The flow rate was 2 mL/min. Fractions of 1 mL were collected and measured for tritium. The elution positions of the internal standards are marked by the arrows: 1, 13-*cis*-4-keto-RA; 2, 4-keto-RA; 3, 13-*cis*-RA; 4, *trans*-RA.

The RP-HPLC analyses of liver [^{3}H]-13-*cis*-RA metabolites in experiments I and II are depicted in Figure 2. Three large and several minor radioactive peaks were observed in the acidic retinoid fraction of liver from untreated rats (Figure 2A). As noted in mucosa, two of the observed peaks comigrated with authentic 13-*cis*-RA (80 mL) and *trans*-RA (85 mL). In fact, putative *all-trans*-RA represented the most abundant [^{3}H]-retinoid in normal liver under these conditions. The third large peak, which eluted at 70 mL, was designated peak E since it was similar to peak E of mucosa. Analysis of 13-*cis*-RA-pretreated rat liver (Figure 2B) revealed increased radioactivity associated with peaks D (3-fold) and E (3-fold) compared to that seen in untreated rat liver. The level of [^{3}H]-13-*cis*-RA was half that observed in liver of untreated rats.

The [^{3}H]-13-*cis*-RA metabolites observed in intestinal mucosa and liver of rats treated chronically with pharmacological doses are shown in Figure 3 (experiment III). The peak E metabolites (59 and 61 mL) were the major [^{3}H]retinoids in the mucosa. Small, but significant, amounts of 13-*cis*-RA (72 mL) and *all-trans*-RA (75 mL) were also detected. A slightly different methanol–water gradient was used to generate the profiles in this experiment compared to that used in Figures 1 and 2. As a result, all retinoids eluted earlier. When aliquots of the intestinal metabolite extracts obtained in experiment III were analyzed with the original gradient system, the peak E metabolites eluted at 67 and 70 mL. These results demonstrate that peak E2 (70 mL) is a major intestinal metabolite under each of the three experimental conditions

Table II: Tissue Levels of *all-trans*-[^{3}H]Retinoic Acid and ^{3}H-Labeled Metabolites E in 13-*cis*-[^{3}H]Retinoic Acid Treated Rats

tissue	pretreatment	[^{3}H]-13-*cis*-RA dose[c] (μg)	[^{3}H]-*all-trans*-RA (ng/g)	^{3}H-labeled metabolite E (ng/g)
liver	none (I)[a]	1	4.5	6.7
	1 mg/kg; 3 days (II)	1	4.4	15.8
	1 mg/kg; 14 days (III)	200	14.5	114
intestine (mucosa)	none (I)	1	0.05	7.7
	1 mg/kg; 3 days (II)	1	0.06	7.0
	1 mg/kg; 14 days (III)	200	30.1	3138[b]

[a] The experiment number is shown in parentheses. [b] This value represents the sum of peaks E1 and E2 in the intestinal mucosa of rats given 200 μg of 13-*cis*-[11-^{3}H]retinoic acid. [c] The specific radioactivity of the dosed [^{3}H]-13-*cis*-RA was 1.3×10^7 dpm/μg in experiments I and II and 1.0×10^5 dpm/μg in experiment III.

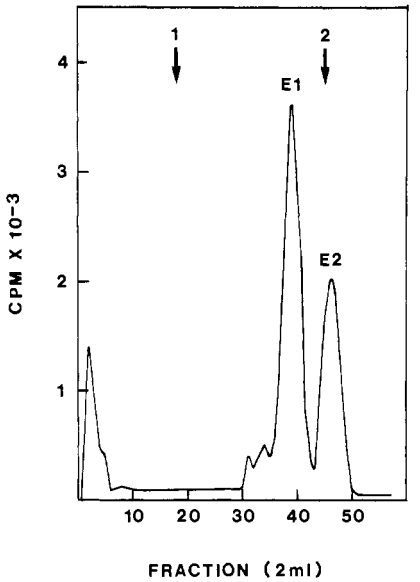

FIGURE 4: Resolution of intestinal mucosa metabolites E1 and E2 by reverse-phase, semipreparative HPLC. An aliquot (500 μL of 2 mL) of the acidic retinoid fraction prepared from intestinal mucosa of 13-*cis*-RA-treated rats was chromatographed on a Whatman Magnum-9 ODS-2 column eluted with methanol–water (65:35) containing 10 mM ammonium acetate. The flow rate was 5 mL/min, and 2-mL fractions were collected. Radioactivity was determined on 20 μL of each fraction. The elution positions of 4-keto-RA and 5,6-epoxy-RA in this solvent system are indicated by arrows 1 and 2, respectively.

