paper on methodology

Chemical synthesis of all-*trans* retinoyl β-glucuronide

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Abstract All-*trans* retinoyl fluoride reacts with the 3,6-lactone of glucuronic acid in slightly alkaline solution to give the lactone of retinoyl β-glucuronide, along with other retinoyl glucuronolactones, in about 60% yield. Hydrolysis of the lactone with very dilute alkali gives the free acid, retinoyl β-glucuronide, in about 80% yield. Pure all-*trans* retinoyl β-glucuronide (overall yield: 20–25%) was obtained free from other isomeric and anomeric forms by reverse-phase high pressure liquid chromatography. Retinoyl β-glucuronide was characterized by UV-visible, infrared, and ^{1}H-NMR spectra, by elementary analysis, by mass spectra, and by its susceptibility to hydrolysis by bacterial β-glucuronidase. —**Barua, A. B., and J. A. Olson.** Chemical synthesis of all-*trans* retinoyl β-glucuronide. *J. Lipid Res.* 1985. **26:** 1277–1282.

Supplementary key words retinoyl fluoride • vitamin A • glucuronolactone • ^{1}H-NMR spectra

Among metabolites of vitamin A, the β-glucuronides of retinol and retinoic acid have a unique position. Although secreted in significant amounts in the bile (1, 2) along with many other biologically inactive, oxidized, and/or conjugated metabolites of vitamin A (3–6), the β-glucuronides are involved in an enterohepatic circulation of vitamin A derivatives (7), are synthesized in discrete amounts in the intestine (8) as well as in the liver (9), and show high biological activity in the quantitative vaginal smear assay (10) as well as in the growth assay (11) in rats. Because of their interesting properties, the β-glucuronides of retinol and retinoic acid may well play a more significant role in vitamin A metabolism and function (12) than originally suggested (13).

In the past, retinoyl β-glucuronide has been available only in small amounts after its laborious isolation and purification from the bile of bile duct-cannulated rats (3, 4, 11). To stimulate further studies on this interesting conjugated form of retinoic acid, we have chemically synthesized (by the pathway shown in **Fig. 1**) and characterized its β-isomer.

MATERIALS AND METHODS

Chemicals and solvents

All-*trans* retinoic acid, β-glucuronidase (from *E. coli*, activity 570,000 units/g of solid), and D-glucurono-3,6-lactone were purchased from Sigma Chemical Co. (St. Louis, MO). Reagent grade methanol (Fischer Scientific Co., Pittsburg, PA) was used. All other chemicals and solvents have been described previously (14).

Chromatographic technique

Reverse-phase high pressure liquid chromatography (HPLC) was conducted with a Waters Associates U6K injector and 6000 A pump equipped with a Perkin-Elmer LC-75 variable-wavelength detector, and a Hewlett-Packard 3390 A integrator or a Houston Instrument Omniscribe recorder. The solvent systems used were methanol or methanol–water (4:1 or 7:3) containing 10 mM ammonium acetate at a flow rate of 1.5 to 3 ml/min. The columns used were a Waters Associates C18 μBondapak column (4.6 mm i.d. × 25 cm), a Whatman Partisil-10 ODS-2 column (4.6 mm i.d. × 25 cm), and, for semi-preparative separations, a Whatman Partisil-10 ODS-3 M9 column (9.4 mm i.d. × 50 cm). Peaks were detected either with the Perkin-Elmer LC-75 detector or with a refractive index detector. Silica gel for dry-column chromatography, activity III/30 mm (Woelm Pharma, Eschwege, West Germany, obtained from Universal Scientific Inc., Atlanta, GA) was wet-packed with hexane before sample addition. Thin-layer chromatography (TLC) was conducted on silica gel plates (0.25 mm × 20 cm × 20 cm) obtained from Brinkmann Instruments Co. (Westbury, NY) with hexane–acetone (4:1 or 2:1) as the developing solvent.

Abbreviations: TLC, thin-layer chromatography; CC, column chromatography; HPLC, high pressure liquid chromatography.

