Broach, J. R., Strathern, J. N., & Hicks, J. B. (1979) *Gene 8*, 121–133.
Ciriacy, M., & Breitenbach, I. (1979) *J. Bacteriol. 139*, 152–160.
Clifton, D., & Fraenkel, D. G. (1981) *J. Biol. Chem. 256*, 13074–13078.
Clifton, D., Weinstock, S. B., & Fraenkel, D. G. (1978) *Genetics 88*, 1–11.
Fraenkel, D. G. (1982) *The Molecular Biology of the Yeast Saccharomyces* (Strathern, J. N., Jones, E. W., & Broach, J. R., Eds.) Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (in press).
Gancedo, J. M., & Gancedo, C. (1979) *Eur. J. Biochem. 101*, 455–460.
Henry, S. A., Donahue, T. F., & Culbertson, M. R. (1975) *Mol. Gen. Genet. 143*, 5–11.
Herrmann, K., Diezel, W., Kopperschläger, G., & Hofmann, E. (1973) *FEBS Lett. 36*, 190–192.
Huse, K., Kopperschläger, G., & Hofmann, E. (1976) *Biochem. J. 155*, 721–723.
Jones, E. W. (1977) *Genetics 85*, 23–33.
Kopperschläger, G., Bär, J., Nissler, K., & Hofmann, E. (1977) *Eur. J. Biochem. 81*, 317–327.
Laurent, M., Chaffotte, A. F., Tenu, J.-P., Roucous, C., & Seydoux, F. J. (1978) *Biochem. Biophys. Res. Commun. 80*, 646–652.
Laurent, M., Seydoux, F. J., & Dessen, P. (1979) *J. Biol. Chem. 254*, 7515–7520.
Navon, G., Shulman, R. G., Yamane, T., Eccleshall, T. R., Lam, K.-B., Baronofsky, J. J., & Marmur, J. (1979) *Biochemistry 18*, 4487–4499.
Orozco de Silva, A., & Fraenkel, D. G. (1979) *J. Biol. Chem. 254*, 10237–10242.
Plietz, P., Damaschun, G., Kopperschläger, G., & Muller, J. J. (1978) *FEBS Lett. 91*, 230–232.
Reuter, R., Eschrich, K., Schellenberger, W., & Hofmann, E. (1979) *Acta Biol. Med. Ger. 38*, 1067–1079.
Tamaki, N., & Hess, B. (1975a) *Hoppe-Seyler's Z. Physiol. Chem. 356*, 399–415.
Tamaki, N., & Hess, B. (1975b) *Hoppe-Seyler's Z. Physiol. Chem. 356*, 1663–1669.
Tijane, M. N., Seydoux, F. J., Hill, M., Roucous, C., & Laurent, M. (1979) *FEBS Lett. 105*, 249–253.
Tijane, M. N., Chafotte, A. F., Seydoux, F. J., Roucous, C., & Laurent, M. (1980) *J. Biol. Chem. 255*, 10188–10193.
Towbin, H., Staehlin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.
Wieland, O. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., Ed.) Vol. 3, pp 1404–1409, Verlag Chemie, Weinheim, and Academic Press, New York.

Metabolism of 5,6-Epoxyretinoic Acid in Vivo: Isolation of a Major Intestinal Metabolite†

Joseph L. Napoli,* Hoda Khalil, and Anne M. McCormick

ABSTRACT: The major metabolite in the small intestinal mucosa of vitamin A deficient rats dosed intrajugularly with 5,6-epoxy[^{3}H]retinoic acid has been identified as 5,6-epoxyretinoyl β-glucuronide. The assignment was based on the metabolite's chemical, spectral, and chromatographic properties. Incubation of the metabolite with β-glucuronidase released 5,6-epoxyretinoic acid. Incubation of 5,6-epoxyretinoic acid with rat liver microsomes in the presence of uridine-5′-diphospho-1α-D-glucuronic acid produced the metabolite. 5,6-Epoxy[^{3}H]retinoyl β-glucuronide was observed in the liver, small intestinal mucosa, and intestinal contents but not in kidney of vitamin A deficient rats. Its concentration was greatly diminished in liver and small intestinal mucosa, and it was not observed in kidney of vitamin A deficient rats dosed orally with retinoic acid for several days before administration of 5,6-epoxy[^{3}H]retinoic acid. Generally, oral retinoic acid treatment accelerated 5,6-epoxyretinoic acid metabolism and enhanced accumulation of highly polar metabolites. Moreover, 5,6-epoxyretinoic acid metabolism was more rapid than that of retinoic acid and did not result in production of retinoic acid.

Retinoic acid is a quantitatively significant metabolite of retinol under physiological conditions (McCormick & Napoli, 1981). It is also more active than retinol in directing differentiation in vitro, by at least an order of magnitude (Sporn et al., 1976; Strickland & Mahdavi, 1978), but unlike retinol, it does not support vision or mammalian reproduction (Dowling & Wald, 1960; De Luca, 1978; Goodman, 1980). Retinoic acid undergoes rapid metabolism in vivo (Fidge et al., 1968; Geison & Johnson, 1970) to several more polar metabolites which include glucuronides (Dunagin et al., 1965, 1966; Lippel & Olson, 1968a,b) in a tissue-specific manner (Napoli & McCormick, 1981). These facts suggest that retinoic acid, or one of its metabolites, rather than retinol, is the form of vitamin A that directs epithelial differentiation.

5,6-Epoxyretinoic acid was the first target-tissue metabolite of retinoic acid isolated and identified (Napoli et al., 1978; McCormick et al., 1978). Like retinoic acid, 5,6-epoxyretinoic acid has been established as a metabolic product of retinol under physiological conditions (McCormick & Napoli, 1981). Moreover, synthetic racemic 5,6-epoxyretinoic acid is equipotent with retinoic acid in inhibiting the induction of ornithine decarboxylase activity, and the promotion of skin tumors by phorbol esters (Verma et al., 1980). The epoxide is also equipotent with retinoic acid in the induction of differentiation

† From the Department of Biochemistry, The University of Texas Health Science Center, Dallas, Texas 75235. *Received October 13, 1981.* This work was supported by National Institutes of Health Grant AM 26535, Robert A. Welch Foundation Grants I-797 and I-810, and Shared Instrumentation Grant GM 27506.

0006-2960/82/0421-1942$01.25/0

of cultured embryonal carcinoma cells (Strickland, 1978). However, the growth-promoting activity of 5,6-epoxyretinoic acid is well-known to be much less than that of retinoic acid (Morgan & Thompson, 1966; Mallia et al., 1970). The latter results may be attributable to pharmacological effects. Nevertheless, the function of retinoic acid epoxidation is not yet known.

Knowledge of 5,6-epoxyretinoic acid's disposition in vivo could aid in developing an understanding of the epoxide's role in retinoid metabolism. This paper reports such a study and shows that the epoxy derivative of retinoic acid is metabolized more rapidly than retinoic acid itself. However, like that of retinoic acid, 5,6-epoxyretinoic acid metabolism is tissue specific and is sensitive to retinoid induction. Furthermore, the major metabolite in intestines of vitamin A deficient rats dosed with 5,6-epoxyretinoic acid has been identified as 5,6-epoxyretinoyl β-glucuronide.

