Retinoic Acid 5,6-Epoxidase

PROPERTIES AND BIOLOGICAL SIGNIFICANCE*

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A retinoic acid epoxidase is present in rat kidney homogenates. It is found in the particulate fraction, and is dependent on ATP, NADPH, and oxygen. It is stimulated by Fe^{2+} and inhibited by Mn^{2+}, Zn^{2+}, Cu^{2+}, EDTA, and *N,N'*-diphenyl-*para*-phenylenediamine. Its properties are closest to those of microsomal lipid peroxidases previously studied. Epoxidase activity is not affected by the rat's vitamin A status, nor is it inducible by retinoic acid. In *N,N'*-diphenyl-*para*-phenylenediamine-feeding experiments, it was shown that blocking epoxidation *in vivo* does not inhibit the function of retinoic acid. It is concluded that retinoic acid epoxidation is not required for retinoic acid function.

Retinoic acid epoxides were first synthesized and studied in the 1960's by Cama and his group (1, 2), as well as Morgan and Thompson in England (3). Initial studies on the biological activity of 5,6-epoxyretinoic acid suggested it was 157% as active as retinyl acetate (2). However, later experiments indicated that initial measurements were in error, and its biopotency was only 80% that of retinyl acetate (4).

No further work on retinoic acid epoxides appeared until 1978, when Napoli *et al.* (5) reported finding 5,8-oxyretinoic acid in the intestines of rats dosed with retinoic acid. It was suggested that the 5,8-oxyretinoic acid isolated may have been a rearrangement product of 5,6-epoxyretinoic acid (6). Subsequent studies determined that, in fact, 5,6-epoxyretinoic acid was the real metabolite and that 5,8-oxyretinoic acid was an artifact (7). From physiological dosing experiments, it was found that 5,6-epoxyretinoic acid was produced in very small amounts in the tissues analyzed (8).

Studies were undertaken in this laboratory to determine the nature of the epoxidation reaction as it occurs *in vitro*. The reaction was found to occur in many tissues at a rapid rate (9). Rat kidney had the highest activity, and was therefore used in subsequent studies. It was found to be dependent on NADPH, Mg^{2+}, ATP, and oxygen, while being inhibited by *n*-propyl gallate and EDTA. Activity was located in mitochondrial and microsomal fractions, but not in the soluble fraction (10).

More recent studies have shed some light on the biological activity of 5,6-epoxyretinoic acid. Newton *et al.* (11) have found that its activity is only 1% that of all-*trans*-retinoic acid in the tracheal organ culture assay. This assay measures the ability of a test compound to maintain differentiation of an epithelial tissue. Zile *et al.* (12) performed a carefully controlled growth assay on pure 5,6-epoxyretinoic acid and found its activity to be only 0.5% that of all-*trans*-retinoic acid. 5,6-Epoxyretinoic acid has also been tested in a vaginal smear assay, which measures its ability to maintain epithelial tissue differentiation, and found to be only 1% as active as all-*trans*-retinoic acid.[1] 5,6-Epoxyretinoic acid does have activity in the inhibition of ornithine decarboxylase induction following mitogen stimulation (13), but the relevance of this phenomena to vitamin A activity *in vivo* remains unclear.

The experiments reported in this paper may be divided into two categories. The first set was performed to characterize the epoxidation reaction as it occurs *in vitro*. The second set was designed to determine the importance of epoxidation *in vivo*, and involves evaluation of retinoic acid activity under conditions which inhibit epoxidation.

MATERIALS AND METHODS

General Techniques—The retinoids used in these experiments are sensitive to light and to air oxidation.[2] In order to prevent isomeric destruction of these compounds, they were at all times handled under yellow light. They were also kept in solution under butylated hydroxytoluene and nitrogen whenever possible.

Materials—Tris[3] and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid were the buffers used in these experiments. They were used at 100 mM concentration and adjusted to pH 7.4. Tris, Hepes, NADPH, ATP, sodium azide, metyrapone, EDTA, and α-naphthoflavone were obtained from Sigma. All-*trans*-retinoic acid and *N,N'*-diphenyl-para-phenylenediamine were from Eastman Kodak Co. (Rochester, NY). Carbon monoxide was obtained from Matheson Gas Products (Joliet, IL). ADP and AMP were purchased from Pabst Laboratories (Milwaukee, WI). $MgCl_2 \cdot 6H_2O$, $CaCl_2$, and $CuCl_2 \cdot 2H_2O$ came from Baker Chemical Co. (Phillipsburg, NJ). Na_2HPO_4, $ZnCl_2$, and $MnCl_2 \cdot 4H_2O$ were from Mallinckrodt Chemical Works. $NH_4CO_2CH_3$, $Na_4P_2O_7 \cdot 10H_2O$, $FeSO_4$, and high pressure liquid chro-

[1] W. K. Sietsema and H. F. DeLuca, unpublished results.