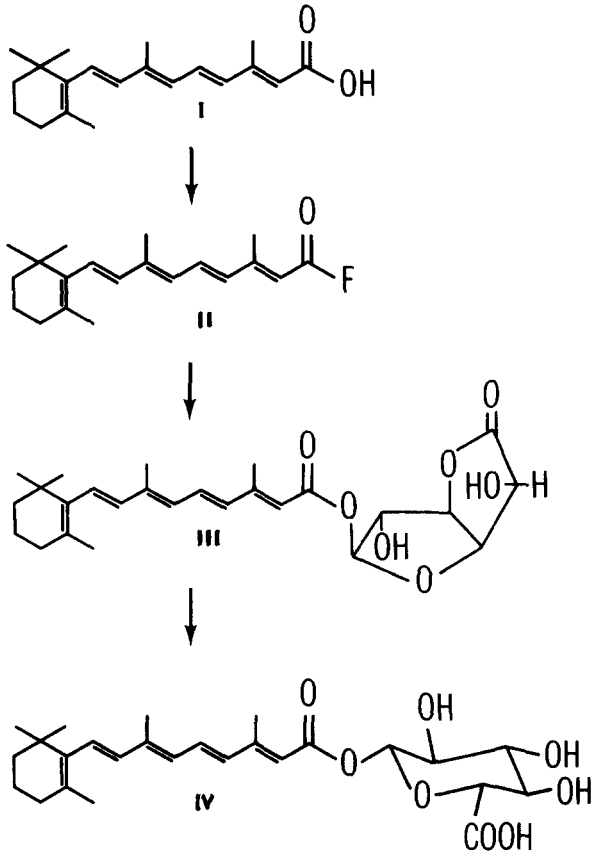

Fig. 1. The chemical synthesis of all-*trans* retinoyl β-glucuronide; I, all-*trans* retinoic acid; II, all-*trans* retinoyl fluoride; III, all-*trans* retinoyl glucuronolactone; IV, all-*trans* retinoyl β-glucuronide.

Physicochemical and spectrometric analysis

Ultraviolet spectra were recorded with a Shimadzu model UV 240 recording spectrophotometer. Mass spectra were obtained by using a direct-inlet probe with a Finnegan model 4000 GC/MS instrument operating in the EI mode. The mass spectrum of underivatized retinoyl β-glucuronide was obtained by desorption technique in the chemical ionization source using methane as the reagent gas. The mass and the relative intensities (in parentheses) of only major and diagnostic peaks are given. The molecular ion is denoted as M^+. The ^{1}H-NMR spectra were determined in $CDCl_3$, CD_3OD, or DMSO-D_6 with tetramethylsilane as the reference by use of a Nicolet 300-MHz instrument. Infrared spectra were recorded in an IBM FT-IR spectrometer. Melting points were determined by the open capillary tube method.

EXPERIMENTAL SECTION

Preparation of all-*trans* retinoyl fluoride (II)

All-*trans* retinoic acid (I) (3 g, 10 mmol) was dissolved in 105 ml of diethyl ether and cooled to −70°C in a mixture of dry ice and acetone. Diethylaminosulfurtrifluoride (1.61 g, 10 mmol) was dissolved in 5 ml of diethyl ether, cooled to −70°C, and added dropwise with stirring to the solution of retinoic acid. After being warmed to room temperature, the solvent was evaporated off, and argon was passed through the red oil to remove white fumes. The oil was dissolved in hexane and passed through a short column of silica gel (1.5 × 3 cm) to remove other products. Retinoyl fluoride was then eluted with a mixture of hexane–diethyl ether 9:1. Removal of solvent gave a red oil consisting of all-*trans* and *cis* isomers of retinoyl fluoride (3 g). When the oil was dissolved in a small volume of hexane and kept at −20°C overnight, all-*trans* retinoyl fluoride crystallized out. Yield 2.3 g (76%); UV_{max} (hexane) 382 nm. Other properties of retinoyl fluoride have been described previously (15).