Materials and Methods

General. Ultraviolet spectra were taken in 0.3 mL of methanol in a 1-mL quartz cuvette with a Beckman Model 25 recording spectrophotometer.

High-performance liquid chromatography (HPLC)[1] was performed with a Waters Associates ALC/GPC 204 liquid chromatograph equipped with a second pump and a Model 660 solvent programmer. Retinoids were detected at 340 nm. The analytical reverse-phase column used was a Du Pont Zorbax ODS C-18 (0.25 × 25 cm). The semipreparative reverse-phase column used was a Whatman ODS-2 Magnum 9 (1 × 25 cm). Radially compressed reverse-phase columns were Waters C-18 Radial-Pak liquid chromatography cartridges (8-mm diameter, 10-μm particles). The normal-phase column used was a Du Pont Zorbax Sil (0.25 × 25 cm). All solvents were distilled in glass and filtered through a 0.45-μm filter.

Radioactivity was measured in a solution composed of 770 mL of toluene, 330 mL of Triton X-100, 0.1 g of 1,4-bis[2-(5-phenyloxazoyl)]benzene, and 6 g of 2,5-diphenyloxazole. Measurements were made with a Beckman LS-330 liquid scintillation counter equipped with an automatic external standard system.

Gas chromatography/mass spectroscopy (GC/MS) was performed with a Finnigan Model 9610 gas chromatograph connected to a Finnigan Model 4021 mass spectrometer served by an Incos data system. Gas chromatography was performed on a glass column (0.25 in. × 6 ft) packed with 3% OV-1 on S-port (60/80) available from Finnigan, Palo Alto, CA. The helium flow was 20 mL/min, and the column temperature was 245 °C. The injector, transfer line, and jet separator were held at 250 °C. Electron-impact spectra were taken at 70 eV. Methane was used as the reagent gas for chemical ionization spectra.

Compounds. 5,6-Epoxy[11-^{3}H]retinoic acid (1.2 Ci/mmol) was obtained from the Chemopreventive Program, Division of Cancer Cause and Prevention, National Cancer Institute. It was >98% pure as determined by HPLC. Unless stated otherwise, unlabeled retinoids were a gift from Dr. Beverly Pawson, Hoffmann-La Roche, Nutley, NJ. UDPGA, arylsulfatase (aryl-sulfate sulfohydrolase, type V, from limpets), β-glucuronidase (β-D-glucuronide glucuronosohydrolase, type VII, from *Escherichia coli*), and saccharo-1,4-lactone were purchased from Sigma Chemical Co., St. Louis, MO.

Methyl 13-*cis*-5,6-epoxyretinoate was synthesized by epoxidation of methyl 13-*cis*-retinoate. To a solution of methyl 13-*cis*-retinoate (4 mg, 12.7 μmol) in diethyl ether (4 mL) was added *m*-chloroperoxybenzoic acid (6 mg, 35 μmol). The solution was stirred at ambient temperature. After 3 h, thin-layer chromatography (10% diethyl ether/hexane, silica gel) showed a spot at R_f 0.24 and no material comigrating with methyl-13-*cis*-retinoic acid (R_f 0.50). The ether solution was washed 3 times with 5% aqueous potassium carbonate, once with water, and once with saturated aqueous sodium chloride and dried over magnesium sulfate. The ether was evaporated, and the residue was purified on a normal-phase analytical HPLC column eluted with tetrahydrofuran/hexane (0.5:99.5), yielding 2 mg of methyl 13-*cis*-epoxyretinoate: UV (hexane) λ_{max} 340 nm, shoulders at 355 and 286 nm; NMR (90 MHz, $CDCl_3$) δ 0.94 (s, C-18 methyl), 1.09 and 1.14 (2 s, C-16 and C-17 methyls), 1.96 (s, C-19 methyl), 2.09 (s, C-20 methyl), 2.71 (s, ester methyl), 5.65 (s, C-14 proton), 6.93 (dd, J = 10 and 16 Hz, C-11 proton), 6.93 (d, J = 16 Hz, C-8 proton), 7.79 (d, J = 16 Hz, C-12 proton), 6.33 (d, J = 16 Hz, C-7 proton), 6.23 (d, J = 10 Hz, C-10 proton). As expected, the chemicals shifts of the C-20 methyl group, C-14 proton, and C-12 proton were different from those of methyl *all-trans*-5,6-epoxyretinoate (δ 2.34, 5.79, and 6.30, respectively), whereas the chemical shifts of the other protons were virtually identical; positive ion chemical ionization mass spectrum, m/e (rel intensity) 331 (100, $M^+ + H$), 315 (30, $M^+ - 15$), 299 (15, M^+ − methanol).

Animals. Male weanling Sprague-Dawley rats were purchased from Charles River Breeding Laboratories, MA. They were fed a vitamin A deficient purified diet purchased from Ziegler Bros., Inc., Gardners, PA. The diet consisted of the AIN-76 base diet, mineral mix, and vitamin mix excluding retinyl palmitate and with an additional 1 g/kg of ascorbic acid (Bieri et al., 1977). Animals usually took 8 weeks to become vitamin A deficient as judged by cessation of growth. The animals were continued without supplementation on the vitamin A deficient diet or were treated orally with *all-trans*-retinoic acid (200 μg per rat per day) in 0.1 mL of Wesson oil for 4 days. Dosing with radiolabeled 5,6-epoxide took place 24 h after the last oral dose of unlabeled *all-trans*-retinoic acid. 5,6-Epoxy[11-^{3}H]retinoic acid was administered intrajugularly in 0.1 mL of ethanol to rats anesthetized with ether. Two hours after being dosed, the animals in all experiments were sacrificed under ether anesthesia by cardiac exsanguination.

Tissues. Liver, kidney, and small intestines were removed. Kidneys were decapsulated. The contents were rinsed from the small intestines with 0.9% sodium chloride, and the mucosa was scraped from the intestinal wall. Tissues were minced and homogenized in 1 volume of glass-distilled water containing EDTA and propyl gallate (50 μg/mL each). Homogenates were frozen and lyophilized. Each residue was extracted with five portions of methanol containing butylated hydroxytoluene (50 μg/mL). Previous work has demonstrated that this procedure results in virtually complete extraction of the radioactivity (Roberts et al., 1978; McCormick et al., 1980). The samples were concentrated to 50 mL, and aliquots in triplicate were measured for radioactivity.

Chromatography. Samples were applied to DEAE-Sephadex A-25 columns (2.5 × 10 cm) in the hydroxyl form equilibrated with methanol. Neutral compounds were eluted with an additional 125 mL of methanol. The charged retinoids

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; UDPGA, uridine-5′-diphospho-1α-D-glucuronic acid; HPLC, high-performance liquid chromatography.

were eluted with 150 mL of 0.25 M ammonium bicarbonate in 10% aqueous methanol. Aliquots in triplicate from both eluants were counted for radioactivity. Of the recovered radioactivity, 91–95% was charged. The solvents were evaporated, and the residues were stored in ethanol (15 mL) under nitrogen at −70 °C.