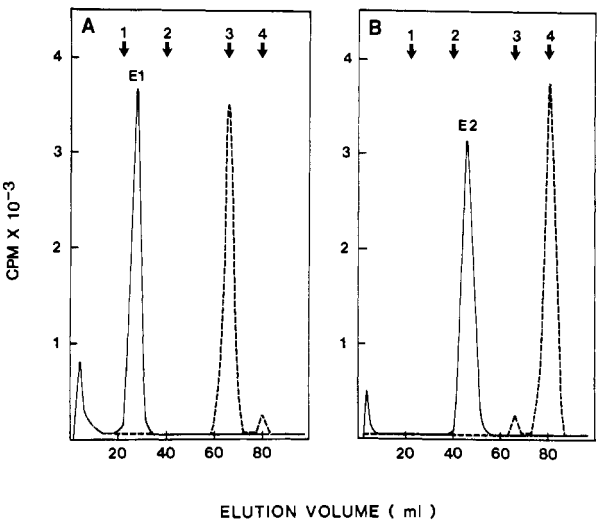

FIGURE 5: β-Glucuronidase sensitivity of intestinal mucosa metabolites E1 and E2. (A) Reverse-phase HPLC profile of native E1 treated with heat inactivated (—) and active β-glucuronidase (---). Metabolites were chromatographed on a Radial-Pak C_{18} cartridge eluted with methanol–water (65:35) containing ammonium acetate (10 mM). The flow rate was 2 mL/min. The elution positions of the internal standards are marked by the arrows: 1, 4-keto-RA; 2, 5,6-epoxy-RA; 3, 13-*cis*-RA; 4, *trans*-RA. Fractions of 1 mL were collected and measured for tritium. (B) Reverse-phase HPLC profile of native E2 treated with heat-inactivated (—) and active (---) β-glucuronidase. The chromatographic conditions were as described in (A).

studied. The data also establish that peak E1 (67 mL) is a novel metabolite, found only in the intestines of chronic 13-*cis*-RA treated rats.

In the livers of chronically treated rats, two metabolite peaks which comigrated with 13-*cis*-4-keto-RA (48 mL) and 4-keto-RA (51 mL) were detected in the peak D region of the chromatogram (Figure 3). Peak E (61 mL) was the most abundant [^{3}H]retinoid. Substantial amounts of 13-*cis*-RA (72 mL) and putative *all-trans*-RA (75 mL) were also detected. Analysis of the acidic liver retinoids with the 45–80% methanol–water gradient used in experiments I and II established the coidentity of the peak D and peak E metabolites in liver under the three experimental protocols studied.

The peak E metabolites, which represented the most abundant 13-*cis*-RA metabolites in mucosa and liver under physiological conditions, and the peak tentatively identified as *trans*-RA were chosen for further characterization. The concentration of tritiated peak E metabolites under the various conditions investigated are summarized in Table II. The highest levels of these metabolites were observed in the chronically treated rats. Since the mucosa evidenced a 30-fold higher level of the peak E metabolites and also contained both E1 and E2, it was chosen as the source of these metabolites for isolation and chemical characterization. The levels of putative [^{3}H]-*all-trans*-RA in mucosa and liver of [^{3}H]-13-*cis*-RA dosed rats are shown in Table II. The mucosa of rats given chronic 13-*cis*-RA treatment (experiment III) contained the highest concentration of the radiolabeled compound. Therefore, it was chosen as the source of putative *trans*-RA for purification.

