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5,6-Epoxyretinoic Acid is a Physiological Metabolite of Retinoic Acid in the Rat

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5,6-Epoxyretinoic acid was detected in small intestine, kidney, liver, testes and serum of vitamin A-deficient rats 3 h after a single physiological dose of [^{3}H]retinoic acid. The maximum concentration of 5,6-epoxide in intestinal mucosa was observed 3 h after intrajugular administration of retinoic acid. However, at 7 h post administration, no 5,6-epoxyretinoic acid was detected in mucosa, demonstrating the rapid intestinal metabolism or excretion of this metabolite. No 5,6-epoxy[^{3}H]retinoic acid was detected in mucosa, liver or serum of retinoic acid-repleted rats 3 h after administration of 2 μg of [^{3}H]retinoic acid.

In 1945, Karrer and Jucker synthesized hepaxanthin by reacting retinol with monoperphthalic acid (Karrer, 1948; Karrer & Jucker, 1950). The spectral characteristics of the compound led to speculation that hepaxanthin might be a retinol epoxide, but the exact chemical structure was not determined. The 5,6-epoxides of retinyl acetate, retinol and retinal have been synthesized in an analogous fashion and were found to have biological activity equal to retinyl acetate (Jungalwala & Cama, 1965; Lakshmanan *et al.*, 1965). 5,6-Epoxyretinoic acid (John *et al.*, 1967) and methyl 5,6-epoxyretinoate (Morgan & Thompson, 1966) have also been synthesized and both support the growth of vitamin A-deficient rats. However, like retinoic acid, the 5,6-epoxide does not support the visual or reproductive functions of vitamin A. Recently, 5,6-epoxyretinoic acid was isolated from intestinal mucosa of vitamin A-deficient rats after a single 450 μg dose of [^{3}H]retinoic acid (Napoli *et al.*, 1978; McCormick *et al.*, 1978*a*), demonstrating that the 5,6-epoxide is a metabolite of retinoic acid in the rat *in vivo* (Fig. 1). Since supraphysiological doses of retinoic acid were used to generate sufficient quantities of the metabolite for identification, the synthesis of 5,6-epoxyretinoic acid under physiological conditions remained to be determined. The present report demonstrates that 5,6-epoxyretinoic acid is formed *in vivo* after administration of physiological amounts of retinoic acid.

Abbreviation used: h.p.l.c., high-pressure liquid chromatography.

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Experimental

Animals

Weanling male rats obtained from Holtzman Co. (Madison, WI, U.S.A.) were maintained on a vitamin A-deficient diet for 6–7 weeks (Zile & DeLuca, 1968). Rats were used for metabolism experiments after reaching a weight plateau.

Chemicals and solvents

All-*trans*-retinoic acid was obtained from Eastman Kodak Co. (Rochester, NY, U.S.A.). [11,12-^{3}H]Retinoic acid (sp. radioactivity 31 Ci/mmol) was generously supplied by Hoffmann–LaRoche and Co. (Nutley, NJ, U.S.A.). The purity of retinoic acid

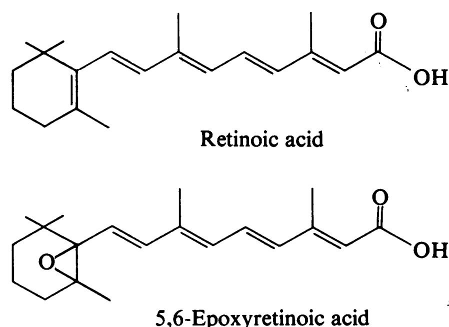

Fig. 1. *Structure of retinoic acid and 5,6-epoxyretinoic acid*

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samples was determined by reverse-phase h.p.l.c. (McCormick *et al.*, 1978*b*, 1979*a*). 5,6-Epoxyretinoic acid was synthesized as previously described (Morgan & Thompson, 1966; John *et al.*, 1967). All solvents used in the extraction and chromatography of retinoic acid metabolites were AR grade and were redistilled in glass before use.

High-pressure liquid chromatography

This was performed with a Waters Associates model ALC/GPC-204 liquid chromatograph. Detection was accomplished with a fixed u.v. monitor at 340 nm. A μC_{18} Bondapak column (0.4 cm × 30 cm) obtained from Waters Associates was used for reverse-phase h.p.l.c.

Radioactivity determinations

Radioactivity 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, MA, U.S.A.) with an efficiency of 25–35% for ^{3}H.

