The in vitro metabolism of all-trans-[11,12-3H]retinoic acid to several more polar compounds has been demonstrated in a hamster tracheal organ culture system. The production of these metabolites is dependent on the presence of tissue. The physiological significance of these compounds is shown by the cochromatography of several of the in vitro formed metabolites synthesized from [carbon-14]retinoic acid with metabolites isolated from the intestine and urine of hamsters previously injected with 0.1 to 1.5 mg of [3H]retinoic acid. One of the metabolites shows about one-tenth the biological activity of all-trans-retinoic acid when tested in a hamster tracheal organ culture assay. This biologically active metabolite is converted by the hamster trachea in vitro to a biologically inactive metabolite.

Thirty-two years ago Arens and van Dorp began the quest for the active form of vitamin A necessary for maintaining proper tissue differentiation by reporting the chemical synthesis of all-trans-retinoic acid (1). While the role of retinoic acid remains unclear, it has not only been shown to be a physiological metabolite of retinyl acetate (2, 3), but several reports have also indicated that it may be further metabolized to a biologically active compound (4-9). These metabolites, however, either have not been identified (4-8) or have been shown to be possible artifacts of isolation (7, 9).

Although little progress has been made in recent years in the identification of active metabolites of retinoic acid, many reports have been published on the in vitro metabolism of all-trans-retinoic acid. From these reports, it appears that retinoic acid may undergo decarboxylation (10-16), esterification (17), glucuronic acid conjugation (M-22), and isomerization (23), as well as hydroxylation and cleavage (16, 23). The physiological significance of these reactions, however, is unclear due to the ease of the production of artifacts during extraction and purification (2, 9, 22, 24) and to the use of milligram quantities of retinoic acid to obtain the metabolite profiles (16, 18-20, 22, 23).

Recently, a hamster tracheal organ culture technique has been developed (25, 26) for assay of vitamin A activity in vitro. The trachea, maintained in a serum-free, chemically defined, vitamin A-deficient medium undergoes keratinized squamous metaplasia characteristic of vitamin A deficiency. Addition of nanomolar concentrations of vitamin A to the organ culture, even after the development of such metaplasia, causes reversal of the process of keratinization and replacement of the squamous cells by columnar ciliated and mucous-producing cells. It was felt that this in vitro target organ system could be advantageously utilized to answer the question of whether retinoic acid itself or one of its metabolites is responsible for the maintenance of normal epithelial differentiation. In this report, the in vitro tissue-dependent metabolism of retinoic acid in the hamster trachea is demonstrated and the in vitro biological activity of several of these metabolites is examined.

**Experimental Procedures**

**Chemicals—**Nonradioactive all-trans-retinoic acid and all-trans-[11,12-3H]retinoic acid (8.4 x 10^6 dpm/mg and 8.2 x 10^6 dpm/mg) were supplied by Hoffmann-La Roche. The tritiated material was purified immediately before use by HPLC utilizing an analytical 10-μm Partisil ODS-2 column (Whatman) as described earlier (27). All-trans-[15-3H]retinoic acid (7.1 x 10^6 dpm/mg) was purchased from Amerham/Searle, and did not require purification before use. CMRL-1066 medium was purchased from Grand Island Biological Co. and the Patho-Cyte-5 bovine albumin was from Miles Laboratories.

**In Vitro Metabolism—**Hamster tracheal organ cultures were prepared as described earlier (25, 26) except that 1.3 to 1.6 x 10^-5 m low specific activity [11,12-3H]retinoic acid in dimethylsulfoxide (final concentration, 0.2%) rather than unlabeled retinoid was added after the 3 to 6-day preincubation period in the absence of vitamin A. Addition of 0.1 to 0.2% Path-O-Cyte-5 bovine albumin and 0.25 to 0.5 mg/ml of L-ascorbic acid was found to be necessary to avoid nonspecific degradation of the [3H]retinoic acid in the aqueous medium over a 24-h period. Addition of these stabilizing agents had little effect on the ability of retinoic acid to reverse the process of keratinization in vitro. Control cultures (containing no tissue) were incubated in the presence of the [3H]retinoic acid-supplemented medium under conditions identical to those used for the experimental cultures containing tracheae. After incubation with the [3H]retinoic acid for 1 to 24 h, the medium was removed and lyophilized, and the residue was extracted with chloroform:methanol (1:1) and then methanol following the slightly modified procedure of Ito et al. (3, 27). The tracheal epithelium was scraped from the supporting tissue (28) into 0.25 ml of homogenization solution (0.028 M sodium ascorbate, 0.014 M trisodium ethylenediaminetetraacetic acid) per trachea, homogenized with a tissumizer (Tekmar, Cincinnati, OH), and extracted using the lyophilization technique described above. Aliquots (0.01 to 0.1 ml) of the final extracts in methanol were analyzed directly by HPLC. 

