Glucuronides of monohydroxylated bile acids: specificity of microsomal glucuronyltransferase for the glucuronidation site, C-3 configuration, and side chain length

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Abstract The ability of rat liver microsomes to catalyze UDP-glucuronic acid-dependent glucuronidation of monohydroxy-bile acids was examined. The following bile acids were used as substrates, each as the 3α and 3β epimer: 3-hydroxy-5β-cholanoic acid (C_{24}), 3-hydroxy-5β-norcholanoic acid (C_{23}), 3-hydroxy-5β-bisnorcholanoic acid (C_{22}), 3-hydroxy-5β-pregnan-21-oic acid (C_{21}), and 3-hydroxy-5β-androstane-17β-carboxylic acid (C_{20}). The corresponding glucuronides were chemically synthesized to serve as standards and were characterized by thin-layer and gas-liquid chromatography as well as by nuclear magnetic resonance. Enzymatic glucuronidation reactions were optimized with respect to pH for each product formed and the kinetic parameters for each reaction were measured. Analytical techniques necessary to separate products from unreacted substrates and to identify them included thin-layer chromatography, gas-liquid chromatography, and nuclear magnetic resonance. It was found that the 3α epimers of the five bile acids listed above enzymatically formed 3-O-glucuronides, C_{24} being the best substrate, followed by C_{21} and C_{20}; C_{22} and C_{23} gave rise to only small amounts of this product. The 3β epimers of all bile acids tested were poorer substrates, although by a factor that varied widely. In addition to the expected hydroxyl-linked glucuronide, three of the 3α-bile acids (C_{23}, C_{22}, and C_{20}) and at least one 3β-bile acid (C_{20}), gave rise to a novel metabolite in which the 1-OH of glucuronic acid was esterified with the steroidal carboxyl group (carboxyl-linked glucuronide).—**Radomińska-Pyrek, A., P. Zimniak, M. Chari, E. Golunski, R. Lester, and J. St. Pyrek.** Glucuronides of monohydroxylated bile acids: specificity of microsomal glucuronyltransferase for the glucuronidation site, C-3 configuration, and side chain length. *J. Lipid Res.* 1986. **27:** 89–101.

Supplementary key words short chain bile acids • chemical synthesis of glucuronides • carboxyl glucuronidation

Hepatic glucuronidation constitutes one of the major pathways of converting a wide variety of substances into polar derivatives in preparation for excretion. Not surprisingly, the UDP-glucuronyltransferase activity (GT) (EC 2.4.1.17) is not due to a single enzyme, but rather it is the expression of multiple isoenzymes with partially overlapping specificities (1–5). This heterogeneity of GT can be discerned by various methods, including immunological techniques (6), competition between substrates (e.g., 7), response to inducing agents such as phenobarbital or methylcholanthrene (e.g., 8–10), or the analysis of mutants (11–13). The most convincing evidence comes from a separation and purification of the various isoenzymes (7, 10, 14–17). Most relevant for the present work is the purification, probably partial (15) and to homogeneity (18), of a bile acid (BA)-dependent GT. This enzyme, named 3-OH androgen GT after its major substrates (18), will also accept certain bile acids.

A variety of BA glucuronides has been detected in biological materials, such as human urine, serum, and bile (19–24). Although BA glucuronides do occur in small amounts in healthy individuals, they are of special importance in relation to liver disease and cholestasis. It is assumed that they, as well as BA sulfates, are formed to prevent a buildup of toxic free BA, although more specific functions of BA glucuronides are also known (25).

Recently, a number of monohydroxylated short-chain BA (C_{20}–C_{22}) have been identified in biological fluids, partly in the form of glucuronides (26–31). Since some of

Abbreviations: C_{20}, 3α- or 3β-hydroxy-5β-androstane-17β-carboxylic acid; C_{21}, 3α- or 3β-hydroxy-5β-pregnane-21-oic acid; C_{22}, 3α- or 3β-hydroxy-5β-bisnorcholan-22-oic acid; C_{24}, lithocholic acid or isolithocholic acid (3α- or 3β-hydroxy-5β-cholan-24-oic acid, respectively). BA, bile acid(s); GT, UDP-glucuronyltransferase(s); Me-Ac, methyl ester-acetate(s); GLC, gas-liquid chromatography; TLC, thin-layer chromatography; R_t, retention time; ^{1}H-NMR, proton magnetic resonance; CMC, critical micelle concentration. Glucuronide types: H, hydroxyl-linked; C, carboxyl-linked; U, unknown.

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these acids (and their glucuronides) exert specific effects on bile flow (Ref. 30 and unpublished results), a study of the enzymology of their glucuronidation appeared worthwhile and was undertaken. The 3-OH androgen GT appears to be the only glucuronyltransferase acting on conventional BA (18). It is, however, not obvious that the same enzyme is responsible for the glucuronidation of short-chain BA, especially since the purified 3-OH androgen GT is strongly influenced by bile acid side chain modifications (18). We therefore decided to use intact microsomes for the present survey of short-chain BA glucuronidation. In this way the information obtained may be less specific, but all potential enzymes acting on BA with a shortened side chain can be measured. This approach led to the discovery of a previously unknown metabolite of BA, the carboxyl-linked glucuronide, that is formed in addition to the conventional 3-OH-linked glucuronide from some of the subtrates used.

MATERIALS AND METHODS

Chemicals

Bile acids were purchased or synthesized as described by Radominska-Pyrek et al. (32) in the accompanying report. D-Saccharic acid-1,4-lactone (saccharolactone), UDP-glucuronic acid disodium salt, β-glucuronidase, and Brij 58 were from Sigma Chemical Co. (St. Louis, MO). Amberlyst-15 resin, silver carbonate, molecular sieves (4A), and all standard reagents were purchased from Aldrich Chemical Co. (Milwaukee, WI).

