Embryotoxic Doses of Vitamin A to Rabbits Result in Low Plasma but High Embryonic Concentrations of All-trans-Retinoic Acid: Risk of Vitamin A Exposure in Humans

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ABSTRACT Retinoid pharmacokinetics were examined in plasma, placenta and embryos of gestational d 12 rabbits following application of an embryotoxic dosing regimen (10 mg retinyl palmitate/kg body wt per day from gestational d 7 to 12). Vehicle-treated or untreated rabbits served as controls. Physiological concentrations of all-trans-retinoic acid (all-trans-RA) and 13-cis-RA in rabbit plasma (5-8.33 nmol/L) were very close to the endogenous levels in human plasma. In addition, we identified endogenous all-trans-RA, 3,4-didehydroretinol and 3,4-didehydroretinoic acid in rabbit embryo. Following the last retinyl palmitate administration, apparent steady-state concentrations of all retinoids were reached in the examined compartments of rabbits. The major polar retinoid in plasma was 9,13-di-cis-RA, but its embryonic concentrations were only about 6% of those in plasma. In the embryo, retinol and its esters were found at high concentrations; lower amounts of all-trans-4-oxo-RA and the newly identified 14-hydroxy-4,14-reiro-retinol could also be measured. Embryonic concentrations of all-trans-RA were about 100% higher than endogenous levels. The overall exposure of the embryo to this retinoid was, however, substantial. Embryonic area under the concentration time curve values strongly suggest that the embryotoxicity of the applied dosing regimen is mainly due to the action of all-trans-RA. A very remarkable finding of this study is the marginal increase of plasma concentrations of all-trans-RA over their endogenous levels, which is comparable to the human situation after vitamin A intake. This analogy indicates that high vitamin A intake may be associated with a higher risk for teratogenic effects in humans even in the absence of high elevation of plasma all-trans-RA levels. J. Nutr. 126: 2159-2171, 1996.

INDEXING KEY WORDS:
- embryotoxicity
- rabbits
- retinoic acid
- retinol
- vitamin A

Vitamin A is an essential micronutrient for humans and animals and is required for a wide variety of biological processes, such as vision, reproduction, cell growth and differentiation, and embryonic development (Gudas 1994, Gudas et al. 1994). Numerous retinoids, a family of molecules structurally and/or functionally related to retinol (vitamin A alcohol), are also effective drugs for therapy of skin and neoplastic diseases and for prevention of carcinogenesis (Bollag and Holdener 1992). Unfortunately, retinoid applicability is limited by their high teratogenic potential. For example, 13-cis-retinoic acid (13-cis-RA, isotretinoin), a very effective
drug for systemic acne therapy, causes severe birth defects with a characteristic pattern ("retinoic acid embryopathy") including craniofacial, central nervous system, cardiovascular and thymus malformations (Nau et al. 1994). Similar effects can also be induced in laboratory animals by 13-cis-RA, all-trans-RA, as well as by vitamin A itself (reviewed by Agnish and Kochhar 1993).

The teratogenic potency of vitamin A in animals has raised concern about the safety of excess vitamin A use during pregnancy in humans. Indeed, 24 cases of fetal malformations possibly associated with daily intake of 25,000 IU of vitamin A (7.5 mg retinol) or more were reported (Rosa 1993). In addition, a retrospectively performed case-control study examined the relation between vitamin A supplementation and the occurrence of certain birth defects (Werler et al. 1990) and suggested a small increased risk of malformations at doses that exceeded by severalfold the dose of vitamin A present in most prenatal multivitamin preparations. Finally, a very recent epidemiological study, performed prospectively, reported an increased risk of birth defects after daily consumption of doses of vitamin A above a certain threshold during early pregnancy (Rothman et al. 1995). It should be noted that substantial exposure to vitamin A can occur not only through supplementation but also through dietary sources such as cooked liver (Buss et al. 1994).

For a more precise assessment of the critical vitamin A dose level, above which an increased risk for teratogenicity exists, information on pharmacokinetics and metabolism of vitamin A could be very helpful. Both of these issues have been addressed following vitamin A supplementation (Eckhoff et al. 1991a) or consumption of a liver meal by humans (Buss et al. 1994). These studies showed that dosing with vitamin A resulted in an appreciable increase of plasma concentrations of mainly 13-cis-RA and 13-cis-4-oxo-RA, and to a lesser degree of all-trans-RA, over their endogenous levels. Remarkably, the same retinoids are found in human plasma during isotretinoin therapy, which is associated with a very high risk of congenital anomalies. These findings support the hypothesis of increased risk of teratogenic effects induced by vitamin A (Eckhoff et al. 1991a). Additional evidence could be provided by data from animal studies, which may prove particularly helpful because they permit determination of the embryonic exposure to retinoids following administration of a vitamin A dose with a known teratogenic activity. Such studies were previously performed in mice and rats. In mice, substantial amounts of all-trans-RA and all-trans-4-oxo-RA were formed and extensively transferred to the embryo following administration of a teratogenic dose of retinol (100 mg/kg body wt), whereas concentrations of the 13-cis-isomers were negligible (Eckhoff et al. 1989). In contrast, the generation of polar retinoids was much more limited in rats administered teratogenic doses of retinyl palmitate (90 mg/kg per day), and no increase of all-trans-RA concentrations in plasma and embryos over the corresponding endogenous levels was observed (Collins et al. 1994). Thus, due to the differences in vitamin A metabolism in mice and rats vs. humans and the huge doses of vitamin A needed to induce teratogenic effects in these rodent species, these are probably not appropriate animal models for extrapolation to the human situation. On the other hand, the metabolism of vitamin A in cynomolgus monkeys is similar to that in humans with respect to the formation of 13-cis-RA, all-trans-RA and their 4-oxo-metabolites (Eckhoff et al. 1991b). The cynomolgus monkey may therefore be a better model; however, no information is available to date about the range of vitamin A doses that elicit teratogenicity in this species.