Preparation of 3',6'-lactone of retinoyl-β-glucuronide (III)

D-Glucurono 3,6-lactone (2 g, 11.3 mmol) was shaken vigorously with 1 ml of H_2O and then added to 10 ml of a solution of retinoyl fluoride (1 g, 3.3 mmol) in acetone. Any undissolved glucuronolactone was transferred to the reaction flask by rinsing with 10 ml of acetone. After the addition of 1 g of $NaHCO_3$, the mixture was stirred at room temperature until no retinoyl fluoride was detected (20–30 hr) by TLC (hexane–acetone 4:1). The solution was then diluted with water, neutralized with 1 N HCl, and extracted with ethyl acetate. The extract was washed several times with water to remove excess glucuronolactone and then dried over anhydrous Na_2SO_4. Upon removal of solvent in a rotary evaporator, a viscous red oil was obtained.

The oil was dissolved in 2 ml of diethyl ether, diluted with 8 ml of hexane, and subjected to column chromatography (CC) on silica gel (2 × 30 cm). The column was developed sequentially with hexane, diethyl ether (5 to 50%) in hexane, acetone (5 to 50%) in hexane, and finally, with mixtures of dichloromethane and methanol (2:1 and 1:1). Five fractions were obtained, as shown in **Table 1**.

Fraction 4, which contained a mixture of retinoyl glucuronolactones in 57% yield, was evaporated to dryness, and the residue was dissolved in 2 ml of diethyl ether. The solution was chromatographed on a silica gel column (2 × 20 cm). After removing some impurities with diethyl ether, the broad zone of retinoyl glucuronolactones was eluted by a mixture of hexane containing acetone (30–50%). Three compounds were present, as indicated by TLC analysis of the eluate. An analytical sample of retinoyl β-glucuronolactone was obtained as follows. A solution of 50 mg of the rechromatographed lactone in 1 ml of acetone was applied as strips to two TLC plates (20 × 20 cm). After development with hexane–acetone 2:1, three lactone zones were found at R_f values of 0.11, 0.22, and 0.44. The major yellow zone (R_f = 0.22), which

TABLE 1. Column chromatographic separation of products formed by the reaction of retinoyl fluoride (1 g) with D-glucurono 3,6-lactone (2 g)

Fraction No.	Compound	Eluent	UV_{max} in MeOH	Yield
			nm	*g*
1	Retinoyl fluoride	Hexane	368	0.08
2	Retinoic acid	30–50% Diethyl ether in hexane	350	0.35
3	Unidentified	30–40% Acetone in hexane	368	0.25
4	3',6'-Lactone of retinoyl glucuronides	40–50% Acetone in hexane	358	0.86
5	Retinoyl glucuronide	Methanol–dichloromethane (1:1)	358	0.12

contained retinoyl β-glucuronolactone, was scraped off and eluted with diethyl ether–CH_2Cl_2 1:1. The solvent was evaporated off, and the residual orange syrupy liquid was dissolved in 4–5 ml of hexane and kept at −20°C. After several days, retinoyl β-glucuronolactone separated as a yellow solid (33 mg). Mp 105–110°C; UV_{max} (methanol) 356 nm (ε 43,500). ^{1}H-NMR (CD_3OD) δ 7.13 (dd, 1, J = 14,11Hz, H-11), 6.43 (d, 1, J = 15Hz, H-7), 6.42 (d, 1, J = 15Hz, H-12), 6.22 (d, 1, J = 11Hz, H-10), 6.18 (d, 1, J = 15Hz, H-8), 5.93 (s, 1, H-14), 5.31 (d, 1, H), 5.21 (d, 1, H), 4.37 (d, 1, H), 3.62–3.81 (m, 4, H), 2.39 (s, 3, C-13 CH_3), 2.07 (m, 2, C-4 CH_2), 2.05 (s, 3, C-9 CH_3), 1.74 (s, 3, C-5 CH_3), 1.68 (m, 2, C-3 CH_2), 1.53 (m, 2, C-2 CH_2), 1.07 (s, 6, C-1 $(CH_3)_2$); ^{1}H-NMR ($CDCl_3$) δ 7.04 (dd, 1, J = 15,11Hz, H-11), 6.31 (d, 1, J = 16Hz, H-7), 6.28 (d, 1, J = 15Hz, H-12), 6.13 (d, 1, J = 11, H-10), 6.12 (d, 1, J = 16, H-8), 5.87 (s, 1, H-14), 5.44 (s, 1, H), 4.99–5.05 (m, 1, H), 4.51 (d, 1, J = 4Hz, H), 4.06–4.10 (m, 1, H), 3.59–3.80 (m, 3, H), 2.31 (s, 3, C-13 CH_3), 2.01 (m, 2, C-4 CH_2), 1.98 (s, 3, C-9 CH_3), 1.70 (s, 3, C-5 CH_3), 1.61 (m, 2, C-3 CH_2), 1.47 (m, 2, C-2 CH_2), 1.02 (s, 6, C-1 $(CH_3)_2$). IR 3440 (broad), 2950, 2900, 2850, 1800, and 1795 (split singlet, lactone C=O), 1722 and 1718 (ester C=O), 1610, 1580, 1450, 1390, 1360, 1250, 1140 (C-O), 1090, 970 cm^{-1}. MS (m/z) 458 (M^+) (0.4), 440 (2), 425 (0.4), 396 (1), 355 (0.3), 300 (94), 285 (26), 255 (29), 201 (39), 185 (61), 175 (70), 159 (100), 145 (72), 133 (67), 119 (87), 107 (73), 105 (58), 95 (85), 81 (41), 69 (59), 55 (27).