[2] The sensitivity of retinoids to air oxidation is best documented by Morgan and Thompson (3), who state that "Epoxides may also be produced by atmospheric oxidation of retinol, retinyl esters or retinoic acid derivatives during chemical manipulation of tissue extracts or storage of fortified diets. We have, in fact, found evidence for the existence of 5,6- and 5,8-epoxides in our stored examples of retinoic acid. The production of these and other biologically active artifacts in vitamin A metabolism experiments must therefore be considered, especially when the isolation of a small quantity of an 'active form' of vitamin A is attempted."

[3] The abbreviations used are: Tris, tris(hydroxymethyl)aminomethane; NPG, *n*-propyl gallate; BHT, butylated hydroxytoluene; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; metyrapone, 2-methyl-1,2-di-3-pyridyl-1-propanone; DPPD, *N,N'*-diphenyl-*para*-phenylenediamine; HPLC, high pressure liquid chromatography.

matography solvents came from Fisher. [11,12-^{3}H]-All-*trans*-retinoic acid (31 Ci/mmol) was generously supplied by Hoffmann-LaRoche, Inc. Retinoid standards (16-hydroxy-4-ketoretinoic acid, 4-hydroxyretinoic acid, 4-ketoretinoic acid, and 5,6-epoxyretinoic acid) were also supplied by Hoffmann-La Roche, Inc. C_{18} Sep-Paks were obtained from Waters Associates (Milford, MA).

Animals—Weanling rats were obtained from the Holtzman Co. (Madison, WI) and raised on a defined experimental diet (14) supplemented with ascorbic acid (0.5 g/kg diet). After 6 weeks on this diet, rats began to show signs of vitamin A deficiency (weight loss, diarrhea, respiratory difficulties, and xerophthalmia). For the DPPD-feeding experiments, rats were weighed every 2 or 3 days and weight gain used as an index of vitamin A deficiency. At the appropriate time, rats were switched to the same diet but containing 0.1% DPPD. All animals were allowed as much food and water as they could consume.

In Vitro Incubation Procedures—These procedures are generally as described elsewhere (9). Minor modifications in some experiments are discussed in the figure legends.

Purification of Substrate—All-*trans*-^{3}H-retinoic acid was purified by reverse-phase HPLC (15) and diluted to an appropriate specific activity immediately prior to use. An aliquot from each batch of diluted label was kept for analysis following the series of experiments in which it was used. Purified retinoic acid was always greater than 95% pure. 5,6-Epoxyretinoic acid was occasionally found as a contaminant, always less than 1%. Two to 3% retinoic acid isomers were typically present.

Extraction Procedures—*In vitro* incubation mixtures were extracted as previously described (9). Tissues analyzed for 5,6-epoxyretinoic acid production *in vivo* were homogenized in 50 ml of methanol containing BHT and NPG (50 μg/ml each) using a polytron. After the tissue was thoroughly homogenized, it was filtered through a medium scintered glass filter and rinsed once with 10 ml of methanol. The methanol phase was mixed with water and extracted with distilled chloroform in the same proportions as the *in vitro* incubations. The chloroform phase was then treated the same as the *in vitro* samples with one exception. Each sample was applied to and eluted from a C_{18} Sep-Pak in methanol prior to HPLC to remove contaminants that might irreversibly bind to a reverse-phase HPLC column.

Chromatography—The chromatography in these experiments was performed on a Beckman (Lincolnwood, IL) model 420 liquid chromatograph. This system is equipped with a microprocessor and two Beckman model 110 pumps for gradient capabilities. Injections were made using a Waters Associates model U6K injector. A Waters Associates model 440 fixed wavelength UV detector was used to monitor the effluent absorbance at 340 or 313 nm. Each sample was chromatographed on a Waters Associates μBondapak C_{18} reverse-phase column. The column was equilibrated in 98% water in methanol, both solvents containing 10 mM ammonium acetate. Immediately following injection, a 2-h linear gradient from 98% to 0% water in methanol was begun. 1.1-ml fractions were collected for scintillation counting. Internal standards were used to mark the elution positions of known compounds.