In this study we describe the transplacental pharmacokinetics of retinol and its metabolites in pregnant rabbits following multiple administrations of retinyl palmitate during organogenesis at a dose within the teratogenic range in this species (Kamm 1982). This dose is closer to those taken by humans through the diet and by supplementation, in contrast to the doses used to induce teratogenic effects in mice (Eckhoff et al. 1989) and rats (Collins et al. 1994). Our goal was to determine the rabbit embryonic exposure to retinoids, relate these findings to the human data available, and attempt to evaluate the teratogenic risk of high vitamin A intake in humans.

MATERIALS AND METHODS

Chemicals. Retinyl palmitate [94% all-trans-retinyl palmitate, 4% 13-cis-retinyl palmitate] used for animal experiments was synthesized and supplied by Hoffmann-La Roche [Basel, Switzerland]. Retinol was purchased from Serva (Heidelberg, Germany) and retinyl esters were synthesized in our laboratory previously (Eckhoff et al. 1989). All other reference retinoids were provided by Hoffmann-La Roche [Basel, Switzerland, and Nutley, NJ]. All-trans-[10,11,12,20-13C]-RA was a gift from CIRD Galderma (Sophia Antipolis, France). All-trans-retinyl-β-glucuronide [ROG] and all-trans-retinol-β-glucuronide [RAG] were gifts from A. B. Barua and J. A. Olson [Iowa State University, Ames, IA]. 14-Hydroxy-4,14-retro-retinol [14-HRR] was kindly provided by J. Buck [Cornell University Medical College, New York, NY] and F. Derguini [Memorial Sloan-Kettering Cancer Center, New York, NY]. Lyophilized analytical grade bovine serum albumin was purchased from Sigma (München, Germany), β-glucuronidase from E. coli was from Boehringer Mannheim (Mannheim, Germany). Organic solvents were of HPLC grade and obtained from Merck (Darmstadt, Germany), which was also the source of all other analytical-grade chemicals. Diazomethane was generated from 1-
methyl-3-nitro-1-nitrosoguanidine [MNNG] with a MNNG-diazomethane kit [Aldrich, Steisenheim, Germany]. Highly pure water for HPLC was obtained from a Milli-Q water purification system (Millipore, Eschborn, Germany). Stock solutions of retinoids were generally prepared in ethanol at a theoretical concentration of 333 μmol/L (for retinol and retinyl palmitate: 3330 μmol/L isopropanol), which was subsequently photometrically corrected. These solutions were kept in glassware at −20°C, tested for stability, and freshly prepared when necessary.

**Laboratory precautions.** All laboratory manipulations involving the retinoids [preparation of dosing solutions, drug administration to rabbits, collection of samples and analytical procedures] were performed in dark rooms under dim yellow light to prevent photodegradation.

**Animals and ethical approval.** Swiss hare rabbits and Himalayan rabbits were obtained from the Biological Research Laboratories (Füllingsdorf, Switzerland) and Karl Thomae GmbH (Biberach, Germany), respectively, and were kept at the Toxicology Department of Hoffman-La Roche, where the in vivo part of the study was performed. Animals were maintained individually in air-conditioned rooms with a temperature of about 18°C and a relative air humidity of 55 ± 10%, and were given free access to pellet diet (Kliba 23-341-4 with 14,000 IU vitamin A/kg diet, from Kliba-Mühlen Klingentalmühle, Kaiseraugst, Switzerland; Nafag No. 9143 with 9,600 IU vitamin A/kg diet, from Nafag Eco-san, Gossau, Switzerland) and tap water. All the rabbits were acclimated to the above conditions and to a 12-h light-dark cycle for at least 4 wk before the beginning of the experiment. For mating, one female was placed with one male in the morning until copulation was observed. After 4 to 6 h, the mating procedure was repeated with another male. The day of copulation was designated GD 0. At this time, females weighed 2.8 to 4.8 kg. Approval for the study was obtained from Kantonales Veterinäramt, Postfach 264, CH-4025 Basel, Switzerland.

**Study protocol.** Retinyl palmitate was administered to mated Swiss hare rabbits by gastric intubation once daily from GD 7 to GD 12. The daily dose was 10 mg retinyl palmitate/kg body wt (corresponds to 5.46 mg retinol/kg body wt), and the dosing volume was 2 mL/kg body wt. This dose was selected based on results of Segment II teratology studies in yellow silver rabbits. In those studies, 5 mg/kg were slightly teratogenic but not embryolethal. A dose of 20 mg/kg resulted in slight embryolethality and some malformed fetuses; 80 mg/kg resulted in 100% embryonic death (Kamm 1982). The dose of 10 mg/kg was expected to induce slight embryotoxic effects. The GD 12 embryos used for kinetic evaluations could not be examined for malformations, and no separate teratology study was performed in Swiss hare rabbits to confirm a teratogenic effect with this strain. However, an embryotoxic effect was confirmed in the present study at 10 mg/kg (see Results). For dose preparation, retinyl palmitate was dissolved in rapeseed oil [Scherer, Eberbach, Germany] using a Polytron mixer at a concentration of 5 g/L. The dosing solution was prepared fresh weekly, stored in the dark at 4°C under N₂, sonicated prior to administration in a 4°C water bath for about 5 min, and constantly mixed during animal treatment by a magnetic stirring bar. The stability of retinyl palmitate under these conditions had thoroughly been validated in the Toxicology Department of Hoffmann-La Roche at earlier times. For intubation, the rabbits were restrained in a wooden box with a firm but elastic rubber cover, and their head looked out of the box. For intubation, rubber catheters (40 cm × 0.6 cm; Rüsch, Waiblingen, Germany) were inserted into the mouth via the margo interalveolaris and passed into the stomach. The does were observed daily for changes in behavior and general condition. At various time intervals following the sixth administration, the rabbits were killed with a captive bolt gun, and blood samples of about 3 mL were collected by heart puncture and drawn into a heparinized syringe. At the time of bleeding, conceptuses were rapidly removed from the uterus, and embryos and placentae were isolated and pooled for each litter after removal of decidua, adhering membranes and fluids. The time intervals for sampling were before dosing (0 h) and 0.5, 1, 2, 3, 4, 6, 10, 24, and 36 h after treatment. Plasma was prepared by centrifugation of the blood for 10 min at 1000 x g and 4°C. Plasma, embryonic and placenta samples were stored in polypropylene vials at −80°C until analysis. Three to five does with viable embryos of sufficient weight were available for each time point except for 0, 4 and 24 h, at which only two litters could be collected.

