Metabolism in vivo of all-trans-[11-3H]retinoic acid after an oral dose in rats

Characterization of retinoyl β-glucuronide in the blood and other tissues

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Soon after [11-3H]retinoic acid (RA) (1.1 × 10^4 d.p.m.) was administered orally to rats either as a large dose (115 μg = 0.38 μmol/rat) or mixed with unlabelled RA as a huge dose (22 mg = 73.33 μmol/rat), retinoyl β-glucuronide (RAG) was identified and characterized as a significant metabolite in the serum and small intestine. Of the administered dose, 70% remained unchanged as retinoic acid in the stomach up to 1 h. Significant amounts of 5,6-epoxyretinoic acid, 4-hydroxyretinoic acid, esters of retinoic acid and several polar retinoids, including 4-oxoretinoic acid, were also detected in the stomach. No significant difference was observed in the nature of the retinoids found after a large or a huge dose; however, the ratio of RAG/RA was higher after a huge dose than after a large dose. Thus RAG, which is biologically active in vivo and in vitro, is formed quickly in significant amounts in tissues after a dose of RA.

INTRODUCTION

Retinoic acid (RA) is an important physiological metabolite of retinol in the control of epithelial-cell growth and cellular differentiation (Roberts & Sporn, 1984). Both vitamin A deficiency and large doses of RA markedly affect embryonic development (Kochhar, 1967; Maden & Summerbell, 1986; Gunning et al., 1989). Both physiological and toxic effects may be mediated at the gene level, at least in part, by nuclear retinoic acid receptors (Petkovich et al., 1987; Giguere et al., 1987).

Metabolites of all-trans-RA in vivo include retinoyl β-glucuronide (RAG), 5,6-epoxyretinoic acid (5,6-ERA), 4-hydroxyretinoic acid (4-HRA) and 4-oxoretinoic acid (4-ORA) (Dunagin et al., 1966; McCormick et al., 1980; Napoli & McCormick, 1981; Zile et al., 1980, 1982; Swanson et al., 1981; Silva & DeLuca, 1982). 4-Oxo-13-cis-RA, its glucuronide, and other oxidized metabolites appear in the blood or bile after an oral dose of 13-cis-RA (Vane & Bugge, 1981; Vane et al., 1990). In the studies cited above, RA usually was given by intravenous or intraperitoneal injection. However, in many of these past investigations, RAG would not have been extracted by the procedures used.

We recently synthesized RAG from RA chemically (Barua & Olson, 1985a,b, 1989a,b) and, thereafter, demonstrated the endogenous occurrence of RAG in human blood (Barua & Olson, 1986).

The purpose of the present study, therefore, was to examine the metabolites of orally administered doses (both large and huge) of all-trans-RA in rats, with particular attention to the formation of RAG and its circulation in the blood. A preliminary communication concerning this study has previously appeared (Barua et al., 1990).

MATERIALS AND METHODS

Chemicals and solvents

All-trans-[11-3H]RA was obtained from SRI International, Menlo Park, CA, U.S.A., through the courtesy of the National Cancer Institute, Bethesda, MD, U.S.A. Other chemicals and solvents used and their purveyors were: all-trans-RA and β-glucuronidase from Escherichia coli (activity 570 000 units/g) (Sigma Chemical Co., St. Louis, MO, U.S.A.); NaHCO3, acetate, methanol, dichloromethane, diethyl ether, ethyl acetate and hexane (Fisher Scientific Co., Fair Lawn, NJ, U.S.A.); silica gel for dry column chromatography (Woelm Pharma, Eschwege, Germany, and supplied through Universal Scientific, Atlanta, GA, U.S.A.); glucuronic acid and p-chloroperoxybenzoic acid (Aldrich Chemical Co., Milwaukee, WI, U.S.A.).

[11-3H]RA (2 μCi) was mixed with unlabelled RA (3 mg) and purified first by column chromatography on a small column of silica gel that was wet-packed with dichloromethane. All-trans-RA, which separated from traces of more- and less-polar compounds as a single major yellow band, was eluted with dichloromethane/diethyl ether (1:1, v/v). The all-trans-[11-3H]-RA thus obtained, when analysed by isotropic reversed-phase h.p.l.c. as described below, was found to be > 99% pure. The specific radioactivity was found to be 9.3 × 10^8 d.p.m./μg.