Preparation of retinoyl glucuronides

The crude mixture of the 3',6'-lactones of retinoyl glucuronides (500 mg, 1.1 mmol), i.e., the reaction mixture just after chromatography on a silica gel column, was dissolved in ethanol (20 ml) and diluted with water (ca. 30 ml) until slightly turbid. The solution was cooled in ice, and aqueous 0.1 N KOH was added dropwise with stirring until the solution was slightly basic to pH paper. During the subsequent hydrolysis at 0–4°C, small volumes of 0.1 N KOH were periodically added (whenever the solution became acid) for 50–60 min until the lactones disappeared, as detected by TLC (hexane–acetone 2:1). Retinoyl glucuronides concomitantly appeared as the major band during TLC (R_f = 0). Thereafter, the solution was acidified with 1 N HCl and extracted with ethyl acetate. The extract was washed with water, dried over Na_2SO_4, and evaporated to dryness in a rotary evaporator. The yellow oil was dissolved in 2–3 ml of diethyl ether and subjected to CC on silica gel (1.5 × 30 cm). After retinoic acid and other products were removed by treatment with hexane and diethyl ether–hexane 1:1, an anomeric mixture of retinoyl glucuronides was eluted with CH_2Cl_2–MeOH 1:1. After solvent removal, the remaining yellow oil, which contained a mixture of retinoyl glucuronides, solidified upon the addition of hexane: 385 mg (75%), mp 120–130°C; UV_{max} (methanol) 358 nm; (H_2O) 365 nm.

Separation of retinoyl glucuronides: preparation of all-*trans* retinoyl β-glucuronide (IV)

The mixture of retinoyl glucuronide, prepared as indicated above, separated into three major peaks with t_R values of 7.0, 9.4, and 11.8 min during HPLC on a Whatman ODS-2 column (methanol–water 7:3). Treatment with β-glucuronidase showed that the fraction with t_R = 9.4 min contained retinoyl β-glucuronide. Semipreparative HPLC on a Whatman ODS-3 column (methanol–water 7:3, 3 ml/min) also resolved the crude mixture dissolved in dimethyl sulfoxide into three major peaks with the following t_R values and relative amounts: 34 min (0.6), 39 min (1.0), and 45 min (0.7). All-*trans* retinoyl β-glucuronide was again identified in the second fraction. After several injections were made, eluates with the same retention times were pooled, acidified with 0.1 N HCl, and extracted with ethyl acetate. The extracts were dried (Na_2SO_4) and evaporated to dryness in a rotary evaporator, and the residual oils were dissolved in the minimum volume of diethyl ether. On the addition of hexane, a yellow solid separated in each of the fractions. HPLC of 100 mg of the mixture of retinoyl glucuronides gave 30–35 mg of pure all-*trans* retinoyl β-glucuronide, free from other isomers and anomers.

