Identification of 9-*cis*,13-*cis*-Retinoic Acid as a Major Circulating Retinoid in Plasma[†,‡]

Ronald L. Horst,*[,§] Timothy A. Reinhardt,[§] Jesse P. Goff,[§] Brian J. Nonnecke,[§] Vineet K. Gambhir,[§] Paul D. Fiorella,[||] and Joseph L. Napoli[||]

United States Department of Agriculture, Agricultural Research Service, National Animal Disease Center, Metabolic Diseases and Immunology Research Unit, Ames, Iowa 50010-0070, and Department of Biochemistry, State University of New York at Buffalo, Buffalo, New York, 14214

Received July 7, 1994; Revised Manuscript Received September 12, 1994[⊗]

ABSTRACT: In the course of measuring the concentrations of retinoic acids (RA) in bovine plasma, a major peak was observed which comigrated with 9-*cis*-RA on normal-phase high-performance liquid chromatography. Rechromatography of this retinoic on reverse-phase high-performance liquid chromatography showed that it was distinct from 9-*cis*-, 13-*cis*-, and *all-trans*-RA, but comigrated with 9-*cis*,13-*cis*-retinoic acid (9,13-di-*cis*-RA). This retinoid was identified as 9,13-di-*cis*-RA based on its chemical, spectral, and chromatographic properties. Plasma concentrations of 9,13-di-*cis*-RA increased from ≤0.5 ng/mL at birth to 5−6 ng/mL by 48 h of age in the calf. The 9,13-di-*cis*-RA was also a major circulating product of 9-*cis*-RA dosed intramuscularly to rats; conversely, intravenous administration of 9,13-di-*cis*-RA produced circulating 9-*cis*-RA in the rat. 9,13-Di-*cis*-RA had little or no affinity for cellular retinoic acid binding proteins types I and II. This study establishes 9,13-di-*cis*-RA as a naturally-occurring retinoid under physiological conditions, shows that it undergoes interconversion with 9-*cis*-RA, and emphasizes a need for careful chromatography to resolve 9-*cis*-RA and 9,13-di-*cis*-RA. This is consistent with *in vivo* 13-*cis* isomerization operating to modify the concentration and perhaps the activity of 9-*cis*-RA *in vivo*.

Retinoic acid (RA)[1] is an activated metabolite of retinol that modulates biological processes involved in embryogenesis, skeletal development, cellular differentiation, and growth (Goss & McBurney, 1992; Morris-Kay, 1993; Richman, 1992; Summerbull & Maden, 1990; Wolf, 1984). Stereoisomers of RA induce transcription by binding to high-affinity intracellular receptors, which include two distinct families; RAR (Giguère et al., 1987; Petrovich et al., 1987) and RXR (Mangelsdorf et al., 1990), each with multiple isoforms (Nagpal et al., 1992). Both *all-trans*-RA and 9-*cis*-RA can bind and activate RAR. The preferred ligand for RXR is 9-*cis*-RA (Heyman et al., 1992; Levin et al., 1992), which can transactivate RXR up to 40 times more efficiently than *all-trans*-RA (Heyman et al., 1992). Although the RAR and RXR act as ligand-activated transcription factors, there is evidence that RXR also acts as an accessory factor for other receptors. Through formation of heterodimers with RAR, vitamin D receptor, and thyroid hormone receptor, the RXR can promote high-affinity binding to respective hormone response elements (Glass et al., 1990; Liao et al., 1990; Murray & Towle, 1989; Yang et al., 1991). While this action requires the cognate ligand for RAR, vitamin D receptor, and thyroid hormone receptor, it is not dependent upon the RXR ligand 9-*cis*-RA.

Because of their ability to activate receptor-mediated transcription events, recent attention has focused on the biosynthesis and metabolism of *all-trans*-RA and 9-*cis*-RA. Derivatives of *all-trans*-RA that circulate in the plasma under physiological conditions include 13-*cis*-RA and the glucuronides of *all-trans*- and 13-*cis*-RA (Barua & Olson, 1986; Eckhoff & Nau, 1990; Tang & Russel, 1990). Although the relationships of these metabolites to retinoid function have not been established, these modifications may provide reservoirs of slowly-metabolized, low-activity retinoids which contribute to maintaining steady-state concentrations of *all-trans*-RA (Barua et al., 1989; Gunning et al., 1993; McCormick et al., 1983; Napoli et al., 1982). Because its role as a ligand for RXR was perceived only recently, less is known about the metabolism of 9-*cis*-RA. The present study demonstrates that 9,13-di-*cis*-RA is a physiologically-occurring retinoid which can be produced from 9-*cis*-RA and can also support 9-*cis*-RA synthesis *in vivo*. Like 13-*cis*-RA, 9,13-di-*cis*-RA has less affinity for CRABP types I and II, than its parent. These data suggest that 13-*cis*-isomerization *in vivo* may operate as a common mechanism for modifying the concentrations and activities of *all-trans*- and 9-*cis*-RA. Nobably 9,13-di-*cis*-RA, which exceeds the concentration of 9-*cis*-RA in newborn bovine plasma, migrates closely to 9-*cis*-RA during normal-phase HPLC. Unless care is taken, 9,13-di-*cis*-RA may be mistaken for 9-*cis*-RA.

MATERIALS AND METHODS

General. High-performance liquid chromatography was performed with a Waters ALC/GPC 204 liquid chromatograph. Retinoids were detected at 340 nm. The analytical

[†] This study was supported in part by NIH Grant DK47839 (J.L.N.).

[‡] Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

* Address correspondence to this author.

[§] United States Department of Agriculture.

[||] State University of New York at Buffalo.

[⊗] Abstract published in *Advance ACS Abstracts,* January 1, 1995.

[1] Abbreviations: CRABP, cellular retinoic acid binding protein; HPLC, high-performance liquid chromatography; MeRA, methyl esters of retinoic acid; NMR, nuclear magnetic resonance; RA, retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor; 9,13-di-*cis*-RA, 9-*cis*,13-*cis*-retinoic acid.

