Biliary Metabolites of *all-trans*-Retinoic Acid in the Rat: Isolation and Identification of a Novel Polar Metabolite†

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ABSTRACT: The biliary metabolites from normal rats dosed with either pharmacological or physiological doses of *all-trans*-[11,12-$^3H_2$]retinoic acid were investigated. Biliary metabolites excreted during the first 24 h account for approximately 60–65% of the radiolabeled dose. A major polar metabolite was purified to homogeneity by using Sephadex LH-20 chromatography and several high-performance liquid chromatographic procedures. This metabolite was negatively charged as revealed by high-performance liquid chromatography on ion-exchange columns and accounts for 10% of the total biliary radioactivity (6% of the dose). The polar compound was positively identified by using Fourier transform proton nuclear magnetic resonance spectroscopy, high- and low-resolution mass spectrometry, fast atom bombardment mass spectrometry, ultraviolet absorption spectrophotometry, Fourier transform infrared spectroscopy, amino acid analysis, and chemical derivatization as 2-[8-[6-(hydroxymethyl)-2,6-dimethyl-3-oxo-1-cyclohexen-1-yl]-2,6-dimethyl-5,7-octadienamido]ethanesulfonic acid. The metabolic transformations required for the generation of this metabolite from *all-trans*-retinoic acid are the following: (1) allylic oxidation at carbon 4 of the cyclohexene ring to produce a 4-keto group, (2) hydroxylation of one of the methyl groups at carbon 1 of the cyclohexene ring, (3) saturation of the two terminal double bonds in the side chain, (4) loss of the terminal carboxyl group of the side chain via decarboxylation, and (5) conjugation of the resulting retinoid with taurine. To our knowledge, this metabolite represents the first taurine conjugate of a fat-soluble vitamin to be identified.

The biotransformation of retinol to retinoic acid under physiological conditions is now well established (Emerick et al., 1967; Roberts & DeLuca, 1967; Kleiner-Bössaler & DeLuca, 1971; Frolik et al., 1981b). This metabolic conversion appears to play a vital function in the expression of vitamin A activity (DeLuca, 1979). Retinoic acid is able to support growth in vitamin A deficient animals and to promote and maintain the differentiation of epithelial tissues and is capable of suppressing carcinogenic transformations in several tissues and thus may be of therapeutic value (Dowling & Wald, 1960; Zile & DeLuca, 1968; Sporn et al., 1976). Recent studies suggest that retinoic acid is not stored in the liver but is rapidly metabolized to products which appear in the bile and plasma (Swanson et al., 1981; Zile et al., 1980). These properties have stimulated renewed interest in the metabolism of retinoic acid with the hope of finding a metabolite of retinoic acid with higher biological activity than the parent compound. Such a discovery would help to establish a molecular mechanism of action for vitamin A.

The development of high-performance liquid chromatographic systems and mild extraction procedures has allowed the isolation and identification of retinoic acid metabolites without the production of artifacts (Frolik et al., 1978; McCormick et al., 1978). The work of Hänni and Rietz led to the identification of several fecal and urinary metabolites of retinoic acid following 27.5-mg doses (Rietz et al., 1974; Hänni et al., 1976; Hänni & Bigler, 1977). The oxidation of retinoic acid to 4-hydroxy- or 4-ketoretinoic acid has been postulated to be the initial step in the inactivation of retinoic acid (Frolik et al., 1979; Roberts et al., 1979).

The rapid disappearance of retinoic acid from tissues followed by the appearance of retinoic acid metabolites in the bile after either a physiological or pharmacological dose suggests that conjugation in the liver may be a major route of metabolism. Indeed, work by Olson and co-workers has shown that up to 95% of an administered dose of retinoic acid is secreted into the bile within 5 days (Zachman et al., 1966a). This group identified one of the biliary metabolites of retinoic acid as retinoyl β-glucuronide (Dunagin et al., 1965, 1966). Recently, a glucuronide of 13-*cis*-4-ketoretinoic acid was isolated and identified from the bile of rats dosed with 5 μg of 13-*cis*-retinoic acid. This metabolite accounted for 8% of the administered dose (Frolik et al., 1981a). Work by two groups has shown that retinoyl β-D-glucuronide represents 10–15% of the total biliary radioactivity after 24 h of collection (Zile et al., 1980; Swanson et al., 1981). Retinoyl β-D-glucuronide also appears to possess 30–100% of the biological activity of *all-trans*-retinoic acid when measured by a growth assay (Nath & Olson, 1967). However, it is not clear whether the glucuronide must first be hydrolyzed to retinoic acid before it shows demonstrable biological activity.

This paper describes the isolation and identification of a novel biliary metabolite of *all-trans*-retinoic acid which represents approximately 10% of the total biliary radioactivity excreted during the first 24 h of collection. This compound was shown to be a taurine conjugate of a retinoic acid metabolite, and hence the trivial name retinotaurine is suggested for this metabolite (Figure 1).

Materials and Methods

General Procedures. High-performance liquid chromatography (HPLC)[1] was performed with a microprocessor-controlled Beckman Model 332 gradient liquid chromatograph (Beckman Instruments, Irvine, CA) equipped with a Waters Model 440 absorbance detector operating at 313 nm and a

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[1] Abbreviations: HPLC, high-performance liquid chromatography; FAB, fast atom bombardment mass spectrometry; FT-NMR, Fourier transform nuclear magnetic resonance spectroscopy; FT-IR, Fourier transform infrared spectroscopy; BSTFA, *N,O*-bis(trimethylsilyl)trifluoroacetamide.

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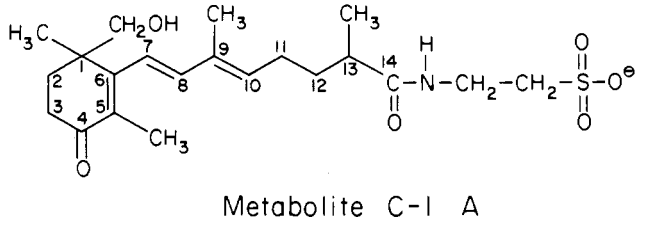

FIGURE 1: Structure of retinotaurine or 2-[8-[6-(hydroxymethyl)-2,6-dimethyl-3-oxo-1-cyclohexen-1-yl]-2,6-dimethyl-5,7-octadienamido]ethanesulfonic acid.

Waters U6K universal injector (Waters Associates, Milford, MA). For reverse-phase semipreparative HPLC, either a 9.4 × 250 mm M9 Partisil-10 ODS-2 column (Whatman, Inc., Clifton, NJ) or a 6.2 × 250 mm Zorbax ODS column (Du Pont, Inc., Wilmington, DE) was used. Analytical reverse-phase chromatography was performed on a 4.6 × 250 mm Zorbax ODS column (Du Pont Instruments, Wilmington, DE). For straight-phase HPLC, a 4.6 × 250 mm Zorbax Sil analytical column was employed (Du Pont, Inc., Wilmington, DE). Ion-exchange high-performance liquid chromatography was performed on an analytical 4.6 × 250 mm Zorbax SAX column eluted with ammonium acetate in methanol.

All solvents used for HPLC were of HPLC grade (Fisher Chemical Co., Itasca, IL) with the exception of water and acetone which were glass distilled before use. All non HPLC grade solvents were degassed and filtered through 0.2-μm filters (Millipore Corp., Bedford, MA). Solvent systems utilized for specific purification steps are noted below. Solvents were removed by rotary evaporation (Brinkmann Instruments, Inc., Westbury, NY) with a water aspirator and a 30–40 °C water bath. Ammonium acetate was removed by a vacuum pump or lyophilization.