**HPLC of Retinoids—**Chromatography of the extracts was performed as described previously (27) using a 10-μm Whatman Partisil ODS-2 column (4.6 mm inside diameter x 25 cm) and a flow rate of 1.1 ml/min. For general metabolite separation, the column was developed for 5 min with acetonitrile:1% ammonium acetate (2-98%), followed by a 35-min linear gradient from 2-98 to 75-25 acetonitrile:1% ammonium acetate with a 10-min hold 6 to 8 min into the gradient. One-minute fractions were collected and counted on a Packard Tri-Carb model 300 liquid scintillation spectrometer as described elsewhere (27).

**Comparison of In Vivo with In Vitro Metabolism—**High specific activity [11,12-3H]retinoic acid (see legend for Figs. 2 and 3 for concentrations) in 50 μl of ethanol:0.9% NaCl (1:1) was injected intraperitoneally into vitamin A-deficient hamsters (♂). Six hours after injection, the small intestine was removed and the urine was collected directly from the bladder. The mucosa was scraped from the intestine producing cells. It was felt that this in vitro target organ system could be advantageously utilized to answer the question of whether retinoic acid itself or one of its metabolites is responsible for the maintenance of normal epithelial differentiation. In this report, the in vitro tissue-dependent metabolism of retinoic acid in the hamster trachea is demonstrated and the in vitro biological activity of several of these metabolites is examined.

* A preliminary report of this work has been presented (Frolik, C., Tavela, T. E., and Sporn, M. B. (1977) Fed. Proc. Abstr. 4573). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Metabolism of Retinoic Acid

and homogenized with the tissumizer in 2 parts homogenizing solution plus 25 \( \mu \)g of unlabeled retinoic acid. To the homogenate was added used tissue culture medium that had been incubated for 24 h in the presence of trachea and 5 \( \times \) 10\(^{-7}\) M [15\(^{14}C\)]retinoic acid. The combined solution was then extracted by the lyophilization procedure and chromatographed as described in the legend to Fig. 2. The urine was also extracted using the lyophilization technique. The resulting chloroform:methanol extract was mixed with a chloroform:methanol extract of tissue culture medium previously incubated for 24 h in the presence of trachea and 4.4 \( \times \) 10\(^{-7}\) M [15\(^{14}C\)]retinoic acid. The combined extracts were chromatographed as described in the legend to Fig. 3.

Biological Activity and Metabolism of Metabolites—In vitro biological activity of three major metabolites of retinoic acid as well as unchanged retinoic acid was determined using the tracheal organ culture technique described elsewhere (25, 26). Briefly, the tracheas were removed from vitamin A-deficient, 30- to 33-day-old hamsters, split longitudinally, and allowed to incubate 3 days in a vitamin A-deficient culture medium (25, 26) at 37°C in an oxygen:carbon dioxide (50:45) atmosphere. After 3 days, some trachea were collected and the rest were cultured for an additional 6 days in medium either containing no added vitamin A or containing one of the compounds to be tested. At time of collection, all trachea were fixed in 10% buffered formalin and embedded in paraffin. Cross-sections of 5 \( \mu \)m were made through the midportion and stained with hematoxylin and eosin. The status of the epithelium was graded in regards to both the extent of squamous metaplasia and to the presence or absence of keratin and keratohyaline granules. The metabolites were obtained from a 24-h incubation of 40 to 80 tracheas in a medium containing 2 to 9 \( \times \) 10\(^{-7}\) M retinoic acid (a mixture of low specific activity [11,12\(^{3}H\)]retinoic acid and nonradioactive retinoic acid for a final specific activity of 1 to 3 \( \times \) 10\(^{6}\) dpm/\( \mu \)g). Extraction and chromatography was as before, except that aliquots of the column effluent were counted and the peak areas were combined, evaporated to dryness, and redissolved in a small volume of 100% dimethylsulfoxide for bioassay. In order to determine whether the metabolites of retinoic acid were further metabolized, the culture medium from the biological activity assay of the various metabolites was extracted and chromatographed as before.

