Identification of a New *all-trans*-Retinol Metabolite Produced through a New Retinol Metabolic Pathway

Xiujuan Jia, Rafal R. Sicinski,[‡] Deneen M. Wellik,[§] Praveen Tadikonda,[||] Heinrich K. Schnoes, and Hector F. DeLuca*

Department of Biochemistry, University of Wisconsin−Madison, 420 Henry Mall, Madison, Wisconsin 53706

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ABSTRACT: In vitro incubation of *all-trans*-retinol (atROL) with kidney homogenate from vitamin A-deficient and retinoic acid-supplemented (VAD−RAS) female rats produces a new retinol metabolite. Reverse-phase (RP) and normal-phase (NP) high-performance liquid chromatography (HPLC) analysis showed that this metabolite coelutes with the unknown *all-trans*-retinol (atROL) metabolite previously found in the day 10 conceptus and kidneys of vitamin A-deficient rats maintained on *all-trans*-retinoic acid (VAD−RA) and given 2 μg of [^{3}H]atROL. Normal-phase (NP) HPLC purification of the metabolite collected from a RP HPLC column further separated the radiolabeled material into two components. The two isolated compounds have identical or very similar spectroscopic properties. Their nuclear magnetic resonance (^{1}H NMR) and mass spectra (MS) indicated that they are isomers. Spectroscopic studies of the metabolites and their derivatives showed that they are nine-carbon fragments resulting from an oxidative cleavage of the side chain of atROL. The cleavage occurs at C-9, and the product is then oxidized to a keto group. The primary hydroxy group from atROL is preserved in the metabolite. A sulfide bridge is formed between C-11 and C-14, which interrupts the conjugation. The formation of the new metabolites, possessing a 2,5-dihydrothiophene ring, is catalyzed by an enzyme(s) located in the cytosolic fraction of kidneys. The process represents a new retinol metabolic pathway; however, its biological significance is unknown.

Retinol and its metabolites participate in many physiological processes, such as growth, development, reproduction, cellular differentiation, and vision (*1*). Except in the visual system where the functional forms of vitamin A have been well characterized (*2*), other biological functions of vitamin A are thought to be mediated by the action of isomers of retinoic acids (RAs)[1] that interact with retinoic acid receptor (RAR) and retinoic acid X receptor (RXR) in the regulation of gene expression (*3*, *4*). The discovery of RARs (*5*−*10*) and RXRs (*11*−*13*) provided a new understanding of RA action at the molecular level.

However, overwhelming data demonstrate that rats fed retinol-free and *all-trans*-retinoic acid (atRA)-supplemented diet fail to reproduce (*14*−*17*). Although vitamin A-deficient, atRA-supplemented (VAD−RAS) female rats can become pregnant after mating with healthy males, they invariably resorb their fetuses at approximately day 15 of gestation. Work by this laboratory (*16*) showed that retinol is required no later than day 10 of gestation in order to prevent fetal resorption in the VAD−RAS female rats, and administration of a dose as little as 2 μg on day 10 of gestation could prevent the resorption and allow parturition to complete.

Wellik and DeLuca (*18*) studied atROL metabolites in day 10 conceptuses of VAD−RAS rats. After administration of a dose of 2 μg of [^{3}H]atROL to the 10-day pregnant female rats, an unknown metabolite was found in the conceptuses. The concentration of this metabolite increases through 6 h post-dose. The metabolite did not react with phosphatase, glucuronidase, or sulfatase; therefore, it was unlikely to be an excretion product. Further study (*19*) showed that the metabolite is also found in several other tissues collected from the VAD−RAS female rats. Kidneys from the VAD−RAS female rats contain 70 times the amount of the metabolite found in the conceptuses. However, the small quantity of the metabolite produced in vivo was not sufficient for isolation and determination of structure.

Based on the previous data by Wellik and DeLuca (*18*), the present study was focused on establishing an in vitro generation method which could allow us to obtain the unknown atROL metabolite in quantities required for its structural identification. The structure of this unknown metabolite was deduced following studies of ultraviolet (UV), infrared (FTIR), and nuclear magnetic resonance (^{1}H NMR) spectra as well as mass spectra.

* No reprints will be available from the authors. Author to whom correspondence should be addressed at Department of Biochemistry, University of Wisconsin−Madison, 420 Henry Mall, Madison, WI 53706. Telephone: 608-262-1620. Fax: 608-262-7122. E-mail: deluca@biochem.wisc.edu.

[‡] Present Address: Department of Chemistry, University of Warsaw, Pasteura 1, 02-093 Warsaw, Poland.

[§] Present Address: University of Utah, Salt Lake City, UT 84132.

[||] Present Address: Biogen, Inc., Cambridge, MA 02142.

[1] Abbreviations: Ras, retinoic acids; RAR, retinoic acid receptor; RXR, retinoic acid X receptor; VAD−RAS, vitamin A-deficient and retinoic acid-supplemented; atROL, *all-trans*-retinol; atRA, *all-trans*-retinoic acid; RP, reverse phase; HPLC, high-performance liquid chromatography; NP, normal phase; MS, mass spectra; IPA, 2-propanol; BHT, 2,6-di-*tert*-butyl-*p*-cresol.