Gas-liquid chromatography

GLC was performed as described (32). Samples of chemically or enzymatically synthesized glucuronides were converted for analysis to methyl ester-acetates as detailed (32) except that acetone-benzene 85:15 (v/v) was used for elution from silica gel during purification.

Thin-layer chromatography

Analytical thin-layer chromatography (TLC) was carried out on precoated silica gel plates (Whatman Chemical Separation Inc., Clifton, NY). Solvent system A (benzene-acetone 95:5, v/v) was used to separate protected (methylated/acetylated) BA and BA glucuronides and Krowicki's reagent (33) was used to visualize the spots. The separation of enzymatically synthesized BA glucuronides from unreacted BA substrate was carried out on preparative precoated silica gel plates (LK5, 0.25 mm × 20 cm × 20 cm, Whatman Chemical Separation Inc., Clifton, NY). The solvent systems used were: solvent B, butanol-acetic acid-water 50:10:5 (v/v); solvent C, chloroform-methanol-acetic acid-water 65:25:2:4 (v/v); and solvent D, ethanol-ethyl acetate-conc. NH_3 45:45:15 (v/v). The compounds were nondestructively visualized as opaque spots on a translucent background by heavily spraying the developed plates with water (34). Radioactivity was localized by fluorography at −80°C on Kodak XAR-5 film after spraying the plates with Enhance (New England Nuclear).

Animals and preparation of microsomes

Rat livers (Sprague-Dawley or Wistar rats, male, 180–230 g) were homogenized in four volumes of 0.25 M sucrose, 1 mM EDTA, 5 mM Tris-HCl, pH 7.6, with a Polytron homogenizer (setting 7.5, 2 × 20 sec). The homogenate was centrifuged for 15 min at 5,000 *g* and the resulting supernatant for 30 min at 17,000 *g*. Crude microsomes were pelleted at 100,000 *g* (1 hr) and washed by resuspending in the homogenization medium and centrifugation as above. The washed microsomes were suspended in 0.25 M sucrose, 5 mM Tris-HCl, pH 7.5 (1 ml per 1 g of liver) and quickly frozen in liquid N_2 in small aliquots. They could be stored at −20°C for at least 4 months without loss of activity. Protein was determined (35) after dissolving the samples in 5% SDS, 2 mM NaOH, with bovine serum albumin as standard.

A mutation greatly reducing the 3-OH androgen GT activity is known to occur in Wistar rats with an incidence of about 50% (11–13). All microsome preparations derived from this strain were therefore tested with lithocholic acid and 3α-hydroxy-5β-androstane-17β-carboxylic acid (C_{20}) and discarded if they had low activity. In the course of this work, 5 out of 13 Wistar rats were found to be GT-deficient. One test preparation made from Sprague-Dawley rats showed the same activity as the high-activity Wistar rats.

Bile acid:UDP-glucuronyltransferase assay

Bile acid UDP-glucuronyltransferase was assayed using a modification and scaling down of methods previously described (8, 36). BA substrates were prepared in the form of mixed micelles with Brij 58 detergent as follows. Sodium salts of BA (1 μmol if not stated otherwise) in methanol were mixed with 0.12 ml of 1% aqueous Brij 58. The use of up to 12 μmol was possible for all bile acids except 3β5β-C_{24}, for which the limit was 1 μmol. The solution was taken to complete dryness under a stream of nitrogen and 1 ml of water was added. The mixture was allowed to swell and was clarified in about 20 min in a bath sonicator at room temperature. The basic reaction mixture contained 100 mM buffer (see below), 5 mM $MgCl_2$, 0.05% Brij 58 (final concentration, adjusted when necessary with 0.12% Brij 58), 5 mM D-saccharic acid 1,4-lactone, 3.3 mM UDP-glucuronic acid, and the BA substrate (0.03–0.4 mM). Na-HEPES of a pH optimal for each substrate was used as buffer, i.e.: C_{20} (3α), pH 7.0 for the carboxyl glucuronide (C) and pH 7.5 for the hy-

droxyl glucuronide (H); C_{20} (3β), pH 6.8(C), C_{21} (3α), pH 7.5 (H); C_{22} (3α), pH 6.8(C) and pH 7.5 (H); C_{22} (3β), pH 6.8; C_{23} (3α), pH 6.5 (C and H), C_{23} (3β), pH 6.5; C_{24} (3α), pH 6.5 (H); C_{24} (3β), pH 6.8. In some early experiments, Tris–HCl, pH 6.5, was used (8), but was later abandoned due to its poor buffering capacity at this pH (at 37°C, pKa = 7.77). The incubation mixture contained approximately 50 μg of microsomal protein in a total volume of 60 μl. In early experiments, radioactive BA (2 μmol) was added in 5 μl of 75% aqueous ethanol directly to the incubation mixture. Later, the radioactive BA was mixed with the unlabeled BA before micelle formation. Although both procedures gave identical results, the latter was preferred (in spite of greater waste of labeled BA) since it avoided introducing ethanol into the reaction mixture. After 10 min at 37°C the reaction was stopped by addition of 40 μl of ethanol containing a synthetic H-glucuronide standard and the entire mixture was directly transferred to a preparative silica gel TLC plate. The reaction tubes were rinsed once with 20 μl of ethanol. Less than 1% of the total radioactivity remained in the tube under these conditions. The plates were developed in solvent B, C, or D. Zones corresponding to the glucuronide band and the unreacted BA substrate (**Fig. 1**) were transferred to glass columns (0.5 cm diam., plugged with glass wool) and eluted with methanol. Alternatively, silica gel was scraped off directly into scintillation vials. Radioactivity was measured in a Mark III (Tracor Analytic) scintillation counter equipped with an automatic quench correction program. Specific activities of enzymes are expressed as nmol of BA glucuronide formed per mg protein per hr.