For generation of chromatographic profiles, 10 μg each of 4-ketoretinoic acid, 5,6-epoxyretinoic acid, 13-*cis*-retinoic acid, and *all-trans*-retinoic acid was added to aliquots (10–40%) of the charged extracts from animals dosed with 1 μg of 5,6-epoxy[11-^{3}H]retinoic acid. The samples were concentrated, filtered, and injected onto HPLC columns. To each fraction were added 6 mL of counting solution and 2 mL of methanol. Disintegrations per minute (dpm) per fraction were adjusted for recovery and aliquot size, and the resulting numbers were divided by grams per tissue so that chromatograms are plotted as dpm per gram of total tissue per fraction. Recoveries from the HPLC columns were greater than 96%.

A control experiment was performed by injecting 1.8 × 10^6 dpm of 5,6-epoxy[11-^{3}H]retinoic acid into 10 g of liver homogenate at 4 °C. After 20 min, the liver was lyophilized and extracted as were the other samples. Of the injected radioactivity, 96% was recovered in the charged fraction after DEAE-Sephadex chromatography. HPLC of the charged material was done as described in Figure 1 (deficient). Of the radioactivity, 94% was recovered as unchanged 5,6-epoxyretinoic acid. The remainder of the radioactivity was found in fractions 2–4 (3%), 45–55 (1%), and 86–89 (2%).

Reactions with Arylsulfatase and β-Glucuronidase. Either arylsulfatase or β-glucuronidase in 1 mL of 50 mM Mops, pH 7.0, was added to the 5,6-epoxyretinoyl conjugate. Enzyme solutions boiled for 10 min were used as controls. The buffer of the β-glucuronidase inhibition experiment contained 5 mM saccharo-1,4-lactone. The tubes were gassed with nitrogen, sealed, and incubated at 37 °C in a shaking water bath. After 1 h, 4-ketoretinoic acid and 5,6-epoxyretinoic acid (10 μg of each) in ethanol (4 mL) were added. The mixtures were filtered through a 0.45-μm filter, and the filtrate was dried under a stream of nitrogen. The residues were dissolved in methanol (150 μL) and analyzed by HPLC.

In Vitro Synthesis of 5,6-Epoxyretinoyl Glucuronide. The liver of a male rat fed a stock diet was used to prepare a 20% (w/v) homogenate in a buffer consisting of 50 mM Mops, 250 mM sucrose, and 2.5 mM EGTA, pH 7.4. The homogenate was spun for 15 min at 5000*g*. The supernatant was collected and spun for 15 min at 18000*g*. The resulting supernatant was spun for 60 min at 105000*g* in a type 30 rotor in a Beckman Model L5-50 ultracentrifuge. The microsomal pellet was homogenized in a buffer consisting of 50 mM Mops, 5 mM EGTA, and 10 mM $MgCl_2$, pH 7.0 (buffer A). Protein was determined by the method of Bradford (1976) with bovine serum albumin as standard. 5,6-Epoxy[11-^{3}H]retinoic acid (38 nmol, 7.9 × 10^4 dpm/nmol), 5 mg of microsomal protein, and UDPGA (0 or 5 mg) in a total volume of 1 mL of buffer A were incubated at 37 °C for 1 h. Unlabeled 4-ketoretinoic acid and 5,6-epoxyretinoic acid (10 μg of each) were added in ethanol (3 mL). The resulting mixture was filtered through a 0.45-μm filter, and the filtrate was dried under a stream of nitrogen. The residue was dissolved in methanol (150 μL) and analyzed by HPLC.

Results

Distribution of the dose among liver, small intestinal mucosa, and kidney in animals administered 5,6-epoxy[^{3}H]retinoic acid (1 μg) intrajugularly is shown in Table I. More of the dose was observed in the liver and kidney of vitamin A deficient

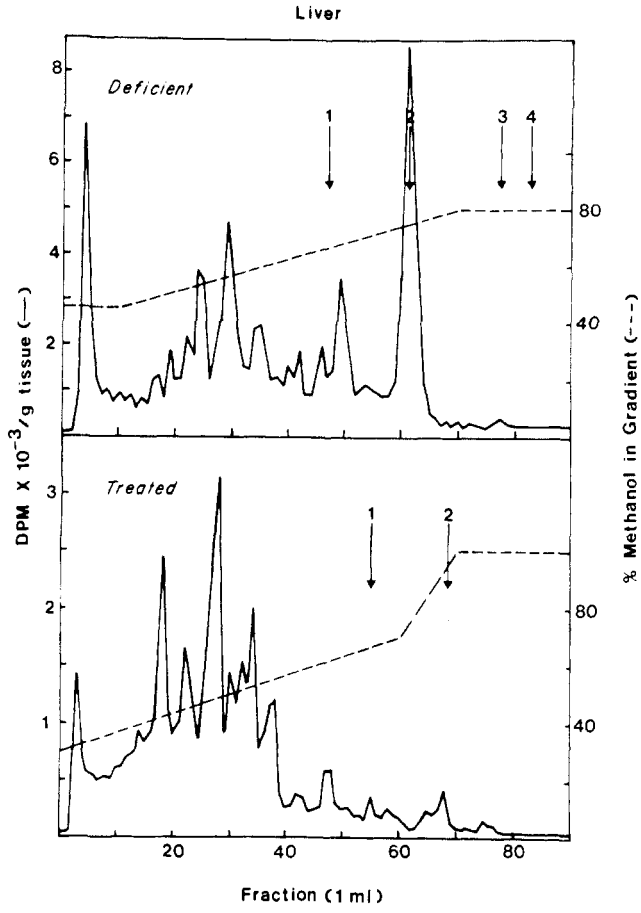

FIGURE 1: HPLC separation of charged tritiated retinoids extracted from liver 2 h after a 1-μg dose of 5,6-epoxy[^{3}H]retinoic acid. Arrows represent the elution positions of the internal standards: (1) 4-ketoretinoic acid; (2) 5,6-epoxyretinoic acid; (3) 13-*cis*-retinoic acid; (4) *all-trans*-retinoic acid. Samples were analyzed on an analytical reverse-phase column. The sample from the vitamin A deficient rats (deficient) was eluted at 1 mL/min with 10 mM ammonium acetate in water/methanol (55:45) for 10 min, followed by a linear gradient reaching 80% methanol in 60 min. The sample from the vitamin A deficient rats treated with retinoic acid (treated) was eluted at 1 mL/min with a linear gradient of 10 mM ammonium acetate in 70/30 water/methanol to 60/70 water/methanol over 60 min, followed by a linear gradient to 10 mM ammonium acetate in methanol over 10 min.

(deficient) animals than in the organs of vitamin A deficient, retinoic acid treated (treated) animals. In contrast, the portion of the dose found in mucosa was not influenced by retinoid status. Furthermore, more radioactivity was found in mucosa than in most of the other samples. With the exception of liver from deficient animals, these results are different from those obtained after dosing [^{3}H]retinoic acid to rats of similar retinoid states. In the latter case, 2–4-fold more radiolabel was found in kidney and liver than in small intestinal mucosa (Napoli & McCormick, 1981).