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EXPERIMENTAL PROCEDURES

Chemicals and Solvents. [11,12-^{3}H]atROL, [15-^{3}H]-atROL, and [20-^{3}H]atROL were obtained from DuPont (New England Nuclear, Boston, MA). atROL was purchased from Fluka Biochemika (Switzerland). The solvents and reagents were obtained from the following sources: pyridine and TBT (a combination of TMS-imidazole, bis-TMS-acetamide, and trimethylchlorosilane) from Pierce (Rockford, IL), acetic anhydride from Fisher (Pittsburgh, PA), chloroform-*d* and acetone-d_6 (100 atom % D) from Aldrich (Milwaukee, WI). Sucrose, Trizma hydrochloride, and other chemicals were purchased from Sigma (St. Louis, MO). Water of HPLC grade was purchased from Fisher (Pittsburgh, PA). All other solvents were B & J Brand high-purity HPLC grade solvents (Baxter, Stone Mountain, GA).

Animals. Female Harlan−Sprague−Dawley rats were obtained at weaning and fed a vitamin A-free synthetic, purified diet (*20*) until they became vitamin A-deficient as revealed by weight loss. Then, the animals were given the same diet supplemented with *all-trans*-retinoic acid (atRA) at a concentration of 5 mg/kg diet. The atRA was dissolved in the vegetable oil component of the diet.

In Vivo Generation of the Unknown Retinol Metabolite. The in vivo production of the unknown retinol metabolite from the VAD−RAS female rats was conducted following the procedure used by Wellik and DeLuca (*18*), except that kidneys instead of conceptuses were collected and extracted.

In Vitro Generation of the Unknown Retinol Metabolite. The female VAD−RAS rats were anesthetized with diethyl ether; kidneys were removed, freed of membranous capsular material, and homogenized in 5 vol of 0.25 M sucrose/100 mM Tris buffer solution (pH 7.4) at 0 °C. An aliquot of 0.5 mL of the homogenate was mixed with 2.5 mL of 100 mM Tris buffer (pH 7.4) in a 25 mL Erlenmeyer flask. An aliquot of 10 μL of ^{3}H-labeled ([11,12-^{3}H] or [15-^{3}H] or [20-^{3}H]) atROL ethanolic solution at a concentration of 7.0 mM and specific activity of 14.0 mCi/mmol was added to each flask. The flasks were placed on a shaker in a 37 °C water bath. They were incubated for different periods of time (from 0 to 60 min), then they were removed from the water bath, and the metabolite was extracted as stated below.

Subcellular Fractionation. Subcellular fractions of the kidney homogenate were separated by following the method of Burgos-Trinidad et al. (*21*). The kidney homogenate was centrifuged at 500*g* for 10 min to separate nuclei (N) and debris from cytosol (C) and mitochondria (M). The supernatants were centrifuged at 10000*g* for 10 min to separate the mitochondria from the cytosol. In one experiment, the cytosol fraction was centrifuged at 100000*g* for 1 h to yield cytosol free of endoplasmic reticulum. The nuclei and mitochondria fractions were washed twice with 0.25 M sucrose/100 mM Tris buffer (pH 7.4) solution, then resuspended in 100 mM Tris buffer solution (pH 7.4). Each of these subcellular fractions was tested for the in vitro generation of this unknown retinol metabolite by following the same procedure stated above. To avoid isomerization of retinoids, all experiments were conducted under yellow light or were shielded from white light.

Extraction and HPLC Purification of the Unknown Metabolite. The extraction procedure was similar to that used by Wellik and DeLuca (*18*). One volume of 0.01% ethanolic solution of 2,6-di-*tert*-butyl-*p*-cresol (BHT) and 0.5 volume of 4.25 M NaCl in 0.015% of *n*-propylgallate aqueous solution were added to the incubation mixture. This aqueous mixture was extracted twice with 1 vol of hexane to remove retinol esters, retinol, and other nonpolar compounds. The aqueous solution was evaporated to dryness in vacuo, and the dry residue was extracted with methylene chloride. The methylene chloride solution was then evaporated in vacuo. The residue was redissolved in reverse-phase HPLC solvent and stored under argon at −20 °C for future HPLC analyses.

The Waters Photodiode Array (PDA) HPLC equipment (Waters Corporation, Milford, MA) was used for both reverse-phase (RP) and normal-phase (NP) chromatography. The instrument includes 600E Pump, 717_{plus} Autosampler, 717 Temperature Controller, 996 PDA Detector and Waters Fraction Collector. The data were acquired and processed by *Millennium v2.15* PDA software. A Phenomenex Zorbax-ODS column (4.6 mm × 25 cm; Terrance, CA) was used for RP HPLC analysis, and an All-tech CN-AQ 5μ column (4.6 mm × 25 cm; Deerfield, IL) was used for NP HPLC analysis. The RP HPLC solvent system was a gradient of methanol in water, and the NP HPLC solvent systems were a gradient of 2-propanol (IPA) in methylene chloride/hexane. The exact gradients used are shown in the figures.

Derivatization Procedures. Acetylation of metabolite was performed by treating a sample with acetic anhydride/pyridine solution (1:2, 30 μL) at room-temperature overnight. The reaction solution was then evaporated twice with 1 vol of hexane to ensure the removal of solvents. The residue was redissolved in NP HPLC mobile phase for NP HPLC purification.

Trimethylsilylation was performed by reaction of a sample with TBT (4 μL) in pyridine (4 μL) for 60 min at room temperature. An aliquot of the reaction mixture was directly analyzed by a gas chromatograph-mass spectrometer (GC-MS, described below). All reactions were conducted under argon atmosphere.