Identification of Intestinal Metabolites E1 and E2. Metabolites E1 and E2 (Figure 4) were treated with β-glucuronidase to determine if these metabolites represented glucuronide conjugates of known retinoids. The β-glucuronidase sensitivity of E1 and E2 is depicted in Figure 5. Metabolite E1 treated with boiled enzyme eluted as a single radioactive peak (Figure 5A) which comigrated with 13-*cis*-retinoyl glucuronide synthesized in vitro. β-Glucuronidase treatment of E1 released a retinoid which comigrated with synthetic 13-*cis*-RA (66 mL). The β-glucuronidase sensitivity of E2 is shown in Figure 5B. Native E2 eluted at 46 mL and comigrated with retinoyl glucuronide synthesized in vitro. β-Glucuronidase treatment released a retinoid which comigrated with synthetic *all-trans*-RA (80 mL).

The methylated derivative of the retinoid liberated from E1 by β-glucuronidase treatment comigrated with synthetic methyl 13-*cis*-retinoate on reverse-phase HPLC (Figure 6A).

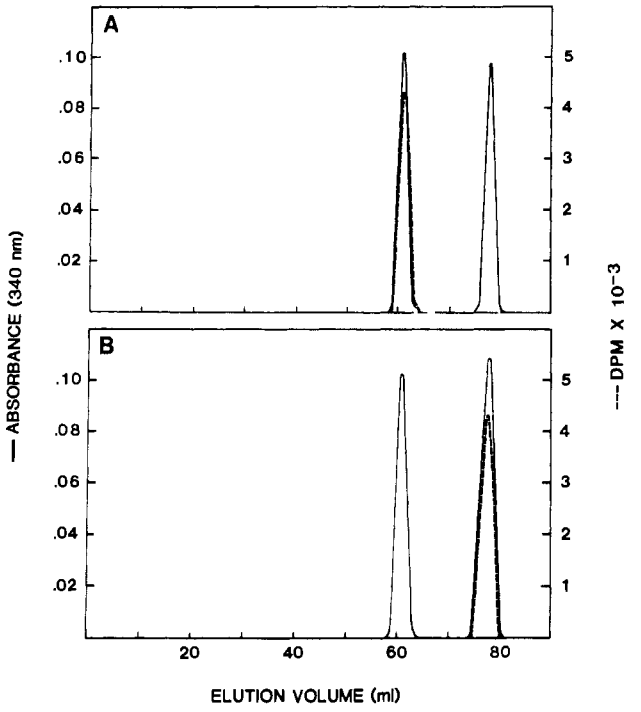

FIGURE 6: Reverse-phase HPLC comigration of the retinoids released by β-glucuronidase treatment of intestinal E1 and E2 with synthetic retinoids. (A) RP-HPLC comigration of the methyl ester of 13-*cis*-RA released from metabolite E1 by β-glucuronidase (---) with authentic methyl 13-*cis*-retinoate. The retinoids were chromatographed on a C_{18} Radial-Pak cartridge eluted with methanol–water (85:15). Fractions of 1 mL were collected, and 10-μL aliquots were counted. The elution positions of authentic methyl 13-*cis*-retinoate (61 mL) and methyl retinoate (78 mL) were monitored at 340 nm (—). (B) RP-HPLC comigration of the methyl ester of *trans*-RA released from metabolite E2 by β-glucuronidase (---) with methyl retinoate. The chromatographic conditions were as described in (A).

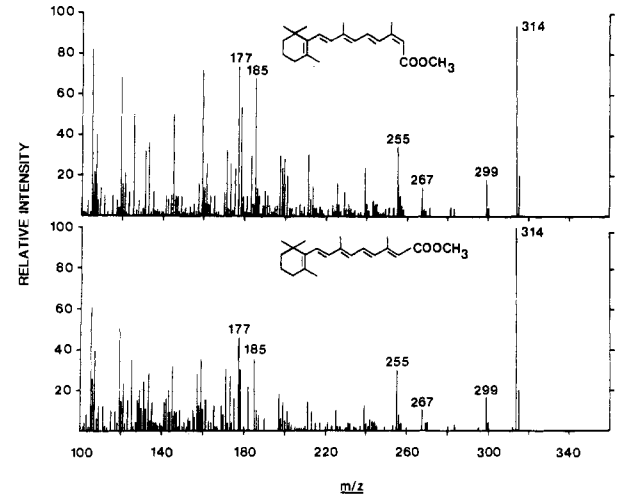

FIGURE 7: Mass spectra of isolated methyl 13-*cis*-retinoate and methyl retinoate. (A) Electron impact mass spectrum of 50 ng of isolated methyl 13-*cis*-retinoate derived from the intestinal glucuronide. (B) Electron impact mass spectrum of 50 ng of methyl retinoate derived from the intestinal glucuronide.