The recovered radioactive materials were examined by HPLC (Table I, Figures 1–3). Clearly, 5,6-epoxyretinoic acid is more rapidly metabolized than retinoic acid. For example, in comparison to the values in Table I, 3 h after [^{3}H]retinoic acid (1 μg) was intravenously dosed, unchanged retinoic acid in liver, kidney, and small intestinal mucosa of vitamin A deficient rats represented 16, 6, and 1% of the total dose, respectively. In vitamin A deficient retinoic acid treated rats, the values were 20, 1, and 1% of the total dose in liver, kidney, and mucosa, respectively (Napoli & McCormick, 1981).

Retinoic acid treatment had the effect of increasing the rate of 5,6-epoxyretinoic acid metabolism and promoting the ac-

Table I: Distribution of Radioactivity after an Intrajugular Dose of 5,6-Epoxyretinoic Acid[a]

tissue: group	% dose[b]	% dose/g of tissue	epoxide (fractions 60–64)			metabolite (fractions 47–49)		
			% ^{3}H[c]	pmol/g of tissue[d]	% total dose	% ^{3}H[c]	pmol/g of tissue[d]	% total dose
liver: deficient[e]	22 ± 1.0	2.4	19	9.2	4.0	7	3.5	1.5
treated[f]	13 ± 0.7	0.9	1	0.3	0.1	*h*	0.3	*h*
mucosa:[g] deficient	5.9 ± 0.3	1.3	3	1.0	0.2	36	14.0	2.1
treated	6.0 ± 0.3	1.4	4	1.3	0.2	3	1.3	0.2
kidney: deficient	1.9 ± 0.1	0.8	30	5.7	0.6	*h*	0.3	*h*
treated	1.4 ± 0.1	0.5	22	2.8	0.3	*h*	0.3	*h*

[a] Rats were each dosed with 1 μg of 5,6-epoxy[11-^{3}H]retinoic acid (2.53 × 10^6 dpm/nmol). The tissues from five animals in each group were pooled. [b] Values are mean ± SD of triplicates. [c] Percent ^{3}H in chromatogram of charged metabolites as epoxide or its metabolite. There was a 5–10% error in the radioactivity measurements. [d] Picomoles of ^{3}H-labeled epoxide or metabolite per gram of tissue. [e] Deficient, vitamin A deficient rats. [f] Treated, vitamin A deficient rats dosed orally with retinoic acid. [g] Small intestinal mucosa. [h] Not detected.

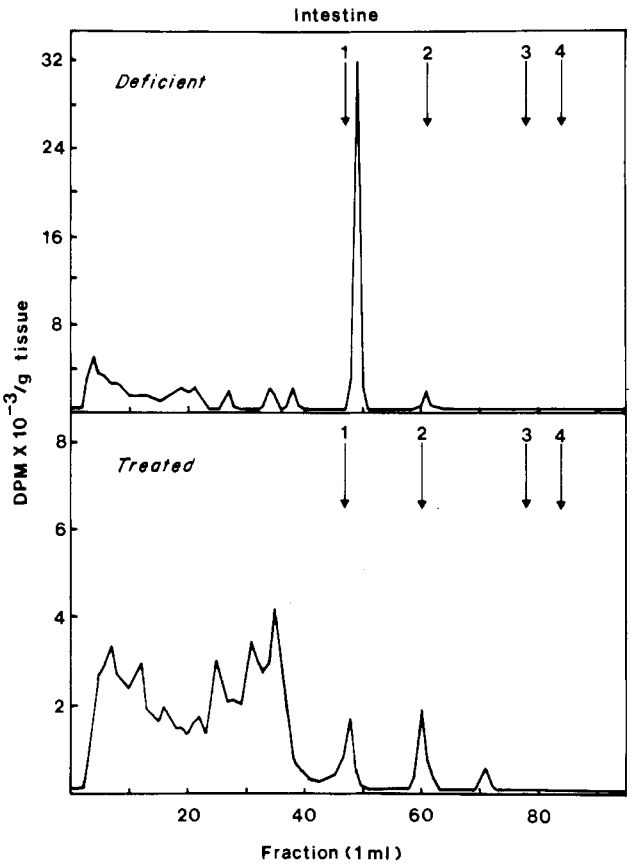

FIGURE 2: HPLC separation of charged tritiated retinoids extracted from the small intestinal mucosa 2 h after a 1-μg dose of 5,6-epoxy[^{3}H]retinoic acid. Both deficient and treated samples were analyzed on the same gradient used for the deficient liver sample.

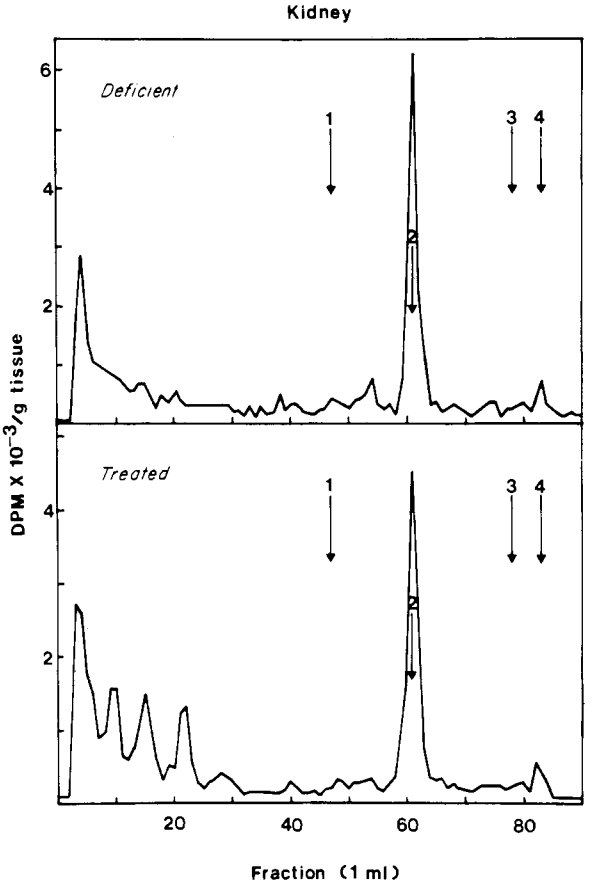

FIGURE 3: Separation of charged tritiated retinoids extracted from kidney 2 h after a 1-μg dose of 5,6-epoxy[^{3}H]retinoic acid. Both samples were analyzed with the same gradient used for the deficient liver sample.

cumulation of much more polar metabolites. Specifically, livers of the deficient group had substantial unchanged 5,6-epoxyretinoic acid (fractions 60–64) and a metabolite in fractions 47–49 that migrated close to the 4-ketoretinoic acid standard that were negligible in the treated animals (Figure 1, Table I). The peaks in fractions 22–24, and fractions 29–32, were present in the livers of both groups, but the large peaks in the livers of treated animals in fractions 17–19, 21–23, and 25–29 were not detected in the livers of the deficient group. These conclusions were confirmed by chromatography of the liver extract from the treated group in the same HPLC system used for the other samples. In the less polar system, most of the radioactivity eluted in the first 20 fractions.

The small intestinal mucosa of the deficient group showed a low amount of 5,6-epoxyretinoic acid regardless of retinoid status (Figure 2). However, the peak in fractions 47–49, like the similar peak in liver, was diminished significantly in the treated group, which had the balance of radioactivity in the very polar region of the chromatogram. It is not clear from these chromatograms, with the exception of the peak in fraction 35, whether these polar 5,6-epoxyretinoic acid metabolites are unique to the intestine of the treated group.