High- and low-resolution mass spectrometry was performed with an AEI Model MS-902 mass spectrometer (Associated Electrical Industries, Ltd., Manchester, England) equipped with a DS-50 data acquisition system (Data General Corp., Southboro, MA). Spectra were obtained by using electron-impact ionization (70 eV) and direct probe sample introduction with a source temperature of 120–240 °C above ambient. Fast atom bombardment mass spectrometry (FAB-MS) was performed by using an MS-9 mass spectrometer equipped with an FAB source. The spectra were obtained with the sample suspended in a sodium cationized glycerol matrix which was bombarded with an Ar^+ beam at 50 μA and 7.5 kV. The source pressure was 1×10^{-5} torr.

Ultraviolet (UV) absorption spectra were recorded in 95% ethanol with a Beckman Model 24 recording spectrophotometer. Fourier transform infrared spectroscopy (FT-IR) was performed by using a Model 7199C dual-beam FT-IR spectrometer operating with 2-cm^{-1} resolution. The FT-IR spectra were obtained by using 25 μg of metabolite in methanol solution on a KBr slide. Prior to plotting, a methanol background spectrum was subtracted from the sample spectrum.

^1H nuclear magnetic resonance (^1H NMR) spectra were recorded on a Brucker WH-270 MHz instrument (Bruker Instruments, Billerica, MA) operating in the Fourier transform mode. All spectra were taken in 100 atom % deuterated methanol (CD_3OD) at 22 °C (60 μg of metabolite in 200 μL of CD_3OD). The ^2H resonance of $CDCl_3$ and CD_3OD was used for internal field frequency locking. Spectra were recorded after accumulation of 2000 scans using a sweep width of 3000 Hz with 16K data points, yielding a digital resolution of 0.19 Hz/point. Quadrature phase detection was used for increased signal to noise ratios. Monoselective proton decouplings were performed with total decoupling of the indicated protons. Water was removed from NMR tubes prior to the addition of deuterated methanol by placing the serum stoppered tubes on an oil diffusion pump for 48 h.

Radioactivity was determined by using liquid scintillation counting with either a Packard Model 3255 liquid scintillation counter equipped with automatic external standardization or a Packard Prias PLD Tri-Carb minivial counter equipped with a microprocessor with a quench curve logged in the memory. All aqueous samples were counted in Aquasol (New England Nuclear, Boston, MA) or 3a70B (Research Products International, Elk Grove Village, IL). For determination of radioactivity in fecal samples, aliquots of the feces from each rat were placed in small paper cups (Combusto-cones, Packard Instrument Co., Downers Grove, IL), treated with Combustaid (Packard Instrument Co.), and combusted to CO_2 and H_2O in a Packard Model B306 Tricarb sample oxidizer. The tritiated water was collected in 12 mL of Monophase 40 scintillation fluid. Samples were counted with a Packard Model 3255 as described above. The values were corrected for recovery (typically 90–95%).

Purification of Radiochemicals and Preparation of Doses. Unlabeled *all-trans*-retinoic acid was obtained from Eastman Organic Chemicals (Rochester, NY) and did not require further purification. *all-trans*-[11,12-$^3H_2$]Retinoic acid of specific activity 31 Ci/mmol and *all-trans*-[15-^{14}C]retinoic acid of specific activity 27.3 mCi/mmol were generous gifts from Hoffmann-La Roche, Inc. (Nutley, NJ). *all-trans*-[14-^{14}C]Retinoic acid of specific activity 4.7 mCi/mmol was purchased from Philips-Duphar Co. (Weesp, Netherlands). The radioactive substrates were purified immediately before use by passing the compounds over an analytical reverse-phase μBondapak C_{18} column (4 × 300 mm, Waters Associates, Milford, MA) eluted with methanol–water (68:32 v/v) containing 10 mM ammonium acetate. Purity of some *all-trans*-[11,12-$^3H_2$]retinoic acid samples was only 15–20% prior to this purification step. However, following HPLC purification, substrates were routinely 93–95% *all-trans*-retinoic acid with the remainder consisting of cis isomers.

Doses were prepared by diluting the appropriate purified radioactive *all-trans*-retinoic acid substrate with unlabeled *all-trans*-retinoic acid to achieve the desired specific activity. The *all-trans*-[11,12-$^3H_2$]retinoic acid used for isolation and identification of biliary metabolites had a specific activity of 15 300 dpm/μg following dilution. For double-label experiments, purified *all-trans*-[11,12-$^3H_2$]- and *all-trans*-[15-^{14}C]retinoic acids were mixed to yield a resultant ^3H to ^{14}C ratio of approximately 1.5. The purified *all-trans*-[14-^{14}C]-retinoic acid was not diluted with unlabeled retinoic acid prior to injection and had a specific activity of 34 500 dpm/μg.

Chemicals. Ammonium acetate used in HPLC solvent systems was HPLC grade (Fisher Chemical Co., Itasca, IL). Methanol used for NMR experiments was 100 atom % D (Aldrich Chemical Co., Milwaukee, WI). Sodium chloride, propyl gallate, and glucose were all reagent grade (Sigma Chemical Co., St. Louis, MO). The silylating reagent BSTFA, a formulation of *N,O*-bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane as catalyst, was acquired from Pierce Chemical Co. (Rockford, IL). Acetic anhydride used for acetylations was obtained from Fisher Chemical Co. Sodium pentobarbital (Nembutal) was obtained from Abbot Laboratories (Chicago, IL). Diazomethane (CH_2N_2) in ether solution was prepared by saponification of *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide by using a Diazald kit (Aldrich Chemical Co., Milwaukee, WI). The polar retinoid standard,

1-(hydroxymethyl)-4-ketoretinoic acid, was a generous gift from Hoffmann-La Roche (Nutley, NJ).

Animals. Male rats (225–275 g) were purchased from the Holtzman Co. (Madison, WI) and housed in groups of four in overhanging wire cages with free access to food and water. The animals were either used immediately upon arrival or after an average of 4 days on a pelleted stock diet (Wayne Lab-Blox, Allied Mills, Inc., Chicago, IL).

Bile Duct Cannulations. Rats were anesthetized by the intrajugular injection of sodium pentobarbital (25 mg/kg of body weight). This injection was given under light ether anesthesia. The bile ducts were then cannulated by using no. 10 polyethylene tubing. After bile flow was established, the abdominal incision was closed with sutures and metal clips. For isolation and identification work, the bile duct cannulated rats were injected intrajugularly with 2–4 mg of *all-trans*-[11,12-$^3H_2$]retinoic acid (20 μCi/rat; specific activity 15 300 dpm/μg) dissolved in 400 μL of dimethyl sulfoxide (Me_2SO). All injections and subsequent collection and isolation procedures were performed under yellow lights to minimize isomerization. Following the injection of labeled retinoic acid, the rats were placed in restraining cages and allowed 10% glucose ad libitum. Bile, urine, and feces were collected over a 24 h interval. The bile samples were collected into round-bottom flasks containing 5 mL of a 50 μg/mL solution of propyl gallate as antioxidant. All collections were at 0 °C, and the flasks were constantly purged with a nitrogen stream.

Lyophilization, Extraction, and Chromatography of Bile. Following the 24 h bile and urine collection, aliquots were removed from each sample for radioactivity determination. Bile volumes were measured, and the bile samples were shell frozen and lyophilized to dryness on a Model 10-100 Uni-Trap lyophilizer (VirTis Co., Inc., Gardiner, NY). Following lyophilization, the dry weight of each bile sample was recorded, and the bile solids were extracted with 100 mL of methanol. The methanol extracts were filtered through sintered glass funnels, and the insoluble residues were washed with several portions of methanol. The washings were combined with the original filtrate, and this extract was concentrated for chromatography by using a rotary evaporator (Figure 2).

The concentrated methanol extract (5 mL) was applied to a 2 × 60 cm column of Sephadex LH-20. The column was eluted with a 1:1 mixture of methanol and acetone. Fractions (3.6 mL) were collected, and 20-μL aliquots were removed for scintillation counting (column profile, Figure 3). Peak L-1 (fractions 25–65) containing retinoic acid, 5,6-epoxyretinoic acid, retinoyl β-D-glucuronide, and other unknown polar metabolites was pooled and concentrated. Peak L-2 (fractions 85–130) was not purified further.