The methylated derivative of the retinoid released by β-glucuronidase treatment of E2 comigrated with methyl retinoate under the same chromatographic conditions (Figure 6B).

The mass spectrum of 50 ng of the isolated methyl 13-*cis*-retinoate derived from E1 is shown in Figure 7 (top panel). As expected a molecular ion at m/z 314 characteristic of a methylated retinoic acid was observed. Prominent peaks at m/z 299 ($M^+ - CH_3$) and 255 ($M^+ - COOCH_3$) were also evident. The bottom panel of Figure 7 depicts the mass spectrum obtained for 50 ng of methyl retinoate derived from E2. A molecular ion at m/z 314 and characteristic fragments at m/z 299 and 255 were observed. Mass spectra obtained for 50 ng of synthetic methyl retinoate and methyl 13-*cis*-retinoate were indistinguishable from those obtained for the biological samples (data not shown).

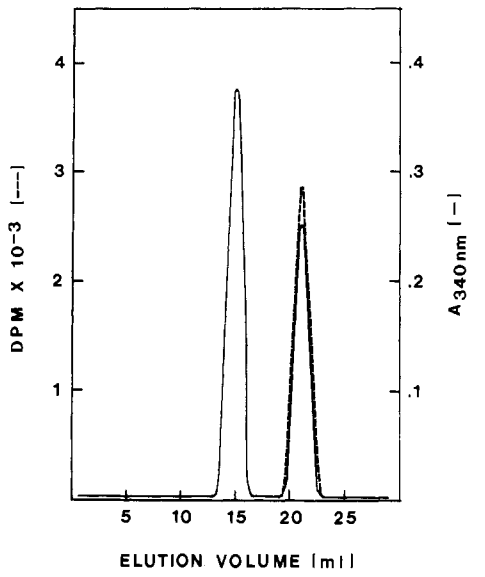

FIGURE 8: Normal-phase HPLC comigration of methyl retinoate isolated from intestinal mucosa with standard methyl retinoate. The retinoids were analyzed on a Du Pont Zorbax-SIL column eluted with hexane–toluene (7:3). The elution of the methyl 13-*cis*-retinoate (15 mL) and methyl retinoate (21 mL) standards were monitored at 340 nm (—). Fractions of 1 mL were collected and counted to determine the elution position of the biological sample (---).

Under the conditions used for storage and isolation of the *all-trans*- and 13-*cis*-retinoyl glucuronides, little isomerization of either compound was noted (<1%). Both retinoyl glucuronides proved to be stable when stored in methanol in the crude acidic retinoid fraction. However, the partially purified glucuronides obtained after reverse-phase HPLC proved to be more labile during storage in methanol at −80 °C. The instability of these compounds resulted from the transesterification of the glucuronides in methanol to yield methyl retinoate or methyl 13-*cis*-retinoate. To overcome this instability problem, the partially purified glucuronides were immediately treated with β-glucuronidase to liberate the free retinoic acids for further purification.

Identification of Liver Metabolite E. The β-glucuronidase sensitivity of liver metabolite E was examined as described for intestinal metabolites E1 and E2. Native E (boiled enzyme control) eluted as a single radioactive peak which comigrated with the retinoyl glucuronide standard. β-Glucuronidase treatment released a single radioactive retinoid which comigrated with synthetic *trans*-RA.

Identification of all-trans-RA. The purified intestinal metabolite (as the methyl ester) was cochromatographed with synthetic methyl retinoate and methyl 13-*cis*-retinoate on a NP-HPLC column eluted with hexane–toluene (7:3) as shown in Figure 8. The biological sample of methyl retinoate comigrated with the synthetic compound (21 mL). Methyl 13-*cis*-retinoate eluted at 15 mL.

