811-825.
Molineux, I. J., Pauli, A., and Gefter, M. L. (1975), *Nucleic Acids Res. 2,* 1821-1837.
Newman, J., Swinney, H. L., Berkowitz, S. A., and Day, L. A. (1974), *Biochemistry 13,* 4832-4838.
Ruyechan, W. T., and Wetmur, J. G. (1975), *Biochemistry 14,* 5529-5534.
Ruyechan, W. T., and Wetmur, J. G. (1976), *Biochemistry 15,* 5057-5064.
Seeman, N. C., Rosenberg, J. M., and Rich, A. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 804-808.
Sigal, N., Delius, H., Kornberg, T., Gefter, M. L., and Alberts, B. M. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 3537-3541.
Stern, O., and Volmer, M. (1919), *Z. Phys. 20,* 183-188.
Takahashi, K. (1968), *J. Biol. Chem. 243,* 6171-6179.
Tao, T. (1969), *Biopolymers 8,* 609-632.
Teale, F. W. J. (1960), *Biochem. J. 76,* 381-388.
Wahl, Ph. (1966), *C. R. Hebd. Seances Acad. Sci. 263,* 1525-1528.
Weiner, J. H., Bertsch, L. L., and Kornberg, A. (1975), *J. Biol. Chem. 250,* 1972-1980.
Witkop, B. (1961), *Adv. Protein Chem. 16,* 221-321.
Wu, F. Y.-H., Nath, K., and Wu, C.-W. (1974), *Biochemistry 13,* 2567-2572.
Yguerabide, J. (1972), *Methods Enzymol. 26,* 498-578.

Isolation and Identification of 5,6-Epoxyretinoic Acid: A Biologically Active Metabolite of Retinoic Acid†

Anne M. McCormick, Joseph L. Napoli, Heinrich K. Schnoes, and Hector F. DeLuca*

ABSTRACT: A highly biologically active metabolite of retinoic acid (8_{III}) has been isolated in pure form from intestinal mucosa of vitamin A deficient rats given [^{3}H]retinoic acid. This metabolite has been positively identified as 5,6-epoxyretinoic acid based on the ultraviolet absorption spectrum and mass spectrum of its methylated derivative. This identification was confirmed by cochromatography of the methylated metabolite and synthetic methyl 5,6-epoxyretinoate on reverse-phase and straight-phase high-pressure liquid chromatography. The 5,6-epoxyretinoic acid is a true in vivo generated metabolite of retinoic acid and not an artifact of the isolation procedure. In addition, 5,8-oxyretinoic acid previously isolated in this laboratory from intestinal mucosa was probably generated from 5,6-epoxyretinoic acid by the acidic conditions used in the extraction and isolation of the 5,8-oxyretinoic acid.

Retinoic acid supports growth (Malathi et al., 1963; Krishnamurthy et al., 1963; Zile & DeLuca, 1968) and maintains epithelial differentiation (DeLuca et al., 1972), but it cannot support the visual (Dowling & Wald, 1960) or reproductive (Thompson et al., 1964) functions of vitamin A. Retinoic acid has been demonstrated to be a major metabolite in several tissues of rats given physiological amounts of retinol or retinyl esters (Kleiner-Bossaller & DeLuca, 1971; Dunagin et al., 1964; Deshmukh et al., 1965; Emerick et al., 1967; Crain et al., 1967). The sparing action of retinoic acid on depletion of liver retinol stores (Krishnamurthy et al., 1963; Krause et al., 1975; Nelson et al., 1972) and the similar metabolite profiles observed after administration of labeled retinol and retinoic acid (Roberts & DeLuca, 1967; Kleiner-Bossaller & DeLuca, 1971; Emerick et al., 1967; Ito et al., 1974) indicate that retinoic acid is a normal intermediate in vitamin A metabolism.

Retinoic acid is rapidly metabolized (Roberts & DeLuca, 1967; Fidge et al., 1968; Geison & Johnson, 1970; Kleiner-Bossaller & DeLuca, 1971) but to data no biologically active metabolites have been isolated from target tissues and positively identified. Retinoic acid is converted in vivo to polar metabolites designated as peaks 8, 9, and 10 which can be separated from retinoic acid by Sephadex LH-20 chromatography (Ito et al., 1974). With the development of high-pressure liquid chromatography (HPLC)[1] systems (McCormick et al., 1978a,b) for these metabolites, their isolation and identification became feasible. Thus a component of peak 8 from intestine of vitamin A deficient rats given retinoic acid was positively identified as 5,8-oxyretinoic acid (Napoli et al., 1978; A. McCormick, J. Napoli, H. Schnoes, & H. DeLuca, unpublished experiments). However, the use of an acidic extraction step in the isolation procedure introduced the possibility that the 5,8-oxyretinoic acid could have originated from 5,6-epoxyretinoic acid by acid catalysis (Morgan & Thompson, 1966; John et al., 1967).

This paper describes the isolation and chemical identification of 5,6-epoxyretinoic acid from intestines of vitamin A deficient rats given retinoic acid. Additionally, it will demonstrate that 5,8-oxyretinoic acid previously isolated from rat intestine is derived from 5,6-epoxyretinoic acid and that 5,6-epoxyretinoic acid is the major peak 8 metabolite of retinoic acid in rat intestine.

