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Isolation and Identification of 4-Hydroxy- and 4-Oxoretinoic Acid. In Vitro Metabolites of *all-trans*-Retinoic Acid in Hamster Trachea and Liver†

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ABSTRACT: Incubation of [^{3}H]retinoic acid in the presence of hamster liver 10000*g* supernatant produces several metabolites that are more polar than the parent compound. Two of these metabolites are identical with synthetic *all-trans*-4-hydroxyretinoic acid and *all-trans*-4-oxoretinoic acid both in ultraviolet absorption and mass spectral characteristics and in migration rates on two different reverse-phase high-pressure liquid chromatographic systems. The metabolites produced in a cell-free liver incubation reaction also migrate on a high-pressure liquid chromatography column together with metabolites isolated from a tracheal organ culture system. Both the metabolites and the synthetic standards show less biological activity than the parent *all-trans*-retinoic acid in a tracheal organ culture assay.

Retinoic acid has been shown to be a normal intermediate in the metabolism of retinol and its esters (Crain et al., 1967; Deshmukh et al., 1965; Dunagin et al., 1964; Emerick et al., 1967; Ito et al., 1974; Kleiner-Bössaler & DeLuca, 1971). It is known to be active in supporting growth (Krishnamurthy et al., 1963; Malathi et al., 1963; Zile & DeLuca, 1968) and in maintaining epithelial differentiation (Dowling & Wald, 1960). The metabolism of retinoic acid has been actively studied in the past [for example, DeLuca & Roberts (1969), Dunagin et al. (1965, 1966), Ito et al. (1974), Lippel & Olson (1968), Nath & Olson (1967), Nelson et al. (1971), Sundaresan & Therriault (1968)], but it is not yet apparent as to whether it is the parent compound itself or a further metabolite that is the final active form of retinoic acid in controlling epithelial differentitation.

With the application of high-pressure liquid chromatography (LC)[1] to the separation of retinoids (Frolik et al., 1978a; McCormick et al., 1978a), there has been a renewed interest in the search for a possible active metabolite of *all-trans*-retinoic acid. This resurgence began with the identification of several rat fecal and urinary metabolites by Hänni and co-workers (Hänni et al., 1976; Hänni & Bigler, 1977). After a single intraperitoneal dose of 27.2 mg of *all-trans*-retinoic acid into vitamin A normal rats, these investigators isolated and identified 4-oxoretinoic acid as well as the all-trans and 9-cis isomers of 5′-hydroxyretinoic acid from the feces. They have also found several decarboxylated metabolites in the urine. These compounds, however, being found in the feces and urine, are most likely excretion products and do not represent an "active" form of retinoic acid.

More recently, 5,8-oxyretinoic acid has been implicated as a possible in vivo metabolite of retinoic acid in the rat intestinal mucosa (Napoli et al., 1978). However, since this compound is readily obtained from 5,6-epoxide under the acidic conditions employed in the isolation procedure (John et al., 1967; Morgan

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[1] Abbreviations used: LC, high-pressure liquid chromatography; Tris, tris(hydroxymethyl)aminomethane.

& Thompson, 1966), it has been suggested that the in vivo product is most likely the 5,6-epoxide of retinoic acid (McCormick et al., 1978b; Napoli et al., 1978).

Most previous investigations have examined the in vivo metabolism of retinoic acid. However, recent studies (Frolik et al., 1978b) have focused on the in vitro metabolism of this retinoid by using a tracheal organ culture system that is responsive to retinoids in vitro (Clamon et al., 1974; Sporn et al., 1974). This technique avoids the complicating effects of absorption, tissue distribution, storage, and excretion. With this method a tissue-dependent metabolism of retinoic acid to several more polar metabolites has been observed (Frolik et al., 1978b). These in vitro metabolites migrated together after LC with either intestinal or urinary metabolites formed in vivo. One of these metabolic regions, peak 5, was shown to retain some biological activity in the tracheal organ culture assay system. This paper reports the identification of two of the metabolites from this peak 5 region as 4-hydroxy- and 4-oxoretinoic acid.