Identification of biosynthetic bile acid glucuronides

Preparative enzymatic reactions (volume 0.6–1.2 ml) were run under conditions very similar to those used in kinetic studies. The BA substrate and protein concentrations were 1.0 mM and 1.5 mg/ml, respectively. The reaction was terminated by addition of ethanol and the mixture was directly applied to a preparative TLC plate. The BA glucuronide zone was eluted with methanol and any BA glucuronide salts in the eluate were converted to the free acids with Amberlyst-15 (H^+ form). The corresponding methyl ester-acetates were prepared as described in ref. 32. The resulting derivatives of BA glucuronides were analyzed by TLC, GLC, and ^{1}H-NMR.

Proton magnetic resonance

Proton magnetic resonance (^{1}H-NMR) spectra were recorded in $CDCl_3$ with a JEOL GX-270 MHz instrument.

Syntheses of bile acid glucuronide methyl ester-acetates

Methyl-3α-O-(methyl-2,3,4-tri-O-acetyl-β-D-glucopyranuronosyl)-5β-androstane-17β-carboxylate (*1c*, see **Fig. 2**). The following procedure describes the general method (37) of preparation of 3-monohydroxy-BA glucuronide dimethyl ester triacetates, on the example of the synthesis of methyl-3-O-(methyl-2,3,4-tri-O-acetyl-β-D-glucopyranuronosyl)-5β-androstane-17β-carboxylate (*1c*).

A solution of methyl-3α-hydroxy-5β-androstane-17β-carboxylate (32) (93.4 mg, 0.28 mmol) and methyl 2,3,4-tri-O-acetyl-1-bromo-1-deoxy-α-D-glucopyranuronate (298

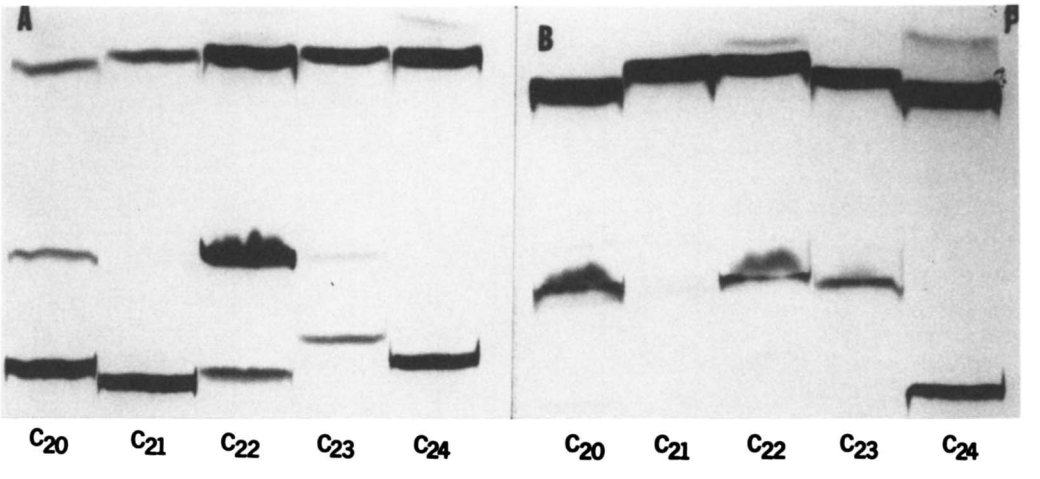
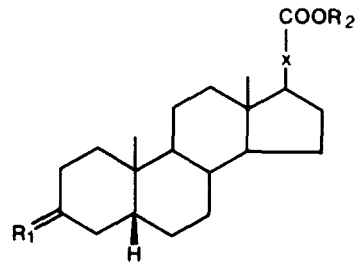
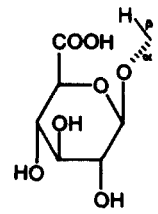
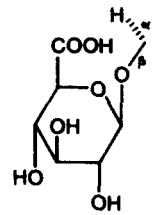
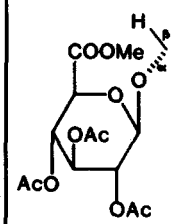
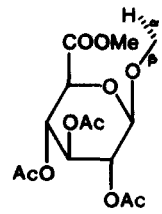


Fig. 1. Radioautogram of a separation of enzymatically formed H- and C-glucuronides by TLC. Enzymatic reactions were performed as described under Methods. Reactions were stopped by methanol addition, applied in their entirety to TLC plates and the plates were developed in solvent D. Radioactive spots were visualized by fluorography. A: 3α-BA; B: 3β-BA. Hydroxyl-linked glucuronides have a relative mobility of 0.1–0.2 and carboxyl-linked glucuronides, of 0.3–0.4. Unreacted free BA migrate close to the solvent front.