Fifty nanograms of the isolated methyl retinoate was used to obtain a mass spectrum of the compound. A spectrum identical with that shown in Figure 7 (bottom panel) was obtained with a molecular ion at m/z 314 and prominent peaks at m/z 299 ($M^+ - CH_3$) and 255 ($M^+ - COOCH_3$).

Discussion

This work characterizes 13-*cis*-RA metabolites in two retinoid target tissues, liver and intestinal mucosa, following oral administration of physiological amounts of [11-^{3}H]-13-*cis*-RA. *all-trans*-RA was identified as an in vivo 13-*cis*-RA metabolite in rat intestinal mucosa and liver. The putative *all-trans*-RA was isolated from mucosa and subjected to chromatographic and mass spectral characterization. Metabolite identification was based on the HPLC comigration of the native metabolite and the methyl ester derivative with synthetic *all-trans*-retinoic acid and methyl retinoate, respectively, and the mass spectral analysis of the methylated metabolite. Under the chromatographic conditions chosen for comigration experiments all of the polar cis isomers of retinoic acid are base line separated from *all-trans*-RA. The *all-trans*-RA metabolite in rat liver was chromatographically identical with reference *all-trans*-RA. On the basis of the combined HPLC and mass spectral data, we conclude that the mucosa and liver metabolites are *all-trans*-RA. An equimolar ratio of tritiated 13-*cis*-RA and *trans*-RA was noted in both liver and mucosa of rats given a physiological dose of radiolabeled 13-*cis*-RA. Tissue homogenates adulterated with [^{3}H]-13-*cis*-RA demonstrated a low level of *trans*-RA (3%) upon experimental manipulation. Therefore, the majority of [^{3}H]-*trans*-RA detected clearly originated from in vivo metabolism of dosed 13-*cis*-RA. Our discovery that a substantial fraction of target tissue associated [^{3}H]RA is present as the all-trans isomer indicates that the isomerization of 13-*cis*-RA to *trans*-RA could be important for the expression of the biological and chemopreventive activity of 13-*cis*-RA.

The major intestinal mucosa metabolite (E2) detected after physiological doses of 13-*cis*-RA was identified as *all-trans*-retinoyl glucuronide. The identification was based on the HPLC comigration of E2 with *all-trans*-retinoyl glucuronide, the β-glucuronidase sensitivity of the native metabolite, and identification of *trans*-RA released by β-glucuronidase treatment. Similarly, one of the major liver metabolites (E) was also identified as *trans*-retinoyl glucuronide by HPLC comigration, β-glucuronidase sensitivity, and the liberation of *trans*-RA by enzyme treatment.

On the basis of our present data and published data on *trans*-RA metabolism in vivo (Napoli & McCormick, 1981; Sundaresan & Bhat, 1982; Zile et al., 1982a), we propose that the following metabolic pathway is involved in the in vivo metabolism of 13-*cis*-RA:

13-*cis*-RA $\rightleftharpoons$ *trans*-RA $\rightarrow$ *trans*-retinoyl glucuronide

The amount of radioactivity recovered as *trans*-RA and *trans*-retinoyl glucuronide in liver and mucosa under each of the three experimental conditions examined demonstrates that the majority of tissue radioactivity is present in the all-trans configuration after oral administration of [^{3}H]-13-*cis*-RA. These data highlight the importance of cis–trans isomerization in the metabolism of 13-*cis*-RA and suggest that the equilibrium of the reversible isomerization reaction lies in the direction of *trans*-RA in vivo. The proposed bidirectional nature of retinoic acid isomerization is consistent with the identification of 13-*cis*-RA as an in vivo *trans*-RA metabolite in several laboratories (Napoli & McCormick, 1981; Sundaresan & Bhat, 1982; Zile et al., 1982a). Moreover, our data demonstrate that isomerization to *trans*-RA followed by glucuronidation is a major route for the in vivo metabolism of physiological as well as pharmacological amounts of 13-*cis*-RA.