Experimental Procedure

Chemicals. Nonradioactive *all-trans*-4-hydroxyretinoic acid, *all-trans*-4-oxoretinoic acid, and *all-trans*-retinoic acid as well as *all-trans*-[11,12-^{3}H]retinoic acid (8.4×10^6 dpm/μg) were supplied by Hoffmann-La Roche Inc., Nutley, NJ. The tritiated material was purified immediately before use by LC on an analytical 10-μm Partisil-10-ODS-2 column (Whatman Inc., Clifton, NJ) as previously described (Frolik et al., 1978a). *all-trans*-[15-^{14}C]Retinoic acid (7.1×10^4 dpm/μg) was purchased from Amersham/Searle, Arlington Heights, IL, and did not require further purification before use.

In Vitro Incubation and Extraction. Male and female vitamin A deficient hamsters (30–33 days old) were raised as described previously (Frolik et al., 1978a). For the liver supernatant incubation, the hamsters were given an oral dose of 1.5 mg of retinoic acid (in ethanol–trioctanoin, 1:3, containing 0.025% butylated hydroxytoluene) per day for 3 days immediately prior to sacrifice. This was shown to be necessary for optimal in vitro metabolism of retinoic acid (Roberts et al., 1979a,b). After sacrifice the liver was removed, homogenized in 5 parts of buffer [0.15 M potassium chloride and 0.05 M Tris (pH7.4)] per g of tissue, and centrifuged at 10000*g* for 10 min. To an 8-mL aliquot of the supernatant (diluted with buffer, 1:1) was added 2.2 μmol of NADP (or its reduced form), 18 μmol of glucose 6-phosphate, 31 units of glucose-6-phosphate dehydrogenase, 1.6 mg of bovine serum albumin, 40 μmol of $MgCl_2$, 1.2 mmol of KCl, and 0.4 μmol of Tris (pH 7.4) to make a final total volume of 16 mL (Roberts et al., 1979a). In the heat-killed control incubations, the supernatant was placed into a boiling water bath for 30 min before the addition of the cofactors. Immediately before incubation, 100 μL of methanol containing [11,12-^{3}H]retinoic acid (diluted to a specific activity of approximately 40 000 dpm/μg with nonradioactive retinoic acid) was added to the reaction mixture, giving a final retinoid concentration of (2–3) $\times 10^{-6}$ M.

The solution was incubated at 37 °C, under air with shaking (120 oscillations per min). At the end of 75 min, 10 μg of unlabeled retinoic acid and 5 mg each of ascorbic acid and Na_3EDTA were added to each flask. The mixture was extracted with methanol containing 50 μg of butylated hydroxytoluene per mL by the lyophilization technique described elsewhere (Frolik et al., 1978a; Ito et al., 1974). The methanol extract was evaporated to dryness under nitrogen, and the residue was redissolved in methanol–1-butanol, 0.05:1, and extracted four times with 1-butanol-saturated water. The final butanol layer was evaporated to dryness in a Vortex evaporator (Buchler Instruments, Fort Lee, NJ) and redissolved in a small volume of methanol for chromatography.

The in vitro tracheal metabolites used for chromatography with the liver peak 5 compounds were obtained from the hamster tracheal organ culture system described previously (Frolik et al., 1978b). The culture medium was extracted with methanol and with butanol–water as described above.

High-Pressure Liquid Chromatography. All chromatography was done on a Spectra Physics Model 3500 B (Spectra Physics, Santa Clara, CA) instrument as mentioned elsewhere (Frolik et al., 1978a). Columns used in this study were a 10-μm Partisil-10-ODS-2 column (4.6 mm i.d. × 25 cm) and a 10-μm Partisil-10-SAX anion-exchange column (4.6 mm i.d. × 25 cm). Both columns were obtained from Whatman Inc., Clifton, NJ. All chromatography was done at ambient temperature with a flow rate of 1.1 mL/min. Exact solvent compositions are described in the figure legends.