0006-2960/95/0434-1203$09.00/0

normal-phase column was an Econosphere Silica 3 μm (0.45 × 15 cm) from Alltech Assoc. (Deerfield, IL). The analytical reverse-phase column was a Suplex pkg-100 5 μm (25 × 4.6 mm) from Supelco, Inc., (Bellefonte, PA). The preparative reverse-phase column was a Zorbax ODS (2.2 × 25 cm) and the preparative normal-phase HPLC column was a Zorbax Sil (2.2 × 25 cm); each was purchased from Dupont Co. (Wilmington, DE). Solvents were HPLC grade and were purchased from Fischer Scientific (Itasca, IL). [^{1}H]NMR were determined on a Varian Unity 500 MHz spectrophotometer.

Animals. Sample collection and processing were performed under yellow lights to minimize isomerization of endogenous RAs. A total of 15 mL of blood was collected into a heparinized tube from each of 12 Jersey calves at 6-h intervals from birth until 48 h of age. The calves received approximately 2 L of maternal colostrum at birth and at 12-h intervals. Blood was immediately spun in a refrigerated (4 °C) centrifuge at 1000*g*, and the plasma was harvested.

Plasma pharmacokinetic studies were conducted with male weanling rats (150−160 g) (Sprague−Dawley, Madison, WI) maintained on a stock diet (Lot 5012, Purina Mills Inc., St. Louis, MO).

Compounds. The 9-*cis*-RA was either a gift from Hoffman-La Roche (Nutley, NJ) or synthesized as described below. The 9-*cis*- and 9,13-di-*cis*-RA were prepared by oxidation of 9-*cis*-retinal (Sigma Chemical Co., St. Louis, MO) with Tollens reagent (Barua & Barua, 1964). The Tollens reagent was prepared immediately prior to use by combining equal volumes of 10% w/w $AgNO_3$ and 10% w/w NaOH. NH_4OH was added dropwise to dissolve the precipitated AgOH. A total of 4 mL of Tollens reagent was added slowly to 10 mL of 9-*cis*-retinal in methanol (5 mg/mL) and incubated in stoppered tubes at 37 °C for 6 h. Isomerization was catalyzed by the strong alkaline conditions. The reaction was quenched by acidification with 4 N HCl. The products were extracted twice with 3 vol of hexane. The hexane fractions were combined, the solvent was evaporated, and the residue was applied to a Zorbax ODS preparative column eluted in acetonitrile/methanol/water/acetic acid (30:50:20:0.6) at 9 mL/min. In this system, the 9,13-di-*cis*-RA eluted 420−460 mL, and 9-*cis*-RA eluted 480−520 mL. The appropriate fractions were combined and submitted for NMR analysis. From 5 mg of 9-*cis*-retinal, 1.2 mg of 9-*cis*-RA and 0.35 mg of 9,13-di-*cis*-RA were obtained routinely. The identities of the products as 9-*cis*-RA and 9,13-di-*cis*-RA were verified by UV and NMR analyses. 9-*Cis*-RA: UV_{EtOH} = 349 nm; [^{1}H]NMR ($CDCl_3$) 1.02 (s, C16,17-dimethyl), 1.73 (s, C18-methyl), 1.95 (s, C19-methyl), 2.35 (s, C20-methyl), 5.78 (s, 14H), 6.05 (d, 10H), 6.24 (d, 7H), 6.63 (d, 8H), 7.1 (dd, 11H), 6.28 (d, 12H). 9,13-Di-*cis*-RA: UV_{EtOH} = 348 nm; [^{1}H]NMR ($CDCl_3$) 1.02 (s, C16,17-dimethyl), 1.73 (s, C18-methyl), 1.99 (s, C19-methyl), 2.07 (s, C20-methyl), 5.65 (s, 14H), 6.16 (d, 10H), 6.27 (d, 7H), 6.63 (d, 8H), 7.09 (dd, 11H), 7.65 (d, 12H).

Methylation. Methylation of the RA stereoisomers was performed using the MNNG-diazomethane kit from Aldrich Chemical Co. (Milwaukee, WI). The methylated RA derivatives were separated using normal- and reverse-phase analytical HPLC. The normal-phase HPLC columns were eluted in hexane/isopropyl ether (99.75:0.25), and the reverse-phase columns were eluted in acetonitrile/methanol/water/acetic acid (60:15:25:0.5).

Pharmacokinetics. Plasma 9-*cis*- and 9,13-di-*cis*-RAs were monitored in two groups of animals (six rats/group) receiving either 9-*cis*-RA (80 μg injected into the semimembranous muscle) or 9,13-di-*cis*-RA (80 μg intrajugularly). The injections were given in 50 μL of ethanol. Each group of six rats was subdivided into two groups of three. Bleeding times were alternated between the two subgroups so that no rat was bled more than four times during the course of the experiments. Blood (1 mL) was collected from the jugular vein in a heparinized syringe while the rats were maintained under isoflurane anesthesia.

Isolation of 9,13-Di-cis-RA from Neonatal Calf Serum. Three liters of newborn calf serum (Lot 30P1029) was purchased from Gibco Laboratories (Grand Island, NY). Aliquots (500 mL) of serum were transferred to 4-L brown glass bottles with screw caps. Proteins were precipitated by the addition of 500 mL of ethanol. The serum/ethanol mixture was acidified with 124 mL of 4 N HCl. The RAs were extracted by adding 2 L of hexane to each bottle and vigorously shaking them for three 30-s intervals, allowing the phases to separate between each interval. The hexane phase was removed, transferred to a round-bottom flask, and evaporated under vacuum. The lipid residue was dissolved in 24 mL of hexane. A total of 0.5 mL of this hexane solution was applied to each of the 48 silica Sep-Paks (Waters Associates, Inc., Milford, MA). The flask was washed with an additional 24 mL of hexane, which was also applied to the Sep-Paks. Neutral lipids were eluted by the addition of 12 mL of hexane methylene chloride (75:25). The RAs were eluted with 8 mL of hexane/methylene chloride/acetic acid (95:5:1) and collected in 13 × 100 glass tubes. The tubes containing the RA fraction were combined, and the solvent was removed under vacuum. The residue was dissolved in 4 mL of hexane/methylene chloride acetic acid (95:5:0.5) and applied to a preparative normal-phase HPLC system, which was preequilibrated and standardized with the same solvent. At a flow rate of 9 mL/min, the RAs eluted between 62 and 70 min. Final purification of the extracted retinoids was achieved with analytical normal- and reverse-phase HPLC. The overall yield of 9,13-di-*cis*-RA from 3 L of newborn calf serum was about 10 μg.