In addition, mated Swiss hare rabbits (n = 9) received daily administrations of rapeseed oil [2 mL/kg body wt] from GD 7 through 12, and collection of maternal plasma, embryos, and placentae occurred 2–4 h following the last vehicle administration, in the same way as described for the treated animals. To confirm the retinoid values of the vehicle-treated animals and to study possible vehicle effects on endogenous retinoid concentrations, an additional control group of mated Himalayan rabbits (n = 7) was retrospectively designed. These does remained untreated, and samples were obtained on GD 12 by the same methods as in the other groups of the study. The reason for conducting this experiment with Himalayan rabbits was that the Swiss hare strain of rabbits could no longer be obtained.

All biological samples were sent frozen on dry ice to the Berlin laboratory for retinoid analysis.

**HPLC analysis.** Retinoids were analyzed by a reversed-phase HPLC method with gradient elution following sample enrichment with liquid-liquid and solid-phase extraction (thoroughly described by Collins et al. 1992). According to this method, plasma, placenta and embryonic samples were extracted with a threefold vol-
volume of isopropanol (placental and embryonic tissue were disrupted by pulsed sonication on ice upon addition of isopropanol), the samples were shaken at room temperature for 2 min, and precipitated protein was pelleted by short centrifugation at 6500 × g and room temperature. The supernatants (0.4 mL) were diluted with a threefold volume of an aqueous ammonium acetate solution (20 g/L) and extracted on AASP C$_2$ cartridges (silica modified with ethyl groups; ICT, Bad Homburg, Germany) using the Varian module (Varian, Darmstadt, Germany) prior to introduction into the HPLC system. Following sample enrichment, cartridges were loaded onto the Varian AASP injector, which was connected on-line with the HPLC system. Retinoids were separated on an octadecyl silica column (i.e., ratio of peak areas or heights) at the two detection wavelengths with that of the standard retinoids.

Recovery and reproducibility of the assay and calibration with an external method were previously reported (Collins et al. 1992, Eckhoff et al. 1989). Peak identification was based on comigration with authentic standards and on coincidence of the absorbance ratio of retinoid peaks collected from the system above and for closer examination of their chromatographic behavior. Briefly, peak eluate was extracted on C$_2$ solid-phase extraction cartridges and analyzed on a 120 × 4 mm Spherisorb ODS2 3 µm column. Retinoids were isocratically eluted with 0.9 mL/min acetonitrile-methanol-isopropanol-water-acetic acid (30:25:15:30:1.2, v/v). This method has recently been proven capable of resolving 13-cis-RA, 9,13-di-cis-RA, 9-cis-RA, and all-trans-RA (Tzimas et al. 1994a).

**HPLC-mass Spectrometry.** The HPLC system for HPLC-MS analysis consisted of a Waters 600-MS pump, a Waters 490-MS UV detector (Waters, Eschborn, Germany) and a Rheodyne 7125 valve (Rheodyne, CA) fitted with a 20-µL loop for sample introduction. Analysis was conducted with a 120 × 4 mm Spherisorb ODS2 3 µm column and isocratic elution using as mobile phase methanol-60 mmol/L aqueous ammonium acetate (87.5:12.5, v/v), at a flow rate of 0.7 mL/min. Following UV detection at 340 nm, the eluate was introduced through a Finnigan MAT atmospheric pressure chemical ionization (APCI) HPLC-MS interface into a Finnigan MAT TSQ 7000 triple-stage quadrupole mass spectrometer (Finnigan MAT, Bremen, Germany). Positive ions were produced in the corona discharge region following exit from the vaporizer, which was heated at 500°C for analysis of 3,4-dDROH and 550°C for analysis of other retinoids. The mass spectrometer was operated either in the full scan mode, with a m/z range 200-350, or in the selected ion detection mode. The HPLC-MS system was integrated with a high performance RISC-based workstation (Finnigan MAT).

**Gas chromatography-mass spectrometry.** Gas chromatography with mass selective detection was utilized for validation of the identity of putative RA isomers. HPLC fractions were evaporated in a Speed Vac Concentrator and methylated with diazomethane dissolved in diethyl ether (100 µL) following addition of 20 µL of ethanol that contained 6 ng of all-trans-$^{13}$C$_4$-RA as internal standard. After derivatization, the samples were evaporated again and reconstituted in 20 µL of hexane. Aliquots (1 µL) were injected into the cool on-column injector of a GC-MS system (HP 5890 gas chromatograph coupled with a mass selective detector 5971A and controlled by a DOS-chemstation, all from Hewlett Packard, Waldbronn, Germany). Separations were achieved on a HP Ultra 1 capillary column (25 m × 0.2 mm i.d., 0.33-µm film thickness) with helium as carrier gas (0.5 mL/min). The oven temperature was initially set at 110°C, then raised with 30°C/min to 230°C and held there for 2 min. The mass selective detector was operated under chemical ionization conditions with isobutane as reagent gas. Chromatograms were recorded in the selected ion mode with the detector focused on m/z = 315 for methyl esters of RA isomers and on m/z = 319 for all-trans-$^{13}$C$_4$-methylretinoate.