Scintillation Counting—Radioactivity was determined with a Prias model PLD Liquid Scintillation counter made by Packard Instruments. Liquid samples (≤1.1 ml) were mixed with 4.0 ml of 3a70b scintillation fluid made by Research Products International (Elk Grove Village, IL) and counted for 1 to 3 min in the external standardization mode.

Mathematical Procedures—Kinetic values were determined using the standard Michaelis-Menten equations with modifications by Lee and Wilson (16) to correct for declining substrate concentrations. The *V versus S* plots and the double reciprocal plots had no unusual features, and are not shown for that reason. Kinetic parameters were determined using a computer program to fit a straight line to the points on a double reciprocal plot by a nonlinear regression method.

Handling of Gases—Incubations with carbon monoxide were carried out in a hood using serum-stoppered flasks. The flasks were first evacuated with a vacuum pump, and the appropriate gases or additions made into the flask with a syringe.

Vaginal Smears—Vaginal smears may be used as an index of vitamin A deficiency (17). These were obtained by rinsing the rat's vagina with about 0.5 ml of normal saline in an eye dropper. A drop of this liquid was applied to a glass slide, covered with a cover slip, and examined with a phase contrast microscope on low power. A vitamin A-deficient rat will have a vaginal smear which is continuously cornified in contrast to a normal rat which manifests an estrous cycle lasting 4 to 6 days.

RESULTS

Effects of NADPH and ATP on Epoxidase Activity—Optimal concentrations of NADPH and ATP were determined as shown in Figs. 1 and 2. In the NADPH experiment, the cofactor was added in the reduced form, and the NADPH-regenerating system was excluded. (In earlier experiments, the cofactor was added in the oxidized form and generated by the action of glucose-6-phosphate dehydrogenase on glucose-6-phosphate.) NADPH saturates at a very low concentration (<0.25 mM), while ATP did not completely saturate even at 20 mM. In subsequent assays, concentrations were set at 0.5 mM for NADPH and 20 mM for ATP.

Effect of Magnesium on Epoxidase Activity—Earlier results indicated that Mg^{2+} activated the epoxidase reaction. However, those experiments were done in the presence of an NADPH-regenerating system. It is known that glucose-6-phosphate dehydrogenase is activated by Mg^{2+} (18), and epoxidase assays done in the absence of the regenerating system show only a weak dependence on Mg^{2+} (see Fig. 3 and Table I).

Effect of Various Forms of Phosphate on Epoxidase Activity—In order to determine whether phosphate derivatives other than ATP would activate the epoxidase, PO_4, P_2O_7, and ADP were tested in the assay at a 20 mM concentration. Each
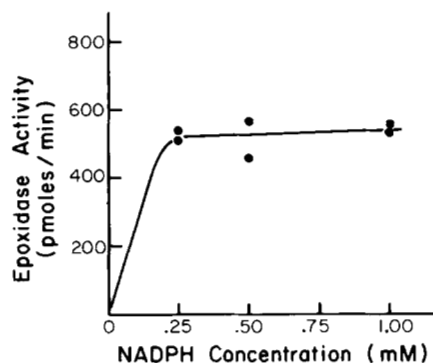

FIG. 1. **Effect of NADPH concentration on epoxidase activity.** *In vitro* assay mixture contained 0.25% kidney homogenate in 100 mM Tris buffer, pH 7.4; 20 mM ATP; 20 mM Mg^{2+}; varying concentrations of NADPH as shown. Retinoic acid concentration was 2.67 μM. Mixture was incubated at 37 °C for 3 min.
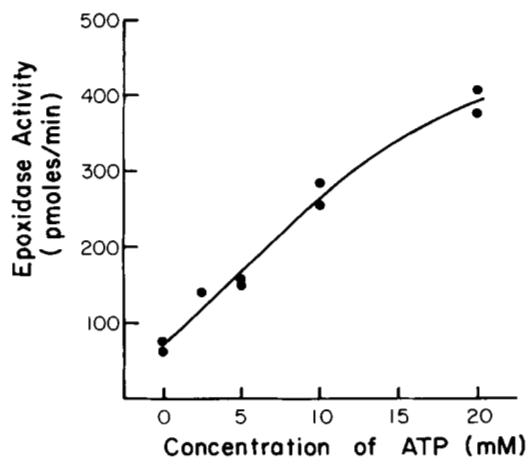

FIG. 2. **Effect of ATP concentration on epoxidase activity.** Conditions were as described in the legend to Fig. 1 except that the NADPH concentration was 0.5 mM and the ATP concentration was varied as shown.

sample was carefully buffered to pH 7.4 before addition to the assay mixture. The results are shown in Fig. 4. It is apparent that only ATP will serve as a cofactor.