RA Assays. Plasma retinoids were measured using a modification of the assay of Napoli (1986, 1990). To 1 vol of plasma was added 15 ng of a RA analog (Ro-23-6457, Hoffman-La Roche, Nutley, NJ) in 50 μL of ethanol as an internal standard. Plasma proteins were precipitated with 1 vol of ethanol, followed by acidification of the mixture with 0.25 vol of 4 N HCl. The lipids were extracted from the mixture with 3 vol of hexane. The hexane phase was evaporated under vacuum. The residue was suspended in 0.5 mL of hexane and applied to a silica Sep-Pak. To optimize recovery, the tube containing the lipid pellet was washed with an additional 0.5 mL of hexane, which was also applied to the Sep-Pak. Neutral lipids were eluted from the Sep-Pak with 10 mL of 75:25 hexane/methylene chloride. The RAs were eluted with 5 mL of 95:5:1:2 hexane/methylene chloride/2-propanol/acetic acid. The solvent was evaporated, and the residue was applied to an analytical normal-phase HPLC column eluted with hexane/methylene chloride/acetic acid (95:5:0.2) at 2 mL/min. Using this system, the 13-*cis*-, 9-*cis*-, 9,13-di-*cis*-, and *all-trans*-RAs eluted at 12.8, 13.4, 13.5, adn 14.4 min, respectively (Figure 1A). Because the 9-*cis*-RA and 9,13-di-*cis*-RA were not

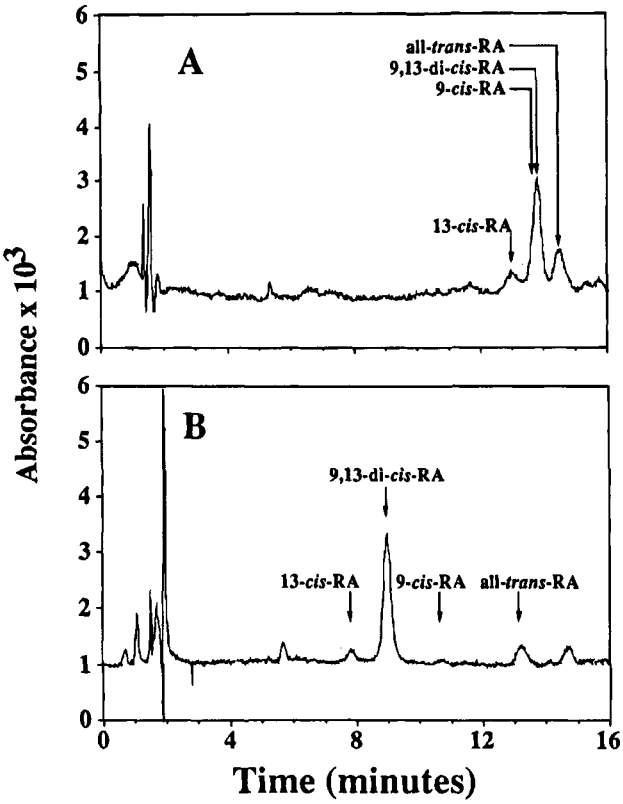

FIGURE 1: (A) Typical HPLC chromatogram of RA isomers extracted from 2 mL of plasma collected from calves at 48 h of age. Arrows indicate the elution of authentic standards. The column used was a normal-phase Econosphere 3 μm column (0.45 × 15 cm) from Alltech, developed in hexane/methylene chloride/acetic acid (95:5:0.2) at 2 mL/min. (B) Second HPLC analysis of RA stereoisomers in bovine plasma. Material corresponding to the elution of the RAs (the 12−15-min region in panel A) was collected and applied to a reverse-phase column (Suplex pkb-100 5 μm, 25 × 4.6 mm) eluted in acetonitrile/methanol/water/chloroform/acetic acid (17:68:10:5:0.6) at 2 mL/min. Elution positions of authentic standards are indicated.

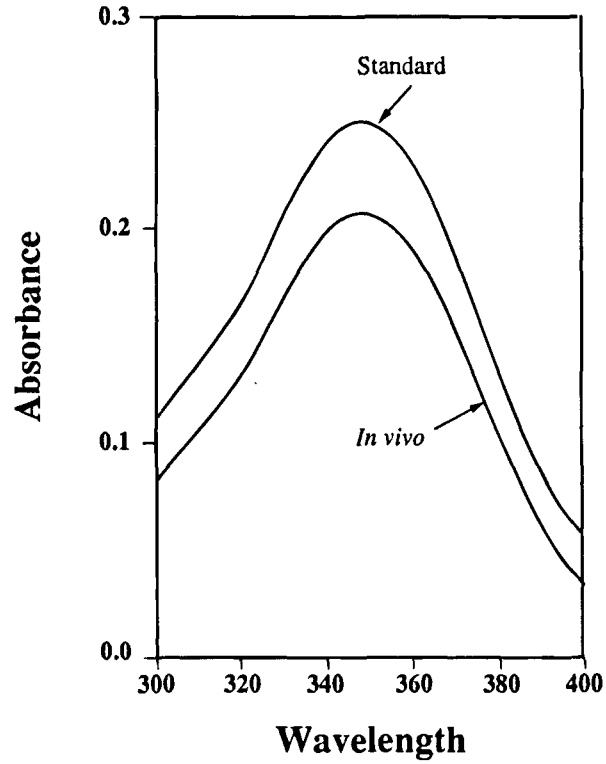

FIGURE 2: UV spectrum of 9,13-di-*cis*-RA purified from newborn calf serum (*in vivo*) or chemically synthesized (standard). The spectra were taken in ethanol with a Beckman DU-70 spectrophotometer.

resolved on the normal-phase system, the region containing the RA isomers (the 12−15-min region) was collected and subjected to a reverse-phase HPLC system eluted with acetonitrile/methanol/water/chloroform/acetic acid (17:68:10:5:0.6). In this system, all the RA isomers of interest were resolved (Figure 1B).

Preparation of CRABP (Types I and II). Expression of *Escherichia coli*-derived apo-CRABP (types I and II), their purification, and fluorescence titration were performed as described previously (Fiorella & Napoli, 1991; Fiorella et al., 1993). The K_d values for RA binding to CRABP were calculated by the method of Cogan et al. (1976).