![Fig. 1. HPLC of the radioactive material extracted into chloroform:methanol after a 24-h incubation of 1.3 \( \times \) 10\(^{-7}\) M all-trans-retinoic acid with hamster trachea. Chromatography was performed on a Partisil ODS-2 column using an acetonitrile:1% ammonium acetate gradient as described under "Experimental Procedures." A, chromatography of the radioactive material present in the chloroform:methanol extract of the tracheal epithelium.](http://www.jbc.org/)
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Comparison of in Vivo and in Vitro Metabolism of Retinoic Acid—The physiological significance of the tissue-dependent in vitro conversion of retinoic acid to more polar compounds rests with a demonstration that these same metabolites are also produced in vivo. In order to determine this, [3H]retinoic acid was injected intrajugularly into vitamin A-deficient hamsters and the intestinal mucosa or urine was collected 6 h later. The radioactive material obtained from the extraction of this mucosa or urine was co-chromatographed with material obtained from an in vitro incubation of carboxy-labeled [14C]retinoic acid with hamster trachea. It can be seen in Figs. 2 and 3 that the in vitro synthesized compounds from the intestine (Fig. 2A) and urine (Fig. 3A) co-chromatograph with the material produced in vitro. In order to further verify this observation, the peak 4 region from the intestinal extract and the peak 2 region from the urinary extract were rechromatographed using a chromatographic system designed for maximal separation of these metabolites. Again the intestinal peak 4 material (Fig. 2B) and the urinary peak 2 material (Fig. 3B) co-chromatographed with the in vitro produced compounds. It therefore appears that the in vitro metabolites do indeed show a very close correspondence to the metabolites synthesized in vivo.

One further point of interest is also demonstrated by these experiments. The in vitro metabolites do not appear to be decarboxylation products of retinoic acid. This is apparent by the similar chromatographic profiles of the extracts from cultures treated with the terminal carboxy-labeled [14C]retinoic acid (Figs. 2A and 3A) as compared to those treated with [3H]retinoic acid (Fig. 1).

Time Course of in Vitro Metabolism—Fig. 4A shows the appearance in the incubation medium of the more polar metabolites of retinoic acid as a function of time. It can be seen that there is a time-dependent decrease in the percentage of radioactive material present in the medium as unchanged retinoic acid with a proportionate increase in peaks 1 through 5. However, in the control incubations (Fig. 4B) the retinoic acid levels remain constant over the 24-h period with little or no radioactive material appearing in the more polar regions of interest. It should be emphasized that this time course study monitored the appearance of metabolites into the medium and may not reflect the time course of production of the compounds in the epithelium. When the epithelial extracts were examined, only approximately 0.3% of the [3H]retinoic acid added to the medium was found to be present at any one time in the epithelium. This was not a sufficient level of radioactive material to obtain an accurate determination of the metabolites present at the various time points.

Biological Activity of the Metabolites of Retinoic Acid—Peaks 2, 4, 5, and 6 (unchanged retinoic acid) were isolated in quantities large enough to test for their biological activity in the tracheal organ culture system (Table I). With this assay, all-trans-retinoic acid displays full activity in the reversal of keratinization at 1 X 10^-9 M. No metabolites were found to be as active or more active than crystalline all-trans-retinoic acid. As seen in Table I, peaks 2 and 4 were inactive in this assay even at 1.4 to 2.6 X 10^-9 M. Peak 5, at 1.3 X 10^-8 M, however, showed activity.

