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89. Isolation and Identification of Three Major Metabolites of Retinoic Acid from Rat Feces

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Summary

Following the intraperitoneal administration of high doses of ^{14}C- and ^{3}H-labelled retinoic acid (**1**) to rats, three major metabolites and the intact compound were isolated from the feces in microgram amounts by use of column, thin-layer and high-pressure liquid chromatography. Their structures were elucidated by mass spectrometry and *Fourier* Transform ^{1}H-NMR. spectroscopy as **2** (all-*trans*-4-oxo-retinoic acid), **3** (7-*trans*-9-*cis*-11-*trans*-13-*trans*-5'-hydroxy-retinoic acid).

Hydroxylation of the 5-methyl group of the cyclohexene ring, oxidation of the cyclohexene ring in position 4 and *cis-trans* isomerisation of the nonatetraenoic acid side chain were the reactions, which produced these products from retinoic acid. The metabolites **2** and **4** each accounted for about 4% of the radioactivity administered. The metabolite **3** and the parent compound accounted for about 16% and 17% of the dose, respectively.

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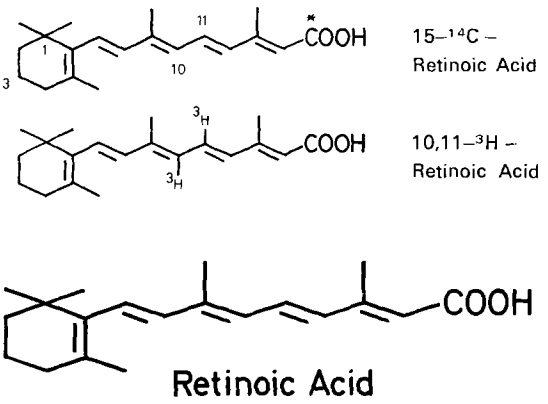
1. Introduction. – Retinoic acid (**1**) (*Scheme 1*) is the active substance of *Airo* Roche applied in acne therapy.

When the vitamine A-like activity of retinoic acid was discovered by *Dowling & Wald* [1], the acid was at first considered to be the 'active form' of vitamin A [2] However it could only be detected in small amounts in the kidney and plasma after administration of physiological doses of retinyl acetate to vitamin A-deficient rats [3]. Apparently it was metabolized extremely rapidly. It was therefore thought that retinoic acid, too, is transferred into actual metabolically active compounds.

Several biologically active metabolites of retinoic acid were described in the literature, but none of these were well characterized [4] [5].

This paper describes the isolation, characterization and identification of three metabolites and the parent compound from the feces, after intraperitoneal administration of large doses of 15-[^{14}C]-retinoic acid and 10,11-[^{3}H]-retinoic acid to rats. Nothing is known about the biological activity of these metabolites.

Scheme 1



2. Experimental Part. – *Radioactive Compounds.* The following labelled retinoic acids were available: 15-[^{14}C]-retinoic acid (59 μCi/mg) and 10,11-Di[^{3}H]-retinoic acid (273 μCi/mg) (*Scheme 1*)[2])

Animal Experiment. To each of 36 male rats (*Füllinsdorf Albino* SPF, 380–420 g) 1 ml of a suspension of 1.07 g retinoic acid (2.7 mCi) and 20.3 mg 15-[^{14}C]-retinoic acid (1.2 mCi) in 40 ml of a 1:2 mixture of Tween 80/NaCl 0.9% were administered intraperitoneally. The animals were kept in metabolism cages and received food once a day (Nafag 850/194) and water ad libitum. Urine and feces were collected separately for five days.

Radioassay Procedure. Radioactivity was measured by the liquid scintillation technique using a *Nuclear-Chicago Mark I* Instrument with external ^{133}Ba standard. The scintillation fluid employed consisted of dioxan (350 ml), toluene (50 ml), ethylene glycol monomethyl ether (100 ml), naphthalene (40 g) and 2-(4′-*t*-butylphenyl)-5-(4″-biphenyl)-1,3,4-oxadiazol (Butyl-PBD, *Ciba*) (3.5 g). Radioactive areas on thin-layer plates were eluted with ethanol before measurement. Eluates, containing radioactive material from thin-layer chromatograms, high-pressure liquid chromatography or samples of urine, were added directly to polyethylene counting vials which were filled up with 15 ml of the scintillation medium.