High-Performance Liquid Chromatography of Peak L-1 Metabolites. Following Sephadex LH-20 chromatography, peak L-1 was divided into six equal portions and subjected to high-performance liquid chromatography on a Du Pont Zorbax ODS semipreparative column. Peak L-1 was injected in methanol and eluted (for the first 40 min) with an isocratic solvent consisting of water–methanol (65:35 v/v) with 10 mM ammonium acetate. Following the isocratic elution, a 20-min linear gradient from water–methanol (65:35) to 100% methanol was initiated. The flow rate was 3 mL/min, and 4.3-mL fractions were collected. Aliquots of 100 μL were removed from each fraction to monitor radioactivity. Figure 4 shows a representative profile of peak L-1 metabolites. The two most polar metabolites (i.e., peak C-1A and peak C-1B) represent approximately 14% of the total biliary radioactivity. This poorly resolved region (fractions 15–22) from the six semipreparative runs was combined and concentrated for further purification.

Injection of this fraction onto an analytical reverse-phase Du Pont Zorbax ODS column eluted with the same solvent program as discussed above gave the results shown in Figure 5. The flow rate was 1.5 mL/min, injection was in methanol, and 1.1-mL fractions were collected. This figure demonstrates base-line resolution between peak C-1A and peak C-1B, with peak C-1A representing 10% and peak C-1B representing 4% of the total biliary radioactivity. Although the radioactivity profile (Figure 5) shows a symmetrical peak shape for metabolite C-1A, the UV profile at 313 nm clearly demonstrated a shoulder on the leading edge of this peak (figure not shown). Thus, another purification step utilizing straight-phase HPLC was required. Injection of peak C-1A from the Zorbax ODS column onto a Zorbax Sil column required 2-propanol for solubilization of the metabolite. The straight phase column was eluted with a linear gradient of 1 h duration of 50% 2-propanol in hexane to 70% 2-propanol in hexane. The flow rate was 2 mL/min, and 1.1-mL fractions were collected, with 100-μL aliquots taken for scintillation counting. Figure 6 shows the radioactivity profile and confirms the existence of another component (designated C-1A′) in the peak C-1A isolated from a reverse-phase analytical column. The purified peak C-1A (fractions 15–19) from the Zorbax Sil column was used for the physical and chemical studies discussed later. A total of 200 nmol of purified metabolite was obtained following the purification scheme outlined above. The ultraviolet (UV) absorption spectrum for metabolite C-1A in 95% ethanol shows that the compound has a broad absorption band with a λ_{max} of 308 nm (Figure 7). Metabolite C-1A′ has an absorbance spectrum identical with C-1A. The lower λ_{max} observed for these metabolites compared to *all-trans*-retinoic acid (λ_{max} 350 nm) indicates that the side-chain double-bond conjugation has been interrupted. Purified C-1B obtained from the analytical Zorbax ODS column (fractions 54–62, Figure 5) shows a λ_{max} of 326 nm in 95% ethanol, also indicating a blue shift in the absorption maximum relative to *all-trans*-retinoic acid.

Derivatization Procedures. The trimethylsilyl derivative of metabolite C-1A was prepared by reacting 5 nmol of metabolite with 100 μL of BSTFA containing 1% trimethylchlorosilane at 65 °C for 45 min under nitrogen. The reaction mixture was evaporated to dryness with a nitrogen stream, dissolved in 50% 2-propanol in hexane, and purified by HPLC on a 4.6 × 250 mm Zorbax Sil column eluted with a 60-min linear gradient from 0% 2-propanol in hexane to 100% 2-propanol. The flow rate was decreased from 2 to 0.5 mL/min at 2-propanol concentrations exceeding 65%. The major radioactive peak eluted between 38 and 41 mL. These fractions were collected, evaporated, and purified further on the same column by using 53% 2-propanol in hexane. The peak eluting between 9 and 12 mL was collected and used for mass spectrometry.

The acetylated derivative of metabolite C-1A was prepared by treating 5 nmol of compound in 20 μL of dry pyridine with 20 μL of acetic anhydride for 13 h at room temperature. Following the reaction, the solvents were evaporated to dryness with a N_2 stream and the products were dissolved in methanol and then applied to a Zorbax ODS column eluted with the following solvent program: 40 min of water–methanol (65:35) followed by a 20-min linear gradient from water–methanol (65:35) to 100% methanol. The radioactive peak eluted between 72 and 77 mL.

The methyl ester of metabolite C-1A was prepared by reacting 12 nmol of compound in 50 μL of methanol with 50

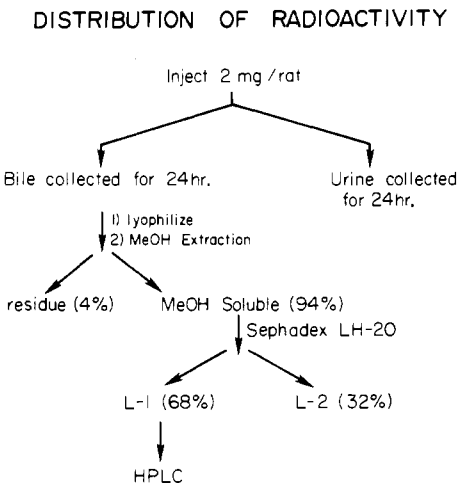

FIGURE 2: Flow chart detailing the methods used to fractionate the metabolites of *all-trans*-retinoic acid from rat bile.

μL of 88% formic acid and a large excess (1.25 mmol) of diazomethane in ether for 30 min at room temperature. The reaction was shown to have a strict requirement for acid since omission of formic acid from the reaction mixture resulted in the recovery of starting material. Following the reactions, solvents were evaporated to dryness with a nitrogen stream, and the products were dissolved in methanol. The formation of methyl esters was monitored by following the appearance of uncharged material at the solvent front of a 4.6 × 250 mm Zorbax SAX column. The uncharged product was purified further on a Zorbax Sil column eluted with 25% 2-propanol in hexane. The methyl ester peak eluting between 17 and 22 mL was saved.

Amino Acid Analysis. Amino acid analyses were performed on 34 nmol of purified metabolite C-1A and 10 nmol of taurocholic acid. The samples were hydrolyzed in 6 N HCl in sealed glass tubes. All hydrolyses were performed under vacuum at 110 °C for 24 h. Following hydrolysis, the samples were concentrated and taken to dryness by using a Savant Speed Vac concentrator/evaporator (Hicksville, NY). For analysis, 40 μL out of a 75 μL total was loaded onto the analyzer in a lithium citrate buffer at pH 2.2 and eluted with a lithium citrate buffer at pH 2.75. Amino acid analyses were performed by using a Beckman–Durham Model D 500 amino acid analyzer equipped with an automatic integrator/plotter. Detection of ninhydrin at 590 nm was used to monitor the effluent. Under these conditions, taurine elutes at 5.78 min between phosphoserine (4.22 min) and phosphoethanolamine (6.7 min).

Results

Bile duct cannulated rats dosed with either pharmacological or physiological levels of *all-trans*-[^{3}H]retinoic acid secreted approximately 60% of the administered dose in the bile within a 24-h collection period. The urine and the feces accounted for 10% and 2% of the dose, respectively.

