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Biochemistry

Identification of 5,8-oxyretinoic acid isolated from small intestine of vitamin A-deficient rats dosed with retinoic acid

(vitamin A metabolite/retinoic acid metabolite/epithelial differentiation)

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ABSTRACT **A retinoid was isolated by a multistep procedure from the small intestines of vitamin A-deficient rats given a single dose of retinoic acid. The compound, designated 8_{II}, was pure, as demonstrated by four high-pressure liquid chromatographic procedures. It was positively identified as 5,8-oxyretinoic acid by ultraviolet spectrophotometry, mass spectrometry, and spectral and chromatographic comparison to known compounds. It is probable that 5,8-oxyretinoic acid was produced from 5,6-epoxyretinoic acid under the acidic conditions used in the isolation. It is highly probable, therefore, that the natural product is 5,6-epoxyretinoic acid.**

Retinoic acid retains the growth-promoting activity of retinol (1–3), but cannot support reproduction (4) or vision (5). When vitamin A-deficient rats are dosed with physiological amounts of retinyl esters, only small amounts of retinoic acid can be detected in their tissues (6) because retinoic acid is not stored but is rapidly metabolized (7–9). Several biologically active metabolites of retinoic acid have been reported (10, 11), but only 13-*cis*-retinoic acid (12) has been isolated in pure form and identified. Metabolic profiles observed after administration of radioactive retinol and retinoic acid suggest that retinoic acid is a normal intermediate in the metabolism of retinol (6, 7, 12–14). Furthermore, a metabolite of retinoic acid may be the active form of vitamin A in promoting growth and controlling epithelial differentiation. Ito and coworkers (15) described several retinoic acid metabolites more polar than the parent compound. The three major metabolites were designated as peaks 8, 9, and 10. Peak 8 was found in liver, kidney, blood, skin, and intestine, whereas peaks 9 and 10 were found only in intestine. Recently, we have separated peak 8 from 9 and 10 in crude form and have tested its ability to reverse keratinization of cultured vitamin A-deficient hamster tracheas. Peak 8 was at least as active as retinoic acid in this assay. This communication describes the isolation of the major component of peak 8 from rat small intestine. The isolated material was identified as 5,8-oxyretinoic acid.

Isolation

Sixty-three vitamin A-deficient rats (15) were each given intrajugularly 450 μg of [*11,12*-$^3H_2$]retinoic acid (provided by Hoffmann-LaRoche, Nutley, NJ; 8.1×10^5 dpm/μg). After 3.5 hr the animals were decapitated, and their entire small intestines were removed and washed with 0.9% NaCl. Mucosa was collected and homogenized in 1 vol of EDTA/propylgallate (50 μg/ml) solution. The homogenate was lyophilized and then extracted with methanol/chloroform containing butylated hydroxytoluene (50 μg/ml) as described (15). The solvent was evaporated and the residue was partitioned between ether and 2% sodium hydroxide (0.2 and 1.0 liter, respectively). After the phases were separated, the aqueous phase was extracted four times with 1 liter of ether. Acidification of the aqueous phase to pH 1 with 6 M HCl and extraction with 0.2 liter of ether five times transferred peak 8 metabolites into the organic phase. The residue from evaporation of the organic phase was partitioned between methanol and hexane (250 ml each). After further extraction with 250 ml of hexane four times, the methanol was evaporated to yield a residue containing 25% of the radioactivity recovered from the small intestine. On the basis of specific activity of the original [^{3}H]retinoic acid, the mass of the metabolite was estimated to be 250 μg.

A portion of the residue equivalent to 140 μg of peak 8 was chromatographed on a Sephadex LH-20 column (2 × 55 cm) that was eluted with acetone. The peak 8 material recovered was placed on a second Sephadex LH-20 column (1 × 148 cm) and was eluted with methanol; it then was applied to a column (1 × 20 cm) of DEAE-Sephadex A-25 in the hydroxide form. The column was washed with 100 ml of methanol followed by two 50-ml portions of 1% formic acid. Peak 8 (101 μg) was eluted with the second portion of formic acid.