[2]) We thank Dr. *Würsch*, of *Hoffmann-La Roche & Co., AG*, Basel, for the synthesis of the radioactive compounds.

Thin-layer Chromatography (TLC.). Glass plates (20 × 20 cm) coated with silica gel 60 F 254 (0.5 mm) were purchased from *E. Merck* (Darmstadt). Prior to use they were washed with acetone and chloroform. The samples were applied to the plates in lines with a microdoser (*Desaga*). Hexane/acetone 60:40 was used as chromatographic solvent. UV.-light absorbing areas were scratched off and eluted with ethanol.

High-Pressure Liquid Chromatography (HPLC.). Pumping System: *Milton Roy* Mini Pump (24–240 ml/h, 5000 psi); Pulse-damping: *LDC* 709 up to 1200 psi; detector: *Cecil CE 212* variable wavelength UV.-monitor with a 10 μl cell; column hardware: stainless steel tubing (internal diameter 3 mm), syringe injection port with septum; stationary phase: silica gel (Partisil 5, *Whatman*), particle diameter 6 μ. Solvent systems: hexane *p.a.*/tetrahydrofuran *p.a.* 99.5:0.5, 88:12 (solvent degassed).

Mass Spectrometry (MS.). All mass spectra were obtained with an *AEI*-MS 9 mass spectrometer using electron impact ionisation. The high resolution data were obtained with an *AEI*-MS 902/DS 30 system.

Proton Magnetic Resonance Spectroscopy (¹H-NMR.). The ¹H-NMR. spectra were run at 90 MHz on a *Bruker* HX 90/15 *Fourier* Transform Spectrometer equipped with a *Nicolet* 1081 computer.

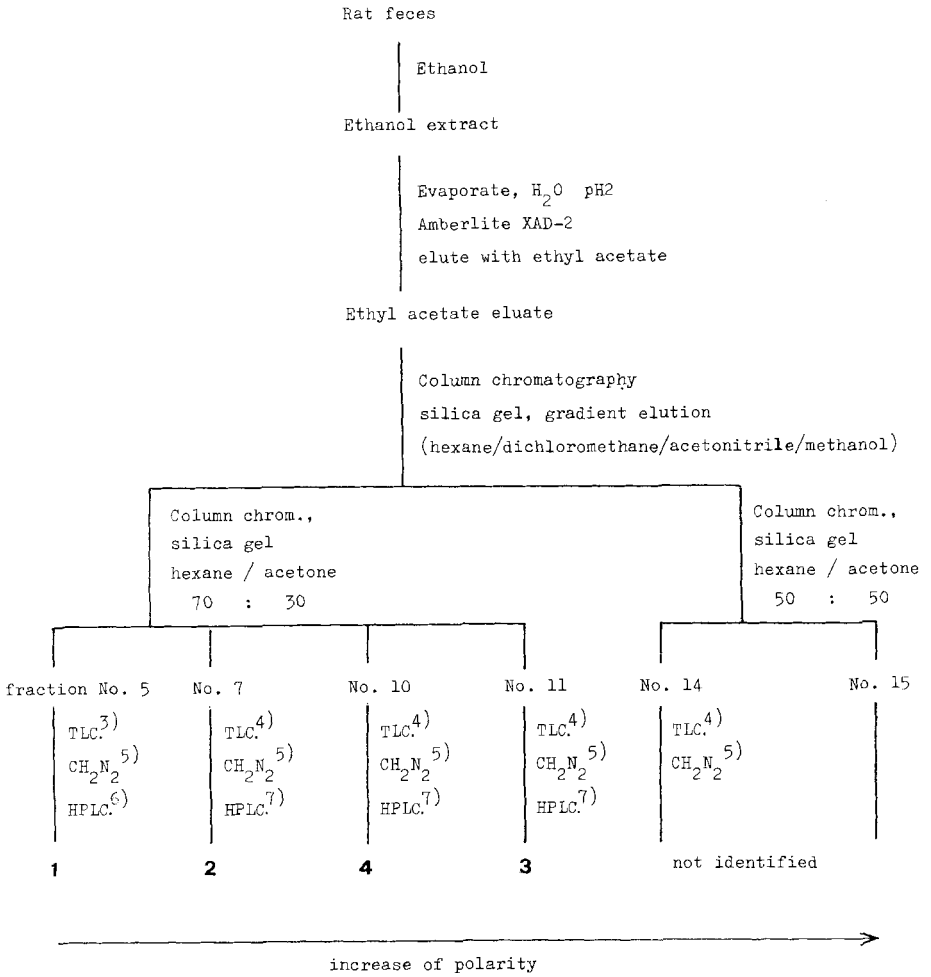
Isolation of the Metabolites **2**, **3**, **4** *and the Parent Compound from the Feces (Scheme 2).* The pooled feces of 36 male rats, collected for a period of five days after administration of a mixture of ^{14}C- and ^{3}H-labelled retinoic acids, were homogenized with a mixer (*Polytron, Kinematica*, Luzern, Switzerland, in 1 l of ethanol). After filtration the fecal residue was homogenized a second time in 0.5 l of ethanol and filtered. The filtrates were evaporated on a rotating evaporator to a volume of approx. 300 ml. Then 1 l of water was added. The resulting suspension was adjusted to pH 2 with 2N HCl and adsorbed on a column (75 × 6.5 cm) of Amberlite XAD-2. After washing with 1.5 l of water a fraction of a mixture of unconjugated metabolites could be eluted with 2.5 l of ethyl acetate accounting for 90% of the fecal tritium (77% of the dose) and 88% of the fecal ^{14}C-activity (72% of the dose). The ethyl acetate eluate was further chromatographed on a column (77 × 6.5 cm) of silica gel 60 (*Merck*, particle size 0.04–0.063 mm) using stepwise gradient elution (hexane/dichloromethane/acetonitrile/methanol). Two fractions which contained most of the radioactivity (80% of the tritium and 78% of the ^{14}C-activity) were separately chromatographed on two columns (50 × 4.0 cm) of silica gel with hexane/acetone 70:30 and 50:50, respectively. By this procedure four radioactive fractions (No. 5, 7, 10 and 11) could be isolated from one column and two further, more polar radioactive fractions (No. 14 and 15) could be isolated from the other. By TLC., methylation with diazomethane and purification by HPLC. the methyl esters of **1** (parent compound) (420 μg), **2** (20 μg), **3** (350 μg) and **4** (10 μg) could be isolated (*Scheme 3*).

