Metabolism of retinoic acid *in vivo* in the vitamin A-deficient rat

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Sample preparation and high-pressure liquid-chromatography separation methods useful for the study of retinoic acid metabolism are reported. The sample preparation procedure does not cause significant degradation of retinoic acid, and the gradient high-pressure liquid-chromatography separation method gives excellent separation of the major metabolites of retinoic acid. These methods were used to examine the metabolites of retinoic acid in blood, trachea and lung, testes, kidneys and small intestine of vitamin A-deficient rats dosed subcutaneously with 2 µg of [11,12-³H]retinoic acid. At 6 h after dosing, a total of eight metabolites of retinoic acid produced *in vivo* were found in the tissues examined. Of these, four were found in most of the epithelial tissues examined, and therefore may be of interest as possible active metabolites in the epithelial functions of vitamin A.

Vitamin A is required for normal growth, vision, reproduction and the maintenance of epithelial differentiation (Moore, 1957). Retinoic acid satisfies the growth and epithelial differentiation functions, but not the visual or reproductive functions of vitamin A (Dowling & Wald, 1960; Thompson *et al.*, 1964). Retinoic acid is a major metabolite of retinol *in vivo* (Emerick *et al.*, 1967; Kleiner-Bossaler & DeLuca, 1971; Ito *et al.*, 1974a) and in a vitamin A-responsive system *in vitro* (Frolik *et al.*, 1981a) when retinol is given at physiological doses. Retinol and retinoic acid are metabolized to similar products (Roberts & DeLuca, 1967; Ito *et al.*, 1974b; Frolik *et al.*, 1981a), and retinoic acid has a slight sparing effect on liver retinol stores (Krishnamurthy *et al.*, 1963; Nelson *et al.*, 1964; Keilson *et al.*, 1979). That retinoic acid is metabolically related to retinol as described and substitutes for retinol in some of its essential functions suggests that retinoic acid is a normal intermediate of vitamin A metabolism closer to the form of the vitamin responsible for maintenance of growth and of epithelial differentiation.

Although much work on the metabolism of retinoic acid has been done, few studies have examined the metabolism of physiological amounts of retinoic acid. Many studies have been done with pharmacological doses of retinoic acid, and others

have been directed to the metabolism of retinoic acid in non-target tissues (Zachman *et al.*, 1966a,b; Dunagin *et al.*, 1966; Lippel & Olson, 1968; Rietz *et al.*, 1974; Hanni *et al.*, 1976; Hanni & Bigler, 1977; Frolik *et al.*, 1980, 1981b; Zile *et al.*, 1980). The relevance of these studies to the physiological metabolism of retinoic acid in its target tissues is not known. Notable exceptions to the above are the studies by McCormick *et al.* (1978, 1980), Frolik *et al.* (1978, 1979) and Ito *et al.* (1974b). McCormick *et al.* (1978, 1980) identified 5,6-epoxyretinoic acid and showed it to be a physiological metabolite of retinoic acid found in epithelial tissues of vitamin A-deficient rats. Frolik *et al.* (1979) identified all-*trans*-4-hydroxyretinoic acid and all-*trans*-4-oxoretinoic acid, and showed that these compounds were produced from low concentrations of retinoic acid in their vitamin A-responsive tracheal organ-culture system. The physiological metabolism of retinoic acid in several tissues of the vitamin A-deficient rat was studied by Ito *et al.* (1974b). Unfortunately, the chromatographic systems available at that time gave poor separation of the polar metabolites of retinoic acid.

Most of the studies done thus far have centred on the isolation and identification of metabolites generated *in vivo* or *in vitro* rather than on the overall metabolism of retinoic acid. A study of the metabolites present in target tissues where retinoic acid exerts the same action may reveal a functionally important metabolite(s). In the present paper we report new sample preparation and chromato-

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

* No reprints are available from the authors.

graphic procedures that have been used to examine retinoic acid metabolites in epithelial tissues of vitamin A-deficient rats given a physiological dose of retinoic acid.