A 33-μg portion of peak 8 from the DEAE-Sephadex column was purified further by high-pressure liquid chromatography (16). Passage through two microparticulate octadecylsilane columns (0.4 × 30 cm each) connected in series, with 0.01 M ammonium acetate in methanol/water (3:2) as eluent, resolved peak 8 into three discrete components. The major component, peak 8_{II} (11.6 μg), was chromatographed on a microparticulate silica gel column (0.4 × 50 cm) that was developed with 0.1% formic acid in 7% tetrahydrofuran/hexane. Recovered peak 8_{II} (9 μg) was methylated with diazomethane and rechromatographed on the microparticulate silica gel column with 2.5% tetrahydrofuran/hexane to yield 7 μg of methyl 8_{II}. 8_{II} and the corresponding methyl 8_{II} were homogeneous in the last two high-pressure liquid chromatography systems, as demonstrated by ultraviolet absorbance (313 nm) and radioactivity.

In control experiments it was demonstrated that radioactively labeled retinoic acid subjected to the isolation procedure used did not produce 8_{II} or any peak 8 metabolites. Thus, 8_{II} or at least its precursor must have arisen by metabolic action.

Identification

Component 8_{II} was retained on the hydroxide form of DEAE-Sephadex and could be eluted only with an acidic sol-

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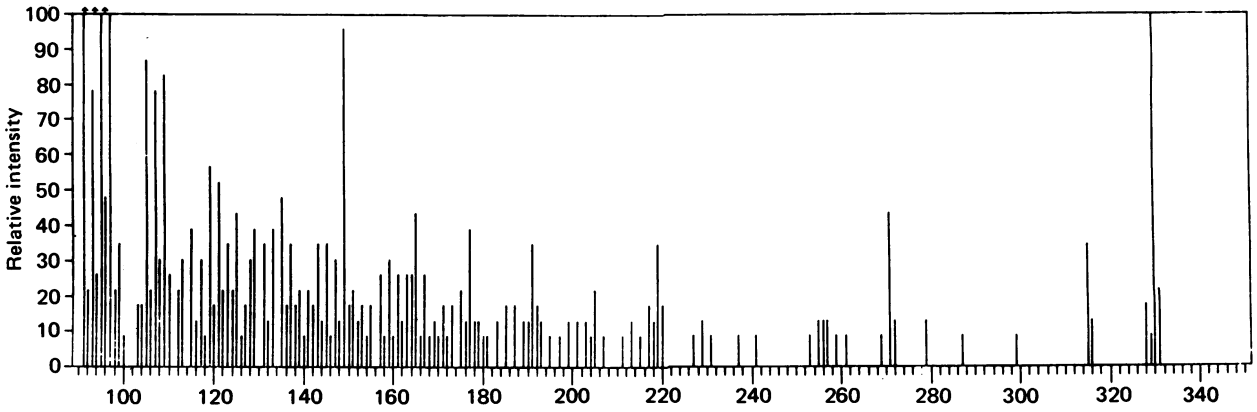

FIG. 1. Mass spectrum of methyl 8_{II}.

vent. Since it was also esterified rapidly (<5 min) when exposed to diazomethane, it is obvious that 8_{II} is an acid. The mass spectrum of methyl 8_{II} (Fig. 1) exhibits a molecular ion at *m/e* 330, which corresponds to a methylated retinoic acid with one additional oxygen atom. Peaks at *m/e* 315 (M^+-CH_3, 34%), 299 (M^+-OCH_3, 9%), and 271 (M^+-CO_2CH_3, 43%) represent loss of fragments from the terminal carboxymethyl function. Absence of *m/e* 312 (M^+-H_2O) suggests that the oxygen atom is not present as a hydroxyl group. Insertion of oxygen into an olefinic bond to form an epoxide, however, is consistent with the mass spectral evidence (Fig. 2). The series of relatively intense peaks at *m/e* 271 (43%), 191 (33%), 177 (39%), 165 (44%), 164 (27%), 149 (96%), and 135 (48%) are indicative of 5,6- or 5,8-oxygenated retinoids (17). Thus, mass spectral evidence suggests that 8_{II} is a 5,6- or 5,8-oxide of retinoic acid. We synthesized oxides **2** and **3** by reported routes (18, 19) and found that the fragmentation pattern of methyl 8_{II} mimics that of authentic 5,8-oxide **3** (R = CH_3).