RESULTS

The most abundant RA isomer in bovine plasma migrated closely with 9-*cis*-RA on normal-phase HPLC (Figure 1A). The migration of this isomer, however, was slightly slower than that of 9-*cis*-RA and was more characteristic of 9,13-di-*cis*-RA migration. Because the normal-phase system did not provide adequate resolution of 9-*cis*-RA and 9,13-di-*cis*-RA, the fraction from the normal-phase system, which corresponded to the elution of 13-*cis*-, 9-*cis*-, 9,13-di-*cis*-, and *all-trans*-RAs, was collected and subjected to reverse-phase HPLC. Initial attempts to separate the RA isomers by reverse-phase HPLC involved an Altech Econosphere 3 μm ODS column (0.45 × 15 cm), eluted in acetonitrile/methanol/water/acetic acid (50:30:20:0.6). Using this HPLC system, the suspected 9,13-di-*cis*-RA comigrated with 13-*cis*-RA (data not shown). Reverse-phase HPLC with the Suplex pkb-100 column resolved 13-*cis*-, 9,13-di-*cis*-, 9-*cis*-, and *all-trans*-RAs and showed that the most abundant isomer comigrated with 9,13-di-*cis*-RA (Figure 1B). With this system, we determined also that plasma 9-*cis*-RA concentrations were low to undetectable. To confirm that the most abundant RA isomer had the 9,13-di-*cis* configuration, the retinoid was purified from newborn calf serum. The UV spectrum of the isolated compound was similar to synthetic 9,13-di-*cis*-RA (Figure 2) with a UV_{max} at 348 nm. Further confirmation of the chemical structure was provided by comigration of the methyl esters of the unknown and synthetic 9,13-di-*cis*-RA on normal- and reverse-phase HPLC (Figure 3).

The relative abundance of 9,13-di-*cis*-RA isolated from newborn calf serum prompted further experiments to evaluate changes in its plasma concentrations during the first 48 h of life in neonatal calves. 9,13-Di-*cis*-RA was present at low (≤0.5 ng/mL) but detectable concentrations at birth. Within 12 h following birth, the plasma concentrations began to rise and reached 5−6 ng/mL by 48 h of age (Figure 4). Because of limited availability of synthetic 9-*cis*-RA, rats instead of calves were used to determine whether 9,13-di-*cis*-RA is a metabolite of 9-*cis*-RA. Following intramuscular injection of 9-*cis*-RA (80 μg), peak plasma concentrations of 9,13-di-*cis*-RA trailed the increase in 9-*cis*-RA by approximately 1.5 h and remained detectable through at least 24 h (Figure 5). In contrast, the 9-*cis*-RA concentrations decreased below detection (<0.5 ng/mL) by 12 h. Attempts were also made to obtain similar data using intravenous and intraperitoneal