Bile was fractionated according to the scheme shown in Figure 2. The methanol extract (containing 94% of the radioactivity) was chromatographed on a Sephadex LH-20 column (Figure 3). This column resolved two major peaks, with the peak designated L-1 accounting for approximately 68% and peak L-2 accounting for 32% of the applied radioactivity. Further purification of the peak L-1 metabolites resolved this material into several peaks (Figure 4), with the two most polar peaks accounting for 14% of the total biliary radioactivity (8.5% of the dose). Further HPLC purification of these polar peaks (Figures 5 and 6) demonstrated the

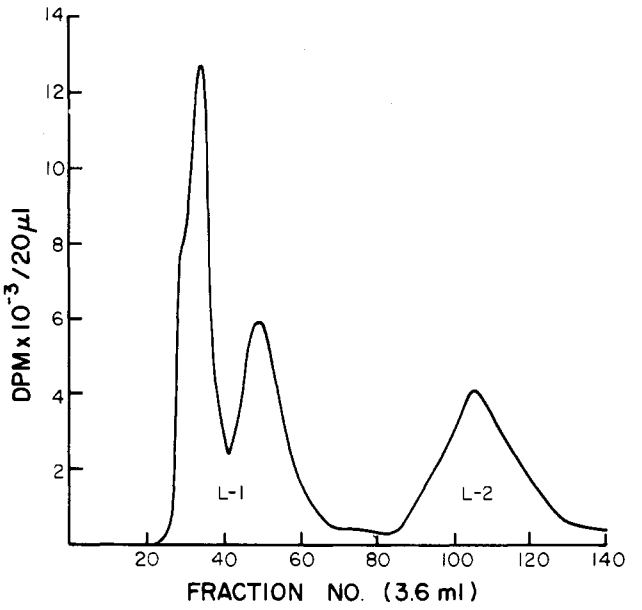

FIGURE 3: Chromatography of the methanol extract of bile from normal rats dosed with radiolabeled *all-trans*-retinoic acid. Chromatography was carried out by using a 2 × 60 cm Sephadex LH-20 column eluted with 1:1 acetone–methanol.

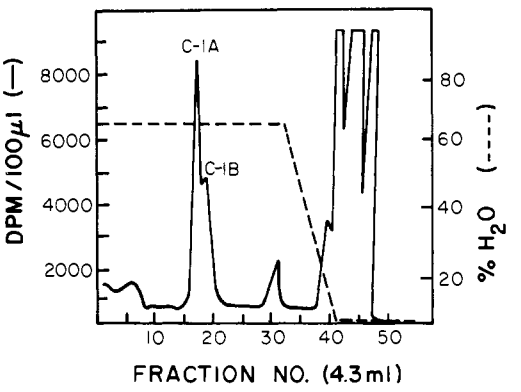

FIGURE 4: Semipreparative high-performance liquid chromatography of peak L-1 (from Figure 3) on a reverse-phase column eluted with a gradient program described under Materials and Methods. The flow rate was 3 mL/min, and 4.3-mL fractions were collected. Fractions 15–22 (peaks C-1A and C-1B) were pooled for further purification.

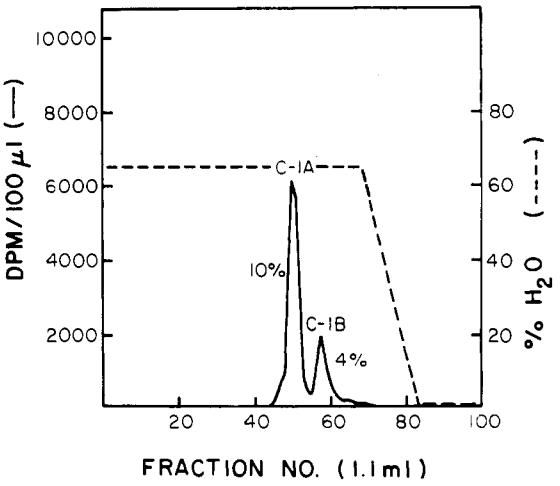

FIGURE 5: High-performance liquid chromatography of peaks C-1A and C-1B (from Figure 4) on a reverse-phase column eluted with the solvent program discussed under Materials and Methods. The flow rate was 1.5 mL/min, and 1.1-mL fractions were collected. Peak C-1A was pooled for further purification.

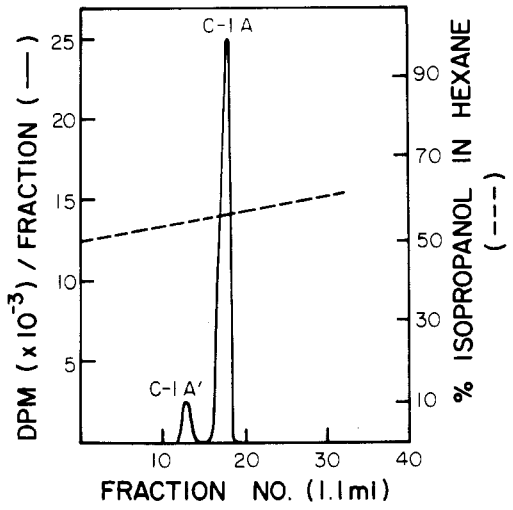

FIGURE 6: High-performance liquid chromatography of peak C-1A (see Figure 5) on a straight-phase column eluted with a solvent program discussed under Materials and Methods. The flow rate was 2 mL/min, and 1.1-mL fractions were collected.

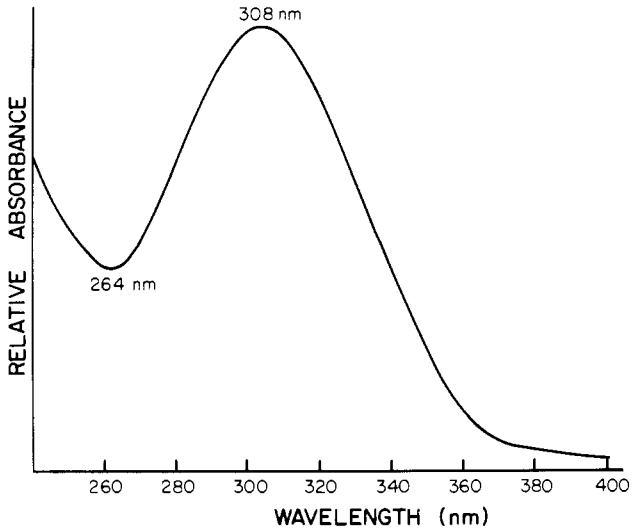

FIGURE 7: Ultraviolet absorbance spectrum of purified peak C-1A (see Figure 6) in 95% ethanol.

presence of three homogeneous metabolites designated C-1A', C-1A, and C-1B. In control experiments, [^{3}H]retinoic acid was added to whole bile or methanol-extracted bile and the entire isolation procedure carried out. The unchanged radioactive retinoic acid was recovered, and no radioactivity was found in the C-1A', C-1A, or C-1B fractions. Thus, C-1A, C-1B, and C-1A' represent true in vivo metabolites. Bile from rats dosed with 2 μg of *all-trans*-retinoic acid also contains metabolite C-1A, and its concentration is comparable to that observed for retinoyl β-glucuronide (K. L. Skare and H. F. DeLuca, unpublished results; Zile et al., 1980; Swanson et al., 1981). For this reason, we chose to further characterize the polar metabolite C-1A.

The purified peak C-1A metabolite (fractions 15–19, Figure 6) exhibits a UV absorbance spectrum (Figure 7) with a λ_{max} of 308 nm. An identical absorbance spectrum was obtained for peak C-1A'. These absorption maxima are not characteristic of the conjugated double-bond system of *all-trans*-retinoic acid since they represent a markedly reduced wavelength absorbance maximum. This shift of approximately 40 nm strongly indicates the loss of two double bonds in the side-chain conjugation of this metabolite compared to *all-trans*-retinoic acid. Calculation of the absorbance maximum

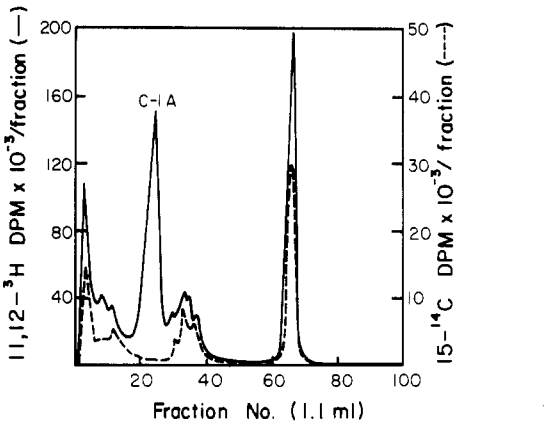

FIGURE 8: High-performance liquid chromatography of bile from normal rats injected with a double-labeled mixture of *all-trans*-[11,12-^{3}H$_2$]retinoic acid (—) and *all-trans*-[15-^{14}C]retinoic acid (---). The column was reverse phase, and the flow rate was 1.5 mL/min. The column was eluted with 60% H_2O in methanol for the first 55 fractions and then stripped with methanol.