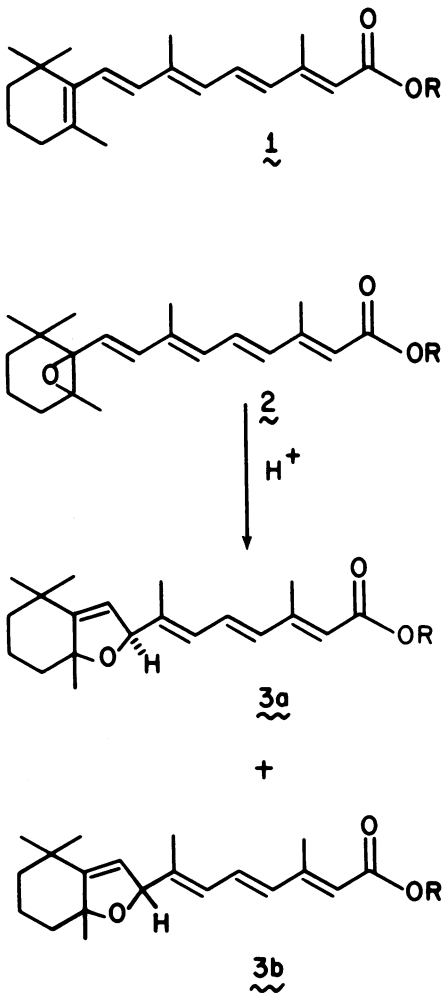

FIG. 2. Structures of retinoic acid (1), 5,6-epoxyretinoic acid (2), and the diastereomers of 5,8-oxyretinoic acid (**3a** and **3b**).

The UV spectrum of methyl 8_{II} in hexane exhibits λ_{max} 306 nm with shoulders at 318 and 294 nm (Fig. 3*A*). In methanol the shoulders are nearly obscured (Fig. 3*B*) because of broadening of the absorption maximum. The decrease in λ_{max} from about 350 nm for methyl retinoate (**1**, R = CH_3) to 306 nm for methyl 8_{II} clearly indicates loss of conjugation. The magnitude of the shift is consistent only with removal of the 5,7-diene unit. The UV spectra of methyl 8_{II} are superimposable with those of authentic methyl 5,8-oxide **3**. The UV spectrum of authentic 5,6-oxide (**2**, R = CH_3) in hexane with absorption peaks at 338 and 354 nm and a shoulder at 326 nm differs from those of methyl **3** and methyl 8_{II}.

Synthetic methyl **3** and methyl 8_{II} were examined further by cochromatography in high-pressure liquid chromatography systems. First, methyl **3** was injected onto a microparticulate silica gel column (0.4 × 50 cm) and was eluted with 1% tetrahydrofuran/hexane (Fig. 4). Note that this system resolves the diastereomers **3a** and **3b**, which have nearly identical UV and mass spectra. Methyl 8_{II} eluted at exactly the positions of synthetic **3**. In each chromatogram the ratio of the larger peak

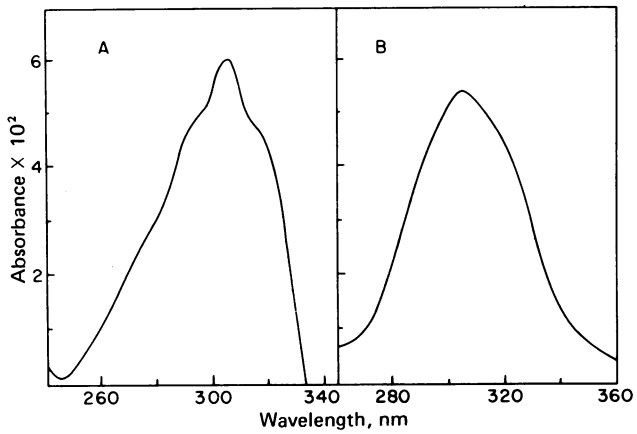

FIG. 3. UV spectra of methyl 8_{II} in hexane (*A*) and methanol (*B*).