Purified all-*trans* retinoyl β-glucuronide had the following physical and chemical characteristics: easily soluble in ethyl acetate, water, and dimethyl sulfoxide; less soluble in methanol; sparingly soluble in diethyl ether and chloroform; and insoluble in hexane. Mp 142–143°C (darkens at

125–130°C); UV_{max} 360 nm in methanol (ε 50,700); 365 nm (ε 33,600) in water. The NMR spectrum of all-*trans* retinoyl β-glucuronide in dimethylsulfoxide-d_6–D_2O 5:1 is presented in **Fig. 2**, and peak assignments are given in **Table 2**. IR 3440 (broad), 2950, 2900, 2850, 1720 (C=O), 1650, 1580, 1440, 1395, 1360, 1240, 1155 (C–O), 1060, 970 cm^{-1}. Anal. calcd. for $C_{26}H_{36}O_8 \cdot 2.5\ H_2O$: C, 59.88; H, 7.86. Found: C, 59.75; H, 7.58. MS (m/z) 477 (M^+ + 1) (1.2), 459 (1.7), 383 (2), 371 (1), 357 (14), 317 (30), 301 (100), 283 (34), 257 (15), 225 (7), 209 (14), 193 (14), 177 (65), 159 (73), 141 (22).

Enzymic cleavage of all-*trans* retinoyl β-glucuronide to retinoic acid

All-*trans* retinoyl β-glucuronide (5–17 μg dissolved in 100 μl of H_2O) was incubated with or without bacterial β-glucuronidase (5 mg ≈ 2850 units) dissolved in 1 ml of 0.1 M phosphate buffer, pH 6.8, at 37°C for 2–17 hr. The retinoids were extracted with ethyl acetate (3 × 1 ml), and the solvent was removed under argon. The residue was dissolved in 100 μl of methanol, and aliquots were subjected to HPLC on an ODS-2 column (methanol-water 7:3, 2 ml/min). Retinoyl glucuronide (t_R = 9.4 min) was cleaved into retinoic acid (t_R = 26 min) when incubated in the presence, but not in the absence, of the enzyme (**Table 3**).

Preparation of the triacetate derivative of retinoyl β-glucuronide methyl ester

All-*trans* retinoyl β-glucuronide (10 mg, 21.8 μmol) was dissolved in diethyl ether; an excess of diazomethane was added and the mixture was left at room temperature for 1 hr. After solvent removal by distillation in a fume cupboard, the residue containing the methyl ester of retinoyl glucuronide was dissolved, without further purification, in pyridine (1 ml). Freshly distilled acetic anhydride (1 ml) was added to the cold solution (0°C), and the mixture was left at 40°C for 2 hr. The product was extracted with diethyl ether, washed with water, and dried (Na_2SO_4). The triacetate methyl ester was purified by TLC on silica gel (hexane–acetone, 2:1; R_f = 0.61) and then by HPLC (t_R = 18 min) on an ODS-3 column (methanol, 1.5 ml/min). UV_{max} 358 nm in hexane. ^{1}H-NMR ($CDCl_3$) δ 7.03 (dd, 1, J = 14,11Hz, H-11), 6.30 (d, 1, J = 16Hz, H-7), 6.28 (d, 1, J = 11Hz, H-10), 6.20 (d, 1, J = 16Hz, H-8), 6.19 (d, J = 14Hz, H-12), 5.79 (d, 1, J = 8Hz, H-1'), 5.70 (s, 1, H-14), 5.19–5.54 (m, 3, H-2', 3', 4'), 4.20 (d, 1, J = 9Hz, H-5'), 3.75 (s, 3, OCH_3), 2.34 (s, 3, C-13 CH_3), 1.99–2.05 (overlapping s and m, 14, C-4 CH_2, C-9 CH_3 and 3, CH_3CO), 1.71 (s, 3, C-5 CH_3), 1.62 (m, 2, C-3 CH_2), 1.46 (m, 2, C-2 CH_2), 1.03 (s, 6, C-1 $(CH_3)_2$. MS (m/z) 616 (M^+) (29), 588 (2), 556 (10), 541 (1), 528 (0.6), 496 (2), 331 (1.0), 317 (18), 300 (11), 299 (37), 283 (15), 282 (30), 267 (18), 255 (42), 254 (69), 239 (44), 197 (44), 155 (100), 139 (35), 127 (30), 111 (32), 97 (29), 85 (33), 71 (33), 60 (49), 56 (40).