1. C_{20} x —
2. C_{21} x – CH_2 –
3. C_{22} x – $CH(CH_3)$ – (20S) "natural"
4. C_{23} x – $CH(CH_3)CH_2$ – (20R) "natural"
5. C_{24} x – $CH(CH_3)CH_2CH_2$ – (20R) "natural"

	R_1	R_2
a.		H
b.		H
c.		Me
d.		Me

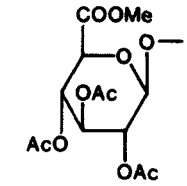
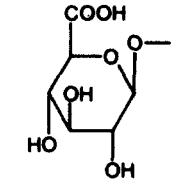
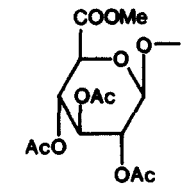
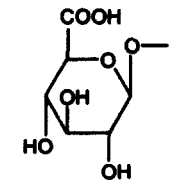
	R_1	R_2
e.	αOAc, βH	
f.	αOH, βH	
g.	βOAc, αH	
h.	βOH, αH	

Fig. 2. Structures of glucuronides chemically and enzymatically synthesized in the course of this work.

mg, 0.75 mmol), prepared according to the procedure of Bollenbach et. al. (38), in anhydrous benzene (10 ml) was stirred at room temperature for 24 hr in the presence of silver carbonate (550 mg) and 4A molecular sieves. The benzene solution was filtered and evaporated. Column chromatography (for small scale reactions, preparative TLC and HPLC separation was utilized) of the residue on silica gel using benzene–acetone 85:15 (v/v) as solvent resulted in the elution of the ortho-ester by-product (31 mg) followed by the desired protected glucuronide (98 mg). An intermediate fraction contained a mixture (42 mg) of these two compounds. Recrystallization of the major fraction from methanol yielded 70.5 mg of pure (as judged by TLC and GLC) protected C_{20} glucuronide.

Alternatively, in order to facilitate chromatographic purification, the ortho-ester by-product was destroyed as follows. The crude product mixture was dissolved in 0.05 N sulfuric acid in acetone (15 ml) and set aside for 15 min at room temperature. The solution was diluted with dichloromethane (20 ml), washed three times with water, dried, and evaporated.

Properties of protected BA glucuronides synthesized by this method are given in **Table 1** (^{1}H-NMR) and **Table 2** (GLC).

(*Methyl-2,3,4-tri-acetyl-β-D-glucopyranuronosyl)-3α-acetoxy-androstane-17β-carboxylate* (*1e*). The methods were similar to that described above for the hydroxyl-linked glucuronide. They are shown on the example of C_{20}, but are applicable to other BA as well.

Method 1. 3-Oxo-5β-androstane-17β-carboxylic acid (see 32) served as the precursor. Approximately 0.1 mmol of this ketoacid was dissolved in benzene (1 ml), solid methyl 2,3,4-tri-O-acetyl-1-bromo-1-deoxy-α-D-glucopyranuronate (50 mg, 0.125 mmol) and 70 mg of silver carbonate were added and the mixture was stirred overnight at room temperature. After filtration through Celite and evaporation, the reaction product was purified by preparative TLC (solvent A), and the glucuronide was eluted from silica gel with acetone. The product (TLC and GLC pure) was reduced with $NaBH_4$, the resulting mixture was acetylated (pyridine/acetic anhydride, 65°C, overnight), and the major product was purified by preparative TLC.

Method 2 (preferred). 3-Acetoxy-5β-androstane-17β-carboxylic acid was prepared as described for lithocholic acid acetate (32). This was coupled with the protected bromoglucuronic acid as in Method 1 above. The product of this reaction was the fully protected glucuronide and did not, therefore, require the additional reduction and acetylation steps of Method 1. The reaction can be carried out for both the 3α and the 3β epimers.

Alkaline hydrolysis of BA glucuronide methyl ester-acetates

The conditions and procedure for the alkaline hydrolysis is exemplified by the alkaline hydrolysis of the protected hydroxyl-linked C_{20} glucuronide (*1c*) and does not apply to carboxyl-attached glucuronides (e.g., *1e*).

To a solution of methyl-3α-O-(methyl-2,3,4-tri-O-acetyl-β-D-glucopyranuronosyl)-5β-androstane-17β-carboxylate (*1c*) (42.1 mg) in 6 ml of ethanol–water 2:1, 0.6 ml of 5 N NaOH was added and the mixture was heated at 70°C for 24 hr. The solution was diluted with water (15 ml), acidified to pH 5 (with diluted HCl), and extracted (3 × 15 ml) with ethyl acetate. The two first extracts contained free glucuronide (*1a*) which was recrystallized from ethyl acetate–ethanol (92%, 27.9 mg).

RESULTS

Preparation and characterization of bile acid glucuronides

Glucuronides of 3-monohydroxy-BA were obtained in the protected form of methyl ester-acetates via the Koenigs-Knorr reaction (37). In one case, the carboxyl group of the BA used as starting material was protected (methyl ester); the reaction led then to the hydroxyl-linked glucuronide *1c–5c* or *1d–5d* (see Fig. 2 for symbols). The reaction could be successfully applied to both 3α (equatorial) and 3β (axial) alcohols; it proceeded with acceptable yields (40%), and, as usually observed, was complicated only by the formation of the acid-labile orthoesters. If required, the deprotected glucuronide *1a–5a*, *1b–5b* could be obtained by alkaline hydrolysis of the methyl and acetyl groups.

Alternatively, the hydroxyl group of the BA substrate was blocked by conversion to a keto function or by acetylation. In this case, the Koenigs-Knorr reaction gave the carboxyl-linked glucuronide of the 3-ketoacid or the 3-acetoxy acid; the former was additionally subjected to borohydride reduction and acetylation of the resulting hydroxyl group to yield the fully protected carboxyl-linked glucuronide (for example, *1e*, *1g*). Due to the known instability of carboxyl-attached β-glucuronides, it is evident that the chemical synthesis of the unprotected form would require an entirely different approach. Thus, the derivatization of the enzymatically formed glucuronides to the same form as that obtained from the chemical synthesis, rather than the troublesome deprotection, appeared to be the most reasonable approach for the unambiguous and straightforward identification of enzymatically formed glucuronides.