5,6-ERA was prepared by treating methyl retinoate (2 mg) dissolved in diethyl ether (1 ml) with a solution of p-chloroperoxybenzoic acid (1 mg) in diethyl ether (1 ml). The crude methyl 5,6-epoxyretinoate was hydrolysed with methanolic NaOH [10%, (w/v); 100 μl]. After acidification with acetic acid, the retinoids were extracted, then purified by isotropic reversed-phase h.p.l.c. 5,6-ERA [retention time (tR) 4 min] was separated as the major band from another earlier unidentified band (tR 2 min) and showed a λmax of 321 nm which shifted to 337 nm when a drop of acetic acid was added to the h.p.l.c. solvent. The purified acidoic form of 5,6-ERA showed a λmax of 337 nm, which shifted to 308 nm, characteristic of the 5,8-furanoid structure, upon addition of a drop of dilute HCl. ORA and 4-hydroxyretinoic HRA were prepared as described previously (Barua & Ghosh, 1972).

H.p.l.c.

Reversed-phase gradient h.p.l.c. was performed on a Waters Resolve 5 μm (3.9 mm × 15 cm) column as described previously (Barua & Olson, 1989a). Reversed-phase isotropic h.p.l.c. was

Abbreviations used: RA, [11-3H]retinoic acid; RAG, retinoyl β-glucuronide; 5,6-ERA, 5,6-epoxyretinoic acid; 4-HRA, 4-hydroxyretinoic acid; 4-ORA, 4-oxoretinoic acid; tR, retention time.
carried out in a similar way, but a solvent mixture of methanol/water (7:3, v/v), containing 10 mm-ammonium acetate, at a flow rate of 1.2 ml/min, was used.

**Retinoic acid solution for oral dosing**

A methanolic solution containing [11-3H]RA (9.3 × 10⁴ d.p.m./μg) in a small test tube was poured under a stream of argon, and the residue was dissolved in ethanol and diluted with an equal volume of peanut oil. The mixture was vortex-mixed to obtain a uniform clear solution, which contained 0.38 μmol (1.1 × 10⁴ d.p.m./100 μl). This solution (100 μl) was fed to rats as a large dose. The huge dose was made by vortex-mixing 73.3 μmol of unlabelled RA in 400 μl of ethanol/peanut oil (1:5, v/v) to which 100 μl of the small dose was added. The huge dose was a thick yellow paste solution. In both instances, the same amount of radioactivity (1.1 × 10⁶ d.p.m.) was given to each rat.

**Animal studies**

Sprague-Dawley rats weighing about 300 g were fed on a normal rat diet. Large doses of the retinoid were given to male rats and huge doses on day 10 of gestation to female pregnant rats, which were concomitantly used for study of teratogenicity. The rats were dosed by means of a Microman positive-displacement pipette (Gilson Medical Electronics, Villiers-le-Bel, France; supplied by Rainin Instruments Co., Woburn, MA, U.S.A.). The volumes of large and huge doses (containing the same amount of radioactivity) were 100 μl and 500 μl respectively, which were pipetted well down into the throats of the animals. Another 50–100 μl of peanut oil were pipetted subsequently into the mouth to rinse the dose down. The rats were anaesthetized with diethyl ether 0.5 or 1 h after the dose, the chest was cut open, and blood was collected from the heart by means of a syringe. Liver, stomach, small intestine, large intestine and kidneys were collected and kept frozen at −27°C until analysis. Blood was allowed to clot for about 15 min and then was centrifuged for 20 min at 1500 rev./min (500 g). Serum was separated and, whenever possible, analysed on the same day; otherwise, it was kept frozen at −72°C.

**Extraction of retinoids**

Serum (0.5 ml) and about 1 g portions of liver, kidney, small intestine and stomach (together with its contents) were extracted with a mixture of dichloromethane, ethyl acetate and methanol as described previously (Barua & Olson, 1989a). The extracts were dissolved in methanol/dichloromethane [3:1 (v/v)]; 100–500 μl], and portions (50–100 μl) were analysed by h.p.l.c.

**Control experiments**

To determine whether the retinoids formed were artefacts of the extraction and isolation procedure, control experiments were conducted by using residual tissues after complete extraction of radioactivity. Thus [3H]RA solution (10⁶ d.p.m.) in methanol was added to residual liver and intestinal tissues that had been mixed previously with five times their weight of anhydrous Na₂SO₄. The mixture was ground, and the retinoids were extracted exactly the same way as described under 'Extraction of retinoids' above. The extract was then analysed by h.p.l.c.