Preparation and administration of [^{3}H]retinoic acid

[11,12-^{3}H]Retinoic acid was diluted with non-radioactive all-*trans*-retinoic acid to give a final specific radioactivity of 4.2×10^{7} d.p.m./μg. It was then purified by Sephadex LH-20 chromatography (Ito *et al.*, 1974). The purity of retinoic acid recovered from Sephadex LH-20 was checked by reverse-phase h.p.l.c. (McCormick *et al.*, 1978*b*). All of the radioactivity co-eluted with authentic all-*trans*-retinoic acid.

Thirty vitamin A-deficient rats were divided into six groups of five animals each. Each rat in group 1 received a 9.2 μg dose of [^{3}H]retinoic acid in 100 μl of ethanol intrajugularly, whereas those in group 2 received 2.2 μg of [^{3}H]retinoic acid in 100 μl of ethanol. Group 3 rats were supplied 100 μg of all-*trans*-retinoic acid in Wesson oil orally for 5 days. At 24 h after the last supplement, they received 2.2 μg of [^{3}H]retinoic acid intrajugularly in 100 μl of ethanol. Animals in groups 1–3 were killed 3 h after administration of [^{3}H]retinoic acid. Liver, kidney, testes, small intestine and serum were collected from these animals. Rats in groups 4–6 received 2.2 μg of [^{3}H]-retinoic acid intrajugularly and were killed at 2 (group 4), 4.5 (group 5) and 7 (group 6) h after dosing. Small intestines were collected from groups 4–6.

Extraction of retinoic acid and 5,6-epoxyretinoic acid

All steps in the extraction and chromatography of these compounds were performed under yellow light. The metabolite-fractionation scheme is shown in Fig. 2. Small intestines were rinsed free of contents with ice-cold 0.15 M-NaCl and mucosa from each group was collected and pooled. Serum, liver, kidney, small-intestinal mucosa, intestinal contents and testes were homogenized and freeze-dried (Napoli *et al.*, 1978; McCormick *et al.*, 1978*a*). Freeze-dried samples were extracted with degassed methanol at 0°C for 4 h. Extractions were performed in the presence of butylated hydroxytoluene (50 μg/ml) to minimize oxidative destruction of the metabolites. Methanol-insoluble material was removed by filtration. The methanol extracts were evaporated to dryness and the residues were taken up in acetone/methanol (1:1, v/v). The soluble metabolites were applied to a column (1 cm × 30 cm) of Sephadex LH-20 equilibrated in the same solvent. Retinoic acid and 5,6-epoxyretinoic acid were batch eluted with 50 ml of column solvent. The solvent was evaporated, the residue was dissolved in 5 ml of methanol and applied to a column (1 cm × 15 cm) of DEAE-Sephadex A-25 (hydroxide form). Neutral lipids were eluted with 50 ml of methanol and the charged components were eluted with 50 ml of 0.1 M-ammonium acetate in methanol. Ammonium acetate was removed by evaporation of the methanol and resuspension of the residue in acetone. The precipitated salt was removed by filtration.

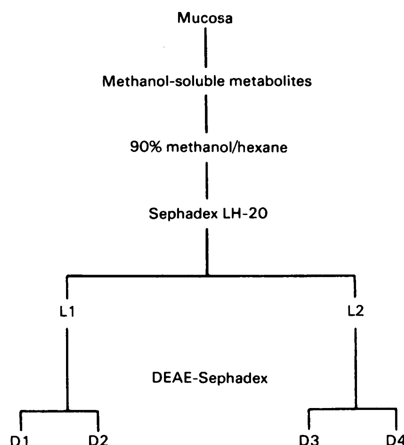

Fig. 2. *Fractionation scheme used for retinoic acid metabolites*

Determination of retinoic acid and 5,6-epoxyretinoic acid by reverse-phase h.p.l.c.

Samples recovered from DEAE-Sephadex were chromatographed on a μC_{18} Bondapak column with a solvent system of 0.01 M-NH_4HCO_3 in methanol/water (1:1, v/v). Retinoic acid and 5,6-epoxyretinoic acid were added to each sample to determine the exact elution volume of the compounds in each chromatographic run. The 5,6-epoxide eluted at 80 ml and retinoic acid was then eluted by changing the solvent to methanol. 5,6-Epoxyretinoic acid and

retinoic acid are radiochemically pure after reverse-phase h.p.l.c. in this solvent system. The concentrations of each compound were calculated from the specific radioactivity of the administered retinoic acid.