TABLE 1. ^{1}H-NMR spectra of bile acid methyl ester-acetates

	Products of Enzymatic Reactions										
	C_{24} 3α5β	C_{24}3β5β	C_{23} 3α5β		C_{22} 3α5β		C_{21} 3α5β	C_{20} 3α5β		C_{20} 3β5β	
	H (5c)	H (5d)	H (4c)	C (4e)	H (3c)	C (3e)	H (2c)	H (1c)	C (1e)	H (1d)	C (1g)
H-1'	4.66 d J = 7.9	4.61 d J = 7.6	4.65 d J = 7.7	*	4.66d J = 8.0	5.73 d J = 8.1	4.66 d J = 7.4	4.66 d J = 7.7	5.74 d J = 8.1	N.F.	5.74 d J = 7.7
H-2'	4.97 t J = 8.4	5.02 dd J = 7.6, 9.2	4.98 t J = 8.7				4.97 t J = 16.8	4.97 t J = 17	4.97 t		
H-3 } H-4' }	5.15–5.35 m	5.17–5.31 m	5.18–5.32 m			5.15–5.35 m	5.17–5.30 m		5.15–5.35m		5.15–5.35m
H-5'	4.03 d J = 9.5	4.02 d J = 9.2	4.02 d J = 9.6		4.04 d	4.16 d J = 9.6	4.03 d J = 9.0	4.03 d J = 9.5	4.16 d J = 9.5		4.15 d J = 9.0
H-3	3.65br(β-H)	4.03bs(α-H)					3.60br(β-H)	3.60br	4.65br(β-H)		5.10bs(α-H)
Me-18	0.63 s	0.64 s	0.68 s		0.66 s		0.57 s	0.63 s	0.71 s		0.66 s
Me-19	0.90 s	0.90 s	0.91 s				0.91 s	0.91 s	0.88 s		0.96 s
MeO:	3.75 s 3.65 s	3.76 s 3.66 s	3.75 s 3.66 s		3.76 s 3.64 s	3.74 s	3.76 s 3.66 s	3.76 s 3.67 s	3.73 s		3.73 s
AcO:	2.05 s 2.02 s (two)	2.020 s 2.025 s	2.05 s 2.025 (two)				2.04 s 2.02 (two)	2.04 s 2.02 s	2.07 s 2.03 s		2.04 s 2.03 s
Other	2.15–2.45 m (CH_2–23) 0.90 d (Me-21)	2.15–2.42 m (CH_2–23) 0.91 d (Me-21)	2.44d, J = 10 (CH_2–22) 0.96 d, J = 7.1 (Me-21)				2.35 dd and 2.14 dd J = 14.1, 5.4 (CH_2–20)				
	Products of Chemical Synthesis										
H-1'	4.65 d J = 7.6	4.60 d J = 8.0	4.65 d J = 7.7			5.73 d J = 7.8		4.64 d J = 7.8	5.75 d J = 7.6	4.61 d J = 7.6	
H-2'	4.96 t J = 8.4	5.02 dd J = 8.0, 9.5	4.98 t J = 8.7					4.95 t J = 7.2		5.03 dd J = 7.6, 9.2	
H-3' } H-4' }	5.18–5.29m	5.17–5.30m	5.18–5.32m			5.15–5.35m		5.0–5.4m		5.15–5.25m	
H-5'	4.02 d J = 8.7	4.02 d J = 9.5	4.02 d J = 9.6			4.16 d J = 9.5		4.02 d J = 7.2	4.15 d J = 9.0	4.02 d J = 8.7	
H-3	3.60 br (β-H)	4.03 bs (α-H)				4.72 tt		3.60 br	4.72m(β-H)	4.04bs(α-H)	
Me-18	0.64 s	0.64 s	0.68 s			0.65 s		0.62 s	0.66 s	0.63 s	
Me-19	0.91 s	0.90 s	0.91 s			0.92 s		0.90 s	0.93 s	0.90 s	
MeO:	3.76 s 3.67 s	3.75 s 3.65 s	3.75 s 3.66 s			3.74 s		3.75 s 3.65 s	3.73 s	3.75 s 3.66 s	
AcO:	2.05 s 2.02 s (two)	2.034 s 2.028 s	2.05 s 2.025 (two)			2.035 s (three) 2.023 s		2.038 s 2.012 s	2.04 s (four)	2.024 s 2.028 s	
Other	2.15–2.45 m (CH_2–23) 0.91 d Me-21)	2.15–2.40 m (CH_2–23) 0.91 d (Me-21)	2.44d, J = 10 (CH_2–22) 0.96 d, J = 7.1 Me-21)			2.45 m (20-H) 1.18 d J = 6.8 Me-21		2.35 t (17-H)			

The spectra were measured in $CDCl_3$ at 270 MHz. Type of glucuronide: H, hydroxyl-linked; C, carboxyl-linked. N.F., not formed.
*, Signals from the carboxyl-linked glucuronide of C_{23} 3α5β were discernible, but the amount was insufficient for a detailed spectrum.