The biochemical significance of the substantial accumulation of [^{3}H]-*trans*-retinoyl glucuronide in liver and mucosa of 13-*cis*-RA dosed rats is not clear. *trans*-Retinoyl glucuronide is not a major biliary metabolite of 13-*cis*-RA (Frolik et al., 1981) or *trans*-RA (Zile et al., 1982b). Therefore, the trans glucuronide does not appear to represent a major excretory product of 13-*cis*-RA. Retinoyl glucuronide may serve as a short-term storage form of *trans*-RA in those tissues which possess β-glucuronidase activity capable of regenerating the aglycone (*trans*-RA). Further experiments are required to test this and other possible functions of *trans*-retinoyl glucuronide.

The livers of rats given physiological doses of 13-*cis*-RA contained minor metabolite peaks which comigrated with 13-*cis*-4-keto-RA and 4-keto-RA (peak D) when analyzed by reverse-phase HPLC. The ratio of the putative 13-cis and all-trans isomers of 4-keto-RA was approximately 3:1. These liver metabolites are currently being isolated for structure identification. 13-*cis*-4-Keto-RA has been identified as an intermediate common to the metabolism of *trans*-RA and 13-*cis*-4-keto-RA in hamsters (Frolik et al., 1980) and has been shown to be less active than *trans*-RA or 13-*cis*-RA in a tracheal organ culture bioassay (Frolik et al., 1979). On the basis of the reduced biological activity of the C(4) oxidized retinoid and the identification of 13-*cis*-4-ketoretinoyl glucuronide as a major biliary metabolite of 13-*cis*-RA in the rat (Frolik et al., 1981), C(4) oxidation probably represents an initial step in the excretion of 13-*cis*-RA (Frolik et al., 1979). The low 13-*cis*-4-keto-RA concentrations detected in rat liver could be explained by rapid conjugation of the ketone with glucuronic acid coupled with the rapid excretion of the glucuronide via the bile.

The in vivo metabolism of 13-*cis*-RA appears to be modulated by the retinoid itself. For example, the concentrations of radiolabeled *all-trans*-retinoyl glucuronide and the putative 13-*cis*-4-keto-RA and *all-trans*-4-keto-RA metabolites found in liver 3 h after physiological doses of [^{3}H]-13-*cis*-RA were elevated by 13-*cis*-RA pretreatment. This apparent induction of the metabolic pathway responsible for C(4) oxidation of 13-*cis*-RA by supraphysiological doses of 13-*cis*-RA is undoubtedly important to the detoxification and excretion of this retinoid. In the intestinal mucosa, 13-*cis*-RA pretreatment did not affect the concentration of tritiated *all-trans*-RA or *all-trans*-retinoyl glucuronide at 3 h after a physiological dose of [^{3}H]-13-*cis*-RA. However, chronic 13-*cis*-RA treatment promoted a marked increase in the levels of radiolabeled *all-trans*-RA and *all-trans*-retinoyl glucuronide in the mucosa. In addition, chronic administration of 13-*cis*-RA resulted in the appearance of substantial amounts of tritiated 13-*cis*-retinoyl glucuronide (E1), a metabolite not detected in mucosa of rats given physiological doses of the retinoid. In fact, the 13-cis glucuronide was the most abundant mucosa metabolite observed in chronically treated rats. The observed accumulation of 13-*cis*-retinoyl glucuronide may have resulted from the enterohepatic circulation of this retinoid. Although significant enterohepatic circulation of *trans*-RA metabolites has been demonstrated (Swanson et al., 1981), similar experiments with 13-*cis*-RA have not been reported. *trans*-RA metabolism studies in bile duct cannulated rats have indicated that the intestine is capable of synthesizing retinoyl glucuronides (Zile et al., 1982b). Intestinal glucuronide synthesis in rats receiving pharmacological amounts of 13-*cis*-RA may function to protect the rat from retinoid toxicity. Further studies are necessary to determine the sites of 13-*cis*-retinoyl glucuronide biosynthesis and to ascertain the role of the enterohepatic circulation of this compound in 13-*cis*-RA metabolism.

This study has established that 13-*cis*-RA is isomerized to *trans*-RA under physiological conditions and that this isom-

erization is a central event in 13-*cis*-RA metabolism in vivo. In addition, we have demonstrated that a significant fraction of the *trans*-RA synthesized in vivo is converted to *trans*-retinoyl glucuronide. Thus, the major 13-*cis*-RA metabolites retained in two retinoid target tissues have the all-trans configuration. Our data strongly suggest that *trans*-RA makes a significant contribution to the observed biological and chemopreventive activity of 13-*cis*-RA.