Bioassay. The in vitro biological activity of the various retinoids was determined by using the tracheal organ culture technique (Clamon et al., 1974; Sporn et al., 1974). Briefly, the tracheas were removed from vitamin A deficient, (30–33)-day-old hamsters and incubated in a vitamin A deficient medium for 3 days and then in a medium containing the retinoid being tested. The retinoid-containing medium was changed every 2–3 days. After 7 days in the retinoid-supplemented medium, the trachea were fixed and hematoxylin- and eosin-stained slides were prepared. The epithelium was graded with respect to the extent of squamous metaplasia as well as the presence or absence of keratin and keratohyaline granules. Peaks 5A–D were obtained from an in vitro liver supernatant reaction that had been incubated with (2–3) $\times 10^{-6}$ M [^{3}H]retinoic acid. The peaks were used for bioassay after chromatography on a column similar to that shown in Figure 2.

Miscellaneous Procedures. Column fractions (1.1 mL) were evaporated by using a Vortex evaporator. Any residue was redissolved in 0.2 mL of NCS (Amersham/Searle, Arlington Heights, IL)–water, 9:1, and 10 mL of Econofluor (New England Nuclear, Boston, MA). The final sample was counted in a Packard TriCarb Model 3385 scintillation counter equipped with an automatic external standard system. Tissue extracts were counted in a similar manner. Methylation of all retinoids was performed by using a diazomethane procedure described elsewhere (Fales et al., 1973). Ultraviolet absorption spectra were determined on a Varian Model 118C recording spectrophotometer. Mass spectra were recorded on a JOEL JMS-01SG-2 spectrometer in the electron impact mode by using an ionization voltage of 70 eV and a solid probe inlet at 105–140 °C.

Results

Generation of Peak 5. It had previously been demonstrated that [^{3}H]retinoic acid can be metabolized by tracheal organ cultures to several more polar metabolites (Frolik et al., 1978b), the major metabolite regions being designated as peaks 2, 4, and 5. The peak 5 region, showing some biological activity, was chosen for initial identification studies. Use of the tracheal organ culture system to generate sufficient quantities of peak 5 for identification proved difficult. Peak 5 metabolites are quickly converted by the trachea to peak 4 region compounds (Frolik et al., 1978b) and therefore never accumulate to any great concentration. In addition, as the concentration of retinoic acid in the medium is increased to obtain a greater quantity of peak 5, toxic levels are soon

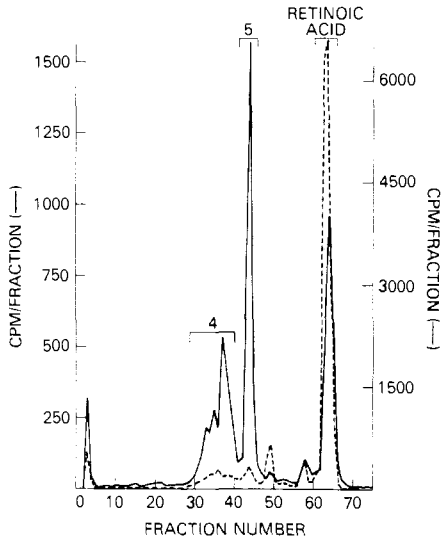

FIGURE 1: In vitro metabolism of [^{3}H]retinoic acid by a 10000g hamster liver supernatant. [^{3}H]Retinoic acid (2.2×10^{-6} M) was incubated with liver supernatant as described under Experimental Procedure. An aliquot of the methanol extract was applied to a Partisil ODS-2 column which was developed 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 8 min into the gradient. One-minute (1.1-mL) fractions were collected. Incubation with normal supernatant (—); incubation with heat-killed supernatant (---).

reached and cell death causes a marked decrease in metabolism. For these reasons, several other in vitro tissue systems were examined for generation of retinoic acid metabolites (Roberts et al., 1979a). As seen in Figure 1, a 10000g hamster liver supernatant is able to metabolize [^{3}H]retinoic acid to compounds migrating in the peak 5 region of the column effluent (fractions 44–47) with an overall yield of peak 5 being 23% of the initial retinoic acid added to the incubation medium. Incubation of heat killed 10000g liver supernatant under identical conditions employed for the normal supernatant yielded only 1.7% of the initial retinoic acid added to the reaction mixture migrating in the peak 5 region of the column effluent (Figure 1), thus demonstrating the enzymatic nature of this process. The in vitro metabolism has also been shown to be cofactor dependent and to be sensitive to a variety of inhibitors (Roberts et al., 1979a).