Effects of Some Divalent Metal Ions on Epoxidase Activity—Several divalent metal ions were tested for their ability to activate the epoxidase. These metal ions were added in the concentrations shown in Table I. Mg^{2+} had only a slight activation effect. Zn^{2+}, Mn^{2+}, and Cu^{2+} inhibited epoxidation. Fe^{2+} activated the epoxidase almost 3-fold at a 10 mM concentration. There was no synergistic effect between Mg^{2+} and Fe^{2+}.

Effects of Some Inhibitors on Epoxidase Activity—At a 1 mM concentration, sodium azide slightly inhibited the epoxi-
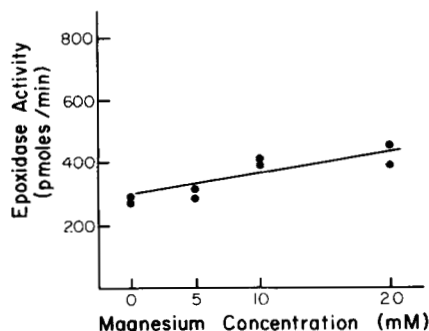

FIG. 3. **Effect of magnesium concentration on epoxidase activity.** Conditions were as described in the legend to Fig. 1 except that the NADPH concentration was 0.5 mM and the magnesium concentration was varied as shown.

TABLE I

Effect of some divalent metal ions on epoxidase activity

Assays were run in the presence of 0.25% rat kidney homogenate at pH 7.5. 100 mM Hepes buffer was used when Fe^{2+} was added. In all other assays, 100 mM Tris buffer was used. NADPH concentration was 0.5 mM and ATP was 20 mM. Retinoic acid concentration was 2.67 mM. Mixtures were incubated for 3 min at 37 °C. Each value is an average of duplicate determinations.

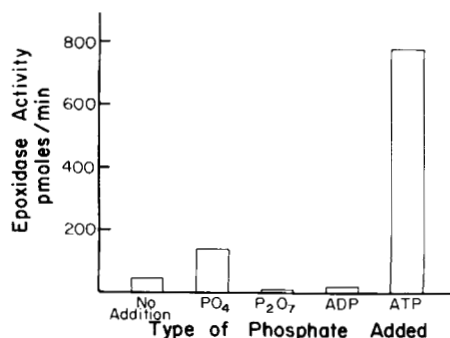| Metal ions added	Epoxidase activity
	pmol/min
None	385
Mg^{2+}, 20 mM	479
Zn^{2+}, 20 mM	76
Ca^{2+}, 20 mM	274
Mn^{2+}, 20 mM	67
Cu^{2+}, 20 mM	105
Fe^{2+}, 10 mM	970
Fe^{2+}, 10 mM, and Mg^{2+}, 20 mM	983

FIG. 4. **Effect of various forms of phosphate on epoxidase activity.** Conditions were as described in the legend to Fig. 1 except that the NADPH concentration was 0.5 mM. Each type of phosphate was tested at 20 mM.

TABLE II

Effect of some inhibitors on epoxidase activity

Conditions were as in the legend to Table I except that 20 mM magnesium was used. Inhibitors were added about 10 min prior to addition of substrate and allowed to equilibrate in the homogenate.

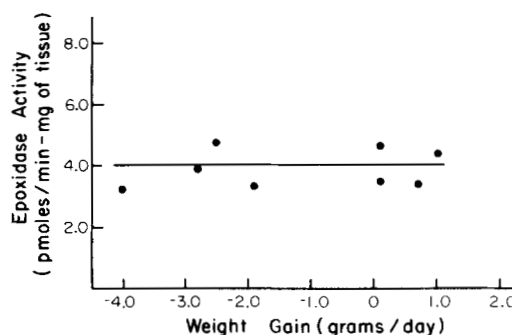| Inhibitor	Epoxidase activity
	% control
Sodium azide, 1 mM	72
Metyrapone, 500 μM	86
EDTA, 2.5 mM	0
DPPD, 200 nM	12
1 μM	10
5 μM	8
α-Naphthoflavone, 4 μM	98
20 μM	96
100 μM	104
Carbon monoxide, 10%	102
33%	85
66%	100
95%	90

FIG. 5. **Epoxidase activity at different stages of vitamin A deficiency.** Male rats were kept on a vitamin A-deficient diet and weighed every 2 days. They were killed at various stages of deficiency, as assessed by a weight gain parameter. Weight gain is assessed by drawing a tangent to the weight curve at the point of killing.

dase (see Table II). This could be a secondary effect if it is inhibiting endogenous ATP production in the assay mixture. It is known that ATP levels in the assay are not saturating, so inhibition of a small amount of endogenous ATP production could significantly reduce the amount of epoxide produced.