Spectroscopic Methods. Ultraviolet (UV) absorption spectra were recorded by PDA Detector, and the corresponding absorption maxima were obtained from the Millenium Spectrum Review Reports generated by *Millenium v2.15* software. Infrared (IR) spectra were recorded on an ATI Mattson Infinity Series, 60AR FTIR spectrometer (Madison, WI) in KBr pellets. ^{1}H nuclear magnetic resonance (NMR) spectra were measured at 500 MHz with a Bruker DMX-500 spectrometer in the solvent noted. A Finnigan Magnum (San Jose, CA) GC-MS was used for low resolution GC-MS measurements. A J & W DB-5MS capillary column (0.25 mm i.d. × 30 m) was coupled directly to the ion source. Helium was used as carrier gas at 15 psi. The split/splitless injector was used in the splitless mode at 280 °C. The GC oven temperature was programmed as follows: initial temperature 40 °C; held at 40 °C for 5 min, then ramped to 280 °C at 10 °C/min; held at 280 °C for 19 min. The transfer line and trap manifold were at 320 °C and 150 °C, respectively. The ion trap was operated in the EI mode under auto gain. High-resolution electron impact mass spectra were recorded at 70 eV on a Kratos DS-50 TC instrument equipped with a Kratos DS-55 data acquisition system.

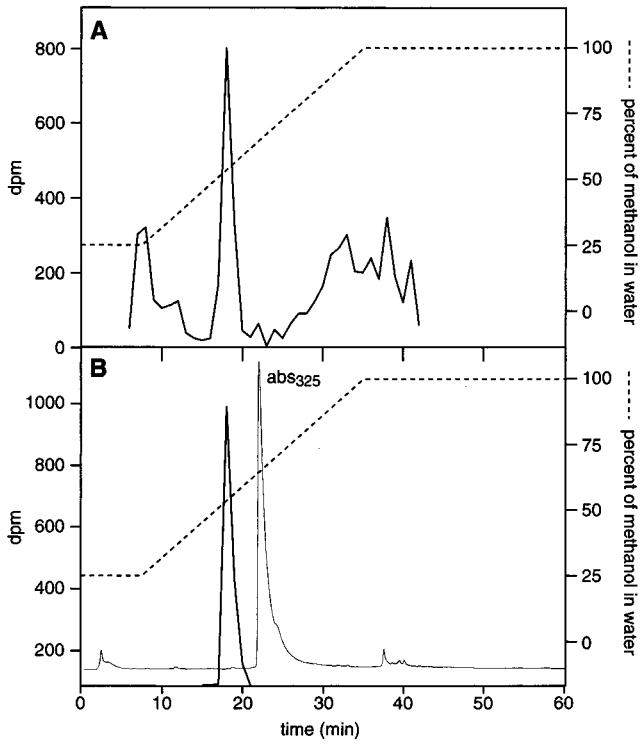

FIGURE 1: RP HPLC purification of the unknown atROL metabolite eluted at about 18 min. Radioactivity profiles of the aqueous/ethanol fraction extracted from (A) in vitro incubation of VAD−RAS rat kidney homogenate in 100 mM Tris buffer (pH 7.4) at 37 °C for 15 min; (B) the VAD−RAS rat kidneys 4 h after oral dosing the rats with ^{3}H-labeled atROL. The dotted line represents HPLC solvent gradient.

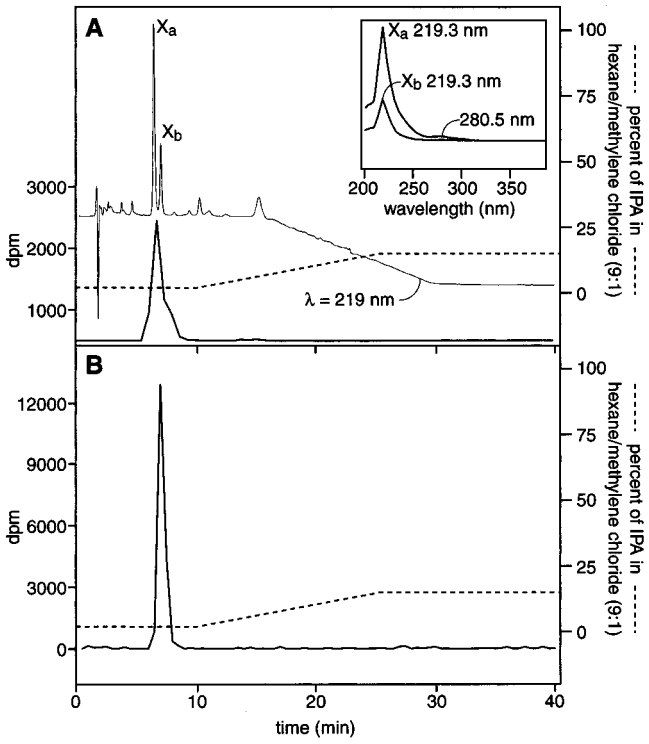

FIGURE 2: NP HPLC purification of the metabolite peak collected from the RP HPLC column. Radiolabel from the in vitro sample (A) comigrates with the in vivo metabolite (B) and coelutes with two UV absorbing peaks (A) that have the same UV profile as shown in the inset of 2A. The dotted line represents the HPLC solvent gradient.

RESULTS

Incubation of ^{3}H-labeled atROL with VAD−RAS rat kidney homogenate in Tris buffer solution (pH 7.4) was carried out for 15 min, and the aqueous extract of the incubation mixture was analyzed on RP HPLC. In vitro incubation produced a peak with radioactivity (Figure 1A) comigrating with the unknown atROL metabolite found from the VAD−RAS rat kidneys 4 h after oral administration of atROL (Figure 1B).