Experimental

Materials

[11,12-³H]Retinoic acid (31–40 Ci/mmol) was a gift from Hoffman–La Roche (Nutley, NJ, U.S.A.). This material was impure and was purified as described below. The non-radioactive retinoid standards all-*trans*-1-hydroxymethyl-4-oxoretinoic acid, all-*trans*-4-oxoretinoic acid, all-*trans*-4-hydroxyretinoic acid, all-*trans*-5,6-epoxyretinoic acid and 13-*cis*-retinoic acid were also generously provided by Hoffmann–La Roche. all-*trans*-Retinoic acid and all-*trans*-retinol were purchased from Eastman Organic Chemicals (Rochester, NY, U.S.A.), and methyl retinoate was prepared by the reaction of all-*trans*-retinoic acid with diazomethane (Zile *et al.*, 1980). All solvents used were either h.p.l.c. grade or glass-distilled and filtered through a 0.45 μ m filter (Millipore, Bedford, MA, U.S.A.). Antioxidants were added to the water and methanol used in tissue sample preparation. The water contained 150 μ g of n-propylgallate/ml, and the methanol contained 50 μ g each of n-propylgallate and butylated hydroxytoluene/ml.

Animals

Male weanling albino rats were purchased from Holtzman Co. (Madison, WI, U.S.A.) and fed on a purified vitamin A-deficient diet (DeLuca *et al.*, 1963). In 5–6 weeks most animals reached a weight-gain plateau, and 2–3 days later were used for experiments on retinoic acid metabolism.

Determination of physiological dose

Three dosages of all-*trans*-retinoic acid, 1.5, 3.0 and 6.0 μ g/day, were given, half of the daily dose every 12 h, by subcutaneous injection. The rats were weighed daily to measure the weight-gain response to retinoic acid supplementation.

Preparation of tissue samples

All work with retinoic acid and tissue samples was done under yellow light, and samples were kept under an N₂ atmosphere whenever possible. The scheme for tissue sample preparation is shown in Scheme 1. In preparation for homogenization, trachea and lungs, kidney and testes samples were rinsed with saline (0.9% NaCl) containing n-propylgallate (150 μ g/ml) and minced. The small-intestine samples were rinsed with n-propylgallate-containing saline to remove the contents, and were split longitudinally. The mucosa was scraped from the visceral wall and was kept. Plasma was

prepared from the heparinized blood samples by two centrifugation steps. The pellet from the first centrifugation (100 g, 5 min) was resuspended in n-propylgallate-containing 0.9% NaCl and centrifuged again (1000 g, 15 min). The supernatants were combined and freeze-dried. All tissue samples and freeze-dried plasma preparations were homogenized on ice in 50 ml of methanol containing n-propylgallate and butylated hydroxytoluene in a Brinkman Instruments (Westbury, NY, U.S.A.) Polytron homogenizer. The homogenates were stirred for 3 h on ice under an N₂ atmosphere to complete the extraction of retinoic acid and its metabolites from the tissue samples. Tissue solids were removed from the methanol extracts by filtration through medium-porosity fritted-glass funnels. About 90–95% of the epithelial tissue radioactivity and 60–70% of the blood radioactivity were recovered in the methanol extract. About 20–30% of the blood radioactivity remained in the 1000 g pellet, and 5–10% of the radioactivity from every sample remained in the homogenate (residue). The methanol extract of each sample was made up to a 75 ml volume with a methanol/water (95:5, v/v) composition and partitioned 1:1 (v/v) against hexane. This step gave recoveries greater than 90%, with only 3% of the recovered radioactivity partitioning in the hexane phase. The methanol/water phase was concentrated

Scheme 1. Procedures used to prepare tissue samples for h.p.l.c.