Results

Time course of appearance of retinoic acid and 5,6-*epoxyretinoic acid in intestinal mucosa*

The total methanol-soluble radioactivity in the mucosa was maximal 3h after intrajugular administration of 2μg of [11,12-^{3}H]retinoic acid. In addition, the highest concentration of retinoic acid (5ng/g) and 5,6-epoxyretinoic acid (100pg/g) were detected at the 3h time point. At 7h post injection, mucosal retinoic acid was decreased by 50%. No 5,6-epoxyretinoic acid was detected at 7h (Fig. 3). The specific radioactivity of dosed [^{3}H]retinoic acid (4.2×10^7 d.p.m./μg) establishes a detection limit of 50pg. The mucosal samples used in this experiment weighed 25g. Thus in terms of concentration 2pg/g would be detected.

Tissue distribution of retinoic acid and 5,6-*epoxyretinoic acid*

The 3h time point was chosen to study tissue distribution of 5,6-epoxyretinoic acid after administration of physiological levels of [^{3}H]retinoic acid. The reverse-phase h.p.l.c. profiles of the D1 metabolite fraction (see Fig. 2) isolated from several tissues after administration of 9.2μg of all-*trans*-retinoic acid is shown in Fig. 4. Retinoic acid and its 5,6-epoxide were detected in serum, intestinal mucosa, intestinal contents, liver, kidney and testes. In addition, a radioactive peak slightly less polar than the 4-oxo-retinoic acid standard was detected in all tissue extracts. This component was also detected in the mucosa after a single 450μg dose of [11,12-^{3}]retinoic acid and designated metabolite 8_I (Napoli *et al.*, 1978; McCormick *et al.*, 1978*a*, 1979*b*). It is interesting to note that no 4-oxoretinoic acid, recently identified as a major urinary and faecal metabolite after pharmacological doses of retinoic acid (Hänni *et al.*, 1976; Hänni & Bigler, 1977; Rietz *et al.*, 1974) was observed in the tissues examined.

The tissue distribution of 5,6-epoxyretinoic acid was also examined 3h after a 2.2μg dose of [^{3}H]-

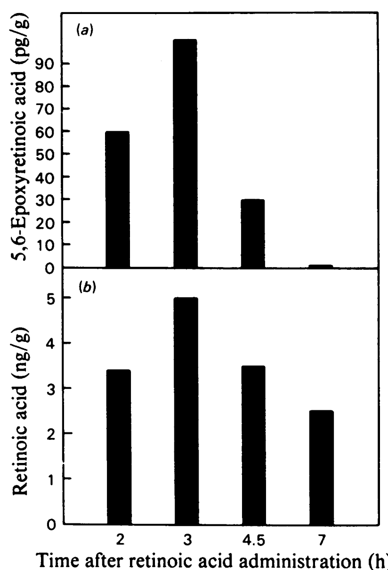

Fig. 3. *Time course of appearance of* 5,6-*epoxyretinoic acid* (*a*) *and retinoic acid* (*b*) *in intestinal mucosa of rats given* 2μg *of* [^{3}*H*]*retinoic acid*

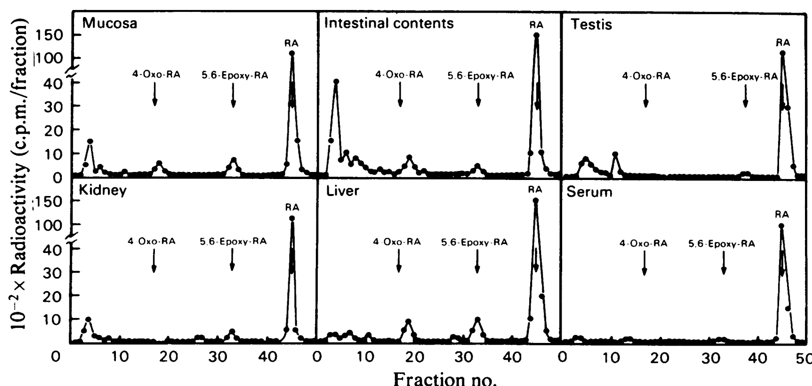

Fig. 4. *H.p.l.c. profiles of retinoic acid* (*RA*) *and its metabolites from tissues taken from vitamin A-deficient rats given* 9.2μg *of* [^{3}*H*]*retinoic acid*

The elution position of the standard compounds 4-oxoretinoic acid (4-oxo-RA) and 5,6-epoxyretinoic acid (5,6-epoxy-RA) is marked. Metabolite 8_I elutes immediately after 4-oxoretinoic acid. The volume of each fraction collected was 2ml.