Purification of Peak 5. The methanol-soluble lipid extract from the in vitro liver supernatant incubation was first dissolved in methanol–1-butanol, 0.05:1, and then extracted four times with 1-butanol-saturated water. The butanol layer, which contained a small amount of the peak 4 region metabolites plus compounds less polar, was evaporated to dryness and applied in a small volume of methanol to a LC reverse-phase Partisil ODS-2 column (not shown) in order to separate peak 5 from peak 4 and the parent retinoic acid. The column was developed with acetonitrile–1% ammonium acetate, 25:75, for 10 min, followed by a 45-min linear gradient from 25:75 to 75:25 acetonitrile–1% ammonium acetate. Seventy-five 1.1-mL fractions were collected. The peak 5 region (fractions 43–46, 190 μg) was pooled and applied to a second reverse-phase column (Figure 2). The use of an isocratic solvent system consisting of acetonitrile–water–acetic acid, 45:55:0.1, for elution separated the peak 5 region from the first column into four metabolites, peaks 5A (7.5% of applied radioactive material), 5B (3.1%), 5C (6.8%), and 5D (79%). That these metabolites retained the terminal carboxyl group was demonstrated by the chromatography of ^{3}H peak 5 with ^{14}C peak 5 obtained by incubation of the liver supernatant with [*carboxy*-^{14}C]retinoic acid. As shown in Figure 2, an identical profile was obtained for both of these labeled compounds.

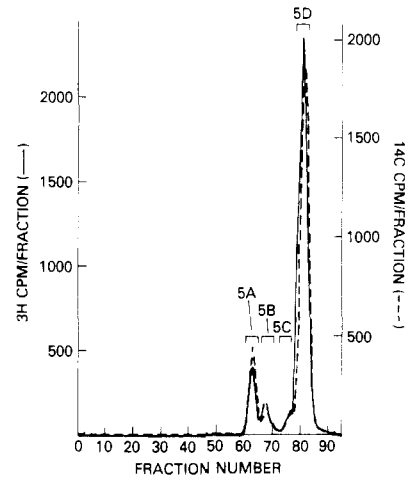

FIGURE 2: Chromatography of in vitro synthesized liver peak 5. A liver supernatant reaction mixture was extracted with methanol, and the methanol extract was reextracted with 1-butanol. The butanol extract of a liver supernatant reaction mixture, prepared as described in the text, was applied to a Partisil ODS-2 column which was developed with an acetonitrile–1% ammonium acetate gradient as described under Results. The peak 5 fractions from the column were pooled and applied to a second Partisil ODS-2 column that was eluted isocratically with acetonitrile–water–acetic acid, 45:55:0.1. One-minute (1.1-mL) fractions were collected. Peak 5 obtained from [11,12-^{3}H]retinoic acid (—); peak 5 obtained from [*carboxy*-^{14}C]retinoic acid (---).

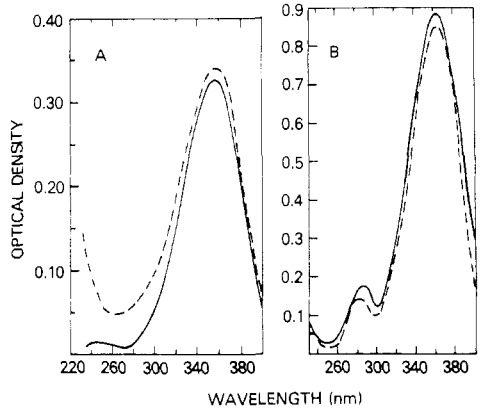

FIGURE 3: Ultraviolet absorption spectrum in methanol of (A) synthetic 4-hydroxyretinoic acid methyl ester (—) and peak 5A methyl ester (---) and (B) synthetic 4-oxoretinoic acid methyl ester (—) and peak 5D methyl ester (---).