For full experimental details see the text.

first on a Buchi Rotavapor-R apparatus (Brinkman Instruments) and then under a stream of N_2 to a volume of 1 ml. This sample was applied to and eluted from a Waters Associates (Milford, MA, U.S.A.) Sep Pak C_{18} cartridge in methanol. An average of 80% of the methanol/water-phase radioactivity was recovered from this step. After concentration under a stream of N_2 , a portion of the sample containing 3×10^5 – 5×10^5 d.p.m. was used in the h.p.l.c. separation of the compounds present. The recovery of sample radioactivity from h.p.l.c. was always greater than 95%.

High-pressure liquid chromatography

The [11,12- 3H]retinoic acid received from Hoffmann-La Roche was purified to be greater than 90% all-*trans* isomer and less than 4% *cis* isomers by using reverse-phase h.p.l.c. The system employed was an Instrument Mini-Pump (Milton Roy Co., Riviera Beach, FL, U.S.A.), a Waters Associates model U6K sample injector and a μ Bondapak C_{18} reverse-phase column (4 mm internal diam. $\times$ 30 cm). The tritiated compounds were eluted isochratically at a flow rate of 1.5 ml/min with a methanol/water (63:37, v/v) solvent mixture containing 10 mM-ammonium acetate. Peak detection was by liquid-scintillation counting of radioactivity. The h.p.l.c. separations of experimental samples were done with a Beckman model 322 Gradient Liquid Chromatograph (Beckman Instruments, Irvine, CA, U.S.A.)

equipped with a Waters Associates model U6K sample injector and model 440 absorbance detector. The reverse-phase column system used was a Whatman (Clifton, NJ, U.S.A.) Guard Column (2.3 mm internal diam. $\times$ 7 cm) packed with μ Bondapak C_{18} /Porasil B resin (Waters Associates) in series with a μ Bondapak C_{18} column. The system was equilibrated with methanol/water (2:98, v/v) containing 10 mM-ammonium acetate. Samples were eluted with a linear 2 h gradient from 2:98 to 100:0 (v/v) methanol/water containing 10 mM-ammonium acetate. The non-radioactive retinoids were used as internal standards and were detected by their absorbance at 340 nm. The chromatographic separation of these compounds is shown in Fig. 1. To detect radioactive compounds, whole fractions (1.1 ml) were collected in 5.5 ml vials and to each was added 4 ml of 3a70b scintillation fluid (Research Products International Corp., Elk Grove Village, IL, U.S.A.). The radioactivity of each fraction was determined with a Packard model PLD Prias scintillation counter (Packard Instruments, Downers Gove, IL, U.S.A.) programmed to calculate sample d.p.m.

Sample-preparation control experiment

The stability of retinoic acid was tested in both systems of sample preparation. To testes and blood samples (each in triplicate) was added 2×10^6 d.p.m. of purified [11,12- 3H]retinoic acid. These samples

Fig. 1. Gradient h.p.l.c. separation of the retinoid internal standards

For full experimental details see the text. The compounds used were: A, all-*trans*-1-hydroxymethyl-4-oxoretinoic acid; B, all-*trans*-4-oxoretinoic acid; C, all-*trans*-4-hydroxyretinoic acid; D, all-*trans*-5,6-epoxyretinoic acid; E, 13-*cis*-retinoic acid; F, all-*trans*-retinoic acid; G, all-*trans*-retinol; H, methyl retinoate. The continuous line (—) shows the relative absorbance at 340 nm and the broken line (---) shows the composition of the methanol/water eluent.

(procedure-check samples) were prepared in the same fashion as the tissue metabolite samples. The h.p.l.c. profiles of the procedure-check samples were compared with profiles obtained from tissue samples (one each: testes, blood and small intestine) to which purified [11,12-³H]retinoic acid was added just before the h.p.l.c. separation step (negative-control samples). Any peak found in or markedly increased in the procedure-check samples over the amount of that in the negative-control samples was assumed to have resulted from retinoic acid by manipulation of samples.