Further purification of this substance on a NP HPLC system with a gradient of 2-propanol (IPA) in a methylene chloride/hexane (1:9) solvent system showed a tailed radioactive peak with retention time of about 6.5 min (Figure 2A), identical to that of the metabolite produced from the in vivo experiment (Figure 2B). The NP HPLC chromatogram (Figure 2A) monitored at the wavelength of 219 nm indicated that in vitro incubation produced two UV peaks coeluting with the radioactive peak. The first peak, Xa, has a retention of 6.4 min, and the second peak, Xb, has a retention time of 7.0 min. Calculation based on radioactivity and UV peak areas gave about a 4:1 ratio of Xa to Xb.

To further confirm the identity of the metabolite produced from in vitro incubation to that isolated from VAD−RAS rat kidneys in vivo, Xa and the in vivo metabolite peaks were collected and reacted with acetic anhydride in pyridine as described in Experimental Procedures. The RP HPLC analysis of the acetylated compounds of Xa and the in vivo generated metabolite showed that they comigrate (Figure 3).

The spectral properties of the in vitro generated compounds are as follows:

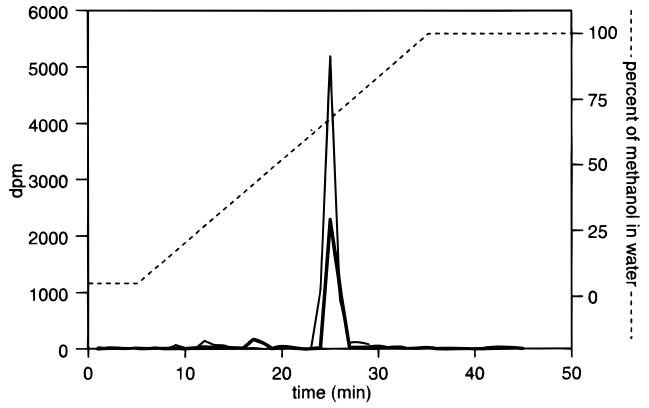

FIGURE 3: RP HPLC analysis of the acetylated Xa (light line) and in vivo metabolite (dark line). The dotted line represents the HPLC solvent gradient.

Xa: UV (Figure 2, inset) λ_{max} 219.3, 280.5 nm, A219/A280 > 30; IR (Figure 4) 3434 (OH), 2925, 2872, 1713 (C═O), 1447, 1393, 1359, 1159, 1070 (C−O) cm^{-1}; ^{1}H NMR ($CDCl_3$) (Figure 5) δ 1.788 (3H, br s, 13-CH_3),[2] 2.17 (3H, s, CH_3CO), 2.77 (1H, dd, J = 17.9, 8.4 Hz, one of 10-H_2), 2.92 (1H, dd, J = 17.9, 5.5 Hz, one of 10-H_2), 3.62 (1H, dd, J = 11.6, 2.9 Hz, one of 15-H_2), 3.75 (1H, dd, J = 11.6, 3.7 Hz, one of 15-H_2), 4.19 (1H, m, 14-H), 4.40 (1H, m, 11-H), 5.48 (1H, br s, 12-H); ^{1}H NMR (CD_3COCD_3) δ 1.798 (3H, br s, 13-CH_3), 2.10 (3H, s, CH_3CO), 2.95 (1H, dd, J = 17.8, 4.8 Hz, one of 10-H_2; the other 10-H overlapped with water peak at 2.85 ppm), 3.60−3.70 (3H,

[2] Numbering system applied for retinoids is used throughout this paper.

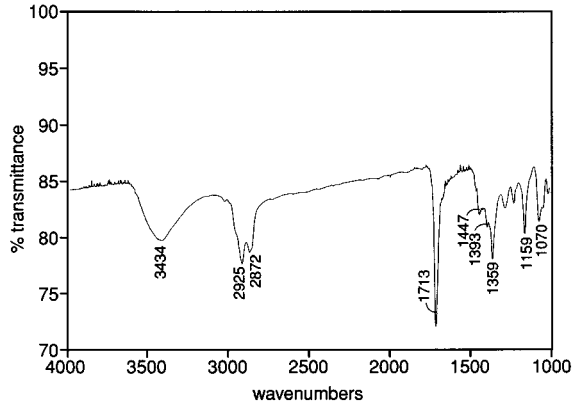

FIGURE 4: FTIR spectrum of Xa in KBr pellet.

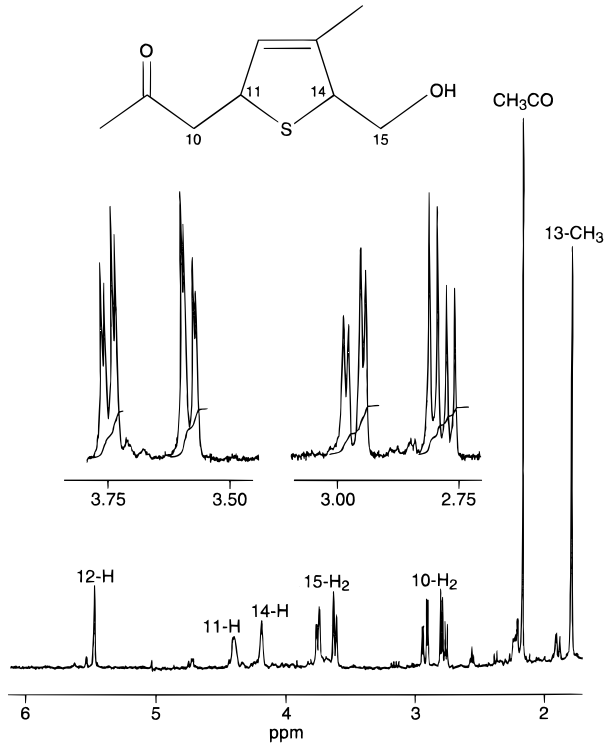

FIGURE 5: ^{1}H NMR spectrum of Xa in chloroform-*d* (100 atom % D) at 4 °C.