Experimental Procedure

Animals. Weanling male rats obtained from Holtzman Co. (Madison, Wis.) were maintained on a vitamin A deficient diet

† From the Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin-Madison, Madison, Wisconsin 53706. *Received April 18, 1978.* Supported by Program-Project Grant AM-14881 and Postdoctoral Training Grant DE-07031 from the National Institutes of Health and by the Harry Steenbock Research Fund.

[1] Abbreviations used: HPLC, high pressure liquid chromatography; EDTA, ethylenediaminetetraacetic acid; BHT, butylated hydroxytoluene; ODS, octadecylsilane; THF, tetrahydrofuran; NH_4OAc, ammonium acetate.

0006-2960/78/0417-4085$01.00/0

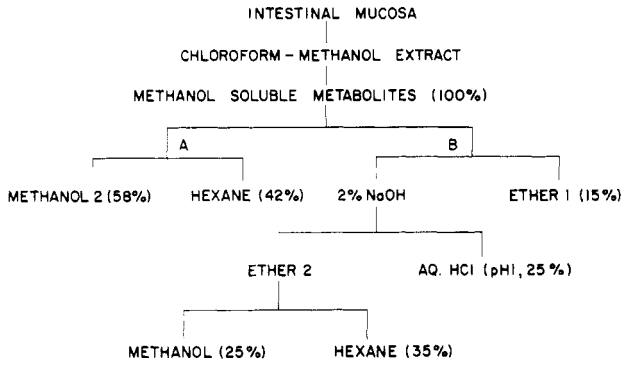

FIGURE 1: Extraction of retinoic acid metabolites from intestinal mucosa.

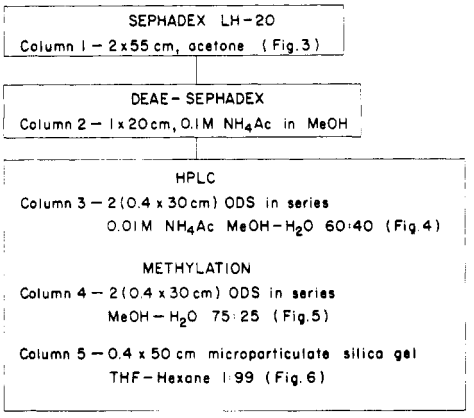

FIGURE 2: Purification scheme for metabolite 8_{III}.

for 5–6 weeks (Zile & DeLuca, 1968). Rats were used for metabolism experiments after reaching a weight plateau.

Substrates. all-trans-Retinoic acid was purchased from Eastman Kodak Co. (Rochester, N.Y.). Its purity was determined by HPLC on a reverse-phase octadecylsilane (ODS) column (McCormick et al., 1978a,b). [11,12-^{3}H]Retinoic acid was generously provided by the Hoffmann-La Roche Co. (Nutley, N.J.). Tritiated retinoic acid was purified by Sephadex LH-20 chromatography and its purity assayed by reverse-phase HPLC (McCormick et al., 1978a,b) immediately before use.

Solvents. All solvents used for extraction and Sephadex chromatography were AR grade and glass distilled before use. In addition, these solvents contained butylated hydroxytoluene (BHT) (50 μg/mL). Glass distilled solvents from Burdick and Jackson Laboratories (Muskegon, Mich.) were used for LC.

Radioactivity Determinations. Radioactive determinations were carried out with a Packard Model 3255 liquid scintillation counter equipped with an automatic external standardization system. Samples were counted in 10 mL of Aquasol (New England Nuclear, Boston, Mass.) with an efficiency of 30–35% for tritium.

Spectroscopy. Ultraviolet absorption spectra were recorded with a Beckman Model 24 recording spectrophotometer. Mass spectra (70 eV) were determined with an AEI MS-9 mass spectrometer using direct probe inlet at 85–95 °C above ambient.

High-Pressure Liquid Chromatography. HPLC was performed on a Waters Associates Model ALC/GPC-204 liquid chromatograph. Detection was accomplished with a fixed ultraviolet monitor at 340 nm. Columns used in this study were two Bondapak C_{18} columns (0.4 × 30 cm) obtained from

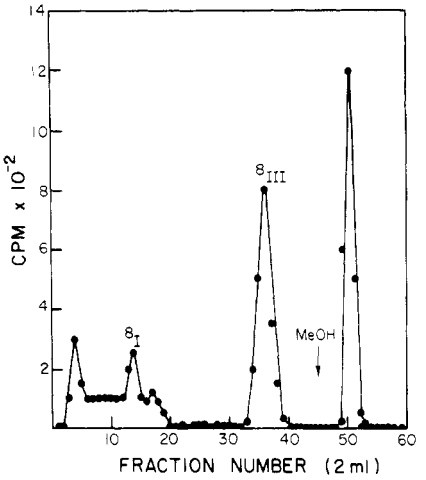

FIGURE 3: Separation of peak 8 components by reverse-phase HPLC (column 3, two μC_{18} columns connected in series). The peak 8 components were injected in 50 μL of MeOH and eluted with 0.01 M ammonium acetate in MeOH:H_2O (60:40). Retinoic acid was eluted after changing column solvent to MeOH. Radioactivity was determined on 5-μL aliquots. Fractions 35–40 were pooled for further purification of 8_{III}.