TABLE 2. Capillary GLC separation of bile acid glucuronide methyl ester - acetates

BA Substrate	Glucuronide Formed	Retention Time	
		Enzymatic	Synthetic
C_{24} 3α5β	H (5c)	8.23	8.20
C_{24} 3β5β	H (5d)	9.56	9.53
C_{23} 3α5β	H (4c)	6.21	6.33
C_{23} 3α5β	C (4e)	7.69	7.75
C_{23} 3β5β	U	N.D.	7.26 (4d)
C_{22} 3α5β	H (3c)	5.13	5.15
C_{22} 3α5β	C (3e)	6.20	6.25
C_{22} 3β5β	U	N.D.	5.76 (3d)
C_{21} 3α5β	H (2c)	4.03	4.05
C_{21} 3β5β	not formed	not formed	4.19 (2d)
C_{20} 3α5β	H (1c)	3.27	3.26
C_{20} 3α5β	C (1e)	3.69	3.75
C_{20} 3β5β	C (1g)	3.59	3.66

Capillary GLC was performed on 2-m DB-1, 0.25-mm d, 0.1 μm. Oven and injector temperature was 285°C, carrier gas N_2, 1 psi.

N.D., not determined; H, hydroxyl- and C, carboxyl-linked glucuronide; U, unidentified. The symbols of glucuronides are as defined in Fig. 2.

Representative synthetic protected glucuronides *1c*, *1d*, *1e*, *3e*, *4c*, *5c*, and *5d* were characterized by proton NMR (Table 1). Well-separated signals of protons belonging to the glucuronic acid moiety were observed in spectra measured at 270 MHz in $CDCl_3$. The configuration at C-1' of glucuronic acid was confirmed by the coupling constant $J_{1',2'}$ = 8 Hz. The configuration of C-3 of the steroidal part was reflected in the characteristic shape of the H-3 signal. Carboxyl-linked glucuronides *1e*, *1g*, and *3e* were easily differentiated from the 3-O-β-glucuronides by the position of H-1' and H-3 signals, both appearing at a much lower field. Moreover, the presence of only one methoxyl and four acetoxyl singlets, as opposed to two methoxyls and three acetoxyls for 3-O-β-glucuronides, was diagnostic. This difference is especially valuable for the identification of enzymatic products present in low amounts.

Derivatized glucuronides (in the form of stable methyl ester - acetates) have another important advantage. These derivatives, despite their relatively high molecular weight (650 to 706), can be successfully separated by GLC on short capillary columns. A 2-m column was optimal because of sufficient separation and negligible decomposition, but columns of up to 15 m could be used. Little thermal decomposition during separation at temperatures 280–300°C was observed, provided the injection system was free from acidic impurities. In the order of R_t, 3-O-β-glucuronides of 3α-hydroxylated BA preceded the corresponding carboxyl-attached glucuronides (Table 2).

Enzymatic formation of bile acid glucuronides

A modified assay system similar to those described previously (8, 36) was used; however, the incubation volume was substantially scaled down to 60 μl. Thus, the entire incubation mixture could be transferred onto a TLC plate, dried, and the plate developed. The above simple modification permitted rapid and efficient separation of the unreacted BA substrates from their glucuronides by preparative TLC and, in the same step, product identification (Fig. 1). The recovery of radioactivity was calculated by the summation of radioactivities of the unreacted BA substrate, the BA glucuronide, and the residual radioactivity in the reaction tube (usually less than 1% of total). The recovery equalled 95 ± 1.5% (mean ± SD for all experiments with all substrates). The simultaneous identification of reaction products by cochromatography with chemically synthesized BA glucuronides constituted another advantage of this assay method.

Under standard reaction conditions, the rate of BA glucuronide synthesis was proportional to protein concentration up to 1.2 mg/ml for all substrates investigated, except lithocholic acid (limit 0.3 mg/ml) and 3β-hydroxy-5β-pregnan-21-oic acid (0.4 mg/ml). At least for the first 30 min, the reaction was linear with respect to time for both the 3α- and 3β-epimers. As originally described, the assay of GT was carried out in the presence of the detergent Brij 58 (8, 36). This is needed mainly to "solubilize" the hydrophobic BA substrate; at the concentration used, the detergent does not solubilize the enzyme. Monohydroxylated BA, even as salts, are poorly soluble in water (39). The formation of mixed detergent-substrate micelles required prolonged sonication of these two components in aqueous solution above the critical micelle concentration (CMC) of Brij 58 (see Methods; CMC of Brij 58 is 0.08%, by interpolation of data from Ref. 40). The stock solution of substrate in detergent was diluted into the assay mixture so that the final concentration of Brij 58 in the assay was below the CMC to minimize effects on the microsomal membrane. By this modification of previously published methods, up to 10 mM concentrations were obtained for almost all BA used, appreciably higher than in Ref. 36. As will be discussed later, Brij 58 at the concentration used appears to have little or no effect on the microsomal glucuronyltransferase except for a stimulation (8, 36) probably due to membrane permeabilization and unmasking of the latent enzyme (41, 42).

One difficulty with BA as substrates is the fact that they are themselves strong detergents. Increasing their concentration would exert a dual effect on the enzyme, viz. a specific (increased enzyme-substrate complex formation) and an unspecific (detergent stimulation or inhibition).

Substrate inhibition curves reported previously (15, 18, 36) were also observed in the present work (not shown). However, the inhibition occurred at much higher substrate concentrations than previously reported (36): at 0.6, 0.5, and 0.43 mM for lithocholic acid and its C_{20} and C_{21} 3α-analogues, respectively. Substrate inhibition by the C_{24} 3β-epimer (isolithocholic) at a concentration greater than 0.72 mM was also observed. In contrast, C_{20} 3β-hydroxy BA caused no inhibition up to 1 mM.