**Other procedures.** For characterization of putative glucuronidated retinoid metabolites, collected peak fractions of the examined retinoids (1 vol.) were diluted with Sørensen buffer of pH 6.8 (1.8 vol.), incubated with β-glucuronidase solution (0.4 vol.; 200 IU/mL) for 1 h at 37°C and further analyzed like regular samples (see above).

**Statistical and pharmacokinetic analysis.** Statistical evaluation of data was performed using the two-tailed Student's t test. Statistical significance was chosen at P < 0.05. Concentrations are presented as means ± SD. The maximum measured concentrations [C$_{max}$] were determined upon inspection of the data. Area-under-the-concentration-time-curve (AUC) values were calculated by the trapezoidal rule for the time interval from 0 to 24 h post-treatment.

**RESULTS**

**Embryotoxic effect of the dosing regimen.** The embryotoxic potency of the applied administration regimen was confirmed by the high embryolethality on GD 12. In the retinoid-treated group, 51 rabbits were employed, and 122 embryos out of 312 implantation...
All-trans-RA and retinyl palmitate were resorbed (resorption rate: 39%). The resorption rate highly varied among the individual does, as shown by the mean ± SD of the relative number of resorptions (no. of resorptions/no. of implantations, as percentage) calculated for every dam (43 ± 35%). The resorption rate in the vehicle-treated group was 10% of the total number of implantation sites, or 9.8 ± 9.4% when expressed as the percentage of implantations per total number of implantation sites, or 9.8 ± 9.4% when expressed as the percentage of implantations per rabbit.

**Endogenous retinoids in plasma, embryos and placenta of rabbits.** Table 1 displays retinoid concentrations in maternal plasma, embryos and placenta of vehicle-treated and untreated rabbits on GD 12. The most prominent retinoid in rabbit plasma was retinol with concentrations of about 3000–3300 nmol/L. In contrast, retinyl esters were found in minimal amounts. Plasma levels of 13-cis-RA and all-trans-RA were between 5 and 8.3 nmol/L, whereas 9-cis-RA was not detectable. The presence of RA isomers in plasma of untreated rabbits was additionally confirmed by GC-MS. Analysis of maternal plasma (cf. Table 1) and with the isocratic analyses, and their identity was further substantiated by following information. First, their A340/A356 ratios were identical to those of authentic retinoids. Second, peak B and peak C comigrated with 3,4-ddROH and all-trans-RA reference standards, respectively, when rechromatographed with a mobile phase with different selectivity (shown in Fig. 1B for 3,4-ddROH only). Third, HPLC-MS analysis in the APCI mode and selected ion monitoring at m/z = 301 (for all-trans-RA) and m/z = 267 (for 3,4-ddROH) showed that peak B and peak C were coeluted with the corresponding reference standards (Fig. 1C). Selection of m/z = 267 for mass selective detection of 3,4-ddROH was based on the mass spectrum of standard 3,4-ddROH, which exhibited a predominant ion at m/z = 267. This probably represents dehydration of the molecular ion MH⁺, i.e., [MH–H₂O]⁺. The mass spectrum of all-trans-RA had a predominant ion at m/z = 301, which corresponds to M + 1 [MH⁺]. We have evidence that peak A represents 3,4-ddRA, based on 1) its coelution with authentic 3,4-ddRA, both with the method used for analysis of embryonic samples (Fig. 1A) and with the isocratic method employed for analysis of collected peak A eluate [Fig. 1B], and 2) on its A340/A356 ratio, which is similar to that of reference standard 3,4-ddRA. The amount of peak A collected from rabbit embryos was not sufficient for further confirmation of its identity by HPLC-MS.

Analysis of placenta samples of vehicle-treated and untreated rabbits showed the high abundance of retinol and its esters; interestingly, the ratio of esterified vs. nonesterified retinol concentrations was even higher in the placenta than in the embryo (1.1 vs. 0.7 in vehicle-treated rabbits; 1.4 vs. 0.4 in untreated rabbits). All-trans-, 13-cis-RA, and the 3,4-didehydroretinoids could be identified: all-trans-RA (peak B), 3,4-ddROH (peak C), and 3,4-ddRA (peak A). Peaks B and C were determined to be all-trans-RA and 3,4-ddROH, respectively, due to coelution with authentic standards under the conditions of the embryonic analyses, and their identity was further substantiated by following information. First, their A340/A356 ratios were identical to those of authentic retinoids. Second, peak B and peak C comigrated with 3,4-ddROH and all-trans-RA reference standards, respectively, when rechromatographed with a mobile phase with different selectivity (shown in Fig. 1B for 3,4-ddROH only). Third, HPLC-MS analysis in the APCI mode and selected ion monitoring at m/z = 301 (for all-trans-RA) and m/z = 267 (for 3,4-ddROH) showed that peak B and peak C were coeluted with the corresponding reference standards (Fig. 1C). Selection of m/z = 267 for mass selective detection of 3,4-ddROH was based on the mass spectrum of standard 3,4-ddROH, which exhibited a predominant ion at m/z = 267. This probably represents dehydration of the molecular ion MH⁺, i.e., [MH–H₂O]⁺. The mass spectrum of all-trans-RA had a predominant ion at m/z = 301, which corresponds to M + 1 [MH⁺]. We have evidence that peak A represents 3,4-ddRA, based on 1) its coelution with authentic 3,4-ddRA, both with the method used for analysis of embryonic samples (Fig. 1A) and with the isocratic method employed for analysis of collected peak A eluate [Fig. 1B], and 2) on its A340/A356 ratio, which is similar to that of reference standard 3,4-ddRA. The amount of peak A collected from rabbit embryos was not sufficient for further confirmation of its identity by HPLC-MS.