Waters Associates for reverse-phase chromatography and a Whatman Partisil 10/50 (0.4 × 50 cm) for straight-phase chromatography.

Preparation of Retinoic Acid Metabolites. The extraction and chromatography of the metabolites were carried out under red light. During the purification procedure, all metabolites were stored under N_2 in degassed solvents at −70 °C. Vitamin A deficient rats were administered 450 μg each of [11,12-^{3}H]retinoic acid (sp act. 8.1 × 10^5 dpm/μg) 3.5 h prior to sacrifice. The preparation of intestinal mucosa and the initial extraction of retinoic acid metabolites were as described for the isolation of 5,8-oxyretinoic acid (Napoli et al., 1978; McCormick et al., 1978b).

Synthesis of Methyl 5,6-Epoxyretinoate. Methyl 5,6-epoxyretinoate and methyl 5,8-oxyretinoate were prepared as previously reported (Morgan & Thompson, 1966; John et al., 1967) and gave spectra (mass spectra, 270-MHz NMR and UV) consistent with their assigned structures.

Results

Comparison of Peak 8 Profiles in Untreated and Acid-Treated Intestinal Extracts. The acid lability of 5,6-epoxyretinoic acid necessitated modification of the partitioning (Figure 1) described for the isolation of 5,8-oxyretinoic acid (Napoli et al., 1978; McCormick et al., 1978b). The methanol soluble lipid extract which contains retinoic acid and metabolites was divided into equal portions; one portion was partitioned by method A and the remaining one by method B. On reverse phase HPLC, peak 8 prepared from acid-treated intestinal extracts (method B) is composed of three discrete components, 8_I (17%), 8_{II} (60%), and 8_{III} (9%). Component 8_{II} has been identified as 5,8-oxyretinoic acid (Napoli et al., 1978; McCormick et al., 1978b). In the intestinal extract not subject to acid treatment (method A) only two peak 8 components were detected after reverse-phase HPLC: 8_I (17%) and 8_{III} (7). Therefore, it appears that 5,8-oxyretinoic acid (8_{II}) is generated from 8_{III} during acid extraction of the metabolites.

Purification of Metabolite 8_{III}. The methanol soluble lipid fraction containing the retinoic acid metabolites was partitioned between methanol:hexane (0.25 L:0.25 L). Fifty-eight percent of the intestinal radioactivity was recovered in the methanol phase with 42% remaining in the hexane phase

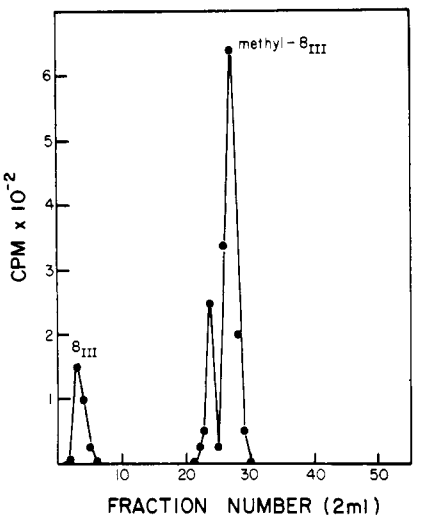

FIGURE 4: Reverse-phase HPLC of methyl-8_{III}. Metabolite 8_{III} was separated from its methylated derivative on 2 (0.4 × 30 cm) μC_{18} columns connected in series using MeOH:H_2O (75:25) as the eluting solvent (column 4). Radioactivity was determined on 5-μL aliquots. Fractions 26–29 were pooled for further purification of methyl-8_{III}.

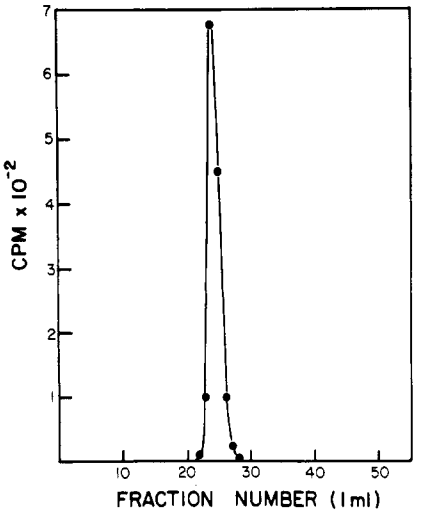

FIGURE 5: Straight-phase HPLC of methyl-8_{III}. The total sample of methyl-8_{III} recovered from column 4 was applied to a microparticulate silica gel column (column 5, 0.4 × 50 cm Partisil) and eluted with THF-hexane (1:99). Radioactivity was determined on 5-μL aliquots of each fraction. Fractions 22–28 were pooled. The recovered methyl-8_{III} was used for determination of the ultraviolet spectrum and mass spectrum of the compound and for comigration experiments.