(λ_{max}) expected for an α,β-unsaturated ketone of the type shown in Figure 1 yields a value of 309 nm (Dyer, 1965). This is in very close agreement with the observed λ_{max} of 308 nm.

Metabolite C-1A was also shown to be negatively charged on anion-exchange HPLC (figure not shown). When a 60-min linear gradient of 0–100 mM ammonium acetate in methanol was used, metabolite C-1A eluted with 60 mM salt whereas *all-trans*-retinoic acid required 47 mM salt. These results suggest that peak C-1A not only is negatively charged but also appears to be more acidic than retinoic acid. Initially, a carboxyl group was suspected as the negatively charged moiety since all other negatively charged retinoic acid metabolites which have been identified contain carboxyl groups. Attempts to methylate the purified compounds with only diazomethane have been unsuccessful. However, the addition of formic acid to the compound prior to the addition of a large excess of diazomethane converts 96% of the compound to its uncharged methyl ester. This strong dependence of the reaction on acid is not generally observed for methylation of carboxyl groups. As discussed in detail later, this metabolite was shown to possess a strongly acidic sulfonate group. A sulfonate group in free taurine has a pK of 1.3, and thus the pH must be very low before protonation occurs. Protonation of the sulfonate group may be required before diazomethane is capable of forming the methyl ester, and this would explain the requirement of acidic conditions for methylation.

Double-label experiments were performed to determine whether the blue shift observed in the λ_{max} of C-1A is due to decarboxylation of the side chain. Retinoic acid labeled with tritium in the 11 and 12 positions was mixed with retinoic acid labeled with ^{14}C in the carboxyl carbon (C-15) to yield a mixture with a resultant ^{3}H/^{14}C ratio of 1.5 as discussed under Materials and Methods. Bile from animals injected with this mixture was fractionated as described in Figure 2. HPLC of these double-label samples using the solvent described under Materials and Methods gave the results shown in Figure 8. This figure clearly demonstrates the loss of the ^{14}C label in metabolite C-1A with a resultant ^{3}H/^{14}C ratio of approximately 75. Decarboxylation of the side chain would result in a lower absorbance maximum of this metabolite, and hence this result is in agreement with the UV spectral data. For examination of the possibility for further decarboxylation of the side chain, *all-trans*-retinoic acid labeled with ^{14}C at carbon 14 was injected into normal rats. Following the purification scheme outlined above, metabolite C-1A was shown to retain

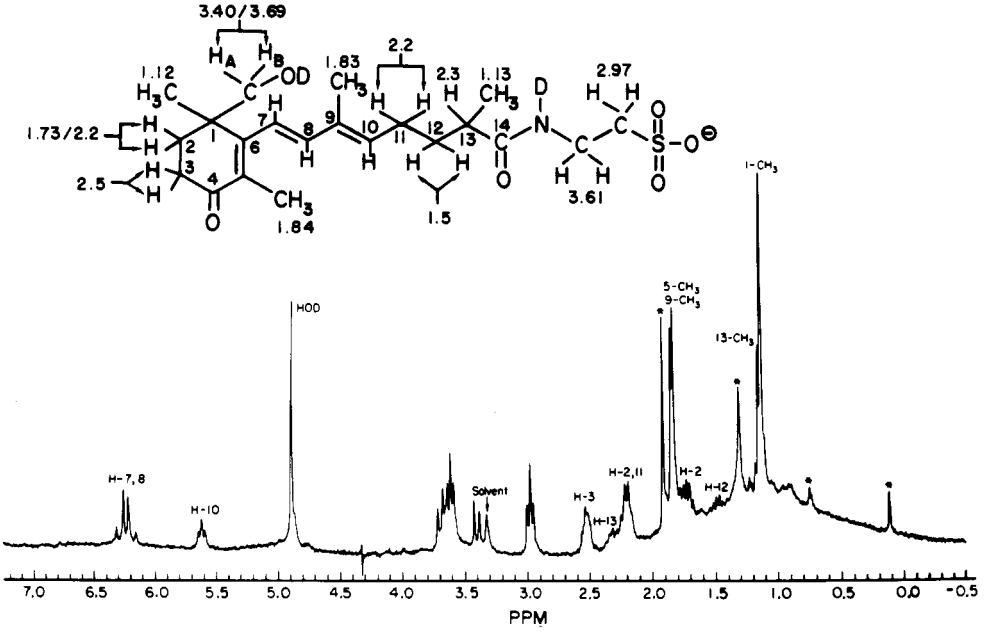

FIGURE 9: Proton nuclear magnetic resonance spectrum of retinotaurine (metabolite C-1A) taken in 100 atom % CD_3OD at 22 °C. Peaks are labeled with assigned protons corresponding to the structure shown. Impurities are indicated by an asterisk.

the ^{14}C label. The 14-^{14}C-labeled metabolite C-1A was shown to comigrate with ^{3}H-labeled C-1A on both reverse- and straight-phase HPLC. The 14-^{14}C-labeled metabolite also exhibited a UV absorbance spectrum identical with that of the ^{3}H-labeled C-1A (λ_{max} 308 nm, ϵ = 13 400). These results conclusively demonstrate that only the carboxyl group of the side chain is lost in the metabolic transformation of *all-trans*-retinoic acid to metabolite C-1A, and the remaining carbon skeleton is intact.

The NMR spectrum of purified metabolite C-1A is shown in Figure 9. This spectrum was obtained from 200 nmol (~75 μg) of purified metabolite, using the conditions described under Materials and Methods. Table I gives a compilation of the coupling constants and chemical shifts for C-1A. Examination of the spectrum reveals three major features which distinguish it from the NMR spectrum of *all-trans*-retinoic acid. First, the olefinic region of the spectrum (δ 5.5–6.5) does not show the characteristic six-proton pattern observed for *all-trans*-retinoic acid. In particular, integration of this region of the spectrum demonstrates the presence of only three olefinic protons for C-1A. Also, the characteristic doublet of doublets seen for H-11 and the singlet seen for H-14 in the NMR spectrum of retinoic acid are absent in the spectrum of metabolite C-1A. Saturation of the two terminal double bonds in the side chain would account for the absence of a C-11 signal in the olefinic region and would also corroborate the UV spectral data discussed above. The loss of the terminal carbon atom via decarboxylation accounts for the absence of the singlet for H-14. Second, protons with chemical shifts between 2.8 and 4.0 ppm are not seen in retinoic acid spectra whereas metabolite C-1A shows six protons in this region. This suggests that C-1A contains protons neighboring heteroatoms which are not present in *all-trans*-retinoic acid. Third, the characteristic *gem*-methyl singlet is absent in the spectrum of C-1A. This signal is replaced by a methyl singlet and a methyl doublet, with very close chemical shifts corresponding to the 1-CH_3 and the 13-CH_3 groups, respectively.