br m, CH_2OH), 4.02 (1H, m, 14-H), 4.26 (1H, m, 11-H), 5.47 (1H, br s, 12-H); GC-MS (Figure 6) (rel intensity) 186 (M^+, 1), 168 ($M^+ - H_2O$, 6), 156 ($M^+ - CH_2O$, 29), 155 ($M^+ - CH_2OH$, 40), 113 ($M^+ - CH_2O - CH_3CO$, 100), 111 (20), 43 (CH_3CO^+, 42); exact mass calcd for $C_9H_{14}O_2S$ 186.0714, found 186.0713, $C_9H_{12}OS$ 168.0609, found 168.0608, $C_8H_{12}OS$ 156.0609, found 156.0621, $C_8H_{11}OS$ 155.0531, found 155.0548, C_6H_9OS 113.0425, found 113.0431;

Xb: UV λ_{max} 219.3, 280.5 nm, A219/A280 > 30; ^{1}H NMR ($CDCl_3$) δ 1.783 (3H, br s, 13-CH_3), 2.18 (3H, s, CH_3CO), 2.77 (1H, dd, $J = 18.1$, 9.3 Hz, one of 10-H_2), 2.95 (1H, dd, $J = 18.1$, 4.9 Hz, one of 10-H_2), 3.64 (1H, dd, $J = 11.7$, 2.8 Hz, one of 15-H_2), 3.78 (1H, dd, $J = 11.7$, 3.7 Hz, one of 15-H_2), 4.20 (1H, m, 14-H), 4.46 (1H, m, 11-H), 5.45 (1H, br s, 12-H); GC-MS (rel intensity) 186 (M^+, 2), 168 ($M^+ - H_2O$, 7), 156 ($M^+ - CH_2O$, 36), 155 ($M^+ - CH_2OH$, 35), 113 ($M^+ - CH_2O - CH_3CO$, 100), 111 (24), 43 (CH_3CO^+, 42); exact mass calcd for $C_9H_{14}O_2S$ 186.0714, found 186.0713.

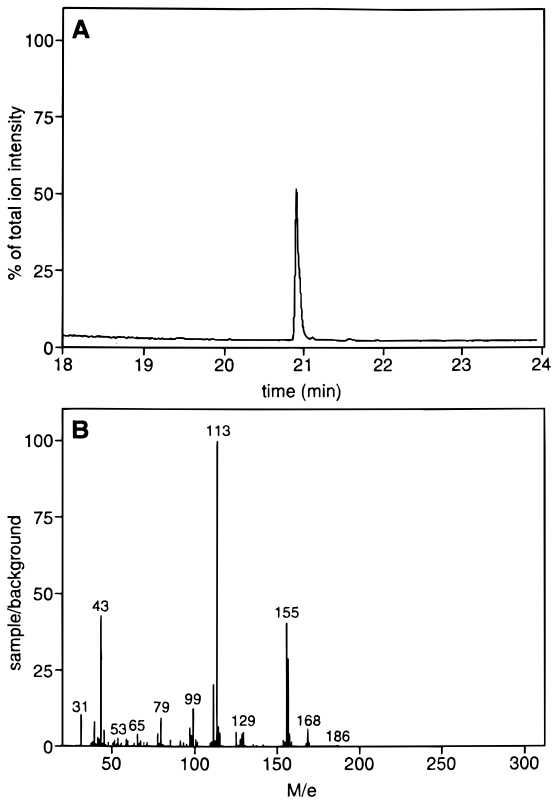

FIGURE 6: GC-MS analysis of metabolite Xa. (A) GC purification of Xa; (B) MS spectrum of the Xa peak purified by GC.

Incubation of 20-^{3}H-labeled atROL with each of the subcellular fractions of the VAD−RAS kidney homogenate at 37 °C for 15 min showed the unknown metabolite was produced only by the C fraction (Figure 7A). Incubation of the ^{3}H-labeled atROL with the combination of C and M (C+M) fractions at 37 °C for 15 min produced the same amount of the metabolite as that with only the C fraction (Figure 7B). Further, sedimentation at 100000*g* of the C fraction showed that all the activity remained in the cytosol (not shown). Thereafter, to simplify the in vitro generation procedure, the C+M fraction, instead of the C fraction, was used for the in vitro generation of the metabolite in the following experiments.

Besides the metabolite peak, a relatively polar peak with a retention time of 7 min (Figure 7A) was produced by each of the three fractions. The biological importance and characterization of this peak are as yet unexplored.