(Figure 1, method A). The methanol phase contains the three metabolite fractions reported by Ito et al. as peaks 8, 9, and 10 in addition to retinoic acid. The purification of metabolite 8_{III} is outlined in Figure 2. The peak 8 metabolites were separated from peaks 9 and 10 on a 2 × 50 cm column of Sephadex LH-20 eluted with acetone (not shown). Peaks 9 and 10 remained on the column and could be eluted with a solvent mixture of acetone:methanol, 1:1. The peak 8 fractions were pooled and applied to a 1 × 20 cm DEAE-Sephadex (hydroxide form) column (column 2). Uncharged lipids were eluted with 100 mL of methanol. The charged peak 8 lipids were eluted with 100 mL of methanol containing 0.1 M ammonium acetate (NH_4OAc). Note that acid elution was avoided in this procedure.

The charged peak 8 metabolites were separated into two components 8_I (17%) and 8_{III} (70%) by reverse-phase HPLC on two (0.4 × 30 cm) ODS columns connected in series and eluted with 0.01 M NH_4OAc in MeOH:H_2O (60:40) (column 3, Figure 3). The radioactive peak obtained at the solvent front probably represents polar breakdown products. Rechromatography of this peak (fractions 3–6) with a solvent of higher polarity (MeOH:H_2O, 50:50) revealed no distinct peaks of radioactivity.

Metabolite 8_{III} (30 μg) recovered from column 3 was methylated with diazomethanane. Methyl-8_{III} was purified by reverse-phase HPLC with a solvent system of MeOH–H_2O (75:25) (column 4, Figure 4). This column separates unreacted 8_{III} (6 mL) from methyl-8_{III} (54 mL). The smaller peak which elutes before methyl-8_{III} was not further characterized. However, we noted that this peak elutes in the region of methyl 5,8-oxyretinoate. Methyl-8_{III} was further purified by straight-phase HPLC on a microparticulate silica gel column (0.4 × 50 cm) with a solvent system of tetrahydrofuran (THF)–hexane (1:99) (column 5, Figure 5). The column profile shows a single peak of radioactivity and ultraviolet absorbance at 340 nm. On this system methyl 5,8-oxyretinoate would elute at fractions 30–40 while methyl 8_{III} elutes between fractions 20–30. Methyl-8_{III} recovered from column 5 was used to determine the ultraviolet and mass spectrum of the compound and in comigration experiments. Twenty micrograms of methyl-8_{III} was obtained in this purification procedure.

Identification of Metabolite 8_{III}. Metabolite 8_{III} was retained by DEAE-Sephadex (hydroxide form) and rapidly esterified with diazomethane. Therefore, 8_{III} is certainly an acid.

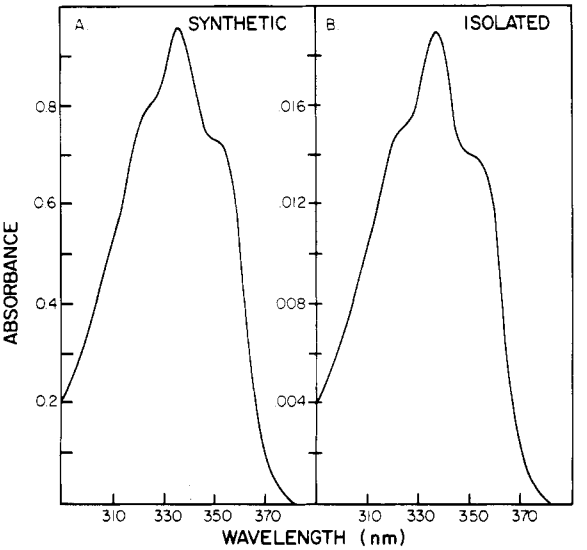

FIGURE 6: Ultraviolet absorption spectra of methyl-8_{III} and methyl 5,6-epoxyretinoate in hexane. (A) Ultraviolet spectrum of synthetic methyl 5,6-epoxyretinoate; (B) ultraviolet spectrum of methyl-8_{III}.

The ultraviolet spectrum of methyl-8_{III} in hexane exhibits λ_{max} 339 nm with shoulders at 352 and 320 nm (Figure 6B). The decrease in λ_{max} from 355 nm for methyl retinoate to 339 for methyl-8_{III} is indicative of the loss of the 5–6 double bond in the β-ionone ring of retinoic acid. Authentic methyl 5,6-epoxyretinoate had an ultraviolet spectrum in hexane (Figure 6A) identical with that of methyl-8_{III}. Both methyl-8_{III} and methyl 5,6-epoxyretinoate have a λ_{max} of 340 nm in methanol.