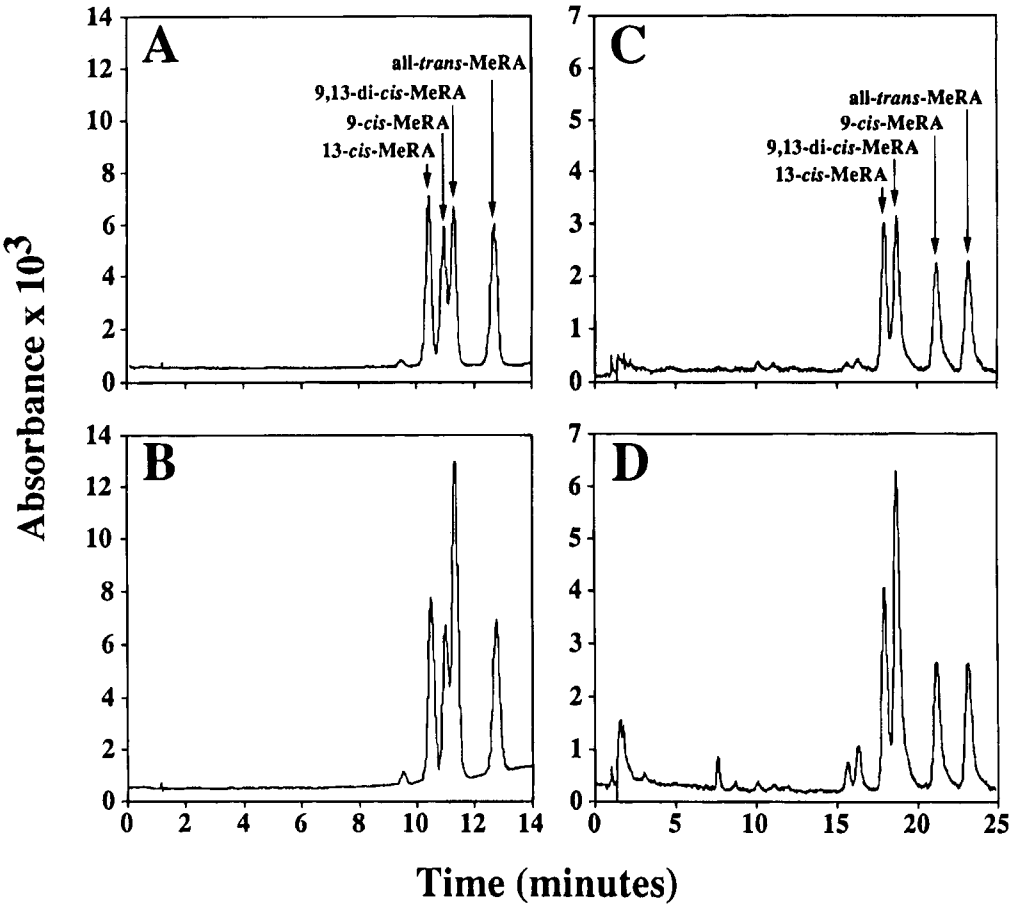

FIGURE 3: Comigration of the methyl esters (Me) of 9,13-di-*cis*-RA with authentic standards on normal- and reverse-phase HPLC. The 9,13-di-*cis*-RA was purified from 2 mL of normal bovine serum as described in Materials and Methods by extraction with hexane, elution through a Sep-Pak, and sequential elution through preparative normal-phase and analytical normal- and reverse-phase HPLC columns. The *in vivo* metabolite was methylated with diazomethane and compared with the ME of authentic standards by normal- and reverse-phase HPLC. (A) Authentic 13-*cis*-, 9-*cis*-, 9,13-di-*cis*-, and *all-trans*-MeRA (50 ng each) eluted from a normal-phase column (3 μm Econosphere, 0.45 × 25 cm) with 0.25% isopropyl ether in hexane at 2 mL/min. (B) Co-injection of 50 ng of 9,13-di-*cis*-MeRA isolated from bovine plasma with 50 ng each of authentic 13-*cis*-, 9-*cis*-, 9,13-di-*cis*-, and *all-trans*-MeRA eluted through a normal-phase column as described in panel A. (C) Authentic 13-*cis*-, 9-*cis*, 9,13-di-*cis*-, and *all-trans*-MeRA (50 ng each) eluted from a reverse-phase column (5 μm Suplex pkb-100, 0.46 × 25 cm) with acetonitrile/methanol/water/acetic acid (60:15:25:0.5) at 2 mL/min. (D) Co-injection of 50 ng of 9,13-di-*cis*-MeRA isolated from bovine plasma with 50 ng each of authentic 13-*cis*-, 9-*cis*-, 9,13-di-*cis*-, and *all-trans*-MeRA eluted through a reverse-phase column as described in panel C.

administration of 9-*cis*-RA. These experiments never resulted in detectable plasma 9-*cis*-RA when the 80-μg dose was given. However, in one of three experiments conducted with intravenously dosed 9-*cis*-RA, plasma 9,13-di-*cis*-RA was detected at the 18−24-h time points (data not shown).

When 9,13-di-*cis*-RA was given intravenously to rats, a 9,13-di-*cis*-RA decay curve was observed with an estimated disappearance half-time of about 4 h (Figure 6). The 9-*cis*-RA concentrations followed the same trend but were only ~3% of the 9,13-di-*cis*-RA concentrations. To determine if sample preparation could account for the presence of 9-*cis*-RA in 9,13-di-*cis*-RA-treated animals, 3 μg of 9,13-di-*cis*-RA/mL (values similar to that observed during the early stages of the 9,13-di-*cis*-RA plasma disappearance study) was added to rat plasma that had no detectable endogenous 9-*cis*- and 9,13-di-*cis*-RA. Concentrations of RA isomers were determined following sample extraction and quantitation by HPLC as described above. The data showed that ≤0.5% conversion of 9,13-di-*cis*-RA into 9-*cis*-RA occurred as a result of extraction and HPLC.

Potential binding of 9,13-di-*cis*-RA with CRABP (types I and II) was measured and compared with the binding of *all-trans*-, 9-*cis*-, and 13-*cis*-RA by quenching of CRABP fluorescence (Figure 7). As expected from previous work (Fiorella & Napoli, 1991; Fiorella et al., 1993), the three retinoids bound to both binding proteins with nominal K_d values of 20, 60, and 213 nM, respectively, for CRABP(I) and 40, 120, and 250 nM, respectively, for CRABP(II). Under the same circumstances, 9,13-di-*cis*-RA in concentrations up to 3 mM (i.e., twice as much ligand as binding protein) produced little quenching of CRABP fluorescence.

DISCUSSION

The transcriptional activation functions of RAR and RXR are mediated in large part by their ligands. *all-trans*-RA serves as a ligand for the RAR, while 9-*cis*-RA can serve as a ligand for both RAR and RXR. The experiments described herein were done to gain further insight into the presence of *all-trans*-RA and *cis*-RA isomers in plasma. We determined that 13-*cis*- and *all-trans*-RA are present in neonatal bovine plasma and were surprised to find that the most abundant circulating RA isomer is 9,13-di-*cis*-RA. Although 9,13-di-*cis*-RA was synthesized chemically as early as 1958 (Matsui et al., 1958), this paper provides the first demonstration of this isomer *in vivo*. The structural assignment of the 9,13-di-*cis*-RA was based on physical information showing

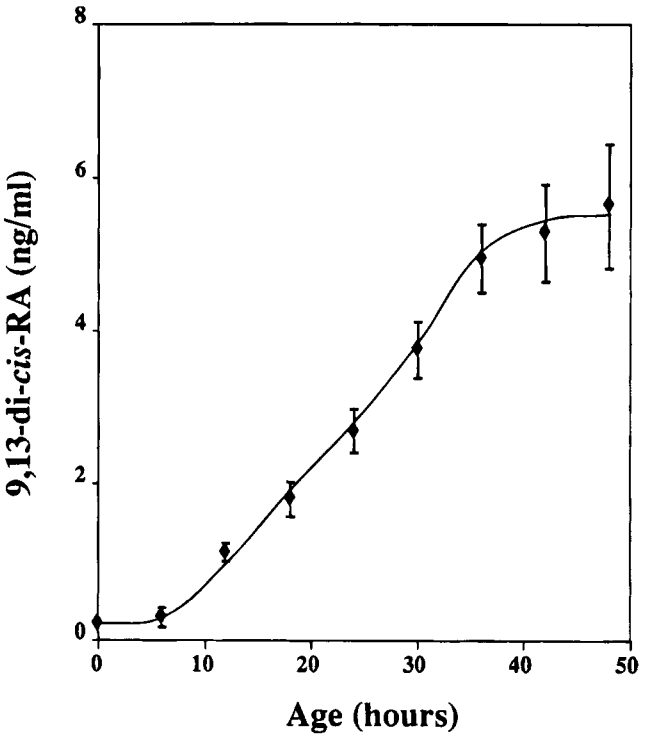

FIGURE 4: Time-dependent appearance of 9,13-di-*cis*-RA in plasma of calves during their first 48 h of life. Each point represents the mean ± SE, $n = 12$. Calf plasma (2 mL) was extracted, and 9,13-di-*cis*-RA was measured as described in Materials and Methods under *RA Assays*.