Metyrapone and carbon monoxide, both cytochrome P-450 inhibitors (19) and α-naphthoflavone, an aryl hydrocarbon hydroxylase inhibitor (20), were tested for their effects on retinoic acid epoxidation. All assays conducted with these inhibitors were within experimental error of the controls.

EDTA, a divalent metal ion chelator, inhibits the reaction completely at 2.5 mM concentration. This inhibition could be due to its chelation of Fe^{2+}, which strongly activates the epoxidation reaction.

DPPD also strongly inhibits the epoxidase reaction, even at very low concentrations. DPPD is a free radical scavenger sometimes used as an antioxidant. It was shown previously that NPG, another free radical scavenger, also inhibits *in vitro* epoxidation (9). It is therefore likely that some sort of a free radical intermediate is involved in the reaction.

Epoxidase Activity as a Function of Vitamin A Status—In one experiment, epoxidase activity was measured in rats under a range of vitamin A deficiency stages. Vitamin A deficiency status was determined by drawing a tangent to the weight gain curve at the point of killing and using the slope in grams/day as a deficiency index. As shown in Fig. 5, epoxidase activity does not change as the rats become deficient.

In another experiment, apparent kinetic parameters were

determined under different dietary conditions. These were determined to confirm that there is no dietary effect on epoxidase activity. It should be stressed that these are not true kinetic parameters for two reasons. First of all, these experiments were done with tissue homogenates, a poorly defined system, so there may be unknown cofactors involved. Secondly, it is known that ATP is not saturating, even at the high levels present in the assay. Kinetic parameters should be determined under saturating conditions. However, measurement of these apparent kinetic parameters is useful for gauging the effect of dietary status on the epoxidase.

Apparent kinetic parameters were determined for rats under three dietary conditions. In one case, the kidney was used from a rat which had been on the vitamin A-deficient diet and was losing weight. In another, kidney tissue was from a normal rat receiving a full vitamin supplement. In the third case, the rat was on a full vitamin supplement, but also received six intraperitoneal injections of all-*trans*-retinoic acid (1 mg in 100 μl of dimethylsulfoxide) spaced 12 h apart. The rat was killed 12 h following the last dose. The third case was included to see if the enzymatic machinery for epoxidation could be induced by high levels of substrate. Samples of kidney from the induced rat were also analyzed for retinoic acid content. It was determined that there was not enough endogenous retinoic acid present to interfere with the determination of apparent kinetic parameters.

The results are shown in Table III. The apparent K_m values for all-*trans*-retinoic acid do not change appreciably under any dietary condition. The variation seen is within experimental error. The apparent V_{max}, a measure of the quantity of enzyme present, is the same for the normal and induced rats. It is slightly depressed in the vitamin A-deficient rat. This could be due to a general debilitation of the rat, as is always seen in the vitamin A-deficient state.

Apparent Kinetic Parameters for 13-cis-Retinoic Acid— The apparent K_m and V_{max} for 13-*cis*-retinoic acid were also determined (see Table III). This experiment was done using a normal rat on a full vitamin supplement. There is no difference between the apparent K_m for all-*trans*-retinoic acid and that for 13-*cis*-retinoic acid. However, the apparent V_{max} is somewhat lower for 13-*cis*-retinoic acid than it is for all-*trans*-retinoic acid.

Blocking Epoxidation in Vivo—It was shown above (see Table II) that DPPD inhibited the epoxidation reaction even at very low concentrations. Since it is known that DPPD can be fed to rats with no immediate ill effects (21), it was possible to assess the effect of blocking epoxidation *in vivo* on the

TABLE III
Apparent kinetic parameters for the retinoic acid epoxidase
Conditions were as in the legend to Table I except that 20 mM magnesium was used, and substrate concentrations were varied from 0 to 2.67 μM. Parameters were calculated from double reciprocal plots as described under "Materials and Methods." Errors shown are standard errors.