**Other procedures**

**Action of β-glucuronidase.** Serum (200–500 μl), or its extract, and other tissue extracts in 50 μl of methanol were diluted with phosphate-buffered saline (0.15 m-sodium phosphate/0.15 m-NaCl, pH 7.5; 0.5 ml) and incubated with β-glucuronidase (5 mg) at 37°C for 1 h. Retinoids were extracted and analysed as described under 'Extraction of retinoids' above.

**Determination of radioactivity.** Portions (50–100 μl) of extracts from serum or other tissues were each mixed with 5 ml of biodegradable-grade Scintiverse BD (Fisher). The h.p.l.c. eluates, collected as 1 ml fractions, were allowed to evaporate in a fume hood for 3–4 h. The residual solution (~ 0.2 ml) was mixed with 5 ml of Scintiverse BD. The ³H content was then quantitatively measured by use of an LS-7500 scintillation counter (Beckman Instruments Co., Fullerton, CA, U.S.A.). Background and quench corrections were made by using H number values of known solvent mixtures (Beckman Instruments Co., 1979).

**RESULTS**

When a solution of all-trans-[11-3H]RA was fed orally to rats, the radioactivity, although remaining largely in the stomach up to 1 h, was found in small amounts in the blood and various tissues within 30 min of dosing. The distribution of radioactivity in serum and in other tissues of rats 30 min and 1 h after the administration of either a large dose (0.38 μmol/rat) or a huge dose (73.3 μmol/rat) of RA is shown in Table 1.

**Distribution and nature of radioactive compounds in serum and other tissues**

**Stomach.** As indicated in Table 1, most (88–95%) of the recovered radioactivity was present in the stomach and its contents. Although the dose of [³H]RA largely remained intact (Table 2), radioactive 5,6-ERA, 4-ERA and 4-ORA were also detected and characterized. Two other non-polar radioactive compounds, one tentatively characterized as an ester of RA, were also present (Table 2). However, no RAG was detected in the stomach.

**Serum.** Although [³H]RA was found to circulate in the blood within 30 min of dosing (Table 3), the predominant radioactive peak found at that time was a polar compound that was eluted close to the solvent front during h.p.l.c. Although the identity of this compound was not established with certainty, 4-ORA exhibits similar h.p.l.c. behaviour. At 1 h after a large or huge dose, however, RA was a predominant compound detected in the blood. Small quantities of 4-HRA and 5,6-ERA (Table 3) were also identified in the blood. Retinoyl β-glucuronide (RAG) was found to be the major metabolite (15.2 nmol/ml) (Table 3; Fig. 1a) 1 h after a huge dose of RA and a significant metabolite (6 nmol of RAG/ml) in the serum 30 min after a large dose. Non-polar radioactive compounds (tₚ = 18–28 min; Fig. 1c), possibly esters of RA, were found in the serum 1 h after either dose of RA.

**Small intestine.** Irrespective of the size of the dose, [³H]RA was the major compound in the small intestine (Table 3). Among all the tissues, however, the highest concentration of RAG was found in the small intestine (Table 3). Smaller quantities of 5,6-ERA, 4-HRA, 4-ORA mixed with polar compounds, and a non-polar compound, presumably an ester of RA, were also present in the small intestine (Table 3).

**Liver.** The pattern of radioactive compounds found in the liver after a large or huge dose was similar (Table 3). At both 30 min and 1 h, RA predominated, followed by a polar fraction, which probably included 4-ORA. A significant amount of 5,6-ERA and a small quantity of RAG were also detected.

**Kidney.** After a 0.38 μmol dose, kidney radioactivity was low (0.1%) at 30 min, but was much higher (2.5%) after 1 h (Table 1). At both doses, administered RA was the major radioactive compound present at 30 min or 1 h after the dose (Table 3).
Table 1. Recovery of radioactivity from serum and other tissues 30 min and 1 h after a large or huge dose of [3H]RA

Rats were fed orally 0.38 μmol or 73.3 μmol of [3H]RA in ethanol/peanut oil and killed 30 min or 1 h after the dose. The retinoids were extracted, and the radioactivity associated with each organ was determined. The volume of serum was assumed to be 4 % of the body weight.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dose...</td>
</tr>
<tr>
<td></td>
<td>Time...</td>
</tr>
<tr>
<td>Stomach + contents</td>
<td>64.7</td>
</tr>
<tr>
<td>Small intestine</td>
<td>1.6</td>
</tr>
<tr>
<td>Liver</td>
<td>1.5</td>
</tr>
<tr>
<td>Serum</td>
<td>0.2</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.1</td>
</tr>
<tr>
<td>Total</td>
<td>68.1</td>
</tr>
</tbody>
</table>