DISCUSSION

Retinoyl fluoride reacts with various amines in methanol–diethyl ether to form retinamides in good yield (16). Because methyl retinoate invariably is a side product, we presumed that retinoyl fluoride might also react with other hydroxylated compounds to give O-retinoyl derivatives. Inasmuch as retinoyl β-glucuronide is a significant, biologically active metabolite of retinoic acid (1–12), we attempted to define favorable reaction conditions for its formation from retinoyl fluoride.

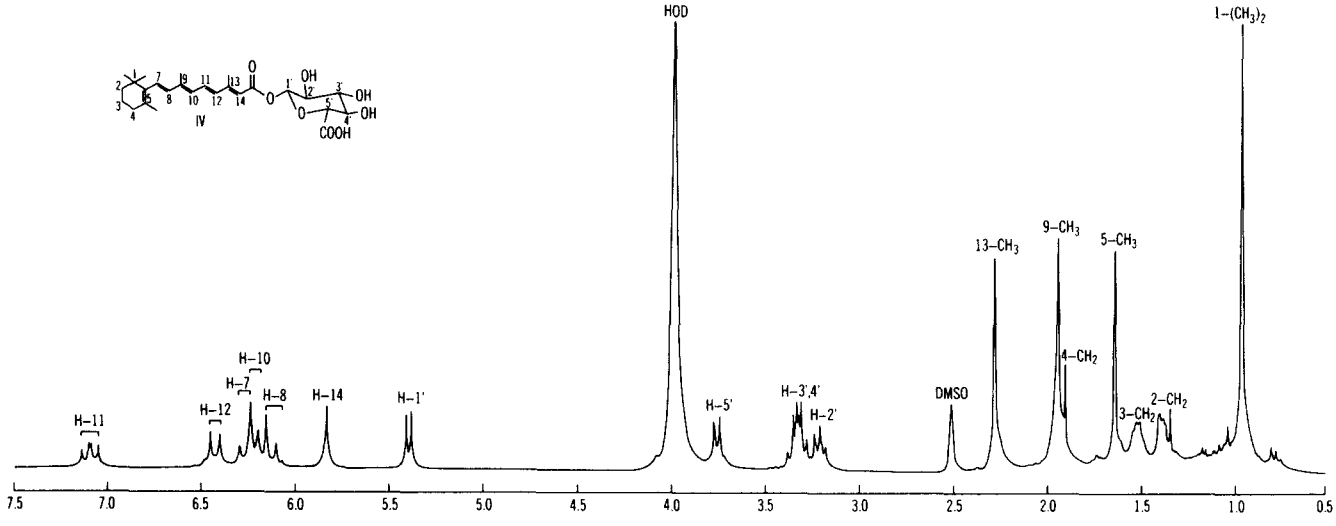

Fig. 2. ^{1}H-NMR spectrum of all-*trans* retinoyl β-glucuronide obtained at 300 MHz in Me_2SO-d_6–D_2O 5:1.

TABLE 2. Proton NMR data for all-*trans* retinoyl β-D-glucuronide obtained at 300 MHz in Me_2SO-d_6 and Me_2SO-d_6-D_2O 5:1

In Me_2SO-d_6			In Me_2SO-d_6-D_2O	
δ ppm	Coupling Constants	Assignment	δ ppm	Coupling Constants
	Hz			*Hz*
7.12	15.0, 11.4	H-11	7.07	15.4, 11.7
6.45	15.0	H-12	6.40	15.3
6.29	15.9	H-7	6.24	16.5
6.24	11.4	H-10	6.19	11.7
6.17	16.2	H-8	6.10	16.2
5.84	singlet	H-14	5.81	singlet
5.40	7.8	H-1'	5.37	8.1
5.37-5.20[a]		OH-2',3'		
3.73	9.0	H-5'	3.73	9.0
		(HOD	3.68)	
3.41-3.15[b]		H-4'	3.32	9.6, 8.7
		H-3'	3.28	8.7, 8.7
		H-2'	3.18	8.4, 8.7
2.31	singlet	C-13 CH_3	2.26	singlet
1.98	singlet	C-9 CH_3	1.92	singlet
1.89	multiplet	C-4 CH_2	1.84	multiplet
1.67	singlet	C-5 CH_3	1.62	singlet
1.52	multiplet	C-3 CH_2	1.48	multiplet
1.43	multiplet	C-2 CH_2	1.39	multiplet
1.00	singlet	C-1 $(CH_3)_2$	0.94	singlet

[a] Broad singlet.
[b] Obscured by overlapping H_2O and OH-4' signals. Assignments of the OH signals may be interchanged.