The other two polar fractions, No. 14 and 15 from the second column, could not as yet be purified well enough for exact identification (*Scheme 2*).

3. Results. – Within the first five days after administration of a mixture of ^{3}H- and ^{14}C-labelled retinoic acids to rats, the tritium and ^{14}C excreted in the feces accounted for about 85% and 82% of the dose, respectively (*Scheme 4*). It was obvious that the compounds **1**, **2**, **3** and **4** were acids since they were methylated by diazomethane within 3 minutes. The $^{3}H/^{14}C$ ratios of **1**, **2**, **3** and **4** were approx. the same as in the administered dose (2.25), revealing the presence of all radioactive labels in the metabolites. As the ^{14}C-atom was located at the end of the side-chain and the UV.-maxima of **2**, **3** and **4** were above 340 nm (hexane/tetrahydrofuran 88:12), it followed that the compounds had an intact tetraen side-chain. The order of the polarity of the methylesters of **1**, **2**, **3** and **4** was deduced from their retention volumes from the HPL-chromatograms (*Scheme 2*). The last polar fraction contained **1**. A 1:1 mixture of **1**, and an authentic sample of the methylester of retinoic acid could not be separated by HPLC.

The retinoic acid fraction accounted for about 20% of the fecal radioactivity (17% of the dose). As deduced from the ¹H-NMR. spectra the methylesters of **2** and **4** were found to be pure after isolation by HPLC., whereas the HPL-chromatogram

Scheme 2. *Isolation of retinoic acid* (**1**) *and the metabolites* **2**, **3** *and* **4** *from rat feces*



of the methyl ester of **3** indicated two peaks. The corresponding fraction could not be separated into a pure form. The metabolites **2** and **4** accounted for about 4% each of the radioactivity administered and the metabolite **3** for about 16% of the dose (*Scheme 4*).

The two very polar fractions No. 14 and 15 were not pure enough for identification. The MS. of the methylated fraction No. 14 showed molecular peaks at *m/e* 346 and 344.