![Fig. 2. Co-chromatography of metabolites formed in vitro with metabolites synthesized in vivo from the intestinal mucosa. Intestinal mucosa obtained 6 h after an intrajugular injection of 1.5 μg (1.3 X 10^-7 dpm/animal) of [11,12-3H]retinoic acid into five vitamin A-deficient hamsters was combined with culture medium that had been incubated for 24 h in the presence of trachea and 5 X 10^-7 M [15-14C]retinoic acid. The combined solution was lyophilized and extracted as described under "Experimental Procedures." A, the chloroform:methanol extract was chromatographed on a Partisil ODS-2 column using the acetonitrile:1% ammonium acetate gradient described under "Experimental Procedures." Aliquots (250 μl) were counted and the peak 4 region of the effluent was rechromatographed (B) on a Partisil ODS-2 column using a 30-min linear gradient from 20:80 to 25:75 acetonitrile:1% ammonium acetate and a flow rate of 1.1 ml/min. 3H radioactivity from in vivo injection, —; 14C radioactivity from in vitro incubation, ——.
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**Fig. 3.** Co-chromatography of metabolites formed in vitro with metabolites synthesized in vivo from the urine. Urine was collected directly from the bladder of five vitamin A-deficient hamsters injected 6 h previously with 0.1 μg (8.4 × 10⁶ dpm/animal) of [11,12-²H]retinoic acid. The chloroform:methanol extract of the urine (see "Experimental Procedures" for extraction procedure) was combined with the chloroform:methanol extract of culture medium previously incubated for 24 h in the presence of trachea and 4.4 × 10⁻⁷ M [15⁻¹⁴C]retinoic acid. A, the combined extracts were chromatographed on a Partisil ODS-2 column using the acetonitrile:1% ammonium acetate gradient described under "Experimental Procedures." Aliquots (250 μl) were counted and the peak 2 region of the effluent was rechromatographed on a Partisil ODS-2 column using an isocratic solvent system consisting of acetonitrile:1% ammonium acetate (7:5:2:5) and a flow rate of 1.1 ml/min. ²H radioactivity from in vivo injection, ---; ¹⁴C radioactivity from in vitro incubation, ----.

**Fig. 4.** Time course of appearance of polar metabolites of retinoic acid in the chloroform:methanol extract of culture medium (A) in the presence of trachea and (B) in the absence of trachea. Vitamin A-deficient hamster tracheas were incubated and the culture medium extracted and chromatographed as described under "Experimental Procedures." The various peak regions correspond to those regions designated in Fig. 1 with peak 6 corresponding to all-trans-retinoic acid. Time points were 1, 6, 12, and 24 h. Retinoic acid, ---; peak 1, ---; peak 2, ••••••; peak 3, ---••; peak 4, ---•; peak 5, ---••••••; peak 6, ---••••••.

**Further Metabolism of the Isolated Metabolites—**Following incubation of the various polar metabolites of retinoic acid at a concentration of 1.3 to 1.7 × 10⁻⁸ M for 48 to 72 h in the presence of trachea, the medium was extracted and the chloroform:methanol extract was chromatographed. Peaks 2 and 4 showed no chromatographic change in elution position.
TABLE I

In vitro biological activity of retinoic acid and its metabolites

The polar metabolites of retinoic acid were isolated as described under "Experimental Procedures" and assayed in the tracheal organ culture system as described elsewhere (25, 26). Numbers in parentheses denote number of cultures used for each concentration. Cultures were graded as to the percentage of their total epithelium showing squamous metaplasia on 4 to 8 cross-sections from the middle of each trachea; if less than 2% of the total epithelial length was squamous, it was graded as having minimal squamous metaplasia; between 2 to 10% was graded as mild; between 10 and 40% was graded as marked; and greater than 40% was graded as severe.

<table>
<thead>
<tr>
<th>Treatment of cultures</th>
<th>Per cent of cultures with respective amounts of squamous metaplasia</th>
<th>Per cent of cultures with keratin and keratohyaline granules</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>None</td>
<td>Minimal</td>
</tr>
<tr>
<td>No retinoid, collected Day 3 (29)</td>
<td>10</td>
<td>48</td>
</tr>
<tr>
<td>No retinoid, collected Day 10 (30)</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>Retinoic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 x 10^{-7} M (10)</td>
<td>10</td>
<td>80</td>
</tr>
<tr>
<td>1 x 10^{-8} M (31)</td>
<td>29</td>
<td>39</td>
</tr>
<tr>
<td>1 x 10^{-9} M (19)</td>
<td>5</td>
<td>26</td>
</tr>
<tr>
<td>Peak 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.4-1.7 x 10^{-4} M (16)</td>
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<td>0</td>
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</tr>
<tr>
<td>1.4-1.7 x 10^{-6} M (6)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Peak 4</td>
<td></td>
<td></td>
</tr>
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<td>1.6-2.6 x 10^{-8} M (18)</td>
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<td>7</td>
</tr>
<tr>
<td>1.6-2.6 x 10^{-9} M (22)</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>Peak 5</td>
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<tr>
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<td>9</td>
<td>55</td>
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<td>0</td>
</tr>
<tr>
<td>1.3-5.3 x 10^{-10} M (14)</td>
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<td>7</td>
</tr>
<tr>
<td>Peak 6</td>
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<td>1.8 x 10^{-8} M (3)</td>
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</tr>
<tr>
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<td>45</td>
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**DISCUSSION**