Conditions	Apparent K_m	Apparent V_{max}
	M	pmol/min/mg
All-*trans*-retinoic acid Vitamin A-deficient rat kidneys	$2.8 \pm 0.5 \times 10^{-6}$	29 ± 4
All-*trans*-retinoic acid Vitamin A normal rat kidneys	$3.7 \pm 0.6 \times 10^{-6}$	45 ± 5
All-*trans*-retinoic acid Vitamin A-induced rat kidneys	$2.6 \pm 0.2 \times 10^{-6}$	51 ± 2
13-*cis*-Retinoic acid Vitamin A normal rat kidneys	$3.2 \pm 1.7 \times 10^{-6}$	24 ± 4

TABLE IV
Weight gain of rats fed DPPD-containing diets
Vitamin A-deficient rats were switched to the DPPD-containing diet after 4 days of weight loss. A group of normal rats the same age was put on a DPPD-containing diet at the same time. Beginning 3 days after the diet change, the vitamin A-deficient rats were given 10 μg of retinoic acid/day by mouth and weight gain monitored for 20 days. Weight gain shown is the average weight gain over a 20-day period $\pm$ standard errors.

Conditions	Weight gain
	g/day
Vitamin A-deficient diet 10 μg retinoic acid/day by mouth	1.2 ± 0.8 ($n = 5$)
Vitamin A-deficient diet + 0.1% DPPD 10 μg retinoic acid/day by mouth	1.4 ± 0.5 ($n = 5$)
Vitamin A normal diet + 0.1% DPPD	1.4 ± 0.3 ($n = 4$)

action of vitamin A. For this experiment, a group of female rats was placed on a vitamin A-deficient diet, and weights monitored until they had plateaued and were losing weight (about 7 weeks). The rats were also monitored by vaginal smear, and when they were losing weight, their vagina epithelium was continuously cornified. The rats were then divided into two groups, the first one for determining the effect of DPPD on vitamin A activity, and the second one to assay for *in vivo* epoxidation in the presence and absence of DPPD.

When deficient, half of the first group was switched to a vitamin A-deficient diet containing 0.1% DPPD. The other half was continued on the vitamin A-deficient diet. At the same time, a group of normal rats the same age was placed on the vitamin A-deficient diet plus 0.1% DPPD and given a full vitamin supplement. These normal rats were treated with DPPD as a control to determine the effects of DPPD on weight gain. Three days after the diet change, the vitamin A-deficient rats were started on a retinoic acid supplement (10 μg/day by mouth). Vitamin A deficiency parameters were carefully monitored for all the rats.

The vitamin A-deficient rats with or without DPPD in their diet reverted to a normal estrous cycle within 48 h of the first retinoic acid supplement (data not shown). The vitamin A normal rats fed the DPPD diet continued to have a normal estrous cycle. Therefore DPPD has no effect on the ability of retinoic acid to maintain differentiation of the vaginal epithelium.

The average weight gain for the 20-day period following the first retinoic acid supplement is shown in Table IV. DPPD had no influence on the growth-promoting effect of all-*trans*-retinoic acid. Rats which were not supplemented with retinoic acid would have lost weight at a rapid rate, and most would not have survived for 20 days.

The second group of rats was used to assay for epoxidase activity *in vivo* in rats fed DPPD. When deficient, half of the rats were placed on a vitamin A-deficient diet containing 0.1% DPPD, and the other half was continued on the vitamin A-deficient diet. After 3 days, two rats from each group were injected intrajugularly with 487 μg of all-*trans*-retinoic acid in 100 μl of ethanol. Each rat was killed 3 h after injection, and the kidneys, small intestine, liver, uterus, and blood collected and stored in methanol containing BHT (50 μg/ml) under nitrogen until processed for analysis. Each tissue was extracted and chromatographed as described under "Materials and Methods." Two chromatograms for the kidney assays are shown in Fig. 6, *A* and *B*.