The pattern of kidney epoxide metabolites was different from those of liver and intestine (Figure 3). Kidney did not have a peak in fractions 47–49. Furthermore, the presence of 5,6-epoxyretinoic acid was not affected to the same extent by retinoic acid treatment as it was in liver and intestine. Like liver and intestine, however, kidney did show an accumulation of more polar epoxide metabolites after retinoic acid treatment.

It is noteworthy that no peaks corresponding to either 13-*cis*- or *all-trans*-retinoic acid appeared in liver or intestine after rats were dosed with 5,6-epoxyretinoic acid. Relatively small peaks in the *all-trans*-retinoic acid fractions (82–84) were

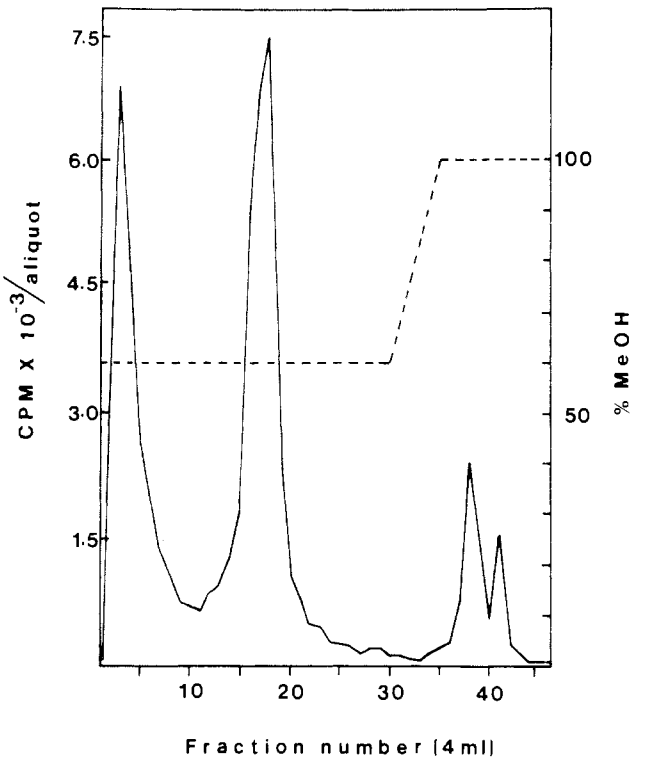

FIGURE 4: Purification of the major 5,6-epoxyretinoic acid metabolite from intestines of vitamin A deficient rats. The semipreparative reverse-phase column was eluted with 10 mM ammonium acetate in water/methanol (40:60) at a flow rate of 4 mL/min for 30 min. Then a linear gradient to 10 mM ammonium acetate in methanol was run over 5 min.

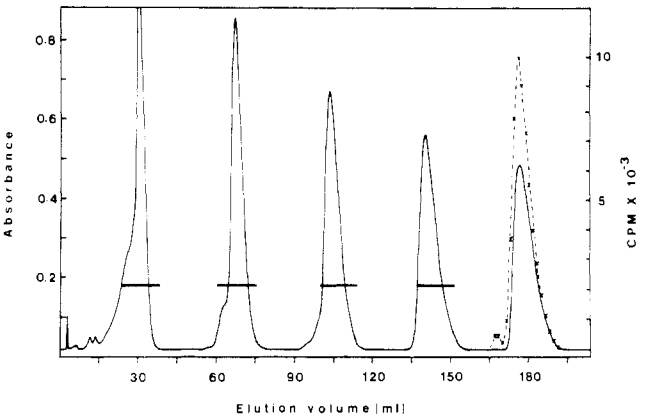

FIGURE 5: Final purification of the major metabolite extracted from the intestinal mucosa of vitamin A deficient rats dosed with 5,6-epoxyretinoic acid. The radially compressed reverse-phase cartridge was eluted with 10 mM ammonium acetate in water/methanol (40:60) at a flow rate of 3 mL/min. The area of the peak represented by the heavy horizontal lines was recycled through the column so that the metabolite passed through a total of 5 times. Fractions (1.5 mL each) were collected beginning at 165-mL elution volume. Aliquots of the fractions (25 μL of each) were measured for radioactivity (×). The materials in fractions 6–13 (172.5–184.5 mL) were combined and used for further studies.

noticed in the kidney samples. Thus, although significant accumulation of retinoic acid in kidney after a dose of 5,6-epoxyretinoic acid was not observed, the possibility that an extremely minor conversion occurs cannot be totally excluded.

The metabolite (fractions 47–49) that appeared predominantly in the intestine and liver of vitamin A deficient rats, which eluted close to 4-ketoretinoic acid, was selected for further study. Each of nine vitamin A deficient rats was dosed with 5,6-epoxy[11-^{3}H]retinoic acid (700 μg, 105 × 10^5 dpm/nmol). The charged metabolites from the small intestinal mucosa and from the intestinal contents were isolated separately by DEAE-Sephadex chromatography, and it was determined that 91% (1.25 × 10^8 dpm) of the total mucosal metabolites (1.38 × 10^8 dpm) and 81% (2 × 10^8 dpm) of the total metabolites in the small intestinal contents (2.5 × 10^8 dpm) were charged. Aliquots of the mucosal material and the material in contents were individually applied to the analytical reverse-phase HPLC system described in Figure 2. Chromatograms similar to those of Figure 2 were observed. Consequently, increasing the dose of 5,6-epoxyretinoic acid apparently did not affect the nature of the major metabolite appearing in the intestine and contents of vitamin A deficient rats.

The metabolites in the mucosa and in the intestinal contents were purified separately, but with identical procedures. The charged materials were applied in four portions to a semipreparative reverse-phase column (Figure 4). The metabolite of interest (fractions 13–21) represented 42.5% (4 × 10^7 dpm) of the total material recovered. The remainder of the radioactivity was in fractions 1–10 (41.4%) and fractions 31–42 (10.7%). Similar results were obtained when the intestinal contents' metabolites were eluted from this column.

Treatment of the mucosal metabolite, recovered from the semipreparative column, with diazomethane in methanol produced methyl 5,6-epoxyretinoate. This was determined, in part, by comigration of the isolated [^{3}H]methyl ester with synthetic unlabeled methyl 5,6-epoxyretinoate in two HPLC systems. First, an analytical reverse-phase column eluted with water/methanol (22:78) was used. This was followed by a normal-phase column eluted with 2-propanol/hexane (0.2:99.8) in the recycle mode for a total of five passes through the column. After being recycled, both synthetic methyl *all-trans*-5,6-epoxyretinoate and the compound generated from the metabolite eluted in 72 mL. In the latter system, base-line resolution between methyl 13-*cis*-5,6-epoxyretinoate (elution volume 12 mL) and its all-trans isomer (elution volume 11 mL) can be achieved in one pass. The isolated methyl 5,6-epoxide displayed ultraviolet and mass spectra identical with those of synthetic methyl 5,6-epoxyretinoate. Both the isolated and synthetic compounds isomerized upon treatment with dilute hydrochloric acid to methyl 5,8-oxyretinoate as confirmed by ultraviolet absorbance and analysis by normal-phase HPLC (Napoli et al., 1978; McCormick et al., 1978).