Experiments on retinoic acid metabolism

Over the course of two experiments, ten rats were injected subcutaneously with 2 μ g of purified [11,12-³H]retinoic acid in 0.3 ml of propylene glycol. The rats were bled to death from the abdominal aorta under light ether anaesthesia 6 h after the dosing. The blood, trachea and lungs, testes, kidneys and small intestine were removed, immediately frozen on solid CO₂ and stored at -70°C. Extracts were prepared and their components separated by h.p.l.c. as described above.

Peak assignment and quantitative determination

Peaks were assigned only if they displayed reproducible peak shape and elution volume relative to the internal standards. A peak was designated as near detection limit if in no more than two of the tissue metabolite determinations the peak was poorly resolved over background. Peak 8 proved to be due to retinoic acid. The amount of metabolite in a tissue was calculated from the specific radioactivity of the [11,12-³H]retinoic acid dose assuming an absence of unlabelled retinoids from the vitamin A-deficient rats and that no ³H was lost in conversion of retinoic acid into the metabolite.

Results

Determination of physiological dose

An experiment to determine the dosage of retinoic acid necessary to elicit a response *in vivo* was performed. The subcutaneous injection of 1.5, 3.0 and 6.0 μ g of all-*trans*-retinoic acid per day into vitamin A-deficient rats gave weight-gain responses (means $\pm$ s.d.) of +0.6 $\pm$ 2.4 (n = 20), +2.6 $\pm$ 2.5 (n = 15) and +5.1 $\pm$ 1.7 g/day (n = 9) respectively. Thus 1.5 μ g of retinoic acid per day, when given in two doses, one every 12 h, resulted in maintenance of rat weight. The higher dosages gave positive weight-gain responses. On the basis of these results, 2 μ g doses of purified [11,12-³H]retinoic acid were administered in the study of tissue metabolites.

Sample-preparation control experiment

The production of metabolites from retinoic acid

as artifacts during the sample preparation was tested. Representative h.p.l.c. profiles from procedure-check and negative-control samples are shown in Fig. 2. A comparison of these profiles shows that no major peaks appeared as artifacts as a result of sample manipulation. The procedure-check samples had a small radioactive peak (0.9% of total radioactivity) that co-migrated with the all-*trans*-5,6-epoxyretinoic acid internal standard and an increase in the amount of radioactivity that was eluted with the 13-*cis*-retinoic acid internal standard. Two small peaks, found only in the blood procedure-check samples, migrated in the same region as tissue metabolite peaks 9 and 10 (Fig. 3a) and accounted for 2.7 and 1.5% of the eluted radioactivity respectively. No radioactive peaks migrating in the regions of tissue metabolite peaks 1–7 were found.

Experiments on retinoic acid metabolism

The metabolites of all-*trans*-retinoic acid in four epithelial tissues and the blood of vitamin A-deficient rats were examined. Table 1 shows the recovery of dose in each of these tissues. The h.p.l.c. separation profiles of the radioactive compounds extracted from the tissues are shown in Fig. 3. Ten metabolite peaks were reproducibly found. Tables 2 and 3 give the percentages of total radioactivity and the values for pmol/g of tissue respectively for the metabolites.

Of the known physiological metabolites of retinoic acid, only 13-*cis*-retinoic acid and all-*trans*-4-oxoretinoic acid co-migrated with tissue-sample radioactivity. The radioactivity that was eluted with the 13-*cis*-retinoic acid internal standard was found in all tissues. A quantitatively important metabolite peak that co-migrated with the all-*trans*-4-oxoretinoic acid internal standard was found only in the small intestine, and represented approx. 2.7% of the tissue radioactivity or 2.0 pmol/g of mucosa. In other tissues this component was present in very small amounts. A comparison of the h.p.l.c. profiles shows that peaks 3, 7 and 8 are present in all of the tissues examined. Also, peaks 4 and 6 are reproducibly present in all of the tissues examined except kidney, where they are present only at the limit of detection.