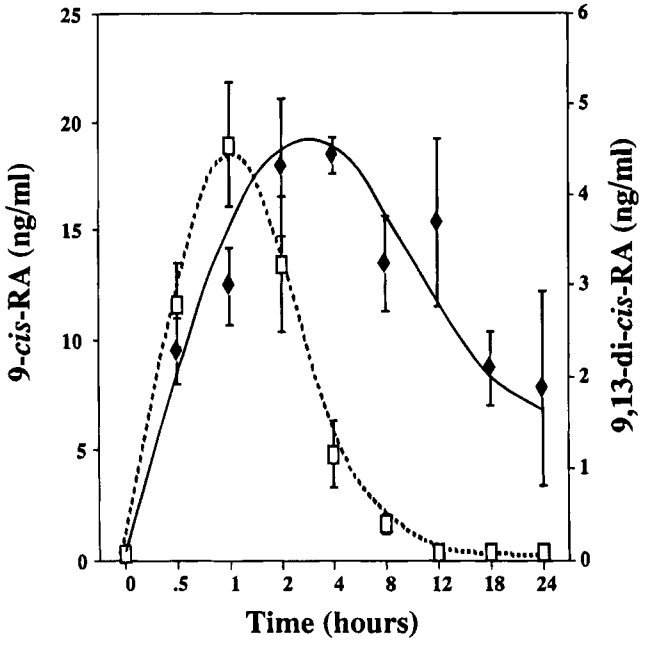

FIGURE 5: Plasma appearance of 9-*cis*- and 9,13-di-*cis*-RA following dosing with 9-*cis*-RA. Rats were given an intramuscular injection of 9-*cis*-RA (80 μg). Each point represents the mean ± SE, $n = 3$. Rat plasma (about 0.5 mL) was analyzed as described in Materials and Methods under *RA Assays*: (□) 9-*cis*-RA; (♦) 9,13-di-*cis*-RA. Note the different scales for 9-*cis*- and 9,13-di-*cis*-RA.

that the retinoid had the spectroscopic properties of authentic 9,13-di-*cis*-RA and, as a free acid, migrated with authentic standard through these HPLC systems (a preparative normal-phase, an analytical normal-phase, and analytical reverse-phase) and, upon methylation, comigrated with authentic

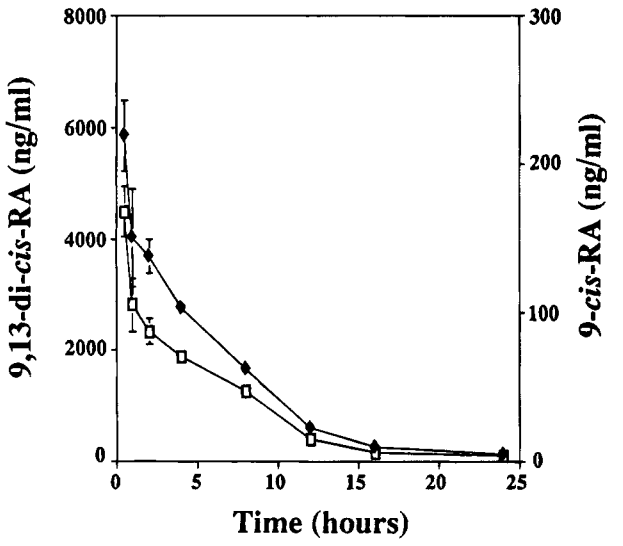

FIGURE 6: Plasma 9-*cis*- and 9,13-di-*cis*-RA following the administration of 80 μg of 9,13-di-*cis*-RA. Each point represents the mean ± SE of three rats dosed intravenously with 80 μg of 9,13-di-*cis*-RA. Note the differences in the two *y*-axis scales. Measurements were made as indicated in the legend to Figure 6: (□) 9-*cis*-RA; (♦) 9,13-di-*cis*-RA.

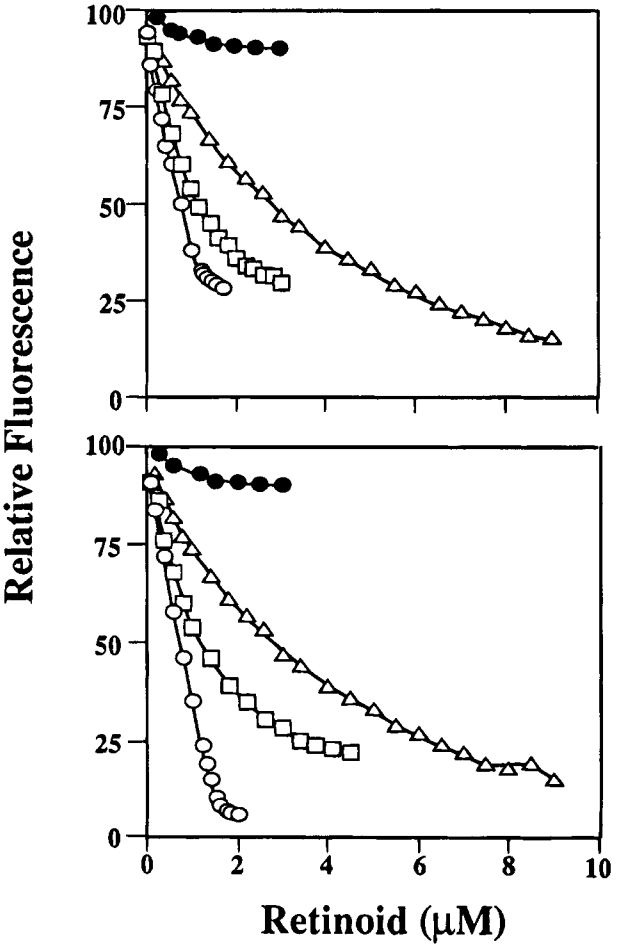

FIGURE 7: Relative affinities of CRABP (types I and II) for RA isomers. apo-CRABP(I), upper panel, and apo-CRABP(II), lower panel, were titrated with *all-trans*-RA (○), 9-*cis*-RA (△), 13-*cis*-RA (□), or 9,13-di-*cis*-RA (●). Experiments were done in 20 mM Tris-HCl, pH 7.4, with 1.5 μM CRABP(I) or 1.7 μM CRABP(II) as described (Fiorella & Napoli, 1991; Fiorella et al., 1993).

standard on reverse- and normal-phase analytical HPLC systems.