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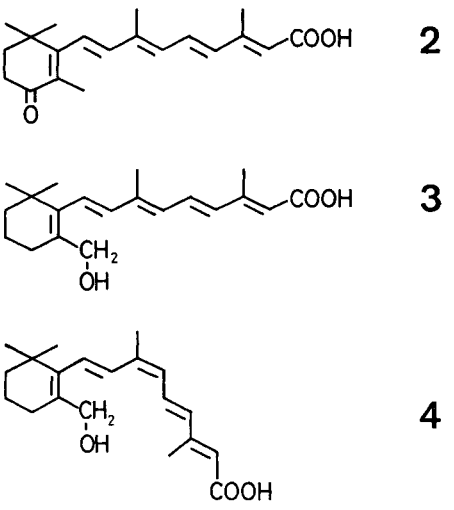
3) Hexane/acetone 80:20.
4) Hexane/acetone 60:40.
5) Diazomethane in ether, 3 min., 20°.
6) Hexane/tetrahydrofuran 99.5:0.5.
7) Hexane/tetrahydrofuran 88:12.

Scheme 3. *Fecal metabolites of retinoic acid*



Metabolite **2**. The elemental composition of the methylester of metabolite **2** was deduced from the mass spectrum taken under high resolution conditions as $C_{21}H_{28}O_3$ (base peak). Characteristic peaks are present at *m/e* 313 (55% relative intensity, *M*-CH_3), *m/e* 281 (95% relative intensity, *M*-CH_3–CH_3OH) and *m/e* 269 (70% relative intensity, *M*-$COOCH_3$).

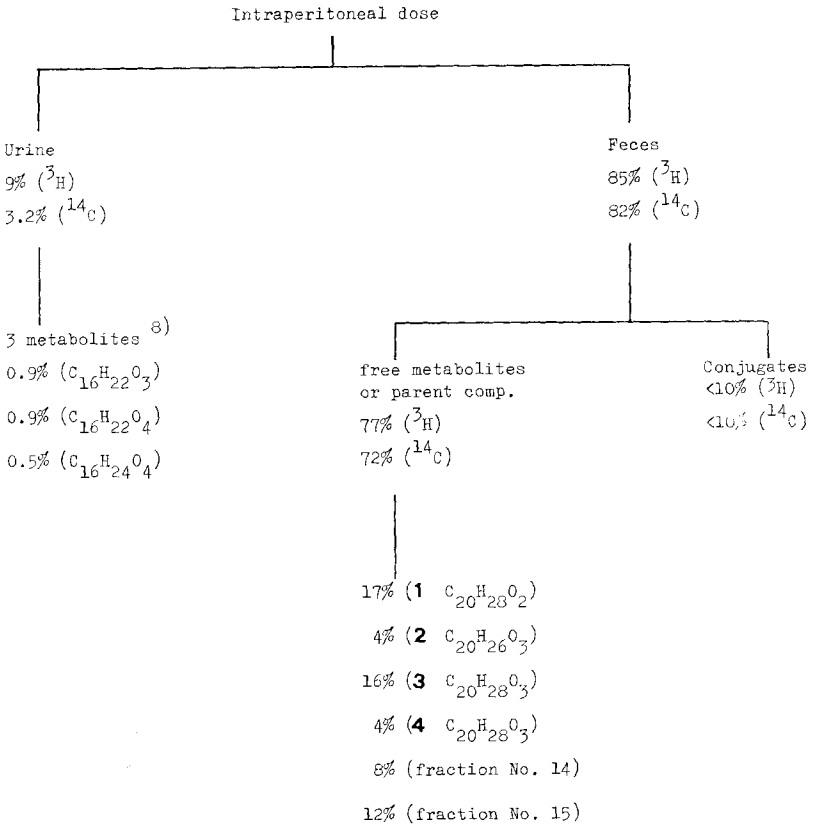
The ^{1}H-NMR. spectrum of 15 μg of the methyl ester of **2** reveals the presence of an intact all-*trans*-tetraen side chain with six olefinic protons and large spin coupling constants in the region of 5.82 ppm [H–C(14)] to 7.0 ppm [H–C(11)]. The part of the spectrum at higher field reveals the presence of six methyl groups appearing at 3.72 ppm (CH_3–OCO), 2.36 ppm [CH_3 at C(13)] slightly coupled (1.5 Hz) with H–C(14), 2.03 ppm [CH_3 at C(9)], slightly broadened probably by interaction with the protons at C(7) and C(10), 1.85 ppm [CH_3 at C(5)] and 1.19 ppm (6H) [2 CH_3 at C(1)]. The remaining multiplets, centered at 2.52 and approx. 1.85 ppm, are caused by the methylene protons at C(3) and C(2), respectively.