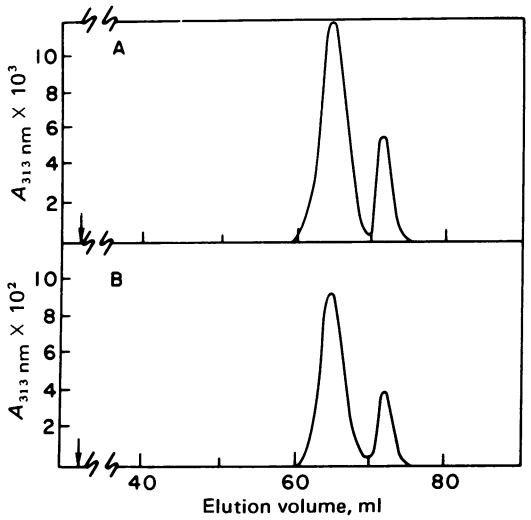

FIG. 4. High-pressure liquid chromatographic profiles of methyl 5,8-oxyretinoic acid 3 (*A*) and methyl 8_{II} (*B*). Arrows indicate injection. The column was microparticulate (10 μm) silica gel (0.4 × 50 cm) eluted with 1% tetrahydrofuran/hexane.

height to the smaller peak height was the same, namely, 2.2. The 5,6-epoxide 2 would elute at 40 ml in this system. Methyl 3 and methyl 8_{II} then were co-injected onto two microparticulate octyldecylsilane columns (0.4 × 30 cm) connected in series and were eluted with methanol/water (3:1) (Fig. 5). After three passes through the two columns (recycle mode; effective column length of 180 cm), fractions were collected and radioactivity was measured. The radioactivity of methyl 8_{II} exactly coincided with the UV absorbance from unlabeled synthetic methyl 5,8-oxide 3. In this system methyl 5,6-epoxide 2 would

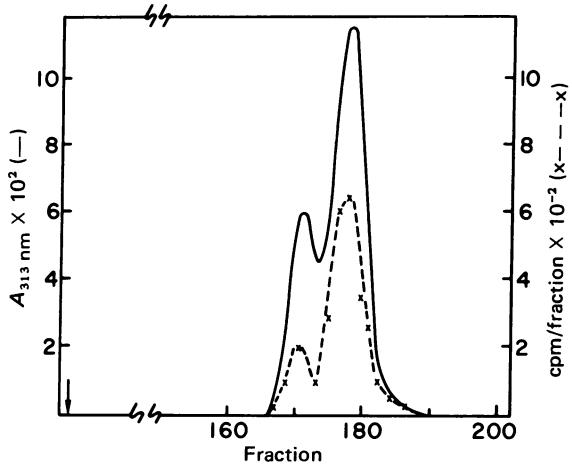

FIG. 5. Comigration of methyl 5,8-oxyretinoate (—) with methyl 8_{II} (× - - - ×). The experiment was done by recycling the compounds through two microparticulate (10 μm) octadecylsilane columns (0.4 × 30 cm) twice after the first pass, for a total of three passes. Each fraction contained 1 ml.

be eluted at 190 ml in comparison to the more prevalent methylated diastereomer of 3 (or 8_{II}), which eluted at 178 ml.

The evidence shows that 8_{II} is an oxidized derivative of retinoic acid with disrupted side-chain conjugation and that it is spectrally and chromatographically indistinguishable from 5,8-oxyretinoic acid. Because the acidic conditions for isolation and chromatographic separation were sufficient to cause the well-known isomerization of 5,6-epoxide 2 to 5,8-oxide 3, we suggest that 5,6-epoxyretinoic acid, 2 (R = H) is the natural metabolite of retinoic acid and that the 5,8-oxide 3, (R = H) was formed during isolation. The fact that both diastereomers of 8_{II} occur, and occur in the same ratio as in the synthetic 5,8-oxide 3, strongly suggests that a nonenzymic mechanism was involved in their synthesis. Moreover, reported biological activity of 2 exceeds that of 3 (18, 19). Although *in vivo* synthesis of 5,8-oxide cannot be absolutely excluded, it is highly likely that it would arise from corresponding 5,6-epoxide.

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