The mass spectrum of methyl-8_{III} (Figure 7, top panel) exhibited a molecular ion at *m/e* 330, indicative of the addition of one oxygen atom to methyl retinoate. Peaks at *m/e* 315 ($M^+ - CH_3$), 299 ($M^+ - OCH_3$) and 271 ($M^+ - COOCH_3$) are indicative of a methylated retinoic acid derivative undergoing loss of C-15 terminal carboxymethyl fragments. Absence of

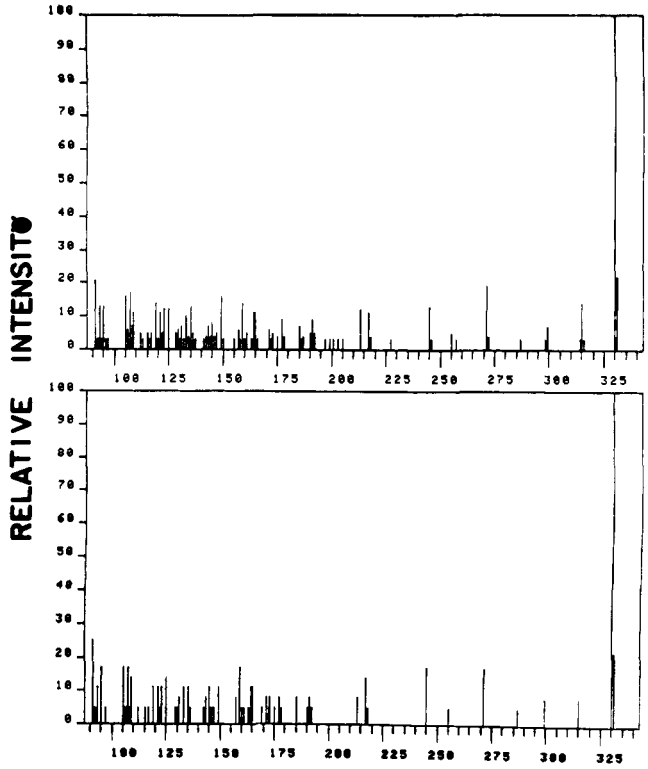

FIGURE 7: (Top) Mass spectrum of isolated methyl-8_{III}; (bottom) mass spectrum of methyl 5,6-epoxyretinoate.

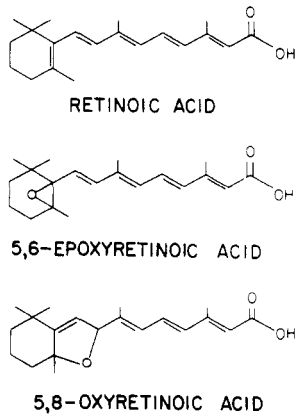

FIGURE 8: Structures of retinoic acid, 5,6-epoxyretinoic acid, and 5,8-oxyretinoic acid.

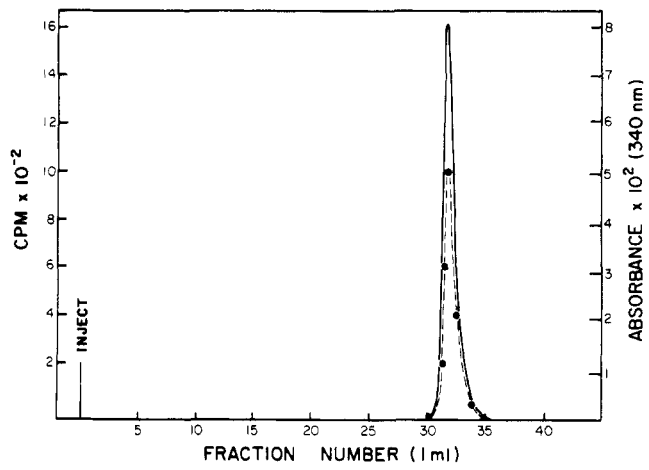

FIGURE 9: Comigration of methyl-8_{III} and methyl 5,6-epoxyretinoate on straight-phase HPLC. Methyl-8_{III} (6000 dpm) and methyl 5,6-epoxyretinoate (800 ng) were coinjected onto a microparticulate silica gel column (column 6, 0.4 × 50 cm Partisil) eluted with THF:hexane (1:99). (—) Absorbance at 340 nm of synthetic methyl 5,6-epoxyretinoate; (- - -) radioactive profile of isolated methyl-8_{III}.

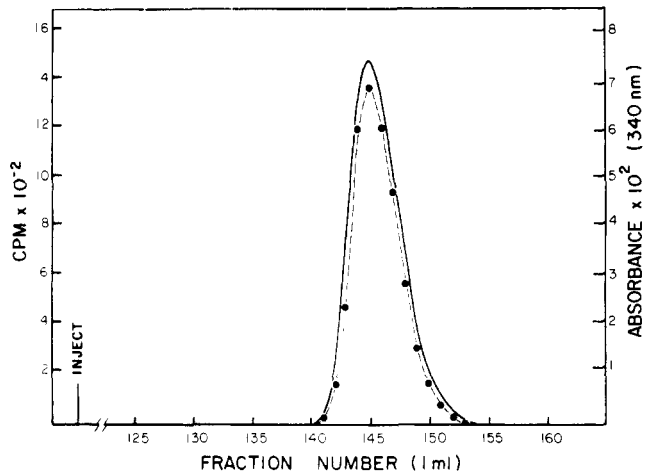

FIGURE 10: Comigration of methyl-8_{III} and methyl 5,6-epoxyretinoate on reverse-phase HPLC. A sample identical with that described in Figure 9 was injected onto two μC_{18} columns (0.4 × 10 cm) connected in series (column 7). The sample was eluted with MeOH:H_2O and recycled one time (effective column length 120 cm). (—) Absorbance at 340 nm of synthetic methyl 5,6-epoxyretinoate; (- - -) radioactive profile of isolated methyl-8_{III}.