So that deconjugation could be avoided, underivatized material, obtained from the semipreparative column, was purified on a radially compressed reverse-phase HPLC cartridge eluted with 1% ammonium acetate in water/acetonitrile (7:3) at a flow of 4 mL/min. The metabolite eluted in 76 mL. The recovered material (6.7 × 10^6 dpm) was injected onto a reverse-phase HPLC system eluted with 10 mM ammonium acetate in water/methanol (40:60) (Figure 5). This provided 60 nmol of pure metabolite from intestinal mucosa. The metabolite isolated from the intestinal contents had chromatographic characteristics identical with those of the mucosal metabolite.

The absorbance spectra of the metabolite were close to those of synthetic methyl 5,6-epoxyretinoate and distinct from those of synthetic 5,6-epoxyretinoic acid. The similarity between synthetic carboxyl derivatized epoxide and the metabolite, and the difference between the metabolic and nonderivatized 5,6-epoxyretinoic acid, was further demonstrated by the spectra obtained after allowing each to react briefly with methanolic hydrochloric acid (Figure 6). Moreover, the molar extinction coefficient (ϵ) of the metabolite was calculated to be 54 600, which is more comparable to the value of 50 560 for synthetic

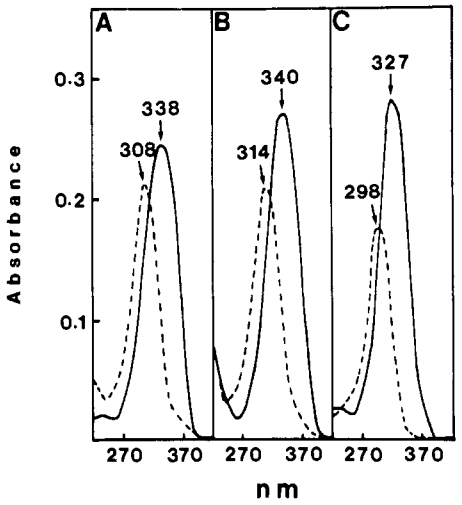

FIGURE 6: Absorbance spectra of 5,6-epoxyretinoids. The solid lines show spectra of native materials. The dashed lines show spectra that resulted after addition of 0.35 mM hydrochloric acid (20 μL) to the cuvettes: (A) synthetic methyl 5,6-epoxyretinoate; (B) epoxide metabolite isolated from intestinal mucosa; (C) synthetic 5,6-epoxyretinoic acid.

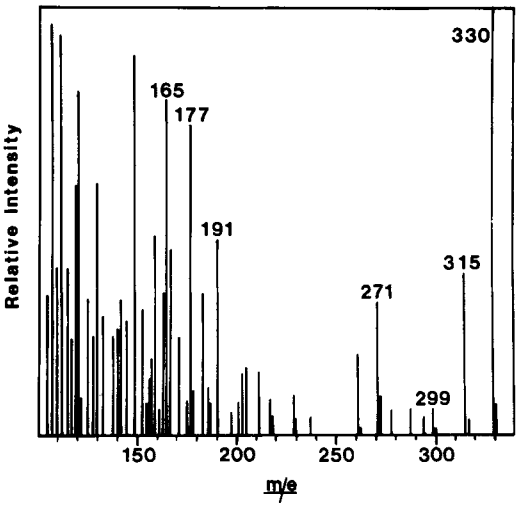

FIGURE 7: Mass spectrum of 5,6-epoxyretinoic acid obtained by treating 5,6-epoxyretinoyl β-glucuronide isolated from rat intestinal mucosa with β-D-glucuronidase and diazomethane, respectively.

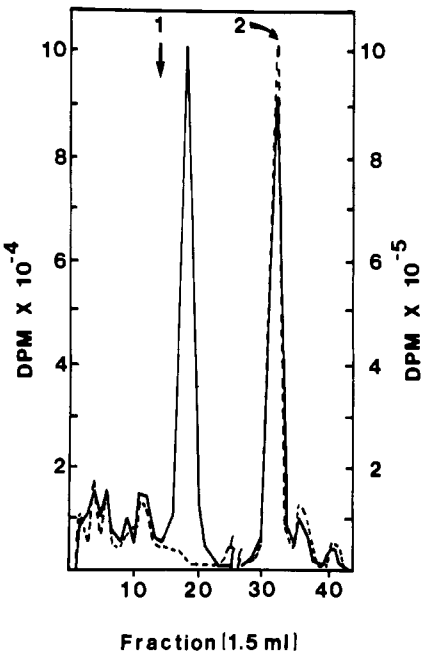

FIGURE 8: HPLC analyses of 5,6-epoxy[11-^{3}H]retinoic acid incubated with microsomes prepared from the liver of a rat on a normal diet. The incubation was done in the presence (solid line) or absence (broken line) of added UDPGA. The radially compressed reverse-phase cartridge was eluted with 10 mM ammonium acetate in water/methanol (38:62). The arrows point to the elution positions of unlabeled 4-ketoretinoic acid (1) and 5,6-epoxyretinoic acid (2) added as carriers and chromatographic markers. The chromatogram represents one of three replicates. Note the change in scale between fractions 25 and 27. The 10^{-4} scale refers to fractions 1–25; the 10^{-5} scale refers to fractions 27–45.

methyl 5,6-epoxyretinoic acid than it is to the value of 45 570 for 5,6-epoxyretinoic acid. Furthermore, the molar extinction coefficient (ε) of the metabolite decreased by 12 500 after hydrochloric acid treatment, as it does for authentic methyl 5,6-epoxyretinoate (John et al., 1967). The material isolated from intestinal contents had similar absorbance characteristics. These data, taken collectively, indicate that the metabolite is a 5,6-epoxyretinoid with a derivatized carboxyl group.

The metabolite was further characterized by a series of enzymic reactions. Incubation of 160 pmol of the metabolite with 280 IU of arylsulfatase resulted in no change in the chromatographic properties of the isolated metabolite. On the other hand, 975 pmol of the metabolite was cleaved by 5 IU of β-glucuronidase to produce a compound which comigrated on HPLC with 5,6-epoxyretinoic acid. This cleavage was 98% inhibited in the presence of saccharo-1,4-lactone. For confirmation of the identity of the compound released by incubation with β-glucuronidase, 2 nmol of the metabolite was incubated with 500 IU of β-glucuronidase for 1 h. The reaction was quenched and extracted in the usual manner (see Materials and Methods), but no retinoid carriers were added. The residue was treated with ethereal diazomethane, and the resulting methyl ester was injected onto a normal-phase HPLC column eluted with tetrahydrofuran/hexane (0.5:99.5). The material eluted in the position of methyl *all-trans*-5,6-epoxyretinoate (34 mL) and was distinct from methyl 13-*cis*-5,6-epoxyretinoate (43 mL). The putative methyl 5,6-epoxyretinoate was analyzed by GC/MS. It eluted from the GC column in the same time as authentic methyl 5,6-epoxyretinoate (2.3 min) and produced a mass spectrum consistent with that of methyl 5,6-epoxyretinoate (Figure 7).