Metabolite **3**. The elemental composition of the methyl ester of the metabolite **3** was deduced from the high resolution mass spectrum as $C_{21}H_{30}O_3$ (45% relative intensity, *M*). The loss of H_2O from the molecular ion, giving rise to a peak at *m/e* 312 (45% relative intensity) reveals the presence of an alcoholic function. The structural proof was based on the observation that a CH_2OH group (doublet at 4.13 ppm, 2H and a triplet at 1.25 ppm, 1H) was present instead of the usual singlet of the CH_3 at C(5) at approx. 1.72 ppm. Upon addition of D_2O the CH_2O-doublet at 4.13 ppm collapsed to a singlet and the OH-triplet disappeared.

As deduced from the ^{1}H-NMR. spectrum the sample contained an impurity (about 10% of **3**), probably the 13-cis-isomer of **3**.

Metabolite **4**. Metabolite **4** had the same molecular weight and elemental composition as metabolite **3** but was less polar. From the ^{1}H-NMR. spectrum it was evident that metabolite **4** was the 9-cis-isomer of **3**.

Scheme 4. *Excretion rates of retinoic acid metabolites in the rat*



4. Discussion. – Following intraperitoneal administration of a mixture of ^{14}C- and ^{3}H-labelled retinoic acids to rats the excretion in the feces accounted for about 85% of the tritium and 82% of the ^{14}C-dose within the first five days. Only 9% of the tritium and 3.2% of the ^{14}C-activity was excreted in the urine (*Scheme 4*). The large amounts of retinoic acid and its metabolites in fecal products are due to the large concentrations of these compounds in the bile. As reported by *Zachman*, *Dunagin*, *Meadows* and *Olson* [6–11] the large amount of radioactivity in the bile, after intraperitoneal or intravenous injection of labelled retinoic acid to bile-cannulated rats, consisted mainly of retinoic acid β-glucuronide. Other metabolites also present in the bile were not identified. The authors suggested that no, or only small amounts of compounds with a cleaved tetraen side chain were present in the bile. These findings about the excretion of retinoic acid metabolites in the bile reported in the literature are in agreement with our results about the excretion of retinoic acid metabolites in the feces. As mentioned before, 85% of the tritium and 82% of the ^{14}C-dose were excreted in the feces after intraperitoneal administration of a mixture of tritium and

[8]) For the structures, see [12].

^{14}C-retinoic acids. The ^{3}H/^{14}C ratio of the administered mixture has therefore not changed. This indicates that the 15-[^{14}C]-carboxy moiety of the fecal metabolites has not been decarboxylated. As a consequence of this we isolated from the feces only compounds with an intact side chain corresponding to ^{3}H/^{14}C-ratios of 2.3. In our previous work with retinoic acid [12] it was found that the unchanged compound was not excreted in the urine after intraperitoneal injection into rats, but exclusively metabolites with a shortened side chain lacking the 15-[^{14}C]-atom. From these results we concluded that metabolites with a shortened side chain are mainly eliminated by the kidney and metabolites with an intact-side chain and the parent compound are eliminated by the liver *via* the bile in the feces. Approx. 90% of the fecal radioactivity was extractable with ethyl acetate, indicating that only small amounts of these fecal elimination products were conjugates (*Scheme 4*).

The glucuronides of retinoic acid and its metabolites present in the bile must therefore have been hydrolysed in the intestine before excretion.

The metabolites **2**, **3**, **4** and the parent compound, isolated from rat feces together with the metabolites isolated previously from rat urine [12] after intraperitoneal injection of retinoic acid, represent approx. half of the administered dose.

The two unidentified more polar fecal fractions No. 14 and 15 (*Scheme 4*) represent a further 20%. About 70% of the elimination products of an intraperitoneal dose to rats have therefore been identified or characterized.

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