retinoic acid. A comparison of the tissue concentrations of retinoic acid and 5,6-epoxyretinoic acid after 2.2 and 9.2 μg doses is shown in Fig. 5. In intestinal mucosa, the concentration of unmetabolized retinoic acid in vitamin A-deficient rats receiving 9.2 μg of retinoic acid was twice that observed in rats given 2.2 μg. Despite the increased retinoic acid concentration, the concentration of 5,6-epoxide in mucosa was comparable in the two groups. Similar results were noted in the serum concentrations of retinoic acid and 5,6-epoxyretinoic acid. The kidney concentrations of retinoic acid and its 5,6-epoxide 3 h after dosing were similar in the 2.2 μg- and 9.2 μg-dosed rats. In contrast, the concentrations of retinoic acid and 5,6-epoxide in liver and testes are 4-fold greater in the group receiving 9.2 μg of retinoic acid.

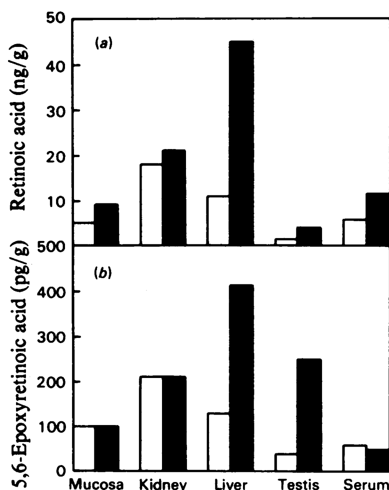

Fig. 5. *Tissue distribution of [^{3}H]retinoic acid (a) and 5,6-epoxyretinoic acid (b) 3 h after a single dose of 2.2 μg (open bars) or 9.2 μg (filled bars) of [^{3}H]retinoic acid*

Metabolism of [^{3}H]retinoic acid in retinoic acid-repleted rats

Vitamin A-deficient rats received daily oral supplements of all-*trans*-retinoic acid (100 μg) for 5 days. During the supplementation period, the rats gained weight at a rate comparable with that observed during the vitamin A-depletion period (3–5 g/day). The D1 metabolite fraction was obtained as previously described and the components were separated by reverse-phase h.p.l.c. The D1 metabolite profiles of mucosa, liver and serum are shown in Fig. 6. In all three tissues, the most striking difference between the metabolism of retinoic acid in deficient and retinoic acid-repleted rats is the absence of detectable 5,6-epoxyretinoic acid in the tissues of repleted rats. Although retinoic acid is still the major metabolite in the D1 fraction, the amount of [^{3}H]-retinoic acid in mucosa, liver and serum after a pulse dose is decreased severalfold in the repleted rats compared with that observed in vitamin A-deficient rats (Fig. 7). Metabolite 8_I appeared in all tissues examined in both vitamin A-deficient and retinoic acid-treated rats.

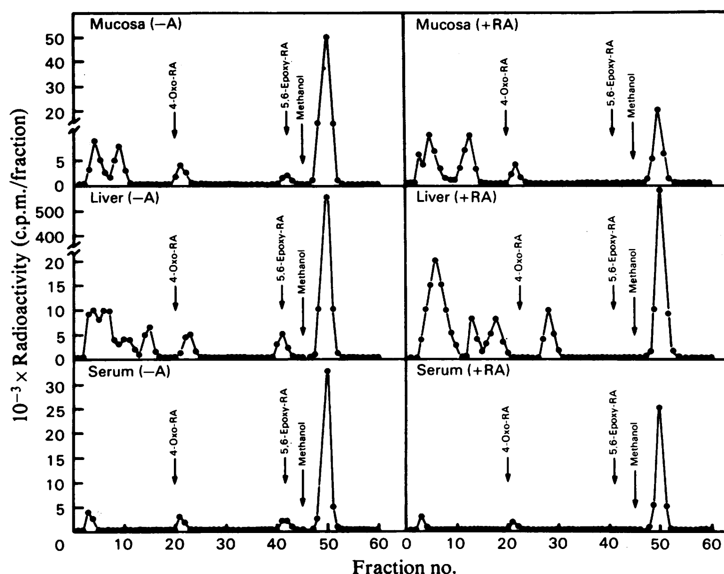

Fig. 6. *D1 metabolites of [^{3}H]retinoic acid appearing in small-intestinal mucosa, liver and serum after a 2.2 μg dose of [^{3}H]retinoic acid to deficient (−A) and retinoic acid-treated rats (+RA)*

The volume of each fraction collected was 2 ml. Abbreviations used: RA, retinoic acid; 4-oxo-RA, 4-oxoretinoic acid; 5,6-epoxy-RA, 5,6-epoxyretinoic acid.