Table 2. Distribution of 3H-labelled retinoic acid and its metabolites in the stomach and its contents of rats 30 and 60 min after oral doses of 0.38 and 73.3 μmol, each containing 1.1 × 10^6 d.p.m. radioactivity

<table>
<thead>
<tr>
<th>Compound</th>
<th>Content (nmol/g of tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dose (μmol)...</td>
</tr>
<tr>
<td></td>
<td>Time (min)...</td>
</tr>
<tr>
<td>Retinoic acid</td>
<td>28</td>
</tr>
<tr>
<td>5,6-ERA</td>
<td>5.4</td>
</tr>
<tr>
<td>4-ORA*</td>
<td>2.0</td>
</tr>
<tr>
<td>4-HRA</td>
<td>2.1</td>
</tr>
<tr>
<td>Non-polar esters†</td>
<td>4.1</td>
</tr>
<tr>
<td>RAG</td>
<td>ND‡</td>
</tr>
</tbody>
</table>

* Mixed with polar metabolites.
† Partially characterized.
‡ ND, not detected.

Table 3. Distribution of [3H]RA and its metabolites in the serum, small intestine, liver and kidney of rats 30 min and 60 min after oral doses of 0.38 and 73.3 μmol

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Serum (pmol/ml)</th>
<th>Small intestine (pmol/ml)</th>
<th>Liver (pmol/ml)</th>
<th>Kidney (pmol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time period (min)...</td>
<td>30</td>
<td>60</td>
<td>60</td>
<td>30</td>
</tr>
<tr>
<td>Dose (μmol)...</td>
<td>0.38</td>
<td>0.38</td>
<td>0.38</td>
<td>0.38</td>
</tr>
<tr>
<td>RA</td>
<td>17</td>
<td>56</td>
<td>13800</td>
<td>410</td>
</tr>
<tr>
<td>5,6-ERA</td>
<td>7</td>
<td>14</td>
<td>2700</td>
<td>40</td>
</tr>
<tr>
<td>4-ORA*</td>
<td>26</td>
<td>35</td>
<td>3500</td>
<td>81</td>
</tr>
<tr>
<td>4-HRA</td>
<td>ND‡</td>
<td>12</td>
<td>3700</td>
<td>29</td>
</tr>
<tr>
<td>RAG</td>
<td>6</td>
<td>14</td>
<td>15200</td>
<td>82</td>
</tr>
<tr>
<td>Non-polar esters†</td>
<td>ND</td>
<td>6</td>
<td>6050</td>
<td>37</td>
</tr>
</tbody>
</table>

* Mixed with polar metabolites.
† Partially characterized.
‡ ND, not detected.
Fig. 1. Reversed-phase gradient h.p.l.c. profiles of (a) serum (500 µl) retinoids 1 h after the administration of a huge (73.3 µmol) oral dose of [3H]RA to a rat, showing the appearance of RAG in blood, (b) the same serum (150 µl) after treatment with β-glucuronidase, showing the conversion of RAG into RA, and (c) radioactivity associated with serum (500 µl and 150 µl) before (----) and after (———) treatment with β-glucuronidase respectively.

Note: absorption units full scale for (a) and (b) are 0.256 and 0.08 respectively. Abbreviation: ROL, retinol.

Fig. 2. Reversed-phase isocratic h.p.l.c. profiles and absorption spectra

(a) and (b) Reversed-phase isocratic h.p.l.c. profiles of (a) standard retinoids and (b) serum retinoids 1 h after a large (0.38 µmol) dose of [3H]RA, showing the appearance of [3H]RAG in the blood of a rat. The mobile phase was methanol/water (7:3, v/v) containing 10 mM-ammonium acetate; the flow rate was 1.2 ml/min. (c–e) Absorption spectra of retinoid fractions (in h.p.l.c. solvent) separated by gradient h.p.l.c. from the blood and stomach of a rat 1 h after a huge (73.3 µmol) dose of [3H]RA. (c): ———, RAG from blood; ———, RA from blood; (d): ———, ERA from blood; ———, RA from stomach; ———, same after treatment with acetic acid; ———, same after treatment with HCl; (e): ———, ORA from stomach; ———, HRA from stomach. Additional abbreviations: cRA, cis-retinoic acid; tRA, all-trans-retinoic acid.