The AB quartet observed at 6.23 ppm is very characteristic for protons at C-7 and at C-8 of the side chain (Hänni et al., 1976; Englert et al., 1978; Hänni et al., 1977). The coupling constant for the spin system is 16.3 Hz and is in very close agreement with values for $J_{7,8}$ reported by others (Hänni et al., 1977; Englert et al., 1978). A coupling constant of 16.3 Hz for these protons also confirms that they are in the trans configuration relative to each other since 7-cis isomers have $J_{7,8}$ ~12 Hz (Englert et al., 1978). The triplet observed for the olefinic proton farthest upfield (δ 5.61) indicates coupling with two chemically equivalent neighboring protons. The coupling constant for this triplet is 7.30 Hz. These data are consistent with the saturation of a double bond between carbons 11 and 12 of the side chain generating methylenes at these two carbon atoms (Figure 9). This arrangement of protons gives the observed triplet for H-10 and a multiplet for the methylene at C-11. No coupling of H-10 with the methyl group at C-9 is observed. The coupling constant $J_{10,11}$ = 7.30 Hz is in the expected range for spin coupling between an olefinic proton and a methylene group separated by a single bond. As mentioned earlier, this would also account for the absence of a doublet of doublets pattern for C-11 normally

Table I: ^{1}H NMR Data for Metabolite C-1A

proton	chemical shift[a]	multiplicity	coupling constant (Hz)
H_3C-C(1)	1.12	s	–
DO—C(H_A)(H_B)—C(1)	3.40/3.69	d/d	11.11/11.17
H-C(2)	1.73	m	14/–[b]
H-C(2)	2.2	m	14/–[b]
H_2-C(3)	2.52	m	–
H_3C-C(5)	1.84	s	–
H-C(7)	6.19	d	16.32
H-C(8)	6.27	d	16.27
H_3C-C(9)	1.83	s	–
H-C(10)	5.61	t	7.30
H_2-C(11)	2.2	dt	7.3/3.5[b]
H_2-C(12)	1.5	dt	3.5/5[b]
H_3C-C(13)	1.13	d	5.88
H-C(13)	2.3	m	5.9/5[b]
$NCH_2CH_2SO_3^-$	3.61	t	6.78
$NCH_2CH_2SO_3^-$	2.97	t	6.74

[a] Chemical shifts are given in ppm downfield from Me_3Si ($CHCl_3$ = 7.88). [b] Chemical shifts and coupling constants were obtained from monoselective decoupling experiments. Hence, the values are approximate due to the complexity of the spin system.

observed for *all-trans*-retinoic acid. Decoupling experiments (data not shown) confirm the above interpretation. In addition, decoupling demonstrates the presence of a methylene at C-12 since irradiation of H-10 causes the H-11 multiplet at 2.2 ppm to collapse to a triplet. The configuration of the double bond between C-9 and C-10 also appears to be trans since 9-cis isomers show a characteristic 0.5-ppm downfield shift of H-8 relative to H-7 (Englert et al., 1978; Vetter et al., 1971). Table I shows that the chemical shift difference between H-7 and H-8 is only 0.08 ppm which is much too small for a 9-cis geometry. Decoupling experiments are also in agreement with a 9-trans geometry since irradiation of H-10 shows no enhancement of the signal for the methyl group at C-9.

The presence of only two vinylic methyl groups in the spectrum of C-1A (i.e., one at 1.84 ppm and the other at 1.83 ppm) is in agreement with the loss of one vinylic methyl at C-13 due to saturation of the double bond between C-13 and C-14. As a consequence of this saturation, the methyl group on C-13 is split into a doublet by the proton on C-13 and has a chemical shift of 1.13 ppm. The proton on C-13 must have a chemical shift of approximately 2.3 ppm since irradiation of this multiplet causes the doublet at 1.13 ppm to collapse to a singlet (data not shown).

Evidence that more than just the two protons at C-11 are under the multiplet at 2.2 ppm was obtained by decoupling experiments (data not shown). When the multiplet at 2.2 ppm was irradiated, the triplet at 5.61 ppm collapsed to a singlet. However, in addition to this change there were also alterations in the multiplets at 2.5, 1.73, and 1.5 ppm. This strongly suggests that one of the two protons at C-2 also has a chemical shift of ~2.2 ppm. The two protons at C-2 must be diastereotopic due to the anisotropy of a neighboring group. Hence, if the C-3 protons have a chemical shift of 2.5 ppm and the other proton at C-2 has a shift of 1.73 ppm, these results are in very good agreement with the proposed structure. The change in the multiplet at 1.5 ppm can be explained by coupling between the side-chain protons at C-11 and C-12, as discussed earlier.

Comparison of the NMR spectrum of 1-(hydroxymethyl)-4-ketoretinoic acid with the spectrum of C-1A greatly aided in the assignment of protons in the cyclohexene ring. The multiplets at 1.73 and 2.5 ppm are virtually identical in the spectra from these two compounds. Hence, the assignment of a chemical shift of 2.5 ppm for the protons at C-3 is consistent with these data since the C-3 protons in 1-(hydroxymethyl)-4-ketoretinoic acid are known to have a chemical shift of 2.5 ppm. Decoupling of the multiplet at 2.5 ppm also agrees with these protons being at C-3 since changes are only observed in the multiplets at 1.73 and 2.2 ppm, as expected. The presence of a hydroxymethyl group at C-1 is also established from the NMR spectrum of C-1A. The same AB quartet is observed for C-1A and 1-(hydroxymethyl)-4-ketoretinoic acid with doublets at 3.40 and 3.69 ppm. The coupling constant of 11.1 Hz (Table I) is virtually identical with that observed for 1-(hydroxymethyl)-4-ketoretinoic acid. Decoupling experiments (not shown) confirm these results. The presence of a hydroxymethyl group at C-1 in metabolite C-1A explains two points mentioned earlier: (1) there is no singlet integrating for six protons normally seen for the *gem*-methyls at C-1, and (2) the difference in the chemical shifts of the two protons at C-2 are due to the anisotropy of the neighboring hydroxymethyl group.

The important question not addressed by the NMR experiments discussed above is the identity of the substituent giving rise to the triplets at 2.97 and 3.61 ppm. Reciprocal

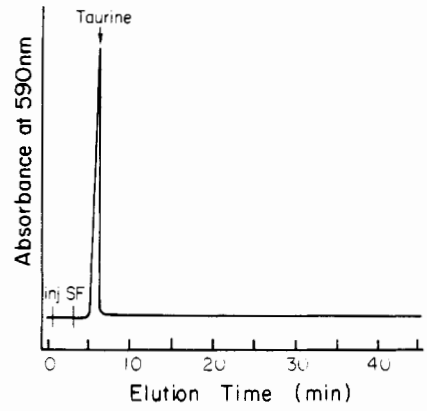

FIGURE 10: Amino acid analysis of retinotaurine. Conditions are as described under Materials and Methods. SF represents the solvent front and inj represents the point of injection.

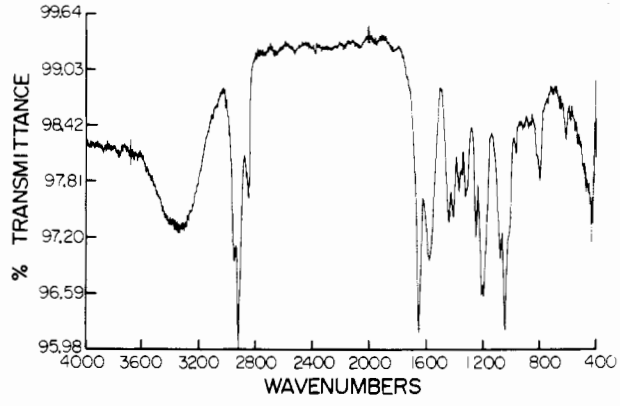

FIGURE 11: Fourier transform infrared spectrum of retinotaurine (metabolite C-1A) taken in methanol on a KBr slide.

decoupling experiments (not shown) indicate that these two triplets are coupled with each other with a coupling constant of 6.76 Hz (Table I). The orientation of the two methylene groups which would give rise to this observed spin system is $X–CH_2–CH_2–Y$ where X and Y are heteroatoms. A conjugate which would conform to these restrictions is the β-amino acid taurine whose structure is $H_2N–CH_2–CH_2–SO_3^-$. This type of conjugate would also agree with earlier results demonstrating the existence of a negatively charged acidic group. An NMR spectrum of taurine in D_2O shows two triplets with a coupling constant of 6.7 Hz (not shown). This is in very good agreement with the coupling constant (J = 6.76 Hz) observed for C-1A. For confirmation of the presence of a conjugated taurine in metabolite C-1A, amino acid analysis was performed. Figure 10 shows the absorbance at 590 nm obtained following analysis. Only one peak, corresponding to taurine, was observed. The elution position of taurine was confirmed by using taurocholic acid as a standard. The molar ratio of taurine to the retinoid was shown to be 1:1 since 17.9 nmol of C-1A was injected (based on specific activity) and 17.1 nmol of taurine was recovered following amino aid analysis.