Discussion

If the metabolism of retinoic acid that is pertinent to its physiological actions in or to its elimination from target tissues is to be examined, three conditions must be met. Metabolites must be examined (1) in retinoic acid target tissues, (2) in an animal given a physiological dose of retinoic acid, and (3) at a time when retinoic acid is exerting its biological effect in the tissues examined. The state of differentiation of many epithelial tissues is

Fig. 2. H.p.l.c. profiles of control-experiment samples

The h.p.l.c. profiles are from: (a) procedure-check samples prepared from testes; (b) procedure-check samples prepared from blood; (c) negative-control samples. For full experimental details see the text. The internal standards used are indicated by arrows. They are (left to right): all-*trans*-1-hydroxymethyl-4-oxoretinoic acid, all-*trans*-4-oxoretinoic acid, all-*trans*-4-hydroxyretinoic acid, all-*trans*-5,6-epoxyretinoic acid, 13-*cis*-retinoic acid, all-*trans*-retinoic acid and all-*trans*-retinol. The continuous lines (—) show the ^3H radioactivity and the broken lines (---) show the composition of the methanol/water eluent.

affected by the vitamin A status of the animal (Moore, 1957; Rojanopo *et al.*, 1980). Administration of retinyl palmitate, retinol or retinoic acid to the vitamin A-deficient rat reverses most of the epithelial lesions brought about by deficiency

(Moore, 1957; Dowling & Wald, 1960; Thompson *et al.*, 1964; Rojanopo *et al.*, 1980), implicating epithelial tissues as a class of vitamin A- and retinoic acid-responsive tissues. For this reason, we have examined the metabolism of retinoic acid in epi-

Fig. 3. H.p.l.c. profiles of samples from the experiments on metabolism of retinoic acid

The h.p.l.c. profiles are from: (a) blood; (b) trachea + lung; (c) small intestine; (d) kidney. For full experimental details see the text. The internal standards are indicated by arrows. They are the same as those shown in Fig. 2. The continuous lines (—) show the ${}^3\text{H}$ radioactivity, and the broken lines (---) show the composition of the methanol/water eluent.

Table 1. *Recovery of radioactivity in tissues after administration of [11,12-³H]retinoic acid*

The values are the percentages of the administered retinoic acid dose that were recovered in each tissue examined in the retinoic acid metabolism experiments. For full experimental details see the text. All results are expressed as means $\pm$ S.D.

Tissue	Recovery (%)
Blood ($n = 9$)	0.83 ± 0.25
Testes ($n = 6$)	0.32 ± 0.10
Trachea + lungs ($n = 10$)	0.22 ± 0.10
Small intestine ($n = 6$)	4.3 ± 1.5
Kidney ($n = 10$)	2.5 ± 1.3

thelial tissues of the rat. It is well known that retinoic acid can also substitute for retinol in the maintenance of growth (Dowling & Wald, 1960; Malathi *et al.*, 1963; Zile & DeLuca, 1968). In the growth assay reported in the present paper, the lowest dosage of all-*trans*-retinoic acid tested, $1.5 \mu\text{g/day}$ ($0.75 \mu\text{g}$ every 12 h), gave weight maintenance in the retinol-depleted rat. The higher supplementation dosages, 3.0 and $6.0 \mu\text{g/day}$ (1.5 and $3.0 \mu\text{g}$ every 12 h), gave positive weight-gain responses. As in the growth assay, the metabolism of retinoic acid was examined in the vitamin A-deficient rat. A $2 \mu\text{g}$ dose was administered to each rat and the animals were

Table 2. *Metabolites of retinoic acid in tissues after the administration of [11,12-³H]retinoic acid*

The values are the percentages of the total tissue radioactivity found in the retinoic acid metabolism experiments. For full experimental details see the text. All results are expressed as means $\pm$ S.D.