A time course experiment showed the amount of the metabolite produced by the C+M fraction of the VAD−RAS kidney homogenate increased with time, and 60 min incubation produced the highest amount of the metabolite (Figure 8). To exclude the possibility that the metabolite was produced, in vitro, by some random degradation, the labeled [20-^{3}H]atROL was incubated in 2.5 mL of 100 mM Tris buffer (pH 7.4) and 0.5 mL of 0.25 M sucrose/100 mM Tris buffer solution (pH 7.4) (control), or 2.5 mL of 100 mM Tris buffer (pH 7.4) and 0.5 mL of the C+M fraction of the VAD−RAS rat kidney homogenate (sample), at 37 °C for 60 min. RP HPLC analysis of the aqueous/ethanol fraction of the 60 min control and sample showed an absence of the metabolite peak from the control (Figure 9).

The acetylated Xa was purified on NP HPLC using methylene chloride/IPA/hexane (10:0.5:89.5) isocratic solvent

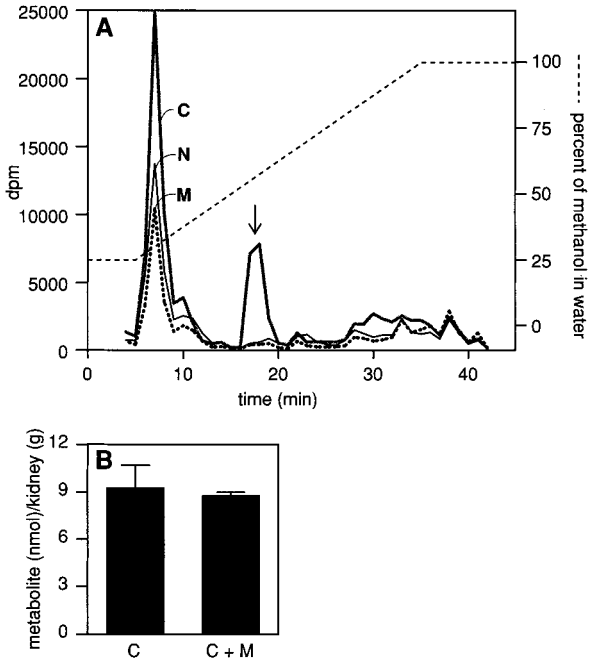

FIGURE 7: In vitro production of the unknown metabolite by each of the subcellular fractions of the VAD−RAS kidney homogenate 15 min after incubation with ^{3}H-labeled atROL at 37 °C. (A) Radiolabel elution of the aqueous/ethanol fraction extracted from each of the subcellular fractions showed the unknown metabolite was produced only by the cytosolic fraction; (B) Comparison of the amount of the unknown metabolite produced by the C fraction and the C+M fraction.The dotted line represents the RP HPLC solvent gradient condition. The arrow indicates the metabolite peak.

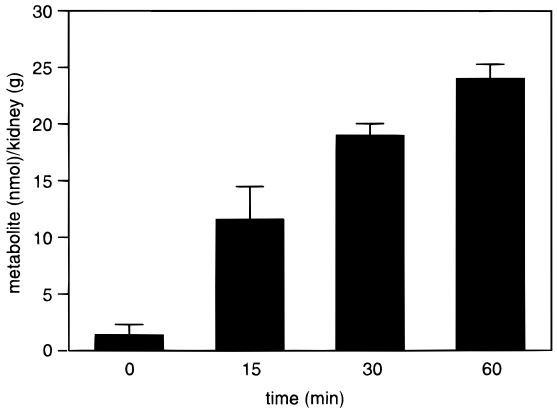

FIGURE 8: The amount of the unknown metabolites (Xa+Xb) produced by the C+M fraction after different time of incubation with [20-^{3}H]atROL at 37 °C.

system. It was eluted at about 6 min (Figure 10). Spectral properties of the acetylated derivative are as follows: UV (Inset of Figure 10) λ_{max} 219.3, 280.5 nm, A219/A280 > 30; ^{1}H NMR ($CDCl_3$) δ 1.790 (3H, br s, 13-CH_3), 2.10 (3H, s, AcO), 2.17 (3H, s, CH_3CO), 2.78 (1H, dd, J = 18.1, 9.2 Hz, one of 10-H_2), 2.90 (1H, dd, J = 18.1, 5.4 Hz, one of 10-H_2), 4.09 (1H, dd, J = 11.3, 7.4 Hz, one of 15-H_2), 4.15 (1H, m, w/2 = 15 Hz, 14-H), 4.30 (1H, dd, J = 11.3, 4.6 Hz, one of 15-H_2), 4.37 (1H, m, w/2 = 19 Hz, 11-H), 5.49 (1H, br s, 12-H); GC-MS (rel intensity) 228 (M^+, 1), 168 (M^+ − AcOH, 37), 155 (M^+ − CH_2OAc, 13), 125 (M^+ − AcOH − CH_3CO, 16), 113 (40), 111 (39), 43 (CH_3CO^+, 100); exact mass calcd for $C_{11}H_{16}O_3S$ 228.0820, found 228.0817, $C_9H_{12}OS$ 168.0609, found 168.0607, $C_8H_{11}OS$ 155.0531, found 155.0526, C_7H_9S 125.0425, found 125.0425.

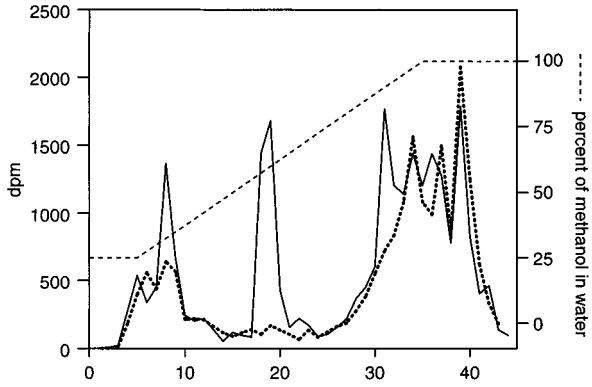

FIGURE 9: RP HPLC purification of the aqueous/ethanol fraction extracted from the sample and the 60 min control showed the absence of the metabolite peak in the control. Sample (—); Control (- - -).