The two major metabolites, peaks 5A (14 μg) and 5D (147 μg), were chosen for further purification. Each compound was chromatographed on a Partisil-10-SAX anion-exchange LC column by using acetonitrile–1 mM ammonium acetate, 73:27, as the eluting solvent. The peak tubes from the anion-exchange column eluting at 25 min for peak 5A and 28–29 min for peak 5D were pooled, methylated via a diazomethane procedure, and further chromatographed on a reverse-phase column in acetonitrile–water, 66:34. The single peak of radioactivity from this column eluting at 29 min for peak 5A and 36 min for peak 5D was used to determine the ultraviolet and mass spectra. The yield of metabolites from this purification procedure was 4.5 μg of peak 5A and 76 μg of peak 5D.

Identification of Peaks 5A and 5D. The fact that these metabolites rapidly reacted with diazomethane suggests that they are free acids.

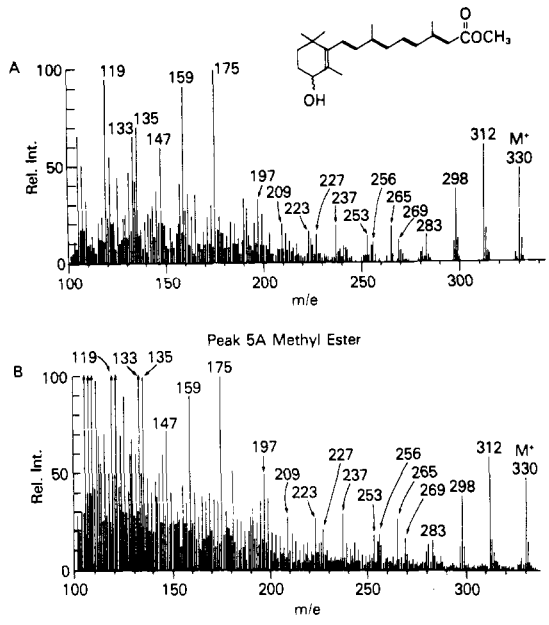

FIGURE 4: Mass spectrum of (A) synthetic 4-hydroxyretinoic acid methyl ester (1.0 μg) and (B) peak 5A methyl ester (0.8 μg).

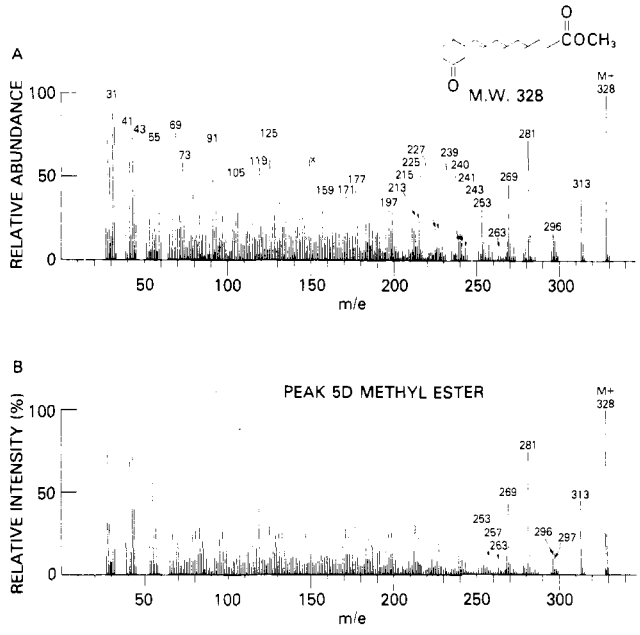

FIGURE 5: Mass spectrum of (A) synthetic 4-oxoretinoic acid methyl ester (2.0 μg) and (B) peak 5D methyl ester (1.3 μg).

Methylated peak 5A in methanol gave an ultraviolet spectrum with a λ_{max} of 352 nm (Figure 3A) while methylated peak 5D exhibited a λ_{max} of 360 nm with a secondary absorption peak at 282 nm (Figure 3B). Both of these λ_{max} values imply that the conjugated double bond system of the parent retinoid has remained intact. Because the initial mass spectral data of these compounds indicated the addition of a single oxygen to the basic retinoid structure, the ultraviolet spectra of several known retinoids containing a single additional oxygen were examined. As indicated in Figure 3, methylated 4-hydroxyretinoic acid gave a spectrum similar to peak 5A methyl ester and the methylated 4-oxoretinoic acid spectrum was identical with that observed for the methylated peak 5D.