Glucuronides of both types (hydroxyl- and carboxyl-linked) were susceptible to β-glucuronidase under conditions described in reference 43. Enzymatic hydrolysis yielded glucuronic acid and the expected BA aglycone that was identified by GLC–mass spectrometry (32).

Characterization of the glucuronidation reaction

Two aspects of the hepatic glucuronidation of monohydroxylated BA have been investigated in the present work. One concerns the glucuronidation site. A monohydroxylated BA has two potential glucuronidation sites: the 3-hydroxy group and the side chain carboxyl group. The other aspect is the selectivity of enzymatic glucuronidation towards the 3-epimeric hydroxyl group of a BA and the side chain length.

Glucuronidation site

Incubation of the series of 3-monohydroxylated BA with UDP-glucuronic acid in the presence of rat liver microsomes yielded two different types of glucuronides: the expected hydroxyl-attached glucuronide (substitution on the 3-OH of the BA) as well as a novel carboxyl-attached glucuronide (substitution on the side chain carboxyl of the BA) (See **Fig. 3** for an example). The ratio of these two products was dependent on the BA used (see below). The fact that the carboxyl glucuronide of bile acids has not been recognized previously may be due to the possibility that C_{24} BA used by most investigators are unlikely to form this derivative: lithocholic acid gives rise to the hydroxyl glucuronide only (this report). Moreover, the separation of BA carboxyl and hydroxyl glucuronides is difficult: both migrate together on the TLC plates in common solvent systems. The following methods capable of the separation and identification of the two types of glucuronides have been developed for the present work.

Thin-layer chromatography. The alkaline solvent system D efficiently separates carboxyl-attached glucuronides from 3-O-β-glucuronides (Fig. 1 and **Table 3**). The reported R_f values are valid only for the conditions used; a change of the buffer cation used in the incubation or the use of a purified glucuronide rather than the whole reaction mixture results in different R_f. It is noteworthy that the C-glucuronides appear to migrate as two spots of almost identical R_f (0.3–0.4; see Fig. 1). However, if both spots are eluted together, they give, after methylation and acetylation, NMR and mass spectra of a homogenous compound. It is, therefore, likely that the two spots represent the same substance, possibly in the form of salts with different cations. As noted above, the cation has an influence on the TLC mobility of glucuronides. It is also noteworthy that, unlike the acyl glucuronides of many organic acids (p. 14 in Ref. 1), BA-carboxyl glucuronides are relatively alkali-stable: no decomposition was observed in aqueous solution at 22°C at pH 11 for 3 hr, (data not shown) or during separation in the ammonia-containing solvent D (no smearing during TLC; see Fig. 1).

Gas–liquid chromatography. The methylated-acetylated BA glucuronides were efficiently separated by capillary GLC on short capillary columns (Table 2) and, as discussed before, both 3-epimeric glucuronides as well as carboxyl-attached derivatives could be unambiguously differentiated. For this purpose the total glucuronide fraction was separated from the enzymatic reaction mixture by preparative TLC in solvent system C. This solvent does not separate or hydrolyze 3-O- and carboxyl-attached glucuronides. Therefore, the GLC (or NMR) analyses of the

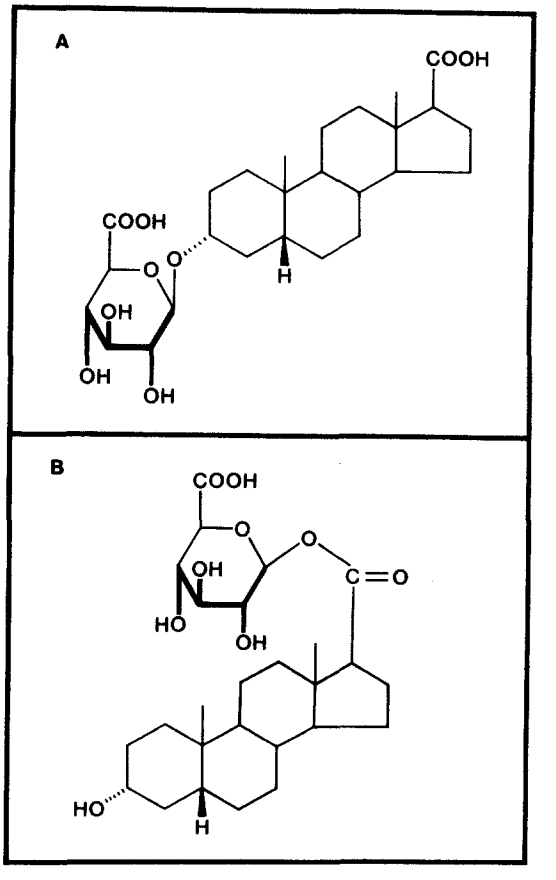


Fig. 3. Structures of the hydroxyl-linked (A) and carboxyl-linked (B) glucuronide of 3α-hydroxy-5β-androstane-17-carboxylic acid.

TABLE 3. TLC separation of enzymatically synthesized bile acid glucuronides

BA Substrate	R_f (Glucuronide Formed) H	C	Not Identified
C_{24} 3α5β	0.15 (5a)		
C_{24} 3α5β	0.09 (5b)		
C_{23} 3α5β	0.20 (4a)	0.38 (4f)	
C_{23} 3β5β			0.36
C_{22} 3α5β	0.11 (3a)	0.38 (3f)	
C_{22} 3β5β			0.42
C_{21} 3α5β	0.08 (2a)		
C_{21} 3β5β			
C_{20} 3α5β	0.11 (1a)	0.38 (1f)	
C_{20} 3β5β		0.37 (1h)	

Enzymatic incubations were stopped by methanol addition and applied in their entirety to the TLC plate as described in Methods. The plate was developed in solvent D. H, hydroxyl-linked glucuronide; C, carboxyl-linked glucuronide. The symbols of glucuronides (in parentheses) refer to Fig. 2.

properly methylated and acetylated fraction reflected the original composition of enzymatically formed products.