Metabolite peak no.	Radioactivity recovered in tissues (% of total)				
	Blood ($n = 9$)	Testes ($n = 6$)	Trachea + lung ($n = 10$)	Small intestine ($n = 6$)	Kidney ($n = 10$)
1	1.2 ± 0.4	2.1 ± 0.2	*	†	*
2	3.3 ± 0.6	5.8 ± 0.6	1.6 ± 0.9	†	*
3	6.6 ± 1.2	9.0 ± 0.5	4.4 ± 2.8	$4.6 \pm$	2.6 ± 1.1
4	9.6 ± 2.2	13 ± 1	5.4 ± 2.8	$5.7 \pm$	*
5	2.5 ± 0.8	3.1 ± 0.3	—	†	—
6	15 ± 5	14 ± 2	7.6 ± 3.7	$3.6 \pm$	*
7	2.8 ± 0.8	6.6 ± 1.4	3.3 ± 0.6	12 ± 4	2.9 ± 0.5
8	36 ± 10	14 ± 9	38 ± 9	11 ± 3	60 ± 8
9	1.2 ± 0.8	—	*	—	4.2 ± 2.3
10	1.2 ± 1.0	—	—	—	—

* Peak present but near the detection limit in no more than two of the tissue determinations.

† Presence of peak not determinable owing to high background radioactivity.

‡ Approximate value owing to incomplete peak separation.

Table 3. *Concentration of metabolites of retinoic acid in tissues*

The values given are those found in the retinoic acid metabolism experiments. For full experimental details see the text. All results are expressed as means $\pm$ S.D.

Metabolite peak no.	Concentration of metabolite in tissues (pmol/g or pmol/ml)				
	Blood ($n = 9$)	Testes ($n = 6$)	Trachea + lung ($n = 10$)	Small intestine ($n = 6$)	Kidney ($n = 10$)
1	0.12 ± 0.08	0.23 ± 0.07	*	†	*
2	0.30 ± 0.18	0.63 ± 0.16	0.22 ± 0.18	†	*
3	0.60 ± 0.36	0.97 ± 0.22	0.62 ± 0.51	$3.3 \pm$	3.0 ± 2.1
4	0.90 ± 0.57	1.4 ± 0.3	0.74 ± 0.57	$4.0 \pm$	*
5	0.22 ± 0.13	0.33 ± 0.09	—	†	—
6	1.4 ± 1.0	1.5 ± 0.4	1.0 ± 0.8	$2.4 \pm$	*
7	0.23 ± 0.09	0.75 ± 0.31	0.41 ± 0.25	8.6 ± 5.0	3.1 ± 1.5
8	3.1 ± 1.4	1.5 ± 0.4	4.3 ± 2.0	8.8 ± 4.5	64 ± 28
9	0.10 ± 0.06	—	*	—	4.5 ± 3.3
10	0.11 ± 0.08	—	—	—	—

* Peak present but near the detection limit in no more than two of the tissue determinations.

† Presence of peak not determinable owing to high background radioactivity.

‡ Approximate value owing to incomplete peak separation.

killed 6 h later. This 6 h time point was chosen as the midpoint of the 12 h interval over which a growth effect was seen as a result of supplementation with low doses (0.75 μ g) of retinoic acid. It was assumed that the maintenance of epithelial differentiation and overall rat growth were of comparable sensitivity to retinoic acid supplementation, and therefore that the metabolism of retinoic acid pertinent to its action in epithelial tissues would be seen.