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Registry No. 13-*cis*-RA, 4759-48-2; *trans*-RA, 302-79-4; *all-trans*-retinoyl glucuronide, 401-10-5; 13-*cis*-retinoyl glucuronide, 78147-42-9.

References

Becci, P. J., Thompson, H. J., Grubbs, C. J., Squire, R. A., Brown, C. C., Sporn, M. B., & Moon, R. C. (1978) *Cancer Res. 38*, 4463–4466.

Becci, P. J., Thompson, H. J., Strum, J., Brown, C. C., Sporn, M. B., & Moon, R. C. (1981) *Cancer Res. 41*, 917–932.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Frolik, C. A., Roberts, A. B., Tavela, T. E., Roller, P. P., Newton, D. L., & Sporn, M. B. (1979) *Biochemistry 18*, 2092–2097.

Frolik, C. A., Roller, P. P., Roberts, A. B., & Sporn, M. B. (1980) *J. Biol. Chem. 255*, 8057–8062.

Frolik, C. A., Roller, P. P., Roberts, A. B., & Sporn, M. B. (1981) *Arch. Biochem. Biophys. 208*, 344–352.

Jetten, A. M., Jetten, M. E. R., & Sherman, M. I. (1979) *Exp. Cell Res. 124*, 381–391.

Lasnitzki, I., & Goodman, D. S. (1974) *Cancer Res. 34*, 1564–1571.

Lotan, R., Neuman, G., & Lotan, D. (1980) *Cancer Res. 40*, 1097–1102.

Mayer, H., Bollag, W., Hänni, R., & Ruegg, H. (1978) *Experientia 34*, 1105–1119.

McCormick, A. M., & Napoli, J. L. (1982) *J. Biol. Chem. 257*, 1730–1735.

McCormick, A. M., Napoli, J. L., Schnoes, H. K., & DeLuca, H. F. (1978) *Biochemistry 17*, 4085–4090.

Napoli, J. L., & McCormick, A. M. (1981) *Biochim. Biophys. Acta 666*, 165–175.

Napoli, J. L., Khalil, H., & McCormick, A. M. (1982) *Biochemistry 21*, 1942–1949.

Port, C. D., Sporn, M. B., & Kaufmann, D. G. (1975) *Proc. Am. Assoc. Cancer Res. 16*, 21.

Sporn, M. B., Dunlop, N. M., Newton, D. L., & Henderson, W. R. (1976) *Nature (London) 263*, 110–113.

Strickland, S. (1978) *Cold Spring Harbor Conf. Cell Proliferation 6*, 671–676.

Strickland, S., & Mahdavi, V. (1978) *Cell (Cambridge, Mass.) 15*, 393–403.

Sundaresan, P. R., & Bhat, P. V. (1982) *J. Lipid Res. 23*, 448–455.

Swanson, B. N., Frolik, C. A., Zaharevitz, D. W., Roller, P. P., & Sporn, M. B. (1981) *Biochem. Pharmacol. 30*, 107–113.

Thompson, H. J., Becci, P. J., Grubbs, C. J., Shealy, F., Stanek, E. J., Brown, C. C., Sporn, M. B., & Moon, R. C. (1981) *Cancer Res. 41*, 933–936.

Vane, F. M., & Buggé, C. J. L. (1981) *Drug Metab. Dispos. 9*, 515–520.

Vane, F. M., Buggé, C. J. L., & Williams, T. H. (1982) *Drug Metab. Dispos. 10*, 212–219.

Verma, A. K., Shapas, B. G., Rice, H. M., & Boutwell, H. K. (1979) *Cancer Res. 39*, 419–425.

Zile, M., & DeLuca, H. F. (1968) *J. Nutr. 94*, 302–308.

Zile, M. H., Inhorn, R. C., & DeLuca, H. F. (1982a) *J. Biol. Chem. 257*, 3544–3550.

Zile, M. H., Inhorn, R. C., & DeLuca, H. F. (1982b) *J. Biol. Chem. 257*, 3537–3543.