On the basis of similarities in the chemical shifts of the C-3 protons and C-5 methyl group of C-1A and 1-(hydroxymethyl)-4-ketoretinoic acid, a 4-keto group is proposed for C-1A. Fourier transform infrared spectroscopy (FT-IR) was used to verify this structural feature. The FT-IR spectrum of C-1A is shown in Figure 11. A strong absorption band at 1658 cm^{-1} is entirely consistent with a 4-keto group since 1-(hydroxymethyl)-4-ketoretinoic acid shows an absorption band at 1657 cm^{-1} due to the 4-keto group. The broad absorption band centered at 3353 cm^{-1} is due to O–H and N–H

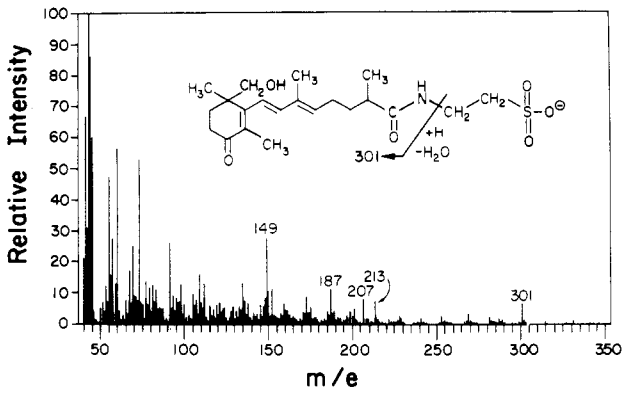

FIGURE 12: Low-resolution mass spectrum of retinotaurine.

stretching and demonstrates the lack of a free carboxyl group. The double bond and amide II stretching frequencies are at 1582 cm^{-1} and are very near the ketone stretch. The intense absorption at 1055 cm^{-1} is probably due to the sulfonate group on taurine.

A low-resolution mass spectrum of the intact metabolite is shown in Figure 12. One salient feature of this spectrum is the absence of a peak corresponding to the molecular ion at *m/e* 427. This suggests that the strongly acidic sulfonate group on this metabolite greatly decreases the volatility of the intact compound. This metabolite gives a spectrum like that shown in Figure 12 only when the temperature is raised above 210 °C. At such high temperatures pyrolysis may produce a fragmentation pattern which is difficult to interpret. However, a reproducible high molecular weight peak at *m/e* 301 is seen in low-resolution spectra of C-1A. High-resolution mass spectrometry (not shown) of metabolite C-1A gave an exact mass of 301.2036 for this peak corresponding to a formula of $C_{19}H_{27}O_2N$ (calculated *M* 301.2041). The high-resolution mass spectrum thus confirmed the presence of a nitrogen atom in C-1A. High resolution also gave peaks at 286.1829 ($C_{18}H_{24}O_2N$; loss of CH_3), at 269.1572 ($C_{18}H_{22}O_2$; loss of CH_3 and NH_3), and at 149.0964 ($C_{10}H_{13}O$). Figure 13 shows a proposed hydrogen rearrangement reaction which eliminates the charge sulfonate group to yield the peak at *m/e* 301.

So that a mass spectrum of a derivative of C-1A could be obtained, the intact metabolite was treated with BSTFA to generate the trimethylsilyl (Me_3Si) derivative. The low-resolution mass spectrum of Me_3Si C-1A is shown in Figure 14. This derivative also did not give a molecular ion peak. However, the peak at *m/e* 373 corresponds to the addition of one trimethylsilyl moiety to the *m/e* 301 peak observed for the underivatized metabolite. A large peak at *m/e* 147 [$(CH_3)_3Si—O^+{=}Si(CH_3)_2$] was also observed in the spectrum of Me_3Si C-1A. Peaks at *m/e* 301, 207, and 149 are common fragments for both Me_3Si C-1A and C-1A. In agreement with the presence of hydroxyl and amide groups in C-1A, the acetylated derivative was much less polar than C-1A on reverse-phase HPLC.

A mass spectrometer equipped with a fast atom bombardment (FAB) source was used to circumvent the problem of volatility. This technique relies on soft ionization and does not cause extensive fragmentation, so it allows for the determination of molecular ions. An FAB spectrum was run on 9 μg of purified C-1A, and it showed peaks at *m/e* 428, 450, and 472 (data not shown). The peak at *m/e* 428 corresponds to the protonated molecular ion ($M + H^+$). The peaks at higher molecular weight are interpreted as ions arising from the addition of either one or two sodium atoms to the molecular ion (i.e., $M + Na^+ = 450$ and $M–H^+ + 2Na^+ = 472$). Since

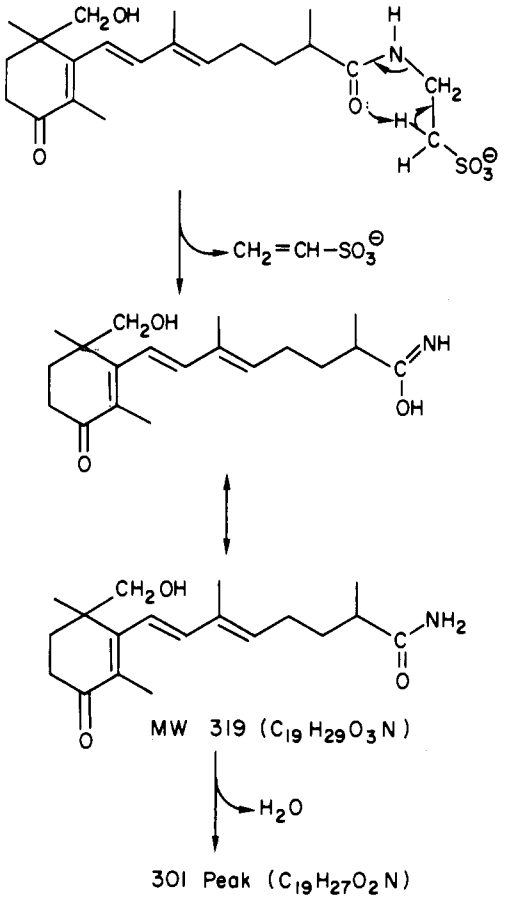

FIGURE 13: Proposed hydrogen rearrangement reaction to account for the *m/e* 301 peak in the low-resolution mass spectrum of retinotaurine (see Figure 12).

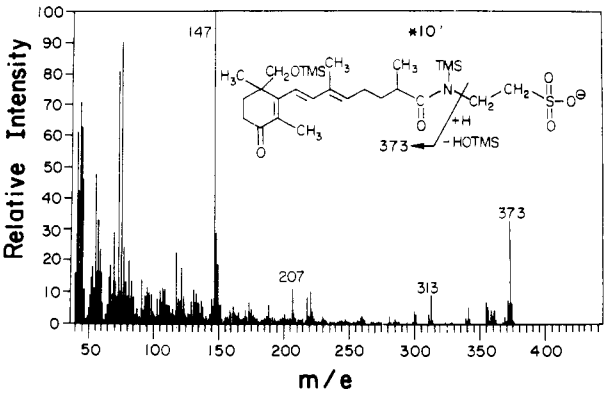

FIGURE 14: Low-resolution mass spectrum of the trimethylsilyl derivative of retinotaurine. Intensities of all ions above *m/e* 300 have been expanded 10-fold to facilitate examination.

the FAB mass spectrum was run in a sodium-cationized glycerol matrix, it is reasonable to postulate that Na^+ can replace easily dissociated hydrogen atoms or attach to nonbonding orbitals of the oxygen atoms.