m/e 312 ($M^+ - H_2O$) suggests that the additional oxygen atom is probably not present as a hydroxyl group. In addition, the series of ions at *m/e* 271, 217, 177, 165, 164, 149, and 135 represents a pattern observed for 5,6 or 5,8 oxygenated retinoic acid derivatives (Reid et al., 1973). The mass spectrum of methyl 5,6-epoxyretinoate (Figure 7, bottom panel) is indistinguishable from that of methyl-8_{III}. Thus, spectral evidence indicates that metabolite 8_{III} is 5,6-epoxyretinoic acid. The structures of retinoic acid, 5,6-epoxyretinoic acid, and 5,8-oxyretinoic acid are shown in Figure 8.

Comigration Experiments. A mixture of methyl-8_{III} (6000 dpm) and methyl 5,6-epoxyretinoate (800 ng) was subjected to straight-phase HPLC (0.4 × 50 cm microparticulate, silica gel) with a THF:hexane (1:99) solvent system (Figure 9). The two compounds comigrate in this solvent system. Methyl 5,8-oxyretinoate elutes at 65 mL in this HPLC system (McCormick et al., 1978b, Napoli et al., 1978). In addition, methyl-8_{III} and methyl 5,6-epoxyretinoate comigrate on reverse-phase HPLC. If the compounds are recycled once through two ODS columns in series eluted with MeOH:H_2O (60:40) (effective column length = 120 cm), they both elute at 145 mL (Figure 10). In an identical system, methyl 5,8-oxyretinoate elutes at 120 mL (Napoli et al., 1978; McCormick et al., 1978b).

Control Experiments. [11,12-^{3}H]Retinoic acid (10^6 dpm) was added to an intestinal homogenate carried through the described extraction procedure. The radioactivity recovered in the methanol phase was subjected to reverse-phase HPLC (2 (0.4 × 30 cm) ODS columns in series) with 0.01 M NH_4OAc in MeOH:H_2O (60:40) as the solvent system. No peak 8 metabolites were detected by HPLC. Ninety-eight percent of the added radioactivity was recovered by changing the eluting solvent to methanol whereby [^{3}H]-retinoic acid coeluted with unlabeled *all-trans*-retinoic acid.

Discussion

Polar metabolites of retinoic acid in intestine are partially resolved by Sephadex LH-20 chromatography (Ito et al.,

1974). Peaks 8-10 represent metabolites more polar than the parent retinoic acid. In the present experiments, unmetabolized retinoic acid represents the largest fraction of intestinal radioactivity (42%) 3.5 h after administration of 450 μg of [^{3}H]retinoic acid. The polar metabolites account for an additional 50% of intestinal radioactivity: peak 8 (25%), peak 9 (20%), and peak 10 (5%).

In previous papers (Napoli et al., 1978; McCormick et al., 1978b), we reported the isolation and identification of 5,8-oxyretinoic acid from rat intestinal mucosa. The well-known conversion of 5,6-epoxyretinoic acid to 5,8-oxyretinoic acid under acidic conditions (Morgan & Thompson, 1966; John et al., 1967) and the use of acid in the isolation of 5,8-oxyretinoic acid suggested that 5,6-epoxyretinoic acid might be the true tissue generated metabolite of retinoic acid. To attempt the isolation of the acid-labile 5,6-epoxide, several modifications in the procedure employed in the isolation of 5,8-oxyretinoic acid were necessary. First, the acid–base partition technique was omitted and replaced by a methanol:hexane partition procedure (Figure 1). In addition, the DEAE-Sephadex chromatography and straight-phase HPLC were modified so that acidic solvent was no longer necessary for chromatography of the metabolite.

The non-acid-treated peak 8 fraction was resolved into two components, 8_I (17%) and 8_{III} (70%), by reverse-phase HPLC. Following the purification procedure outlined in Figure 2, 20 μg of methyl-8_{III} was isolated in pure form from 95 g of intestinal mucosa. The ultraviolet and mass spectra of methyl-8_{III} are identical with those of authentic methyl 5,6-epoxyretinoate. Moreover, methyl-8_{III} comigrates with methyl 5,6-epoxyretinoate on reverse-phase and straight-phase HPLC. On this basis, metabolite 8_{III} has been unequivocally identified as 5,6-epoxyretinoic acid. Thus, in the present report we have demonstrated that the major in vivo generated intestinal peak 8 metabolite of retinoic acid is 5,6-epoxyretinoic acid; and that the isomerization of the native metabolite to 5,8-oxyretinoic acid occurred during the acidic extraction procedure used in the isolation of the 5,8-oxyretinoic acid.

Several observations establish that 5,6-epoxyretinoic acid is a true tissue generated metabolite of retinoic acid. If [^{3}H]-retinoic acid is added to intestinal homogenates and carried through the extraction procedure, only retinoic acid is detected in the extracts by reverse-phase HPLC. Moreover, no polar compounds are generated during Sephadex LH-20 or DEAE-Sephadex chromatography or by HPLC. Therefore, the polar metabolites of retinoic acid are not artifacts of the isolation procedure. The peak 8 metabolites appear in the intestine of bile duct ligated and bile duct cannulated rats (unpublished observations). Thus, these metabolites are not generated in the enterohepatic circulation of the rat.