The plasma concentrations of 9,13-di-*cis*-RA were low (about 0.5 ng/mL) but detectable at birth and were elevated to 5−6 ng/mL by 48 h of age (Figure 4). The reasons for the dramatic rise are unknown, and the relative high abundance of this RA isomer in the bovine neonate prompted several questions regarding precursors and potential role(s) in either directly-mediating or indirectly-reflecting cellular events. For example, 9-*cis*-RA given intramuscularly to rats consistently produced elevations in plasma 9,13-di-*cis* RA. This suggests that one source of 9,13-di-*cis*-RA was metabolism of 9-*cis*-RA. The presence of 9,13-di-*cis*-RA may, therefore, be an indirect indicator of the cellular concentration and/or metabolism of 9-*cis*-RA. Conversely, 9,13-di-*cis*-RA appeared to act as an *in vivo* precursor for 9-*cis*-RA (Figure 6). Based on this latter observation, 9,13-di-*cis*-RA may be exploited therapeutically as a prodrug supporting *in vivo* 9-*cis*-RA synthesis and thereby indirectly controlling RXR and/or RAR-specific gene regulation. The 9,13-di-*cis*-RA could also have inherent biological activity mediated through a known or unidentified receptor.

There are few studies of 9,13-di-*cis*-RA activity or function. One study concluded that 9,13-di-*cis*-RA is at least as active as *all-trans*-RA in inhibiting growth and stimulating the differentiation of HL-60 cells (Murayama et al., 1993). Another study (Yen et al., 1986) concluded that 9,13-di-*cis*-RA is not effective at inducing HL-60 cell differentiation. Perhaps the different findings are attributable to the degree that the test retinoid isomerized in culture. It seems reasonable to propose that 13-*cis*-isomerization would have the same effect on 9-*cis*-RA potency as 13-*cis*-isomerization has on the potency of *all-trans*-RA. For example, conversion of *all-trans*-RA into 13-*cis*-RA reduces the ability to induce F9 cell differentiation by 10-fold (Williams et al., 1987). Indeed, 13-*cis*-RA seems to have <10% of the potency of *all-trans*-RA in disrupting human development with respect to central nervous system malformations and postnatal death, growth reduction, and behavioral dysfunction (Adams, 1993). A combination of relatively low toxicity and activity, a longer elimination half-life than *all-trans*-RA, and conversion into *all-trans*-RA *in vivo* suggests that 13-*cis*-RA serves as a reduced-activity reservoir of *all-trans*-RA (McCormick et al., 1983; Sandberg et al., 1994). The current work has demonstrated that 9,13-di-*cis*-RA is derived from and more abundant than 9-*cis*-RA and apparently is eliminated more slowly than 9-*cis*-RA (Figure 5). In these respects and with respect to binding to CRABP(I and II), the relationship between 9-*cis*- and 9,13-di-*cis*-RA parallels the relationship between *all-trans*- and 13-*cis*-RA. These data are consistent with the hypothesis that 13-*cis*-isomerization may serve to reversibly modify and, therefore, maintain the concentrations of the more active *all-trans*- and 9-*cis*-RA.

The order of binding affinities for both CRABP(I) and CRABP(II) was *all-trans*-RA ≫ 9-*cis*-RA > 13-*cis*-RA ≫ 9,13-di-*cis*-RA. This agrees with previous work which concluded that *all-trans*-RA binds stoichiometrically with excess CRABP (types I and II) (Bailey & Siu, 1988; Ong & Chytil, 1978; Sani & Banerjee, 1978) and isomerization greatly decreases affinity for CRABP(I) and CRABP(II), with 13-*cis*-isomerization having more impact than 9-*cis*-isomerization (Fiorella et al., 1993). Although these relative affinities are apparent, the precise differences in affinities between stoichiometric binding retinoids (*all-trans*-RA) and equilibrium binding retinoids (9-*cis*-RA and 13-*cis*-RA) cannot be determined by equilibrium binding methods because in the presence of excess CRABP, *all-trans*-RA does not equilibrate between binding protein and solvent. If estimates of CRABP affinity for retinol can be used as a general guide for affinity between stoichiometrically binding retinoids and retinoid binding proteins, then the underestimates of affinity for stoichiometric binding retinoids may be 2 orders of magnitude (Li et al., 1991). Thus, the K_d of *all-trans*-RA would be closer to 0.2 and 0.4 nM than the 20 and 40 nM indicated by equilibrium binding techniques for CRABP(I) and CRABP(II), respectively. On the other hand, the K_d values of 9-*cis*-RA and 13-*cis*-RA of 60 and 213 nM, respectively, for CRABP(I) and 120 and 250 nM, respectively, for CRABP(II) probably are reasonably accurate because these retinoids are "equilibrium binding" retinoids. Therefore, 9-*cis*-RA and 13-*cis*-RA would likely have ~300- and ~600−1000-fold less affinity for CRABP(I) and CRABP(II), respectively, than *all-trans*-RA. The negligible affinity of 9,13-di-*cis*-RA supports this inference by revealing that dual isomerization increases the K_d values for CRABP(I) and CRABP(II) to more than the 600 nM and 1.2 μM, respectively, predicted (3-fold for 9-*cis*-isomerization times and 10-fold for 13-*cis*-isomerization) if the nominal K_d values were accurate for *all-trans*-RA.

The lack of binding of 9,13-di-*cis*-RA to CRABP should be considered in view of the relationship among CRABP(I) concentrations, the rate of *all-trans*-RA metabolism, and the potency of *all-trans*-RA. CRABP(I) seems to attenuate RA action by sequestering the hormone and acting as a conduit for its efficient metabolism (Boylan & Gudas, 1991, 1992; Fiorella & Napoli, 1991; Williams & Napoli, 1985; Winston, 1989). The inconsequential binding of 9,13-di-*cis*-RA to CRABP(I) indicates that it would not affect the metabolism of *all-trans*-RA by displacing it from CRABP.

The presence of 9,13-di-*cis*-RA *in vivo* does not confound measurement of *all-trans*-RA because either normal- or reverse-phase HPLC can resolve *all-trans*-RA from its *cis* isomers. But 9,13-di-*cis*-RA does pose potential problems for quantifying 9-*cis*-RA and 13-*cis*-RA in biological samples. The normal-phase HPLC column used in this work as well as columns from other vendors (data not shown) did not resolve 9,13-di-*cis*-RA from 9-*cis*-RA. We have also found that the reverse-phase Suplex HPLC column used in this work was atypical, insomuch as it could readily resolve all four isomers in question (*all-trans*-RA, 9-*cis*-RA, 13-*cis*-RA, and 9,13-di-*cis*-RA); reverse-phase columns from several other vendors could not resolve 9,13-di-*cis*-RA from 13-*cis*-RA. Consequently, analysis with a single HPLC column could result in misidentification of 9,13-di-*cis*-RA as 9-*cis*-RA or 13-*cis*-RA.