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### Table 1

**Endogenous retinoid concentrations in maternal plasma, embryos and placenta of rabbits on gestational d 12**

<table>
<thead>
<tr>
<th>Retinoid</th>
<th>Plasma (nmol/L)</th>
<th>Embryo (nmol/kg)</th>
<th>Placenta (nmol/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle-treated</td>
<td>Untreated</td>
<td>Vehicle-treated</td>
</tr>
<tr>
<td>All-trans-RA</td>
<td>5.0 ± 1.0</td>
<td>4.7 ± 1.0*</td>
<td>76.3 ± 13.0</td>
</tr>
<tr>
<td>13-cis-RA</td>
<td>8.3 ± 3.3</td>
<td>5.7 ± 3.7</td>
<td>—7</td>
</tr>
<tr>
<td>3,4-ddRA</td>
<td>—7</td>
<td>—7</td>
<td>25.5 ± 6.7</td>
</tr>
<tr>
<td>3,4-ddROH</td>
<td>—7</td>
<td>—7</td>
<td>127 ± 18.3</td>
</tr>
<tr>
<td>Retinol</td>
<td>3339 ± 937</td>
<td>3007 ± 490</td>
<td>818 ± 75.2</td>
</tr>
<tr>
<td>Retinyl palmitate</td>
<td>10.3 ± 2.1</td>
<td>67.9 ± 25.4*</td>
<td>433 ± 246</td>
</tr>
<tr>
<td>Retinyl stearate</td>
<td>7.7 ± 2.4</td>
<td>60.6 ± 22.5*</td>
<td>133 ± 52.5</td>
</tr>
<tr>
<td>Retinyl linoleate</td>
<td>—7</td>
<td>—7</td>
<td>—7</td>
</tr>
</tbody>
</table>

1 Values are means ± SD, n = 6–7. *Significantly different from corresponding values of vehicle-treated rabbits (P < 0.05).
2 Swiss hare rabbits were treated daily with rapeseed oil at a dosing volume of 2 mL/kg body wt from gestational d 7 to 12.
3 Himalayan rabbits were untreated.
4 Additional analysis by GC-MS for validation of endogenous all-trans-RA gave 4.3 ± 0.5 nmol/L (n = 4).
5 A broad peak with retention time similar to that of authentic all-trans-RA hindered the detection of all-trans-RA in placenta samples.
6 Confirmed by GC-MS.
7 Below limit of detection.
FIGURE 1 Identification of endogenous retinoids in gestational d 12 rabbit embryos. (A) HPLC chromatogram of vehicle-treated rabbit embryo (sample mass = 100 mg). Arrows show the elution positions of authentic retinoids: 1, all-trans-4-oxo-RA; 2, 13-cis-4-oxo-RA; 3, ROG; 4, 3,4-ddRA; 5, 13-cis-RA and 9, 13-di-cis-RA; 6, all-trans-RA; 7, 14-HRR; 8, 3,4-ddROH; 9, retinol; 10, retinyl linoleate; 11, retinyl palmitate and retinyl oleate; 12, retinyl stearate. RAG is eluted 0.2 min prior to ROG and 9-as-RA 0.2 min prior to all-trans-RA. The eluates of peak A (putative 3,4-ddRA), peak B (putative all-trans-RA) and peak C (putative 3,4-ddROH) were collected for further characterization. (B) Overlays of HPLC chromatograms demonstrating coelution of peak C with authentic 3,4-ddROH (left; retention time = 13.52 min) and of peak A with authentic 3,4-ddRA (right; retention time = 16.40 min), after rechromatography with an isocratic method. The solid line chromatograms are for peaks A and C, and the dotted line chromatograms for authentic 3,4-ddRA and 3,4-ddROH. (C) APCI HPLC-MS mass selective chromatograms of peak C and authentic 3,4-ddROH at m/z = 267 (left) and peak B and authentic all-trans-RA (as well as its 13-cis-isomer) at m/z = 301 (right).
FIGURE 2 Identification of 9,13-di-cis-RA in rabbit plasma following retinyl palmitate administrations. (A) HPLC chromatogram of retinyl palmitate treated rabbit plasma [sample volume = 100 μL]. Arrows show the elution positions of authentic retinoids [see legend of Fig. 1 for labeling]. Coelution was observed for peak D and 13-cis-4-oxo-RA, peak E and ROG, peak F and 9,13-di-cis-RA, peak G and all-trans-RA, peak H and retinol, peak I and retinyl linoleate, peak J and retinyl palmitate/oleate, and peak K and retinyl stearate. Eluate of peak F was collected for further characterization. (B) Overlay of HPLC chromatograms showing coelution of peak F with authentic 9,13-di-cis-RA after rechromatography with an isocratic method. The solid line chromatogram is for peak F, the dotted line chromatogram for a mixture of four authentic RA isomers (from left to right: 13-cis-RA, 9,13-di-cis-RA, 9-cis-RA, and all-trans-RA). The structural formula of 9,13-di-cis-RA is shown in the insert. (C) Mass spectra of authentic 9,13-di-cis-RA [left] and of purified peak F [right] by APCI HPLC-MS.
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not be detected in rabbit placenta, however, the detection of all-trans-RA may have been hindered by the presence of a broad interfering peak with retention time similar to that of all-trans-RA.