5,6-Epoxyretinoic acid is a highly biologically active metabolite. If given intraperitoneally, synthetic 5,6-epoxide has 100-157% of the activity of retinyl acetate in supporting growth of vitamin A-deficient rats (John et al., 1967). However, upon oral administration, the 5,6-epoxide has only one-third the activity of retinyl acetate, probably due to isomerization of the compound to 5,8-oxyretinoic acid by gastric secretions. This is supported by the fact that 5,8-oxyretinoic acid is three times less active than retinyl acetate when dosed intraperitoneally. Thus, 5,6-epoxyretinoic acid is the first metabolite of retinoic acid for which biological activity greater than the parent compound has been demonstrated.

It is particularly significant that this metabolite was isolated from intestine where vitamin A has been reported to affect DNA synthesis (Krause et al., 1975), RNA synthesis (Johnson et al., 1969; Zachman, 1967; Zile & DeLuca, 1970), protein synthesis (DeLuca & Wolf, 1969; DeLuca et al., 1969), the cell cycle and cell migration (Zile et al., 1977), and goblet cell production (Fidge et al., 1968). In the present experiments, 5,6-epoxyretinoic acid accounted for 13% of the radioactivity (400 ng/g of mucosa) in intestine after [^{3}H]retinoic acid administration. The discovery of significant amounts of this highly active metabolite in a target organ suggests that 5,6-epoxyretinoic acid may be a functional form of vitamin A in the intestine. In fact, the 5,6-epoxide could be the "active form" of vitamin A in the intestine or at least closer to the active form than retinoic acid.

Crude peak 8 (mixture of 8_I (15%), 8_{III} (35%), and polar breakdown products (50% by HPLC)) is as active as *all-trans*-retinoic acid in the reversal of keratinization in tracheal organ cultures.[2] If the fact that only 50% of the tritiated compounds assayed represent true metabolites is taken into account, the estimate of peak 8 activity would be low by 50%. Since the peak 8 sample tested was a mixture of 8_I and 5,6-epoxide, an accurate assessment of the biological activity of 5,6-epoxyretinoic acid in reversal of keratinization cannot be made at this time. However, the enhanced activity of peak 8 in this bioassay suggests that 5,6-epoxyretinoic acid may be active in epithelial differentiation. The activity of the purified metabolite in tracheal organ culture is currently being investigated.

Acknowledgment

We are grateful for the expert technical assistance of Mary Fivizzani, Mel Micke, Doreen Moriarity, and Connie Smith.

References

Crain, F. D., Lotspeich, F. J., & Krause, R. F. (1967) *J. Lipid Res. 8,* 249-254.

DeLuca, L., & Wolf, G. (1969) *Am. J. Clin. Nutr. 22,* 1059-1065.

DeLuca, L., Little, E. P., & Wolf, G. (1969) *J. Biol. Chem. 244,* 701-711.

DeLuca, L., Maestri, N., Nonanni, F., & Nelson, D. (1972) *Cancer 30,* 1326-1331.

Deshmukh, D. S., Malathi, P., & Ganguly, J. (1965) *Biochim. Biophys. Acta 107,* 120-122.

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

Dunagin, P. E., Jr., Zachman, R. D., & Olson, J. A. (1964) *Biochim. Biophys. Acta 107,* 120-122.

Emerick, R. J., Zile, M., & DeLuca, H. F. (1967) *Biochem. J. 102,* 606-611.

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

Geison, R. L., & Johnson, B. C. (1970) *Lipids 5,* 377-378.

Ito, Y., Zile, M., DeLuca, H. F., & Ahrens, H. M. (1974) *Biochim. Biophys. Acta 369,* 338-350.

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

Johnson, B. C., Kennedy, M., & Chiba, N. (1969) *Am. J. Clin. Nutr. 22,* 1048-1052.

Kleiner-Bossaller, A., & DeLuca, H. F. (1971) *Arch. Biochem. Biophys. 142,* 371-377.

Krause, R. F., Beamer, K. C., McCormick, A. M., Canterbury, R. J., & Tryfiates, G. P. (1975) *Br. J. Nutr. 33,* 73-80.

Krishnamurthy, S., Bieri, J. G., & Andrews, F. L. (1963) *J. Nutr. 79,* 503-510.

[2] These assays were done by Diane Newton in the laboratory of Dr. Michael B. Sporn, National Cancer Institute, National Institutes of Health, Bethesda, Maryland (unpublished observations).