Two things are clear from these plots. First, there are no apparent differences between the two metabolic profiles shown, and second, levels of 5,6-epoxyretinoic acid are so low
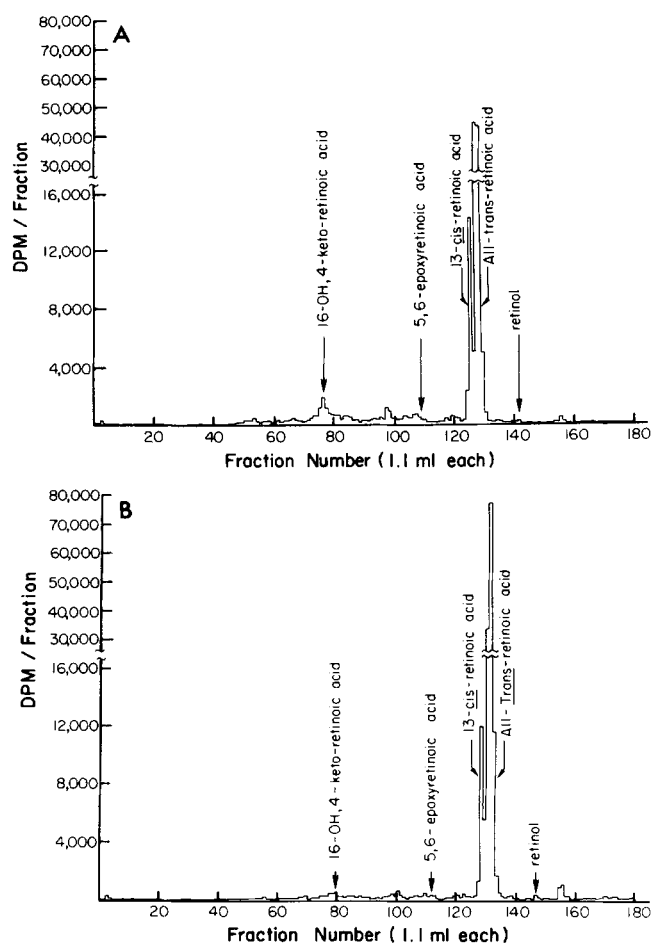
FIG. 6. *In vivo* assay for 5,6-epoxyretinoic acid production. Rats were injected with 487 μg of all-*trans*-[11,12-³H]retinoic acid and killed 3 h later. Kidneys were removed and processed as described under "Materials and Methods." These samples were chromatographed on a Waters Associates μBondapak C₁₈ reverse-phase column in methanol:water plus 10 mM ammonium acetate. A linear gradient from 98% to 0% water was used to elute metabolites from the column. Flow rate was 1.5 ml/min. UV absorbing internal standards are marked with *arrows*. Fractions were 1.1 ml each. *A*, rat fed a vitamin A-deficient diet until weight loss had occurred for 7 days; *B*, rat fed a vitamin A-deficient diet until weight loss had occurred for 4 days, then put on a 0.1% DPPD diet for 3 additional days.

as to be undetectable. The same observations were made on the metabolic profiles for all the other tissues. In no tissue was there a peak of radioactivity that consistently co-migrated with the unlabeled 5,6-epoxyretinoic acid present in each of the assays. It is probable that this assay is not sensitive enough to detect the low levels of 5,6-epoxyretinoic acid produced *in vivo* (8).

Since epoxidase activity was undetectable *in vivo* by these methods, an experiment was performed to measure the *in vitro* epoxidase activity in the kidneys of rats fed a DPPD-containing diet. Vitamin A-deficient rats were put either on the vitamin A-deficient diet or the DPPD-containing diet. After 3 days, kidneys were removed from a rat in each group and epoxidase assays performed as usual. It was found that epoxidation was inhibited by 91% in the rats fed the DPPD-containing diet. This shows that enough DPPD is getting into the tissue to inhibit epoxidation.

DISCUSSION

It has been difficult to attribute the epoxidation of retinoic acid to any currently known enzyme. Epoxidations are known

to be mediated by microsomal cytochrome P-450 systems (22), but lack of inhibition by carbon monoxide and metyrapone indicate that cytochrome P-450 is not involved here. Likewise, microsomal aryl hydrocarbon hydroxylases are not responsible for this reaction, because there is no inhibition by α-naphthoflavone. Epoxidation of retinoic acid does not appear to occur by the same mechanism as it does for vitamin K, since the latter system can use either NADH or reduced vitamin K as a cofactor (23), and retinoic acid epoxidation has a strict requirement for NADPH (10). Similarly, the prostaglandin cyclooxygenase has no requirement for ATP or NADPH, and is therefore not a suspect (24).

There are some similarities between retinoic acid epoxidation and lipid peroxidation reactions. Ernster and co-workers described a microsomal lipid peroxidase which requires oxygen, NADPH, Fe^{2+}, and a diphosphate (25). However, their enzyme can use ADP or P_2O_7. Those cofactors do not support retinoic acid epoxidation. A similar microsomal lipid peroxidase studied by McCay and his group requires NADPH, oxygen, and Fe^{2+}, while being inhibited by Mn^{2+} and EDTA (26, 27). However, no need was demonstrated for ATP or any form of diphosphate, so the analogy is not complete.