9,13-di-cis-Retinoic acid is a major plasma retinoid following vitamin A administrations. Following the sixth daily administration of 10 mg retinyl palmitate/kg body wt on GD 12, various retinyl esters, retinol and a number of more polar retinoids were found in rabbit plasma, placenta and embryos. **Figure 2A** shows a representative HPLC chromatogram of a rabbit plasma 1 h after treatment. The most abundant peak, after retinol, retinyl palmitate, and retinyl stearate, was peak F with a retention time of about 10.1 min; thus, it was coeluted with authentic 13-cis-RA and 9,13-di-cis-RA. Therefore, peak F eluate was collected and subjected to isocratic analysis on a system that provides adequate resolution of four RA isomers. Putative 14-HRR comigrated with authentic 14-HRR under these conditions of analysis (not shown). In addition, equal amounts of either the examined metabolite or reference standard 14-HRR were repeatedly analyzed at different wavelength settings between 332 and 368 nm. This kind of analysis revealed a UV$_{max}$ at 348 nm for both entities as well as very similar absorbance ratios for A$_{340}$/A$_{356}$ (0.91 vs. 0.94), A$_{340}$/A$_{356}$ (0.91 vs. 0.94), and A$_{340}$/A$_{359}$ (1.38 vs. 1.33) for putative and authentic 14-HRR, respectively. These data suggest that 14-HRR is generated in vivo from retinol.

**Identification of 14-hydroxy-4,14-retro-retinoL** Another retinoid peak which was coeluted with 14-HRR was observed in chromatograms of placenta and embryos of retinyl palmitate treated rabbits. For further characterization, the eluate of this peak was collected from several HPLC analyses and analyzed with the isocratic method used for validation of the identity of other retinoids. Putative 14-HRR was submitted to APCI HPLC-MS analysis, which yielded essentially identical mass spectra for peak F and authentic 9,13-di-cis-RA (Fig. 2C). The major ion observed for both substances had m/z = 301, corresponding to MH$^+$. The identity of ROG was substantiated by incubation of the corresponding HPLC fraction (peak E) with β-glucuronidase. Subsequent HPLC analysis of the incubation products showed the absence of the putative ROG peak and the occurrence of a peak with retention time and absorbance characteristics similar to those of retinol (not shown).

**Retinoid distribution in plasma, placenta and embryos following vitamin A administrations.** Concentration-time profiles for retinoids following the last of six daily administrations of 10 mg retinyl palmitate/kg body wt are shown in **Figure 3** for maternal plasma and **Figure 4** for placenta and embryo. Table 2 displays AUC of retinoids in the three compartments for the time interval 0-24 h post-treatment.

Multiple retinyl palmitate administrations resulted in very high plasma concentrations of retinyl esters on GD 12 [50- to 200-fold over control values]. Levels of retinyl esters were also prominent in placenta and embryos; however, their relative increase over control values was much lower than that in plasma. This is demonstrated by the placental and embryonic AUC values of retinyl esters, which were about 3.2- to 4.8-fold the "endogenous" AUC obtained by extrapolation of the control concentrations over a 24-h period. Interestingly, retinol levels were elevated to a much lower degree (only 70% higher than those in vehicle-treated rabbits), and the relative increase was similarly high in all three compartments.

The oxidative metabolism of retinol gave rise to considerable amounts of 9,13-di-cis-RA in plasma. A C$_{max}$
of 607 nmol/L for this retinoid was found before dosing on GD 12, and its mean levels ranged between 213 and 327 nmol/L post-treatment. All-trans-RA levels were markedly lower, and this was also reflected by its AUC, which was 2.4% of that of 9,13-di-cis-RA (Table 2). The reverse situation was observed in the embryo, where the AUC of all-trans-RA was about sevenfold that of the 9,13-di-cis-isomer. This mainly resulted from the limited transfer of 9,13-di-cis-RA from the maternal circulation to the embryo, as the low embryo to maternal plasma (E/M) AUC ratio of 9,13-di-cis-RA (0.064) indicates. On the other hand, the E/M AUC ratio for all-trans-RA was much higher, but it should be kept in mind that a considerable portion of the embryonic AUC of all-trans-RA simply resulted from the physiological occurrence of this retinoid in the rabbit embryo (cf. Table 1). 13-cis-4-oxo-RA was found only in maternal plasma (concentration up to 85 nmol/L), not in placenta or embryos, whereas all-trans-4-oxo-RA was present in the embryos but not in the other two compartments.

Glucuronidation was another metabolic pathway of retinol, as shown by the presence of ROG in rabbit plasma and placenta. Mean plasma concentrations of this glucoconjugate ranged between 16.7 and 95.2 nmol/L, whereas placenta levels were about 100% higher. In contrast, ROG was not detectable in rabbit embryo. The hydroxylated metabolite of retinol, 14-HRR, also displayed differential distribution in the examined compartments, because it could not be detected in maternal plasma, but measurable amounts of it were found in placenta \([C_{\text{max}} = 28.5 \ \text{nmol/kg}]\) and embryo \([C_{\text{max}} = 124 \ \text{nmol/kg}]\).

**DISCUSSION**

The aim of the present study was to examine whether the rabbit is an appropriate animal model for...
the assessment of the teratogenic risk of vitamin A in humans. For this purpose, we studied retinoid metabolism in pregnant rabbits, both under physiological conditions as well as following administration of teratogenic doses of retinyl palmitate, and we compared our findings with corresponding data on retinoid metabolism in humans.

**Endogenous retinoid status in rabbits and similarity to the human situation.** We determined endogenous concentrations of retinoids in rabbit maternal plasma and embryos to be able to precisely determine the exposure to retinoids arising from the administered retinyl palmitate. Furthermore, information on endogenous retinoids may be helpful in understanding the physiological role of retinoids across species.