Malathi, P., Subba Rao, K., Seshadri Sastry, P., & Ganguly, J. (1963) *Biochem. J. 87,* 305-311.
McCormick, A. M., Napoli, J. L., & DeLuca, H. F. (1978a) *Anal. Biochem.* (in press).
McCormick, A. M., Napoli, J. L., Schnoes, H. K., & DeLuca, H. F. (1978b) *J. Biol. Chem.* (in press).
Morgan, B., & Thompson, J. N. (1966) *Biochem. J. 101,* 835-842.
Napoli, J. L., McCormick, A. M., Schnoes, H. K., & DeLuca, H. F. (1978) *Proc. Natl. Acad. Sci. U.S.A.* (in press).
Nelson, E. C., Dehority, B. A., Jengon, H. S., Grip, A. P., & Sanger, V. D. (1962) *J. Nutr. 76,* 325-329.
Reid, R., Nelson, E. C., Mitchel, E. D., McGregor, M. L., Walker, G. R., & John, K. V. (1973) *Lipids 8,* 558-565.
Roberts, A. B., & DeLuca, H. F. (1967) *Biochem. J. 102,* 600-611.
Thompson, J. N., Howell, J. M., & Pitt, G. A. J. (1964) *Proc. R. Soc. London, Ser. B 159,* 510-535.
Zachman, R. D. (1967) *Life Sci. 6,* 2207-2210.
Zile, M., & DeLuca, H. F. (1968) *J. Nutr. 94,* 302-308.
Zile, M., & DeLuca, H. F. (1970) *Arch. Biochem. Biophys. 140,* 210-215.
Zile, M., Bunge, E. C., & DeLuca, H. F. (1977) *J. Nutr. 107,* 552-559.

Transition-Metal Binding Site of Bleomycin A_2. A Carbon-13 Nuclear Magnetic Resonance Study of the Zinc(II) and Copper(II) Derivatives†

James C. Dabrowiak,* Frederick T. Greenaway, and Robert Grulich

ABSTRACT: The ^{13}C NMR spectra at 25.2 MHz of the Zn(II) and Cu(II) complexes of the antitumor antibiotic bleomycin A_2 are discussed. Complexation of the drug to Zn(II) causes 38 of the 52 resonance lines of bleomycin A_2 to shift to new positions. All but ten of these shifted lines have been assigned in the Zn(II) bleomycin complex. Although the specific donor sites of the drug cannot be identified from the ^{13}C NMR data, the analysis clearly shows that the pyrimidine–imidazole portion of the molecule is affected by chelation. This finding is in agreement with the previously reported metal-binding site of the antibiotic. The analysis also shows that carbon atoms which have large through-bond distances from the binding site can experience substantial chemical-shift changes upon metal binding. Complexation of the drug to Cu(II) eliminates 23 resonances from the spectrum of the molecule. All of these resonances emanate from carbon atoms which are located in the pyrimidine–imidazole portion of the drug.

The utility of ^{13}C NMR as a probe of structure is well recognized. Perhaps the best evidence of the power of this technique can be seen in its application to structural studies on large molecules (Allerhand, 1975). Where conventional ^{1}H NMR techniques yield featureless spectra made up of a large number of overlapping resonances, ^{13}C NMR spectroscopy exhibits a high degree of resolution. More often than not, each of the carbon atoms in the molecule produces a single, distinct, well-resolved resonance line. Since the position and width of the resonance line are sensitive to electronic changes which can occur within the molecular framework, the technique is well suited for studying metal–ligand interactions.

Metal complexation can affect the ^{13}C NMR spectrum of an organic molecule in a number of ways. If the metal ion is diamagnetic, the carbon resonances are generally narrow and well defined but are shifted from their original positions in the unbound molecule (Chisholm and Godleski, 1976). Most studies to date show that the magnitude of the shift is a strong function of the through-bond distance between the metal ion and the carbon atom (Stockton and Martin, 1974; Fuhr and Rabenstein, 1973; Yasui and Ama, 1975; Ama and Yasui, 1976; Fedarko, 1973). Carbon atoms remote from the metal-binding site remain unshifted or experience only a small shift upon metal complexation. Although changes in molecular conformation, as would result from metal binding, should have a significant effect on the position of a ^{13}C resonance line, this aspect of ^{13}C NMR vis-a-vis metal complexation has been largely unexplored.

The ^{13}C NMR spectrum of a complex containing a paramagnetic metal ion is different than the previously described case. For this type of compound, the observed carbon shifts are about one order of magnitude greater than those found for diamagnetic systems (Doddrell and Roberts, 1970; Anderson and Matwiyoff, 1972). In addition, the paramagnetic ion broadens the carbon resonance line. Depending on the spin–lattice relaxation time (LaMar et al., 1972) and the distance between the metal ion and the carbon atom, the resonance line may be so wide so as to go undetected in the ^{13}C NMR spectrum of the compound.

The use of ^{13}C NMR to study metal binding to large biological or biological-type molecules is just beginning. Ohnishi et al. (1972) examined the ^{13}C NMR spectrum of the potassium complexes of valinomycin and nonactin. Although the largest shifts upon complexation occurred for the carbonyl carbon atoms of these antibiotics, the carbon atom closest to the metal-binding site, other shifts occurred for carbon atoms which were far from the metal ion. The authors suggested that induced changes in conformation of the molecule as a whole were as important as direct interactions with the potassium ion in determining the chemical shifts for the complex.

† From the Department of Chemistry, Syracuse University, Syracuse New York 13210 (J.C.D. and F.T.G.), and Bristol Laboratories, Syracuse, New York 13206 (R.G.). *Received June 9, 1978.*

0006-2960/78/0417-4090$01.00/0