Further evidence for the structure of the metabolite was provided by its in vitro synthesis. The rat liver microsomal synthesis of the metabolite was unmistakably UDPGA dependent (Figure 8). The reaction, done in triplicate, produced 9 ± 1% (±SD) product. The in vitro reactions were repeated with unlabeled 5,6-epoxide to confirm that the in vitro and in vivo metabolites were identical. The unlabeled metabolite produced in vitro was purified with the HPLC system described in Figure 8. The isolated metabolite had an ultraviolet absorbance spectrum identical with that of the in vivo metabolite. The in vitro metabolite (22 nmol) was compared to the ^{3}H-labeled metabolite produced in vivo (124 000 dpm, 1.2 nmol) on the HPLC system described in Figure 5. In this experiment, a mixture of the in vitro and in vivo metabolites was recycled through the column twice for a total of three passes. After the third pass, fractions (1.5 mL) were collected. The 5,6-epoxyretinoyl glucuronide produced in vitro (absorbance) coeluted with the metabolite isolated from rat intestine (radioactivity).

Discussion

A complex spectrum of 5,6-epoxyretinoic acid metabolites was observed in vivo, but no one metabolite predominated, with the exception of 5,6-epoxyretinoyl β-glucuronide in the intestinal mucosa of vitamin A deficient rats. Moreover, al-

though some peaks were observed in all tissues examined, some others were dependent on the tissue source and retinoid status of the animals. Generally, oral pretreatment of vitamin A deficient rats with retinoic acid caused more rapid metabolism of 5,6-epoxyretinoic acid and accumulation of more polar metabolites. These findings are consistent with those of previous experiments which showed that retinoic acid metabolism in vivo is partially tissue specific (Napoli & McCormick, 1981) and is modulated by retinoic acid treatment (Roberts et al., 1979).

Neither 13-*cis*- nor *all-trans*-retinoic acid was observed as significant 5,6-epoxyretinoic acid metabolites in this study. Apparently, in vivo retinoid epoxidation is irreversible. Thus, the biological activity of the epoxide cannot be accounted for by postulating deoxygenation to the parent retinoid. Moreover, under similar experimental circumstances, the metabolism of 5,6-epoxyretinoic acid in vivo is much more rapid than that of its precursor, retinoic acid. Consequently, estimates of 5,6-epoxyretinoic acid biological activity, especially obtained with in vivo assays, may be low (Morgan & Thompson, 1966; Mallia et al., 1970; Strickland, 1978; Adamo et al., 1979; Newton et al., 1980; Strickland & Mahdavi, 1980).

The identity of the major 5,6-epoxyretinoic acid metabolite in the small intestinal mucosa and intestinal contents as 5,6-epoxyretinoyl glucuronide is based on several facts. The metabolite undergoes cleavage in methanol to produce methyl 5,6-epoxyretinoate. This behavior is similar to that of retinoyl β-glucuronide, which also undergoes methanolic alcoholysis (Lippel & Olson, 1968a,b). The metabolite possesses a chromophore like that of methyl 5,6-epoxyretinoate and isomerizes in the presence of acid to yield a chromophore like that of methyl 5,8-oxyretinoate (Morgan & Thompson, 1966; John et al., 1967; Napoli et al., 1978). Low concentrations of β-D-glucuronidase release 5,6-epoxyretinoic acid from the metabolite. This cleavage by β-D-glucuronidase can be prevented by the specific β-D-glucuronidase inhibitor saccharo-1,4-lactone (Levy, 1952) and is not catalyzed by relatively high concentrations of arylsulfatase. Finally, UDPGA-dependent synthesis of the metabolite in vitro provides material that is identical in chromatographic and spectral characteristics with that isolated from intestine. Thus, the evidence for the structural assignment is firm.

The fact that intestine, intestinal contents, and apparently liver contain the metabolite suggests that it undergoes enterohepatic circulation. This suggestion is supported by the finding that liver endoplasmic reticulum is one site of 5,6-epoxyretinoyl β-glucuronide synthesis. This situation is similar to those of retinol (Lippel & Olson, 1968a), retinoic acid (Dunagin et al., 1965, 1966; Zile et al., 1980), and 13-*cis*-retinoic acid (Frolik et al., 1981) which are excreted in bile as their β-glucuronides. Glucuronide formation apparently represents a common pathway of retinoid metabolism.

The significance of higher 5,6-epoxyretinoyl β-glucuronide concentrations in vitamin A deficient rats in contrast to vitamin A deficient retinoic acid treated rats is unclear. This could be the result of a mechanism to conserve necessary retinoic acid like compounds in vitamin A deficiency. Alternatively, vitamin A deficient animals may lack or have low levels of retinoid-metabolizing enzymes. On the other hand, treatment with pharmacological doses of retinoic acid may acclerate turnover of most vitamin A compounds in vivo. Further research is needed to determine which of these possibilites is most plausible.

A radioactive peak that migrates in the position of 5,6-epoxyretinoyl β-glucuronide has been noted previously in the intestinal mucosa of vitamin A deficient rats dosed with [^{3}H]retinoic acid (peak f) but not in the mucosa of vitamin A deficient rats orally pretreated with unlabeled retinoic acid before the intrajugular dose of [^{3}H]retinoic acid was given (Napoli & McCormick, 1981). Preliminary data from this lab also indicate that 5,6-epoxy[^{3}H]retinoyl β-glucuronide can be observed in liver and small intestinal mucosa, but not in kidney, after an oral dose of [^{3}H]retinol to rats on a normal diet (J. L. Napoli, A. M. McCormick, and C. Watson, unpublished results). Confirmation of these data would indicate that the epoxy glucuronide, like retinoic acid and 5,6-epoxyretinoic acid (McCormick & Napoli, 1981), is a physiological intermediate in retinol metabolism.

Whether epoxidation of retinoic acid is an activation mechanism or a tolerated modification remains to be determined. 5,6-Epoxyretinoic acid does possess some of the attributes of an activated metabolite, however. It is an oxidized metabolite that itself is rapidly metabolized. It is present in vitamin A target tissues (McCormick et al., 1980). It has potent biological activity in vitro that may be underestimated, not only because racemic mixtures of synthetic material, rather than distinct enantiomers, have been tested but also because it is more rapidly cleared than retinoic acid. Moreover, it is not an excretion product per se (Rietz et al., 1974; Hanni et al., 1976; Hanni & Bigler, 1977). Although the role of epoxidation in retinoic acid function cannot be established from these data, they do indicate that retinoid epoxidation is an interesting topic for future research. We should be alert to the possibility that epoxidation directs retinoic acid to a specialized function.

Acknowledgments

We are grateful to Bikash Pramanik for his assistance in obtaining the mass spectra.

References

Adamo, S., De Luca, L. M., Akalorsky, I., & Bhat, P. V. (1979) *JNCI, J. Natl. Cancer Inst. 62*, 1473–1477.

Bieri, J. G., Stoewsand, G. S., Briggs, G. M., Phillips, R. W., Woodard, T. C., & Knapka, J. J. (1977) *J. Nutr. 107*, 1340–1348.

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

De Luca, L. M. (1978) in *Handbook of Lipid Research* (De Luca, H. F., Ed.) Vol. 2, pp 1–67, Plenum Press, New York.