The in vitro tissue-dependent metabolism of all-trans-retinoic acid to more polar metabolites has been demonstrated. These metabolites formed in vitro behave similarly to metabolites formed in vivo in several different chromatographic systems. It is interesting to note that the intestine contains few or no radioactive compounds migrating in the peak 2 from the column after their exposure to the tissue (data not shown). Peak 5, however, showed a significant conversion to material migrating in the peak 4 region of the chromatogram (Fig. 5B). This shift in the elution position of peak 5 did not occur when the peak 5 was incubated in the absence of trachea for a similar length of time (Fig. 5B). It therefore appears that the biologically active peak 5 can be converted to material migrating similarly to the biologically inactive peak 4.

**Fig. 5.** Metabolism of peak 5 in vitro by hamster tracheal organ cultures. Peak 5 was isolated from tracheal organ culture medium and reincubated as described under "Experimental Procedures." The chloroform:methanol extract of the culture medium was chromatographed on the same chromatographic system used in Fig. 1. A, chromatography of peak 5 prior to reincubation. B, chromatography of the chloroform:methanol extract of the culture medium after incubation in the presence of trachea (---) and in the absence of trachea (----).
region of the column effluent (Fig. 2A) while this appears to be the major metabolite present in the urine (Fig. 3A). This, together with its apparent lack of biological activity (Table I) and its inability to be further metabolized, suggests that peak 2 is an excretion form of retinoic acid. Preliminary experiments treating in vitro formed peak 2 with sulfate or β-glucuronidase did not change its elution position from HPLC. It does not appear, therefore, to be a sulfate or glucuronic acid conjugate of retinoic acid.

From the metabolism studies shown in Fig. 5, a tentative metabolic pathway can be postulated in which retinoic acid is converted to peak 5 which in turn is metabolized to peak 4. It is still too early to determine whether this would be an activation or an inactivation pathway. Although peak 5 does show some biological activity, it should be kept in mind that the apparent inactivity of peaks 2 and 4 and the decreased activity of peak 5 may be due to the inability of these metabolites to reach the proper active site inside the cell. Alternatively, at least for peak 5, the further metabolism of this compound may be too rapid for it to reach its full biological potency. Also because the compounds are still impure, the presence of various lipids or other factors may inhibit the full activity of the metabolites.

It has been demonstrated in this report that none of these metabolites have undergone decarboxylation, although there are many references in literature showing decarboxylation of all-trans-retinoic acid (10–16). This apparent discrepancy could perhaps best be explained by examining the levels of retinoic acid administered. In this report, the in vitro studies employed a maximum concentration of 8.7 x 10^{-7} M (0.26 μg/ml) retinoic acid and the in vivo experiments involved injection of 0.1 to 1.5 μg of retinoic acid/animal. In past experiments, levels of retinoic acid administered ranged from 14.5 μg (11) to 27.5 mg (16, 23). This could possibly indicate a difference in the metabolism of retinoic acid at levels close to the physiological levels compared to that occurring at higher pharmacological concentrations. Furthermore the experiments described in this paper employed vitamin A-deficient hamsters, while the experiments demonstrating decarboxylation utilized either vitamin A-deficient, vitamin A-deficient supplemented with retinoic acid, or normal rats.

Since the in vitro system described in this report has been shown to be capable of the tissue-dependent metabolism of all-trans-retinoic acid to metabolites similar to those found in vivo, it should prove useful in the work currently underway involving identification of the metabolites of retinoic acid and the search for a possible "active form."

**Acknowledgment**—We thank Dr. Peter Roller for the mass spectral analysis of retinoic acid.

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