The results of the present study reveal several similarities between rabbits and humans. First, the high abundance of retinol in contrast to much lower concentrations of retinyl esters, observed in rabbit plasma, is in concordance with similar findings in human plasma [Eckhoff et al. 1991a]. The situation is different in mice and rats, in which retinyl esters account for a considerable portion (up to 50%) of total vitamin A in plasma of pregnant animals [Eckhoff et al. 1989, Collins et al. 1994].

Second, concentrations of all-trans-RA and 13-cis-RA in rabbit plasma (Table 1) were very close to those in human plasma (4.40 ± 1.53 and 5.43 ± 2.83 nmol/L, respectively), as reported previously [Eckhoff and Nau 1990]. Again, mice and rats are different in this regard, because corresponding concentrations were much lower [Collins et al. 1994, Tzimas et al. 1995].

Third, we have demonstrated the physiological occurrence of 3,4-didehydroretinoids in the rabbit embryo (Fig. 1 and Table 1). 3,4-Didehydroretinol has previously been identified as an endogenous retinoid in human embryonic and fetal tissues [Creecraft et al. 1993] and in embryos of chickens [Scott et al. 1994, Thaller and Eichele 1990] and frogs [Creecraft et al. 1994], however, it could not be found in embryos of mice [Scott et al. 1994] or rats [Collins et al. 1994]. On the other hand, 3,4-ddRA has previously been found only in embryos of chickens [Thaller and Eichele 1990] and not in any other species examined. Our observation that 3,4-ddROH and 3,4-ddRA are endogenously present in the rabbit embryo is compatible with the view that didehydroretinoids may play an important physiological role. 3,4-ddRA is a biologically active retinoid: it displays morphogenetic activity in the chicken limb bud assay with equivalent potency to all-trans-RA [Thaller and Eichele 1990] and exhibits substantial biological activity in various models of epidermal differentiation [Törmä et al. 1994].

Finally, appreciable amounts of all-trans-RA were found in rabbit embryos, similarly to what was found in human embryonic and fetal tissues [Creecraft et al. 1993]. Rabbit embryonic concentrations of all-trans-RA were about 15-fold higher than those in plasma (Table 1). The high abundance of all-trans-RA in the embryo seems, however, to be a universal feature of vertebrates, as suggested by earlier findings in rodents [Collins et al. 1994, Scott et al. 1994], chickens [Scott et al. 1994, Thaller and Eichele 1990], and frogs [Creecraft et al. 1994]. Neither 13-cis-RA nor 9-cis-RA was detected in rabbit embryos.

**All-trans-RA and all-trans-4-oxo-RA are proximate teratogens of vitamin A administrations in rabbits.** A very important aspect of the present study was to determine the rabbit embryonic exposure to various retinoids with the idea of delineating the proximate teratogens. The applied dosing regimen was clearly embryotoxic and is expected also to be teratogenic, as previous studies in rabbits suggested [Kamm 1982]. Embryonic AUC of retinoids have been considered to be the most appropriate pharmacokinetic marker of the embryonic exposure to retinoids, rather than plasma or embryonic C\text{max} values or plasma AUC. This was based on the results of recent studies, which examined retinoid pharmacokinetic profiles in plasma and embryos following application of mildly teratogenic dosing regimens of all-trans-RA or 13-cis-RA to rabbits [Tzimas et al. 1994b] and rats [Collins et al. 1994].

Our data show that following retinyl palmitate administrations to rabbits, embryos were substantially exposed to all-trans-RA as well as to retinol and its esters (Table 2). A considerable portion of the high embryonic AUC of all-trans-RA is attributed to the endogenous pool of this retinoid (estimated as 76.3 ± 24 = 1831 nmol x h/kg, i.e., ca. 60% of the AUC value of 3097 nmol x h/kg). The remainder of the AUC, representing additional all-trans-RA, is still much higher than the plasma AUC of all-trans-RA. This may be explained by the rapid and extensive transfer of all-trans-RA from the maternal circulation to the embryo, as numerous studies in rabbits and other species have already well established [Collins et al. 1994, Eckhoff et al. 1989, Tzimas et al. 1994b, 1995]. Additionally, all-trans-RA may be locally synthesized from retinol; this oxidation could be accomplished by embryonic retinol dehydrogenase and oxidase activities, such as those recently described [Chen et al. 1995]. In contrast, 9,13-di-cis-RA, the major polar retinoid metabolite in rabbit plasma, displayed a very limited transplacental passage, as indicated by its low E/M AUC ratio (0.064). This is in agreement with recent observations in mice and rats [Tzimas et al. 1994a]; in that study, 9,13-di-cis-RA was shown to be the main polar metabolite of 9-cis-retinaldehyde and 9-cis-RA in maternal plasma, but its embryonic levels were 3% [mouse] and 1% [rat] of plasma concentrations.

Is the rabbit embryonic exposure to all-trans-RA sufficient to explain the embryotoxic potency of the applied dosing regimen? To answer this question we compared embryonic AUC values of retinoids following administrations of either retinyl palmitate [this study] or all-trans-RA [earlier study, reported by Tzimas et al. 1994b]
All-trans-4-oxo-RA, All-trans-RA, and 9,13-di-cis-RA were evaluated for the time interval 0-24 h post-treatment. Multiple comparisons test showed that there is no significant difference (P > 0.05) in the resorption rate among the retinyl palmitate- and all-mimns-RA-treated groups. Thus, the overall embryotoxic potency of these two dosing regimens was comparable. Remarkably, embryonic AUC of both all-trans-RA and all-frons-RA were very similar in the two studies. This comparison reveals a number of interesting similarities between rabbits and humans.