Dowling, J. E., & Wald, G. (1960) *Proc. Natl. Acad. Sci. U.S.A. 46*, 587–608.

Dunagin, P. E., Meadows, E. H., & Olson, J. A. (1965) *Science (Washington, D.C.) 148*, 86–87.

Dunagin, P. E., Zachman, R. D., & Olson, J. A. (1966) *Biochim. Biophys. Acta 124*, 71–85.

Fidge, H. H., Shiratori, T., Ganguly, J., & Goodman, D. S. (1968) *J. Lipid Res. 9*, 103–109.

Frolik, C. A., Swanson, B. N., Dart, L. L., & Sporn, M. B. (1981) *Arch. Biochem. Biophys. 208*, 344–352.

Geison, R. L., & Johnson, B. C. (1970) *Lipids 5*, 371–378.

Goodman, D. S. (1980) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 39*, 2716–2722.

Hanni, R., & Bigler, F. (1977) *Helv. Chim. Acta 60*, 882–887.

Hanni, R., Bigler, F., Meister, W., & Englert, G. (1976) *Helv. Chim. Acta 59*, 2221–2227.

John, K. V., Lakshmanan, M. R., & Cama, H. R. (1967) *Biochem. J. 103*, 539–543.

Levy, G. A. (1952) *Biochem. J. 52*, 464–472.

Lippel, K., & Olson, J. A. (1968a) *J. Lipid Res. 9*, 168–175.

Lippel, K., & Olson, J. A. (1968b) *J. Lipid Res. 9*, 580–586.

Mallia, A. K., John, J., Lakshmanan, M. R., Jungalwala, F. B., & Cama, H. R. (1970) *Indian J. Biochem. 7*, 102–103.
McCormick, A. M., & Napoli, J. L. (1982) *J. Biol. Chem. 257*, 1730–1735.
McCormick, A. M., Napoli, J. L., Schnoes, H. K., & De Luca, H. F. (1978) *Biochemistry 17*, 4805–4090.
McCormick, A. M., Napoli, J. L., Yoshizawa, S., & De Luca, H. F. (1980) *Biochem. J. 186*, 475–481.
Morgan, B., & Thompson, J. N. (1966) *Biochem. J. 101*, 835–842.
Napoli, J. L., & McCormick, A. M. (1981) *Biochim. Biophys. Acta 666*, 165–175.
Napoli, J. L., McCormick, A. M., Schnoes, H. K., & De Luca, H. F. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2603–2605.
Newton, D. L., Henderson, W. R., & Sporn, M. B. (1980) *Cancer Res. 40*, 3413–3425.
Rietz, P., Wiss, O., & Weber, F. (1974) *Vitam. Horm. (N.Y.) 32*, 237–249.
Roberts, A. B., Nichols, M. D., Frolik, C. A., Newton, D. L., & Sporn, M. B. (1978) *Cancer Res. 38*, 3327–3332.
Roberts, A. B., Frolik, C. A., Nichols, M. D., & Sporn, M. B. (1979) *J. Biol. Chem. 254*, 6303–6309.
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. (1980) *Cell (Cambridge, Mass.) 15*, 393–403.
Verma, A. K., Slaga, T. J., Wertz, P. W., Mueller, G. C., & Boutwell, R. K. (1980) *Cancer Res. 40*, 2367–2371.
Zile, M., Schnoes, H. K., & De Luca, H. F. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3230–3233.

Kinetic and Mechanistic Analysis of Prothrombin–Membrane Binding by Stopped-Flow Light Scattering†

G. Jason Wei, Victor A. Bloomfield, Robert M. Resnick, and Gary L. Nelsestuen*

ABSTRACT: We have investigated the kinetics and mechanism of prothrombin–membrane vesicle interaction by using stopped-flow light scattering. Under conditions of approximately physiological protein concentration (≤3 μM prothrombin), prothrombin interaction with the vesicles was modeled according to a simple bimolecular process with noninteracting prothrombin binding sites on the vesicle. The association rate constant (per protein binding site) for interaction of prothrombin with vesicles containing 20% phosphatidylserine–80% phosphatidylcholine at 10 °C, in buffer containing 3 mM calcium, is $(1 \pm 0.1) \times 10^7$ $M^{-1} \cdot s^{-1}$. This corresponds to a 10% collision efficiency. The reverse process is a first-order dissociation with a rate constant of 3 ± 1 s^{-1}. Off-rate experiments conducted by sample dilution were consistent with these values. With varying membrane compositions the association process was found to be somewhat cooperative with respect to phosphatidylserine, but dissociation was unaffected by phosphatidylserine density. The activation energy for prothrombin–membrane association varied with the amount of acidic phospholipid in the membrane. Membranes of 10% phosphatidylserine gave an activation energy of about 9 kcal/mol while those of 40% phosphatidylserine gave a value of 4 kcal/mol. For these same membranes the collision efficiency was estimated to be 3 and 20%, respectively. This trend in the activation energy suggests that as the acidic phospholipid content increases, the association energy of activation becomes characteristic of a diffusion-controlled reaction. Dissociation rate constants were obtained by mixing prothrombin–membrane complexes with ethylene glycol bis-(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA). It was found that a necessary population of calcium ions exchanged rapidly from the prothrombin–membrane complex but that this population could be replaced by magnesium or manganese. A second population of essential ions, specific for calcium, exchanged slowly and, under certain conditions, appeared to be released at the rate of prothrombin–membrane dissociation. Under conditions of high free-protein concentrations the association process became complex and had lower rate constants. The anomalous binding characteristics were observed under conditions that are not likely to be physiologically important.

The blood coagulation cascade contains several enzymatic steps that are dependent on the presence of a phospholipid membrane component [see Jackson & Nemerson (1980) for a review]. Both the substrates and enzymes in these reactions are vitamin K dependent proteins that show calcium-dependent binding to the phospholipid membrane in a process that depends on γ-carboxyglutamic acid residues. Understanding of the kinetic processes involved in blood coagulation therefore requires knowledge of these protein–membrane interactions.

Previous studies have revealed much about the interaction of vitamin K dependent proteins with membranes at equilibrium. For the protein prothrombin, binding constants under a variety of conditions have been reported (Nelsestuen & Lim, 1977; Nelsestuen & Broderius, 1977; Nelsestuen et al., 1978; Resnick & Nelsestuen, 1980). Independent studies have dealt with the binding of prothrombin fragment 1 (residues 1–156 of prothrombin) to phospholipid vesicles (Dombrose et al., 1979). These proteins appear to bind to the surface of the phospholipid without penetration into the hydrophobic region of the membrane (Lim et al., 1977; Hanahan et al., 1969). Dissociation constants for binding to membranes containing approximately physiological amounts of acidic phospholipid, which are similar to cytosolic membranes, at physiological calcium concentration, are very near to the plasma concen-

† From the Department of Biochemistry, College of Biological Sciences, University of Minnesota, St. Paul, Minnesota 55108. *Received June 11, 1981.* This work was supported in part by Grant HL 15728 from the National Institutes of Health (to G.L.N.) and Grant PCM 78-06777 from the National Science Foundation (to V.A.B.).

0006-2960/82/0421-1949$01.25/0