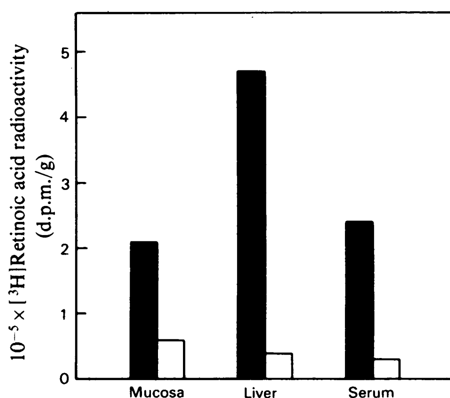

Fig. 7. *Retinoic acid concentrations in vitamin A-deficient (filled bars) and retinoic acid-repleted (open bars) rats given a single 2.2μg dose of [^{3}H]retinoic acid 3h before killing*

Control experiments

To ensure that the D1 metabolites are truly generated metabolites *in vivo* and not artefacts produced during extraction and chromatography, 10^6 d.p.m. of all-*trans*-[11,12-^{3}H]retinoic acid was added to tissue homogenates and subjected to freeze-drying, extraction and all chromatographic procedures. The reverse-phase h.p.l.c. profile of the D1 fraction of control samples revealed a single peak of radioactivity that co-eluted with unlabelled all-*trans*-retinoic acid. In addition, the radioactivity recovered from reverse-phase h.p.l.c. co-eluted with all-*trans*-retinoic acid in a straight-phase h.p.l.c. system capable of resolving *cis*- and *trans*-isomers of retinoic acid.

Discussion

A thorough study of the metabolism of labile vitamin A compounds requires that the methods used give quantitative recovery of all metabolites and do not produce artefacts that could be mistaken for actual metabolites formed *in vivo*. The combination of freeze-drying, methanol extraction, Sephadex chromatography and h.p.l.c. fulfils these requirements (Ito *et al.*, 1974; Napoli *et al.*, 1978; McCormick *et al.*, 1978*a*,*b*, 1979*a*,*b*). In the present study we have examined the biosynthesis of the D1 metabolites (Fig. 2) in vitamin A-deficient rats given physiological doses of retinoic acid to determine if 5,6-epoxyretinoic acid and metabolite 8_I are physiological metabolites of retinoic acid. The methods employed for determination of tissue concentrations of retinoic acid, 5,6-epoxyretinoic acid and metabolite 8_I parallel those developed for purification of these metabolites. Thus each metabolite peak after reverse-phase h.p.l.c. is homogeneous.

At 3h after intrajugular injection of [^{3}H]retinoic acid, the substrate represents the largest component in all tissues surveyed. Tissue concentrations were in the 1–50ng/g range in all organs examined. The highest concentration of retinoic acid was observed in the kidney after a 2μg dose, but after a 9μg dose, liver contained the highest concentration. The increases in hepatic retinoic acid are consistent with the proposal that excess retinoic acid is eliminated via the bile (Fidge *et al.*, 1968; Lippel & Olson, 1968*a*,*b*).

5,6-Epoxyretinoic acid was detected in liver, mucosa, kidney, testis, serum and intestinal contents of vitamin A-deficient rats 3h after administration of physiological amounts of retinoic acid. The concentration of 5,6-epoxide in the tissues examined was in the 50–250pg/g tissue range, in contrast with the nanogram amounts of unmetabolized retinoic acid. In kidney, mucosa and serum, 5,6-epoxide concentrations were comparable in animals receiving either 2 or 9μg of retinoic acid, despite a 2-fold increase in the tissue substrate concentration at the higher dosage. However, in liver and testis, the increased substrate concentration is paralleled by increased 5,6-epoxide concentrations. These data suggest that 5,6-epoxyretinoic acid concentrations are more tightly regulated in kidney and mucosa than in liver or testis. The increased concentrations in liver and testis could result from accumulation of the metabolite in tissues not capable of further metabolism of the compound or concentration of the metabolite in those tissues responsible for its further metabolism and/or excretion.