The mass spectrum of methylated peak 5A (Figure 4B) indicated a molecular ion at *m/e* 330. This molecular weight corresponds to the addition of a single oxygen to methyl retinoate. The next significant ion was found at *m/e* 312

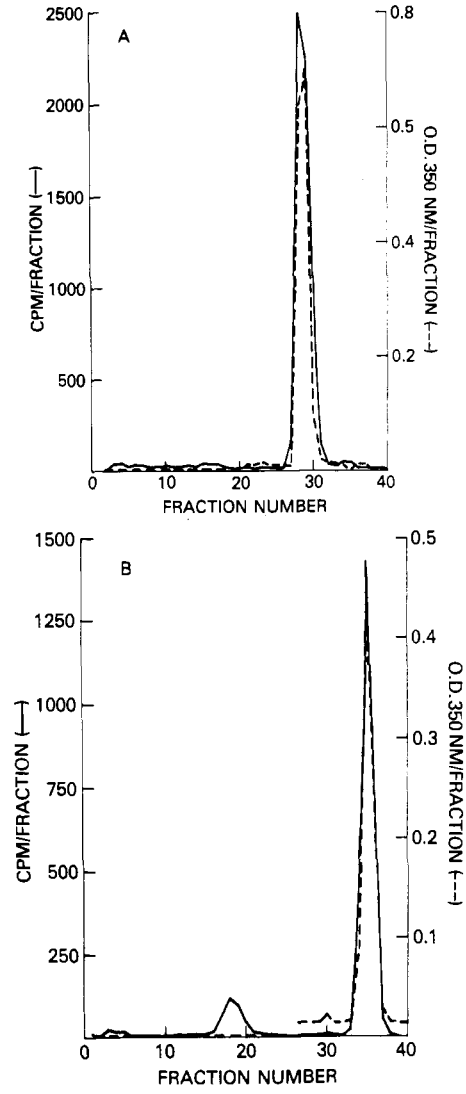

FIGURE 6: Chromatography of peaks 5A and 5D methyl esters with 4-hydroxy and 4-oxoretinoic acid methyl ester standards. A mixture of the standard compound and the ^3H peak 5 metabolite was chromatographed on a Partisil ODS-2 column in acetonitrile–water, 66:34, collecting 1-min fractions (1.1 mL). The ultraviolet absorption spectrum of each fraction was determined on a Cary 118 recording spectrophotometer, and each fraction was then counted as described in the text. Chromatography of (A) 15 μg of 4-hydroxyretinoic acid methyl ester standard (---) measured by ultraviolet absorbance at 350 nm with 15000 dpm in vitro ^3H peak 5A methyl ester (—) and (B) 20 μg of 4-oxoretinoic acid methyl ester standard (---) measured by ultraviolet absorbance at 350 nm with 8000 dpm in vitro ^3H peak 5D methyl ester (—).

which corresponds to the loss of water from the molecular ion. Such loss is indicative of hydroxyl compounds. The spectrum is identical with that of the synthetic 4-hydroxy derivative (Figure 4A). Some of the lower mass ions in the spectrum of the metabolite are due to background and to paraffinic impurities and are not considered to be significant. In addition, the spectrum of the metabolite was found to differ from the spectra of the synthetic 5,6-epoxy- or the 5,8-oxyretinoic acid derivatives, two compounds which have a molecular weight identical with that of peak 5A. The mass spectrum of a structure with a hydroxyl on one of the geminal methyl groups would be expected to show a significant loss of 30 mass units corresponding to loss of formaldehyde. A *m/e* of 300 was not observed in the mass spectrum of the metabolite.