Because of the co-elution of ORA with unidentified compounds that absorb strongly at shorter wavelengths, the small peak at 280 nm, characteristic of the 4-oxo group, was masked. Thus the exact concentration of 4-ORA could not be determined. The presumed presence of non-polar esters of RA was based on the tR of standard methyl retinoate during h.p.l.c. and on the disappearance of radioactivity in tissue extracts from this region after alkaline hydrolysis. Because of the very small amounts involved, further characterization of these presumed esters was not possible.

DISCUSSION

Retinoyl glucuronide was first recognized as a metabolite of retinoic acid in the bile (Olson, 1968; Zile et al., 1980) and small intestine of the rat (Zile et al., 1982). Recently, after a 80 mg dose of 13-cis-RA, the presence of small amounts of the glucuronide conjugate of 13-cis-RA along with other oxidized and glucuronidated metabolites in human bile has been reported (Vane et al., 1990). With the availability of synthetic RAG (Barua & Olson, 1985a, 1989a), the earlier finding that RAG
supported the growth of vitamin A-deficient rats (Nath & Olson, 1967) was confirmed (Barua & Olson, 1985a). RAG, like RA, induces the differentiation of HL-60 cells, but is less cytotoxic (Gallup et al., 1987; Zile et al., 1987; Janick-Buckner et al., 1991) and less teratogenic (Gunning et al., 1989, 1990) than RA. The occurrence of RAG in human blood as an endogenous compound has been clearly demonstrated (Barua & Olson, 1986), but the appearance of RAG in the blood after administration of RA has not previously been reported.

In the present study we have been able to show that RAG is synthesized rapidly from administered RA and can be detected in the blood within 30 min after the administration of RA. The ratio of RAG/RA in the serum was much higher after a huge dose (1.10) of RA than after a large dose (0.25). After a large dose of RA, the RAG peak was best resolved from RA and other metabolites by isocratic h.p.l.c.

The highest concentration of RAG was found in the small intestine (Table 3). In this regard, Zile et al. (1982) earlier found that the small intestine was a major site for the biosynthesis of RAG from RA. Although we demonstrated previously that RAG was the major retinoid in the liver up to 24 h after an intraperitoneal dose of [3H]RAG (Barua & Olson, 1989a), only very small amounts were found after oral dosing with RA (Table 3). In the present study, we could not detect any RAG in the kidney within 1 h after the dose of RA.

After the oral administration of a large dose of RA, radioactivity recovered from the blood and the major tissues (e.g. liver, small intestine and kidney) accounted for only 3.5–9.9% of the total dose (Table 1). To locate the rest of the administered radioactivity, the stomach and its contents were extracted and analysed. Indeed, 64–70% of the administered radioactivity, which equalled 88–95% of the total recovered radioactivity, was still present in the stomach 0.5–1 h after the dose. H.p.l.c. analysis of the extract from the stomach and its contents showed that, besides administered RA, three other radioactive compounds (namely 4-ORA, 4-HRA and 5,6-ERA) were present in amounts sufficient for characterization by their u.v. spectra. Furthermore, 5,6-ERA was characterized by demonstrating the expected shifts in its absorption maximum in the presence of acetic acid and HCl. Thus the 5,6-epoxy ring clearly does not isomerize to the furanoid form in the presence of stomach acid.

5,6-ERA was previously characterized as a physiological metabolite of RA (McCormick et al., 1980, 1982; McCormick & Napoli, 1982; DeLuca et al., 1981; Leo et al., 1984) on the basis of its molecular ion (M+), which is the same for both the 5,6- and the 5,8-epoxide of RA. In our studies, the small intestine and its contents also contained lesser amounts of 5,6-ERA. Whether 5,6-ERA, 4-ORA and 4-HRA found in the intestine and other tissues originated primarily in situ or were derived in part from those formed in the stomach merits further study.

As far as we are aware, this is the first study where the stomach and its contents have been analysed after a dose of RA. It seems unlikely that 5,6-RA, 4-ORA, 4-HRA and some non-polar esters are produced metabolically in stomach cells. Moreover, because a total of < 1% of oxidized derivatives of RA were formed in our inactivated-control experiments, it seems unlikely that their formation can be attributed to an artefact of the process of extraction and analysis. Thus the most plausible explanation is that they are formed in the stomach by a process of chemical oxidation. Because RA is only a very minor component of foods, whether ingested retinyl ester, the major dietary form of vitamin A, is affected similarly merits attention.

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