Metabolite 8_I, originally isolated from intestinal mucosa of vitamin A-deficient rats after a single 450μg dose of retinoic acid (McCormick *et al.*, 1978*a*; A. M. McCormick, J. L. Napoli, H. K. Schnoes & H. F. DeLuca, unpublished work) was detected in mucosa, liver, serum and intestinal contents after physiological doses (2–10μg) of retinoic acid. The amounts of this as yet unidentified metabolite are highest in liver and intestinal contents.

Since 5,6-epoxyretinoic acid was detected in all tissues examined, the present data provide no clues to the site(s) of synthesis of this metabolite unless it is indeed made in all tissues in which it was observed. The fact that retinoic acid was present in high concentrations in all tissues where the 5,6-epoxide was detected suggests that this possibility cannot be overlooked. The fact that retinoic acid and 5,6-epoxyretinoic acid are found in the intestinal contents raises the question of the mechanism by which they appear in contents. Their presence could be explained by: (1) sloughing of mucosal cells into the contents fraction during the washing procedure used to separate intestinal tissue from contents; (2)

excretion of these compounds via the bile or other mechanism; (3) enterohepatic circulation of the compounds. The data available are compatible with any of the three possible explanations.

In contrast, metabolite 8_I was observed only in mucosa, liver, intestinal contents and serum. These data suggest that this metabolite may be circulated in the rat via the enterohepatic circulation. However, the site of synthesis has not yet been located.

Intestine, a known target organ of vitamin A (Johnson *et al.*, 1969; Zile & DeLuca, 1970; DeLuca *et al.*, 1969), was chosen for further study of the D1 metabolite fraction. The time course of the D1 metabolites was studied over a 7h period after administration of a physiological dose of retinoic acid. The concentration of unmetabolized retinoic acid reached a peak at 3h and declined to 50% of the maximum concentration by 7h. The concentration of 5,6-epoxyretinoic acid in mucosa was also highest at 3h, but in contrast with retinoic acid, none of the epoxide metabolite was detected at 7h. The rapid disappearance of the 5,6-epoxide from mucosa suggests that it is either rapidly metabolized or excreted by the rat. From these data, it does not appear that a steady-state concentration of 5,6-epoxyretinoic acid is maintained in mucosa unless it is below the detection limits of the present experiments (2pg/g). The concentration of metabolite 8_I in mucosa remained at a constant value (400pg/g) during the time period studied.

In retinoic acid-repleted rats, decreases in total methanol-soluble radioactivity and in tissue concentrations of [^{3}H]retinoic acid were noted in mucosa, liver and serum. The observed decreases could be the result of: (1) decreased uptake of [^{3}H]retinoic acid at the tissue level due to adequate endogenous concentrations of retinoic acid and/or further metabolites in liver and mucosa; (2) accelerated metabolism and excretion of retinoic acid and further metabolites; (3) a combination of the two processes. No 5,6-epoxy-[^{3}H]retinoic acid was detected in serum, liver or mucosa of retinoic acid-repleted rats. The endogenous concentrations of retinoic acid and 5,6-epoxide in the repleted rats were not determined since a suitable method for measuring low amounts (100pg–5ng) of non-radioactive metabolites in tissue samples is not yet available. However, assuming that 60% of the retinoic acid supplements are excreted in 24h (Lippel & Olson, 1968*a*,*b*; Geison & Johnson, 1970), the maximum dilution of dosed [^{3}H]retinoic acid would raise detection limits to 20pg/g. Thus although the actual tissue concentrations of retinoic acid and 5,6-epoxide cannot be calculated from these data, it is evident that the amounts of the tritiated compounds are decreased in liver, serum and mucosa in retinoic acid-repleted rats. The observed decrease in 5,6-epoxy[^{3}H]retinoic acid could be explained by accelerated metabolism or excretion of the metabolite. However, an equally viable alternative is that endogenous 5,6-epoxide may have repressed the synthesis of the ^{3}H-labelled 5,6-epoxide.

Synthetic 5,6-epoxyretinoic acid has 80–100% the biological activity of all-*trans*-retinoic acid in supporting the growth of vitamin A-deficient rats (John *et al.*, 1967). In addition, the 5,6-epoxide is equally as effective as retinoic acid in blocking the induction of ornithine decarboxylase activity in mouse epidermis and in preventing the appearance of skin papillomas after dimethylbenzanthracene administration in mice (A. K. Verma, J. L. Napoli, R. K. Boutwell, A. M. McCormick & H. F. DeLuca, unpublished work). Despite the high biopotency of this metabolite established in two quite different vitamin A-responsive test systems, the biological significance of 5,6-epoxyretinoic acid remains uknown.

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