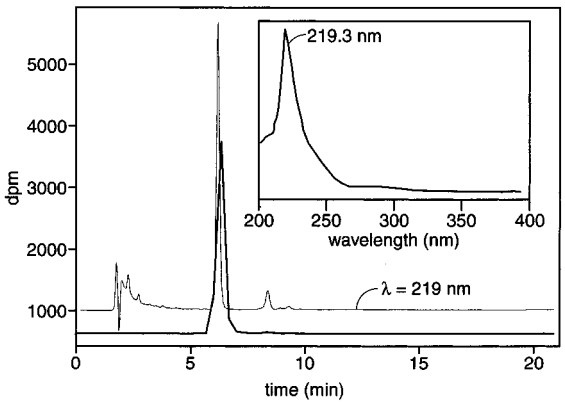

FIGURE 10: NP HPLC purification of the acetylated metabolite Xa. Solvent was a methylene chloride/IPA/hexane (10:0.5:89.5) isocratic solvent system. Inset shows the UV spectrum of the acetylated compound.

The silylated Xa was purified by GC and analyzed by MS (GC-MS conditions described in Experimental Procedures) and exhibits the following mass spectrum: GC-MS (rel intensity) 258 (M^+, 2), 168 (M^+ − Me_3SiOH, 43), 155 (M^+ − CH_2OSiMe_3, 35), 113 (100), 111 (34), 103 (21), 73 (23), 43 (CH_3CO^+, 34).

DISCUSSION

atRA is able to support early fetal development, but itself at a concentration of up to 100 μg per gram of diet is not sufficient to allow successful gestation in vitamin A-deficient rats (*14*, *16*, *17*). The mechanism by which retinol is required for a successful gestation is not clear. A study by Kurlandsky et al. (*22*) showed that the ability of atRA uptake from circulation by different tissues varies dramatically. One may argue the possibility that atRA or its metabolite(s) is the active form in supporting gestation but not able to cross the maternal−fetal barrier at a sufficient level. However, work in our laboratory (*18*) showed that no detectable atRA was formed from labeled retinol in the fetal tissue 1, 3, 6, and 12 h post-administration to the VAD−RAS pregnant rats on day 10 of gestation. This might indicate that the embryos can obtain atRA from the circulation, eliminating the need for in situ synthesis of atRA. In their study, Wellik and DeLuca (*18*) found a new, early appearance polar retinol metabolite in the kidneys and 10-day old conceptus after oral administration of 2 μg [^{3}H]atROL to the VAD−RAS mother.

In this study, in vitro incubation of atROL with the kidney homogenate of the VAD−RAS rat produced a metabolite which coeluted with the unknown metabolite reported by Wellik and DeLuca (*18*) on both the RP HPLC and NP HPLC systems. The coelution of the acetylated products of the in vivo and in vitro (Xa) generated metabolites further confirmed that they are very likely identical.

The metabolite peak was absent from the 0 min incubation and 60 min control. Therefore, the metabolite was produced specifically by kidney tissue. NP HPLC purification of the metabolite peak collected from the RP HPLC column separated the radioactive materials into two peaks, Xa and Xb. In vitro incubation produced predominantly metabolite Xa with a small quantity of Xb. Whether Xb can also be generated in vivo is not certain at this point.

Both retinol metabolites Xa and Xb, isolated in this study, absorb UV light; however, their absorption maxima differ drastically from those observed for retinoids. They both exhibited a strong peak at 219.3 nm, suggesting the absence of double bond conjugation. GC-MS analyses of the two metabolites provided low resolution spectra which were virtually identical and displayed molecular ions at *m*/*z* 186, i.e., 100 mass units lower than the molecular mass of atROL. Even more surprising was the result of examining the high-resolution mass spectra, suggestive of the presence of one sulfur atom in the metabolites ($C_9H_{14}O_2S$). Accurate mass measurements of the remaining major fragmentation ions in their mass spectra confirmed the presence of a sulfur atom in a majority of them. The intense peaks at *m*/*z* 155 ($M^+ - CH_2OH$) and 43 (CH_3CO^+) suggested a presence of hydroxymethyl and acetyl moieties. This conclusion was nicely confirmed by the IR spectrum of Xa exhibiting bands at 3434 (OH) and 1713 (C═O) cm^{-1}. Mass spectra of acetyl and trimethylsilyl derivatives of metabolite Xa show molecular ions at *m*/*z* 228 and 258, respectively, as well as other fragment ions compatible with the presence of a derivatized primary hydroxyl group. The ^{1}H NMR spectrum of the major metabolite Xa showed two methyl signals at 1.788 and 2.17 ppm. The chemical shift of the latter was typical of an acetyl moiety in simple aliphatic acyclic ketones. The former singlet was slightly broadened, and its chemical shift was characteristic of methyl groups connected to double bond systems. The presence of a double bond was confirmed by a signal at 5.48 ppm attributable to a vinyl proton. The NMR spectrum displayed also signals of six protons forming two ABX systems. The AB portion of the first system was centered at ca. 2.85 ppm and, as was shown by double resonance experiments, it was coupled to a methine proton resonating at 4.40 ppm. The signals of the protons belonging to the other AB part were centered at ca. 3.69 ppm and they were shown to be coupled to the other methine proton (4.19 ppm). Considering the chemical shifts and geminal coupling constants of the protons[3] from both the AB parts and taking into account the fact that the deshielded methine protons, those corresponding to the respective X parts of the two ABX patterns concerned, are not coupled to each other, it was reasonable to assume that they belong to the CH_3COCH_2-CH and $CH-CH_2OH$ fragments which are, in turn, linked to a methyl-ethenylene group and a sulfide bridge.[4]