When retinoyl fluoride was incubated with glucuronic acid or its sodium salt in methanol-diethyl ether or in acetone, methyl retinoate and retinoic acid, but no retinoyl glucuronide, were formed. When the 3,6-glucuronolactone was used, however, a highly polar derivative of retinoic acid was formed in small amounts in methanol-diethyl ether and in quite good yield (> 50%) in acetone. The product, characterized as retinoyl glucuronolactone by spectra and chromatographic behavior, could be separated into at least two compounds by TLC and HPLC.

Rather than attempting to purify the retinoyl glucuronolactone, we hydrolyzed the crude product to the free acid by careful treatment with dilute base in aqueous ethanol at room temperature. Under the selected conditions, the 3',6'-lactone bond is cleaved faster than the 1'-retinoyl acetal linkage; thus, the ratio of retinoyl β-glucuronide to retinoic acid formed is approximately 3:1. In stronger base at higher temperatures, the sole products were retinoic acid and glucuronic acid.

After removal of retinoic acid by chromatography, the retinoyl glucuronide fraction was separated into three primary components by HPLC in methanol-water 7:3. The absorption spectra and elementary analysis of all three components were essentially identical, and the melting points were similar. While showing identical ^{1}H-NMR spectra for the retinoyl moiety, the three components showed significant differences in the ^{1}H-NMR spectra of the glucuronide portion (3-6 ppm).

The ^{1}H-NMR spectrum of the glucuronide moiety of retinoyl β-glucuronide in dimethyl sulfoxide-d_6 was somewhat difficult to interpret because the water peak derived from the water of crystallization overlapped with the proton peaks of the glucuronide moiety. In dimethyl sulfoxide, four of the protons of the glucuronide ring (one due to an hydroxyl group) appeared as multiplets along with the water peak in the region δ 3.15-3.41 (Table 2). Moreover, the other two hydroxyl peaks appeared as a broad peak (δ 5.2-5.37). After adding D_2O to the solution, however, the spectrum became simpler and interpretation of the peaks became easier. With D_2O, the three hydroxyl groups and the water molecules were converted to HOD, which appeared as a singlet at δ 3.68 (Table 2). The five protons of the glucuronide moiety then clearly appeared as two doublets (δ 3.73 and 5.37) and as three double doublets (δ 3.18, 3.28, 3.32). Tentative assignments for the proton peaks in the glucuronide ring were made by analogy to other similar compounds (17-20) and particularly to 3-O-β-D-glucopyranuronosyl-cholesterol (21).

The rapid hydrolysis of retinoyl β-glucuronide by bacterial glucuronidase and the ^{1}H-NMR spectra and mass spectra of its triacetyl methyl ester derivative further confirmed its structure. As expected, the synthetic retinoyl β-glucuronide and the natural metabolite isolated from rat bile chromatographed together in a high-resolution HPLC system.

TABLE 3. Enzymic cleavage of retinoyl β-glucuronide to retinoic acid

Experiment Number	Time Incubated	Retinoyl β-Glucuronide		Retinoic Acid		Other Products					
		t_R	%	t_R	%	t_R	%	t_R	%	t_R	%
	h	*min*		*min*		*min*		*min*		*min*	
1	2	9.43	41	25.8	45	19.0	7	22	2	2-5	5
2	17	9.63	8	26.0	70	19.1	13	22.2	4	2-5	5
3	2	9.6	99.8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Retinoyl β-glucuronide was incubated with (experiments 1 and 2) or without (experiment 3) bacterial β-glucuronidase (5 mg = 2850 units) in phosphate buffer (pH 6.8), and the formation of retinoic acid was noted by HPLC. The relative percentages of the various products are shown. n.d., Not detected.

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