The methylated peak 5D gave a mass spectrum with the molecular ion at *m/e* 328, indicative of the addition of one

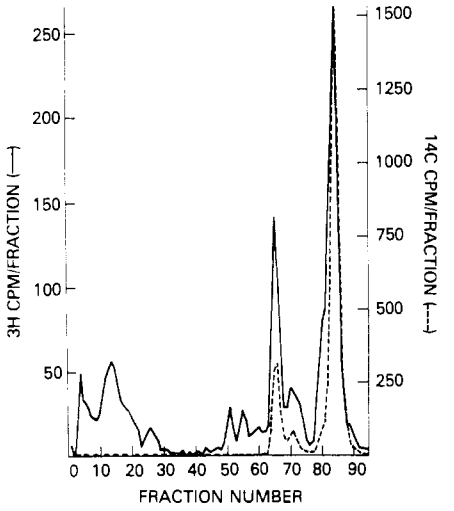

FIGURE 7: Chromatography of in vitro synthesized peak 5 from hamster trachea and hamster liver. Hamster trachea were incubated with 5×10^{-9} M [^{3}H]retinoic acid as described in the text. The methanol extract of the incubation medium was applied to the column described in Figure 1. The tracheal peak 5 region from this column was combined with the peak 5 region from a liver 10000*g* supernatant preparation that had been incubated in the presence of 1.4×10^{-6} M [^{14}C]retinoic acid. The mixture was then chromatographed on the column described in Figure 2. ^{3}H peak 5 from trachea (—); ^{14}C peak 5 from the liver (---).

oxygen and the loss of two hydrogens from methyl retinoate (Figure 5B). This mass spectrum is identical with that of the synthetic 4-oxoretinoic acid (Figure 5A).

Chromatography of Synthetic and Metabolic Compounds. The methyl ester of peak 5A or peak 5D was mixed with the respective methyl ester of the synthetic standard. The samples were chromatographed on a reverse-phase column in acetonitrile–water, 66:34. As is shown in Figure 6, the methyl esters of the purified metabolites and of the synthetic standards migrated together during chromatography. In addition, in vitro synthesized liver peak 5 was mixed with synthetic 4-hydroxyretinoic acid or 4-oxoretinoic acid. The samples were chromatographed in the reverse-phase system (acetonitrile–water–acetic acid, 45:55:0.1) capable of separating the peak 5 region into its component compounds (see Figure 2). Again, the *all-trans*-4-hydroxyretinoic acid migrated together with peak 5A and the *all-trans*-4-oxoretinoic acid migrated together with peak 5D (data not shown).

Therefore, from both chromatographic behavior and mass spectral and ultraviolet absorption spectral characteristics, strong evidence is given for identification of peak 5A as *all-trans*-4-hydroxyretinoic acid and peak 5D as *all-trans*-4-oxoretinoic acid. It should be pointed out that the stereochemistry at position 4 of the 4-hydroxy derivative is still unknown.

Tracheal vs. Liver Peak 5. In order to verify that the liver supernatant cell-free system was producing the same peak 5 metabolites that had been observed in the tracheal organ culture system, liver ^{14}C peak 5 was chromatographed with tracheal ^{3}H peak 5. As shown in Figure 7, the trachea and liver are indeed producing identical peak 5 metabolites, although the proportion of each may be slightly different in the two tissues.

Biological Activity. Liver peaks 5A–D as well as the synthetic standards were assayed for biological activity in the tracheal organ culture assay. As indicated in Figure 8, none of the compounds tested showed an activity greater than that of *all-trans*-retinoic acid. In fact, in this assay system, each metabolite was only about 3% as active as the parent compound.

Discussion

The metabolism of retinoic acid to more polar metabolites has been known for some time. Indeed, there have even been several reports indicating a possibility of finding a metabolite that displays greater biological activity than the parent compound (Krishnamurthy et al., 1963; Sundaresan, 1966; Wolf et al., 1963; Yagishita et al., 1964; Zile et al., 1967; Zile & DeLuca, 1965). However, in all cases, the metabolites of interest have remained unidentified. It was not until the advent of LC, with its capabilities of separating closely related compounds without the production of artifacts, that the metabolism of retinoic acid could be investigated in detail.