Thus, all of the physical and chemical data, taken together, positively identifies metabolite C-1A as the structure shown in Figure 1.

Discussion

This paper describes the isolation and identification of the first taurine conjugate of a fat-soluble vitamin to be reported. This metabolite, with the suggested trivial name of retinotaurine, accounts for approximately 10% of the total biliary radioactivity 24 h following a pharmacological or physiological dose of *all-trans*-retinoic acid (K. L. Skare and H. F. DeLuca,

unpublished results). This metabolite was shown to be very polar and acidic due to the sulfonate group of the taurine conjugate.

The presence of acidic metabolites of *all-trans*-retinoic acid in the bile of guinea pigs, chickens, and rats has been known for several years (Dunagin et al., 1966; Zachman et al., 1966b). However, prior to this report, the only metabolites from bile to be isolated and identified were retinoyl β-D-glucuronide (Dunagin et al., 1965; Zile et al., 1980; Swanson et al., 1981) and 13-*cis*-4-oxoretinoyl β-glucuronide (Frolik et al., 1981a). Although retinoyl β-glucuronide was originally reported to account for up to 80% of the radioactivity in bile following a pharmacological dose of *all-trans*-retinoic acid (Lippel & Olson, 1968), recent reports indicate that it only represents approximately 12% of the biliary radioactivity (Zile et al., 1980; Swanson et al., 1981). These results clearly indicate that other unknown metabolites of *all-trans*-retinoic acid are present in bile. Although retinotaurine and retinoyl β-glucuronide are of comparable concentration following a 24-h collection period, retinoyl β-glucuronide appears at earlier times than retinotaurine (K. L. Skare and H. F. DeLuca, unpublished results). For example, following a physiological or pharmacological dose of *all-trans*-retinoic acid, retinoyl β-glucuronide is at its maxiumum concentration at 2 h of collection (M. H. Zile, R. C. Inhorn, and H. F. DeLuca, unpublished results) whereas retinotaurine does not reach maximum concentrations until 4–6 h after the dose (K. L. Skare and H. F. DeLuca, unpublished results). This lag in the appearance of retinotaurine may be the result of the extensive enzymatic transformations which must occur prior to its secretion into the bile.

The double-label experiment presented in this paper confirms the existence of decarboxylated metabolites of retinoic acid in bile. Earlier work using expiration of $^{14}CO_2$ as an indicator of decarboxylation demonstrated that 18–20% of an administered retinoic acid dose was decarboxylated with loss of only the 15-carbon atom (Roberts & DeLuca, 1967). This route of metabolism was postulated to yield products which are excreted in the feces. These results are in agreement with the present results which show that retinotaurine has lost only the terminal carbon atom of the side chain. Since retinotaurine accounts for 10% of the biliary radioactivity (6% of the dose), it represents approximately one-third of the metabolism by this route. The mechanism of the decarboxylation reaction, which is required prior to conjugation with taurine, is unknown. One cannot invoke a β-keto acid as an intermediate in decarboxylation since the methyl group at carbon 13 prevents oxidation of this carbon to a ketone. Thus, one possible substrate for the decarboxylase is an α-keto acid. Several examples of α-keto acid decarboxylases are known (Walsh, 1979). In the case of retinoic acid, oxidative attack at carbon 14 with a subsequent loss of carbon 15 by decarboxylation would be plausible. Roberts & DeLuca (1968) postulated an aldehyde at carbon 14 following decarboxylation. However, retinotaurine biosynthesis would require oxidation to a carboxyl group prior to the formation of the amide bond with taurine.

The question of enterohepatic circulation of retinotaurine is not examined in this study. However, it was shown several years ago that up to 40% of the biliary metabolites of retinol or retinoic acid undergo enterohepatic circulation (Zachman et al., 1966a). These results were confirmed by a recent study which demonstrated 34% reabsorption of biliary radioactivity following a 1.5-mg dose of retinoic acid (Swanson et al., 1981). This group also found that interruption of the enterohepatic circulation by cannulation caused a reduction in the excretion of radioactivity into the urine. It was concluded that 25% of the retinoic acid metabolites in urine were derived from biliary metabolites. Using double-label studies, Hänni & Bigler (1977) concluded that metabolites with shortened side chains are mainly eliminated by the kidney whereas metabolites with an intact side chain are eliminated by the liver via the bile in the feces. These studies suggest that retinotaurine may be eliminated in the urine. However, recent work in our laboratory has shown no detectable retinotaurine in the intestinal mucosa, blood, or kidney of vitamin A deficient rats given physiological doses of *all-trans*-retinoic acid (D. P. Silva and H. F. DeLuca, unpublished results). Thus, it is unlikely that intact retinotaurine is absorbed by the small intestine and excreted in the urine. More likely is the possibility of hydrolysis of the amide linkage by amidases present in the bacterial flora of the small intestine. The free retinoid liberated upon hydrolysis may then be reabsorbed by the small intestine and excreted in the urine. Approximately 25% of all bile acids are deconjugated and dehydroxylated by bacterial action in the distal ileium (Balistreri & Soloway, 1978). These deconjugated bile acids are then reabsorbed, along with the 75% which are not deconjugated, by active transport mechanisms of the small intestine and returned to the liver.

The relationship of retinotaurine to the excretory metabolites identified by Hänni et al. (1976, 1977) and Rietz et al. (1974) after pharmacological doses (27.5 mg) of *all-trans*-retinoic acid is not clear. Since these metabolites were not examined following smaller doses of retinoic acid, their physiological significance is not known. However, there are several structural features which are common to retinotaurine and these excretory metabolites. First, all of the urinary metabolites which have been identified possess a 4-keto group. Second, several of the urinary metabolites are oxidized to either a carboxyl or a hydroxymethyl group at carbon 1. Third, two of the urinary metabolites have saturated double bonds in the same positions as retinotaurine in the side chain. Finally, several of the urinary metabolites have lost part of the side-chain skeleton via decarboxylation.

One of the first steps which has been proposed for the inactivation of retinoic acid is hydroxylation of the cyclohexene ring to form 4-hydroxyretinoic acid (Roberts et al., 1979; Frolik et al., 1980). This metabolite is then further oxidized to 4-ketoretinoic acid. The oxidation of 4-ketoretinoic acid to more polar metabolites appears to have similar enzymatic properties as the initial hydroxylation event (Roberts et al., 1980). Both reactions are dependent on the vitamin A status of the animal, both require NADPH and O_2, and both are inhibited by carbon monoxide. These requirements led the authors to believe that the hydroxylase(s) responsible for the two metabolic conversions was (were) similar to cytochrome P-450 monooxygenases. These results suggest that hydroxylation of one of the *gem*-methyl groups could be the next step in the conversion of 4-ketoretinoic acid. If this is true, these metabolites may represent intermediates in the biosynthesis of retinotaurine. This paper does not provide any information on the intermediates involved in retinotaurine synthesis. However, retinotaurine was recently shown to be biologically inactive in a vaginal smear assay (K. L. Skare, W. K. Sietsema, and H. F. DeLuca, unpublished results) and hence may represent a metabolite in the inactivation pathway for retinoic acid.

The question of the identity of metabolite C-1A′ is interesting since it has properties which are very similar to those of retinotaurine. As discussed earlier, C-1A′ and retinotaurine have very similar chromatographic properties on both reverse-

and straight-phase HPLC and also have virtually identical chromophores. Since retinotaurine was shown to be in the all-trans configuration, C-1A′ may merely represent a 9-cis isomer of retinotaurine. However, NMR data will be required before one can assign an unambiguous structure to metabolite C-1A′. The data compiled in this paper should also be useful in the identification of other polar metabolites of retinoic acid which may have structural features similar to retinotaurine.

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