The spectroscopic data of the minor metabolite Xb were found to be very similar to those of compound Xa. An interpretation of the NMR spectra of both compounds indicated that the only difference between them is the cis and trans orientation of substituents of the dihydrothiophene ring. 2-Hydroxymethyl-3-methyl-5-(2′-oxopropyl)-2,5-dihydrothiophene[5] has two asymmetric carbons and can, therefore, exist in four isomeric forms. However, minute differences in the spectroscopic properties of the two metabolites and insufficient literature data (*31*) preclude the possibility of determination of their relative stereochemistry at C-11 and C-15. It is also unknown whether the metabolites are formed as single diastereomers, or if they are a mixture of the corresponding optical antipodes.

The carbon skeleton of such 2,3,5-trisubstituted-2,5-dihydrothiophene compounds can be derived from atROL by an oxidative cleavage of its side chain C(8)−C(9) bond with the formation of a keto group at C-9. An incubation experiment using [15-^{3}H]atROL produced, after purification and separation on both RP HPLC and NP HPLC systems (data not shown), the same peaks with radioactivity as in the case of other ^{3}H-labeled ([11,12-^{3}H] or [20-^{3}H]) atROLs. This indicates the origin of the nine-carbon skeleton of metabolites and shows that the primary hydroxy group in the metabolites was derived from atROL.

The physiological importance of the involvement of sulfide in retinol metabolism remains to be determined. From protein to coenzymes to prothetic groups to the iron-sulfur clusters, sulfur is actively involved in many important redox processes (*33*). In mammalian tissues, sulfide can be released from desulfuration of cysteine by *r*-cystathionase and cysteine aminotransferase in conjunction with 3-mercaptopyruvate sulfurtransferase (*34*). Stipanuk (*35*) postulated that the released sulfide was then incorporated into some pool of reduced sulfur that has a relatively long half-life prior to its oxidation.

Study by Ogasawara et al. (*34*) showed that bound sulfur is widely distributed in tissues and highest in kidneys. In kidneys, the bound sulfur is primarily located in the cytosolic fraction. In this study, the new retinol metabolite is exclusively produced by the cytosolic fraction of the VAD−RAS rat kidney cells. This indicates that the enzyme(s) that catalyze(s) the cleavage/oxidation and sulfide formation is/are located in the cytosolic fraction of the kidney tissue.

[3] Methylene protons adjacent to an acetyl group in several 4-alkylthio-2-butanones have usually a geminal coupling constant ca. 18 Hz, and they resonate in the range of 2.6−2.9 ppm; in the 4-vinyl- and 4-phenyl-substituted analogues, the 4-methine proton resonates at 4.15−4.8 ppm (*23*−*26*). Chemical shifts of the methylene protons of the hydroxymethyl moiety in different 2-alkylthio-1-propanols are reported in the range of 3.45−3.8 ppm (*27*−*29*).

[4] Methylene protons of the 2-hydroxymethyl group in the tetrahydrothiophene derivative resonate at 3.69 ppm (*30*). In the 2,3,5-trialkyl-2,5-dihydrothiophenes, the 3-methyl group resonates at ca. 1.8 ppm and olefinic 4-H at ca. 5.35 ppm ($J_{4,5}$ = 2 Hz) (*31*). The ^{1}H NMR spectrum (in CCl_4) of a compound closely resembling the metabolite(s) structure, i.e., 2-hydroxymethyl-4-methyl-2,5-dihydrothiophene is similar to those recorded for Xa and Xb; signals of 4-methyl, the CH_2-O group, 2-H, and vinylic 3-H appeared at 1.75, 3.5, 4.1, and 5.35 ppm, respectively (*32*).

[5] The dihydrothiophene structure is in agreement with the observations of Wellik (*19*) who found the polar products of periodate oxidation of an unknown retinol metabolite. Sodium periodate is known to oxidize sulfides to sulfoxides without subsequent over-oxidation to sulfones. We subjected the major metabolite Xa to a periodate oxidation and isolated two polar sulfoxides which were found to have 203 ($M^+ + 1$) ions in their mass spectra.

During the past decade, the study of cytosolic sulfotransferases has been expanding. This family of enzymes catalyze the transfer of sulfur in sulfate form. Sulfonation has been reported in the metabolism of neurotransmitters, hormones, particularly steroid hormones and bile acids (*36*−*39*). Some of the compounds can be bioactivated by sulfate incorporation. It appears, however, that this represents the first known involvement of sulfide in retinol metabolism. Our knowledge about sulfurtransferases and the characteristics of the reactions they catalyze is rather limited. The metabolic route and structural identity of the other portion of the atROL molecule, i.e., the cyclohexane ring and rest of the side chain, are not known at the present time.

No binding activity of Xa or Xb to the retinoic acid receptors was observed (data not shown). Whether Xa has any in vivo biological activity remains to be tested. However, the early appearance of this metabolite in the VAD−RAS rat conceptus warrant further investigation of this retinol metabolic pathway.

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