First, retinoid pharmacokinetic profiles are quite similar in both species. In particular, apparent steady-state concentrations were reached for all retinoids in rabbit plasma and the other compartments examined (Fig. 3 and 4) following the sixth daily administration of retinyl palmitate, whereas steady-state concentrations of 13-cis-4-oxo-RA only were observed in human plasma following repeated dosing with vitamin A. The steady state of 13-cis-4-oxo-RA in humans probably results from the long plasma half-life of this retinoid, found to be around 30 h in the study of Buss et al. (1994). The steady state of retinoid concentrations in rabbit compartments may accordingly reflect the very slow elimination rates of the retinoids; in addition, co-prophagy by rabbits may account for uptake of non-absorbed retinyl palmitate after the intragastric administration of the dosing solution, but it is unclear to what extent such a phenomenon would affect the pharmacokinetic profile of the administered retinoid and its metabolites.

The second similarity of vitamin A metabolism in rabbits and humans is that the predominant RA isomer in the plasma has not the all-trans-configuration; instead, the main RA isomer was 9,13-di-cis-RA in rabbit plasma (Fig. 2) and 13-cis-RA in human plasma (Buss et al. 1994, Eckhoff et al. 1991a). The rabbit is the first species studied to date in which the oxidative metabolism of exogenously administered retinol yields 9,13-di-cis-RA. In contrast, all-trans-RA was the major RA isomer in mouse plasma and tissues after retinol administration (Eckhoff et al. 1989), whereas appreciable amounts of both 13-cis- and all-trans-RA were found in plasma of cynomolgus monkeys following treatment.

### TABLE 3

Values of area under the concentration-time curves of retinoids in embryos of gestational d 12 Swiss hare rabbits following multiple oral administrations of either 6 mg all-trans-RA/kg body wt per day or 10 mg retinyl palmitate/kg body wt per day from gestational d 7 to 12.

<table>
<thead>
<tr>
<th>Retinoid</th>
<th>All-trans-RA (2) (6 mg/kg per day)</th>
<th>Retinyl palmitate (10 mg/kg per day)</th>
<th>nmol·h·kg(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>All-trans-RA</td>
<td>3180</td>
<td>3097</td>
<td>453</td>
</tr>
<tr>
<td>13-cis-RA</td>
<td>277</td>
<td>561</td>
<td>3</td>
</tr>
<tr>
<td>9,13-di-cis-RA</td>
<td>561</td>
<td></td>
<td></td>
</tr>
<tr>
<td>All-trans-4-oxo-RA</td>
<td>3097</td>
<td>3097</td>
<td>453</td>
</tr>
</tbody>
</table>

1 Areas under the concentration-time curves (AUC) were calculated for the time interval 0-24 h post-treatment.
2 Data from Tzimas et al. (1994b).
3 Below limit of detection.
The generation of 9,13-di-cis-RA may, however, have been underestimated in some of the earlier studies due to the coelution of 13-cis-RA and 9,13-di-cis-RA under standard HPLC conditions. Most recently, we observed that substantial amounts of 9,13-di-cis-RA are indeed present—together with 13-cis-RA—in human plasma following intake of high doses of vitamin A (Arnhold et al. 1996). The high abundance of 9,13-di-cis-RA in rabbit plasma and of 9,13-di-cis-RA and 13-cis-RA in human plasma does not necessarily mean that the total amount of the corresponding cis-isomer(s) synthesized in the body exceeded that of all-trans-RA; it rather may arise from differences in tissue distribution and the elimination rate between the RA isomers.

The high plasma concentrations of 9,13-di-cis-RA in rabbits following retinyl palmitate administrations raise several questions on the possible roles of this retinoid in mediation of retinoid action. This aspect was addressed in two recent studies: 9,13-di-cis-RA was shown to be a physiological constituent of neonatal calf plasma as well as of bovine plasma during the periparturient period, but it exhibited very low affinity for binding to two subtypes of RXR, RXRa and RXRβ (Horst et al. 1995a, 1995b), thus suggesting that 9,13-di-cis-RA is not a biologically active retinoid, at least per se. This may explain results of an earlier study showing that 9,13-di-cis-RA poorly stimulated the differentiation of HL-60 cells (Yen et al. 1986).

A third, and from the toxicological point of view the most interesting, similarity between rabbits and humans with respect to vitamin A metabolism is the marginal increase of plasma all-trans-RA concentrations over their endogenous levels following retinyl palmitate administrations (rabbit: this study, see Table 1, Fig. 3A; human: following 50,000 tu/d, Eckhoff et al. 1991a). In both species, the plasma AUC of all-trans-RA after the last vitamin A administration was only 40% higher than the "endogenous" AUC. However, rabbit embryonic exposure to all-trans-RA was substantial (Table 2) and possibly sufficient to account for the embryotoxic effects of the applied dosing regimen (Table 3). This finding suggests that relatively mild increase of all-trans-RA plasma concentrations in vitamin A-exposed pregnant women may result in substantial, possibly teratogenic, exposure of the embryo to all-trans-RA.

Overall, the present study strongly indicates that rabbits are very similar to humans with respect to 1) the dose of vitamin A needed to elicit embryotoxic effects, which in rabbits is much closer to those that are suspected to be teratogenic in humans than corresponding doses in mice and rats; 2) physiological concentrations of retinoids in plasma and embryos of rabbits and humans; and 3) retinoid pharmacokinetics and metabolism following vitamin A administrations. Most interestingly, the relative increase of plasma all-trans-RA concentrations over endogenous levels in rabbits was marginal and similar to that in humans following prolonged vitamin A supplementation (Eckhoff et al. 1991a) or single consumption of a liver meal (Buss et al. 1994). This analogy indicates that teratogenic effects of excess vitamin A in humans may possibly be induced even if plasma concentrations of metabolically generated all-trans-RA are mildly increased.

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LITERATURE CITED