This paper describes 9,13-di-*cis*-RA as the most abundant RA isomer in neonatal bovine plasma. One metabolic pathway leading to 9,13-di-*cis*-RA likely proceeds through 9-*cis*-RA. The presence of circulating 9,13-di-*cis*-RA may, therefore, be an indirect indicator of 9-*cis*-RA presence or metabolism in tissues. This work also emphasizes the need for the use of chromatographic systems with adequate selectivity when monitoring RA isomers in biological samples.

ACKNOWLEDGMENT

We thank Derrel A. Hoy for excellent technical assistance, Kathleen S. Kelderman for preparation of the manuscript, and Gary D. Fry for care of experimental animals.

REFERENCES

Adams, J. (1993) *Neurotoxicol. Teratol. 15,* 193−202.

Bailey, J. S., & Siu, C. (1988) *J. Biol. Chem. 263,* 9326−9332.

Barua, R. K., & Barua, H. B. (1964) *Biochem. J. 92,* 21C−22C.

Barua, A. B., & Olson, J. A. (1986) *Am. J. Clin. Nutr. 43,* 481−485.

Barua, A. B., & Olson, J. A. (1989) *Am. J. Clin. Nutr. 50,* 370−374.

Boylan, J. F., & Gudas, L. J. (1991) *J. Cell Biol. 112,* 965−969.

Boylan, J. F., & Gudas, L. I. (1992) *J. Biol. Chem. 267,* 21486−21491.

Cogan, V., Kopelman, M., Mokady, S., & Shimitzky, M. (1976) *Eur. J. Biochem. 65,* 71−78.

Eckhoff, C., & Nau, H. (1990) *J. Lipid Res. 31,* 1445−1454.

Fiorella, P. D., & Napoli, J. L. (1991) *J. Biol. Chem. 266,* 16572−16579.

Fiorella, P. D., Giguère, V., & Napoli, J. L. (1993) *J. Biol. Chem. 268,* 21545−21552.

Giguère, V., Ong, E. S., Segui, P., & Evans, R. M. (1987) *Nature 330,* 624−629.

Glass, C. K., Devary, O. V., & Rosenfeld, M. G. (1990) *Cell 63,* 729−738.

Goss, G. D., & McBurney, M. W. (1992) *Crit. Rev. Clin. Lab. Sci. 29,* 185−215.

Gunning, D. B., Barua, A. B., & Olson, J. A. (1993) *Teratology 47,* 29−36.

Heyman, R. A., Mangelsdorf, D. J., Dyck, J. A., Stein, R. B., Eichele, G., Evans, R. M., & Thaller, C. (1992) *Cell 68,* 397−406.

Levin, A. A., Sturzenbecker, L. J., Kazmer, S., Bosakowski, T., Huselton, C., Allenbyt, G., Speck, J., Kratzeisen, C., Rosenberger, M., Lovey, A., & Grippo, J. F. (1992) *Nature 355,* 359−361.

Li, E., Quan, S.-J., Winter, N. S., d'Avignon, A., Levin, M. S., & Gordon, J. L. (1991) *J. Biol. Chem. 266,* 3622−3629.

Liao, J., Ozono, K., Sone, T., McDonnell, D. P., & Pike, J. W. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87,* 9751−9755.

Mangelsdorf, D. J., Ong, E. S., Dyck, J. A., & Evans, R. M. (1990) *Nature 345,* 224−229.

Matsui, M., Okano, S., Yamashita, K., Miyano, M., Kitamura, S., Kobayashi, A., Sato, T., & Mikami, R. (1958) *J. Vitaminol. 4,* 178−189.

McCormick, A. M., Kroll, K. D., & Napoli, J. L. (1983) *Biochemistry 22,* 3933−3940.

Morris-Kay, G. (1993) *Biochemistry 15,* 9−15.

Murayama, A., Takakazu, S., & Matsui, M. J. A. (1993) *Proc. Jpn. Acad. Sci. 69,* 185−190.

Murray, M. B., & Towle, H. C. (1989) *Mol. Endocrinol. 3,* 1434−1442.

Nagpal, S., Saunders, M., Kastner, P., Durand, B., Nakshatri, H., & Chambon, P. (1992) *Cell 70,* 1007−1019.

Napoli, J. L. (1986) *Methods Enzymol. 123,* 112−124.

Napoli, J. L. (1990) *Methods Enzymol. 189,* 470−482.

Napoli, J. L., Khalil, H., & McCormick, A. M. (1992) *Biochemistry 21,* 1942−1949.

Ong, D. E., & Chytil, F. (1978) *J. Biol. Chem. 253,* 4551−4554.

Ong, D. E., MacDonald, P. N., & Gubitosi, A. M. (1988) *J. Biol. Chem. 263,* 5789−5796.

Petrovich, M., Brand, N. J., Krust, A., & Chambon, P. (1987) *Nature 330,* 440−450.

Posch, K. C., Burns, R. D., & Napoli, J. L. (1992) *J. Biol. Chem. 267,* 19676−19682.

Richman, J. M. (1992) *Crit. Rev. Oral Biol. Med. 4,* 93−109.

Sandberg, J. A., Eckhoff, C., Nau, H., & Slikker, W., Jr. (1994) *Drug Metab. Dispos. 22,* 154−160.

Sani, B. P., & Banerjee, C. K. (1978) *Biochem. J. 173,* 643−649.

Summerbull, D., & Maden, M. (1990) *Trends Neurosci. 13,* 142.

Tang, G., & Russell, R. M. (1990) *J. Lipid Res. 31,* 175−182.

Williams, J. B., & Napoli, J. L. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82,* 4658−4662.

Williams, J. B., Shields, C. O., Brettel, L. M., & Napoli, J. L. (1987) *Annu. Rev. Biochem. 160,* 267−271.

Winston, J. H. (1989) The Role of Cellular Retinoic Acid Binding Protein in Retinoic Acid Mediation of Differentiation. Ph.D. Dissertation, Southwestern Medical School, Dallas, TX.

Wolf, G. (1984) *Physiol. Rev. 64,* 874−937.

Yang, N., Schüle, R., Mangelsdorf, D. J., & Evans, R. M. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88,* 3559−3563.

Yen, A., Powers, V., & Fishbaugh, J. (1986) *Leuk. Res. 10,* 619−629.

BI941511Z