The properties of the retinoic acid epoxidase are closest to, but not identical with, the lipid peroxidases cited above. It is unknown whether the epoxidase is a common enzyme, involved in other reactions, or whether it is a specialized enzyme, functioning only in epoxidation of retinoic acid. Two lines of evidence would tend to indicate the former possibility. To begin with, there was no relationship between epoxidase activity and vitamin A status (see Fig. 5). This suggests that there is no need to regulate the epoxidase in response to vitamin A status. If epoxidation were an activation reaction, one might expect enzyme levels to increase with progressive vitamin A deficiency. Second, the epoxidation reaction was not substrate-inducible (see Table III). Such inducibility has been seen for retinoic acid metabolism by other workers (28). The lack of inducibility as well as low levels of epoxidation *in vivo* indicates that epoxidation is not a major deactivation step.

It is interesting to note that *in vitro*, epoxidation occurs with 13-*cis*-retinoic acid in addition to all-*trans*-retinoic acid (see Table III). Isomerization at the 13,14-double bond may be an important reaction *in vivo* since it has been shown to occur in the hamster (29), and 13-*cis*-retinoic acid is also known to have high vitamin A activity (11). However, no evidence was seen in these experiments for the production of 13-*cis*-5,6-epoxyretinoic acid from all-*trans*-retinoic acid either in *in vivo* or *in vitro*. 13-*cis*-5,6-epoxyretinoic acid elutes just after, and only partially resolves from all-*trans*-5,6-epoxyretinoic acid on the reverse-phase HPLC systems used.[4] In no case was such a peak or shoulder ever observed.

The DPPD-feeding experiments reported here were designed to assess the relationship between retinoic acid epoxidation and retinoic acid activity. As shown in Fig. 6, *A* and *B*, it was found impossible to demonstrate *in vivo* inhibition of the epoxidase because the product was undetectable. The retinoic acid injected into these rats had a specific activity of 5.6×10^4 dpm/μg. It would be necessary to have about 1000 dpm to detect a peak with reasonable accuracy. Such a peak would contain about 19 ng of 5,6-epoxyretinoic acid. Since the kidneys were about 1.75 g each, this gives a detection limit of 11 ng /g of tissue. It is known that about 200 pg/g of tissue of 5,6-epoxyretinoic acid is found in rat kidney 3 h following a 9.2-μg dose of retinoic acid (8). Even though the 487-μg dose used in this experiment is 53 times the dose used in that

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[4] W. K. Sietsema and H. F. DeLuca, unpublished results.

experiment, any 5,6-epoxyretinoic acid produced was still below the detection limit.

Even though *in vivo* inhibition was not detectable, *in vitro* epoxidation was strongly inhibited by DPPD feeding of the rats used as a source of enzyme. This indicates that DPPD is getting into the tissue in amounts sufficient to block the epoxidase.

Concurrently with the *in vivo* epoxidase assay, vitamin A-deficient rats on a DPPD diet were re-fed small amounts (10 μg/day by mouth) of retinoic acid. These repleted rats began to gain weight at the same rate as rats not receiving DPPD (see Table IV). In addition, both groups reverted to a normal estrous cycle within 48 h of retinoic acid feeding. These results demonstrate that epoxidation is not necessaryy for either the growth promotion or the epithelial cell differentiation functions of retinoic acid.

Because 5,6-epoxyretinoic acid is made in small amounts, even at high dose levels, and epoxidation is not substrate-inducible, it is unlikely that epoxidation is a deactivation step. It has also been shown that 5,6-epoxyretinoic acid has very little biological activity (11, 12). In addition, epoxidation probably occurs by a nonspecific lipid peroxidation reaction. From these facts, it is concluded that retinoic acid epoxidation is coincidental and does not play a role in retinoic acid function.

Note Added in Proof—One of the retinoid standards used in these experiments was called 16-hydroxy-4-ketoretinoic acid. This compound, obtained from Hoffmann-La Roche, is more correctly called 1-hydroxymethyl-4-ketoretinoic acid. The hydroxyl is located on one of the geminal methyl groups attached to C-1. It is not clear whether the hydroxyl is on C-16 or C-17, or whether it is a mixture of the two possible isomers.

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