A total of nine unidentified metabolite peaks in the h.p.l.c. profiles from epithelial tissues and blood of retinoic acid-dosed vitamin A-deficient rats were found. Additionally, we have found radioactive peaks co-migrating with all-*trans*-4-oxoretinoic acid in the small-intestine preparations and peaks co-migrating with 13-*cis*- and all-*trans*-retinoic acid in all tissue preparations. However, control experiment results show that all-*trans*-retinoic acid can be converted into artifact peaks that are eluted with peaks 9 and 10. These components account for the percentage of the sample radioactivity that is eluted as peaks 9 and 10 in the experimental blood samples. The control experiments also show that the all-*trans*-retinoic acid, to some degree, is converted into 13-*cis*-retinoic acid during preparation of tissue samples. Therefore the 13-*cis*-retinoic acid found in the experimental samples cannot be solely attributed to isomerization *in vivo*. Thus we have found that a total of eight unidentified metabolite peaks and one previously identified compound, 4-oxoretinoic acid, are made in the retinoic acid-dosed vitamin A-deficient rat. A tissue-to-tissue comparison of these metabolites found may point out which of these metabolites belong to a pathway of retinoic acid metabolism common to retinoic acid-responsive epithelial tissues. Metabolite peaks 3, 4, 6 and 7 are found in most of the epithelial tissues examined, and therefore may be of interest for future isolation and identification work.

At first review, the results presented here on the identified target-tissue metabolites of retinoic acid, 5,6-epoxyretinoic acid, all-*trans*-4-hydroxyretinoic acid and all-*trans*-4-oxoretinoic acid, do not appear to agree with the results from previous work. McCormick *et al.* (1980) demonstrated 5,6-epoxyretinoic acid to be a physiological metabolite of retinoic acid in several epithelial tissues and the serum of vitamin A-deficient rats 3 h after a 2.2 μ g dose of retinoic acid. In the present work, 5,6-epoxyretinoic acid was not found when metabolites were examined 6 h after the administration of a 2 μ g dose of retinoic acid to vitamin A-deficient rats. Time-course work reported by McCormick *et al.* (1980) has shown that the tissue concentration of 5,6-epoxyretinoic acid was maximal (100 pg/g) in the small intestine at 3 h, but undetectable 7 h after dosing. Therefore one possible explanation for the apparent absence of 5,6-epoxyretinoic acid in our

experiments is that 6 h after dosing the tissue concentration of this compound had decreased to values below the detection limits of our techniques. We have also failed to find all-*trans*-4-hydroxyretinoic acid and all-*trans*-4-oxoretinoic acid as quantitatively important metabolites of retinoic acid in trachea + lung samples. Frolik *et al.* (1979) showed that these compounds were made in their tracheal organ-culture system from a low (5 nM) concentration of retinoic acid. The difference in results may be due to differences in the retinoic acid-metabolizing systems of the rat and of the hamster, and in the conditions employed. In the work by Frolik *et al.* (1978), retinoic acid metabolism was examined in hamster tracheal organ culture 24 h after the addition of retinoic acid, whereas in the work reported in the present paper the metabolites from the trachea and lung of the vitamin A-deficient rat were examined 6 h after administration of a physiological dose of retinoic acid.

The most thorough work exploring the distribution and disposition of retinoic acid in tissues of the vitamin A-deficient rat has been done by Ito *et al.* (1974b). Their work included studies of the time-course distribution of the dose radioactivity and a study of the tissue distribution of the metabolite peaks found 6 h after dosing. A comparison of the values presented in the present paper and those found by Ito *et al.* (1974b) for the recovery of dose and the ratio of polar metabolites of retinoic acid to retinoic acid found in the tissues were not in close agreement. Our experiment differed from that performed by Ito *et al.* (1974b) in the route and amount of retinoic acid administered. In the study by Ito *et al.* (1974b), vitamin A-deficient rats received 10–13 μ g of retinoic acid by intrajugular injection, whereas we injected 2 μ g doses by the subcutaneous route. These procedural differences may explain the differences found in the comparison of the 6 h time-point data reported by Ito *et al.* (1974b) and the results given in the present paper.

Eight unidentified metabolites of retinoic acid, which account for a large proportion of total tissue radioactivity, have been found in epithelial tissues of the vitamin A-deficient rat. Although four of these metabolite peaks may be part of a common metabolic pathway for retinoic acid, whether they are activation or excretory metabolites of retinoic acid is not known.

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