The in vitro metabolism in hamster tracheal organ cultures of *all-trans*-retinoic acid to several more polar metabolites had previously been reported (Frolik et al., 1978a). One metabolite region, designated as peak 5, was shown to retain some bi-

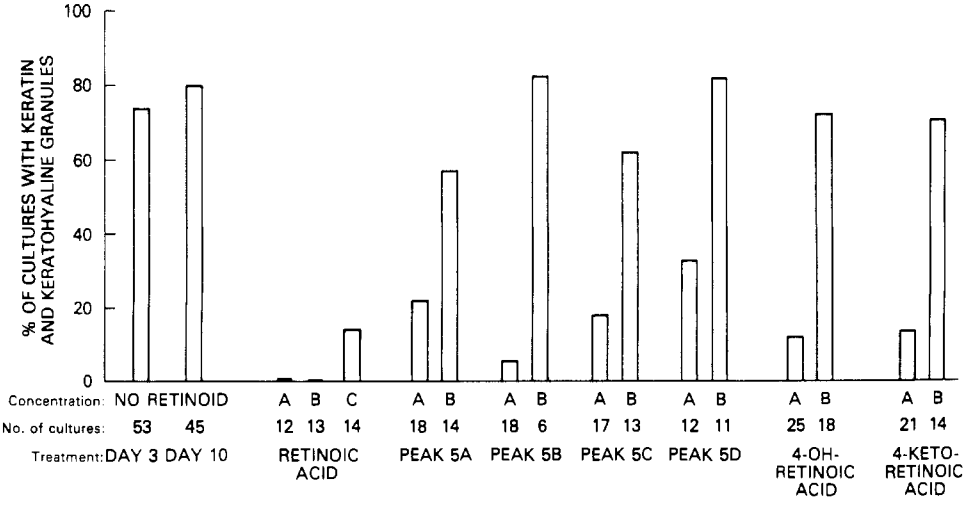

FIGURE 8: In vitro biological activity of retinoic acid and its peak 5 metabolites in hamster tracheal organ cultures. Peaks 5A–5D were isolated from a hamster liver 10000*g* supernatant incubation as described in the text. The metabolites were tested for biological activity after chromatography on the LC system shown in Figure 2. Hamster tracheal organ cultures were treated as described elsewhere (Clamon et al., 1974; Sporn et al., 1974). Cultures were graded as to the percentage of cultures containing keratin and keratohyaline granules. Concentrations of retinoids were (A) 3×10^{-9} M, (B) 3×10^{-10} M, and (C) 1×10^{-10} M.

ological activity in a hamster tracheal organ culture assay. The major metabolites in this region have now been shown to be 4-hydroxy- and 4-oxoretinoic acid.

Because these compounds have been generated in vitro, their in vivo significance is open to question. It has previously been demonstrated that the metabolites produced from retinoic acid in the in vitro tracheal organ culture system migrated together with metabolites formed in vivo (Frolik et al., 1978b). The fact that peaks 5A–D produced in the in vitro liver generating system chromatograph exactly as do the peaks produced in the tracheal cultures (Figure 7) supports the idea that similar metabolic pathways are being utilized in vitro as occur in vivo. In addition, the 4-oxoretinoic acid metabolite has already been identified in vivo in rat feces after administration of a massive dose of retinoic acid (27.5 mg per rat) (Hänni & Bigler, 1977). Experiments are currently underway to determine both the significance of the 4-hydroxy- and 4-oxoretinoic acid metabolites in vivo and the conditions under which they are maximally produced.

The question also arises as to whether these compounds are closer to a possible biologically active metabolite of retinoic acid or whether they are merely excretion products. There is no evidence to date to indicate the role these metabolites play in the metabolic pathway of retinoic acid under physiological conditions. The observed diminished activity of the metabolites in the tracheal organ culture assay could possibly be explained by their rapid metabolism or by their difficulty in reaching the intracellular site of action of retinoic acid when administered extracellularly rather than being synthesized internally. It therefore cannot be definitely ruled out that the 4-hydroxy derivative, the unidentified peaks 5B and 5C, or even a transient precursor to the 4-hydroxy compound could play a role in the mechanism of action of retinoic acid. However, at this time both the diminished biological activity of these compounds relative to retinoic acid and the identification of the 4-oxoretinoic acid metabolite in rat feces after a very large dose of retinoic acid seem to indicate that these compounds are most likely involved in the excretion pathway of *all-trans*-retinoic acid. Only further studies will be able to firmly establish the role of these compounds under physiological conditions.

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