Both separation methods described above permitted a comparison with authentic, chemically synthesized standards and, therefore, positive identification of the compounds derived from enzymatic reactions was achieved. The structure of the latter compounds has been elucidated independently by proton NMR of their methyl ester-acetates (Table 1).

Of the ten BA studied (monohydroxylated C_{20}, C_{21}, C_{22}, C_{23}, and C_{24}, each as the 3α and 3β epimer), the site of glucuronidation could not be elucidated, because of insufficient amount of product available, for the 3β-epimers of C_{22} and C_{23}. The remaining glucuronides were identified by comparison with authentic, chemically synthesized standards in GLC (protected forms, Table 2) and, for H-glucuronides, in TLC (free forms, Table 3), as well as by ^{1}H-NMR (Table 1). The distribution of types and amounts of enzymatically formed glucuronides is shown in **Fig. 4A** for the 3α epimers and Fig. 4B for the 3β epimers of the bile acids tested. The pH optimum for the reaction was determined for each of these acids and, where applicable, for both types of glucuronide (**Fig. 5**). In the case of hydroxyl-linked glucuronides the optimal pH for the 3α epimers of C_{24} and C_{23} was acidic (pH 6.5), as expected for bile acids (18); it is, however, noteworthy that the hydroxyl-directed glucuronidation of 3α-C_{20}, C_{21}, and C_{22} proceeded best under slightly alkaline conditions (pH 7.5). The carboxyl-directed glucuronidation of the 3α BA, as well as the glucuronidation of the 3β epimers, displayed no bimodal distribution; the pH optima fell into the 6.5–7.2 range.

Of the five 3α-BA tested, three gave rise to both types of glucuronide, albeit in a different ratio; C_{22} was the only substrate that formed more carboxyl-linked than hydroxyl-linked glucuronide. In contrast to the above, four of the five 3β-hydroxy-BA gave rise to only one glucuronide each; the fifth, 3β-C_{21}, was not glucuronidated under the conditions used (Fig. 1B; a very weak spot with R_f = 0.35 represents < 0.1% of total radioactivity, which is not sufficient for any characterization). Of the

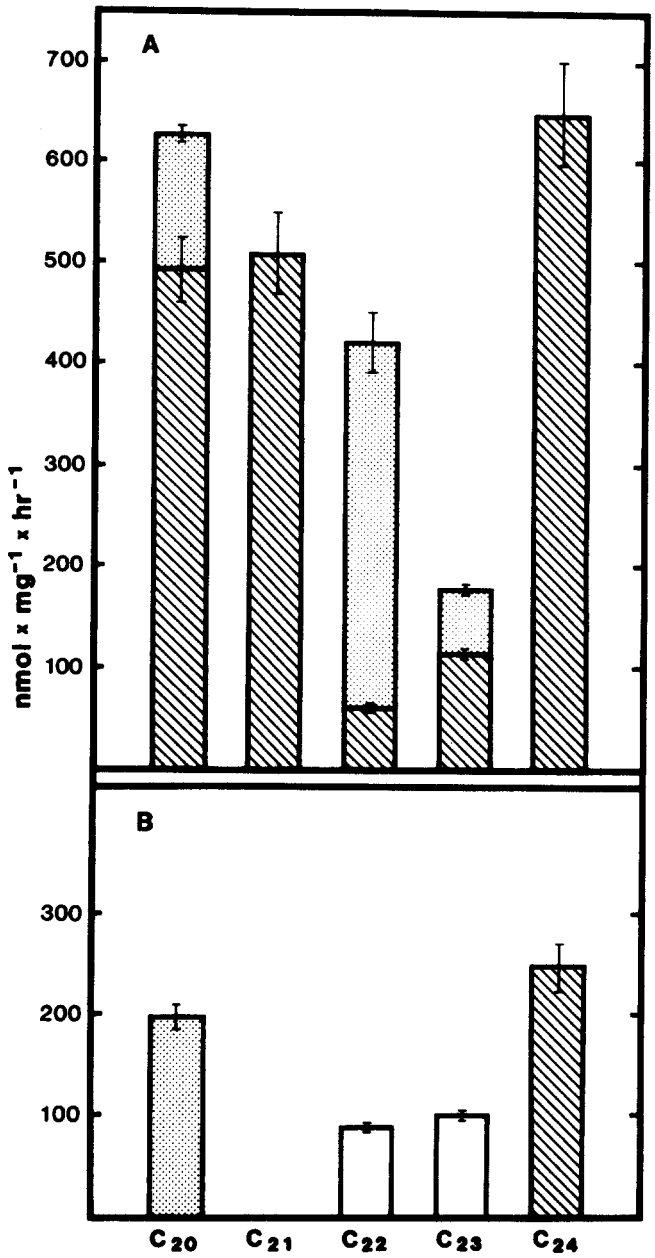


Fig. 4. Rates of enzymatic glucuronidation of various bile acids and the distribution of products. Reactions were carried out at pH values optimal for the formation of each product (see Methods). Hatched bars represent hydroxyl-linked, dotted bars carboxyl-linked, and open bars unidentified glucuronides. Each determination was carried out on at least six substrate concentrations with constant Brij 58 concentration and the calculated V_{max} is plotted. The standard deviation is shown for each product (n being the same as in Table 4). Panel A: 3α5β-BA; panel B: 3β5β-BA.

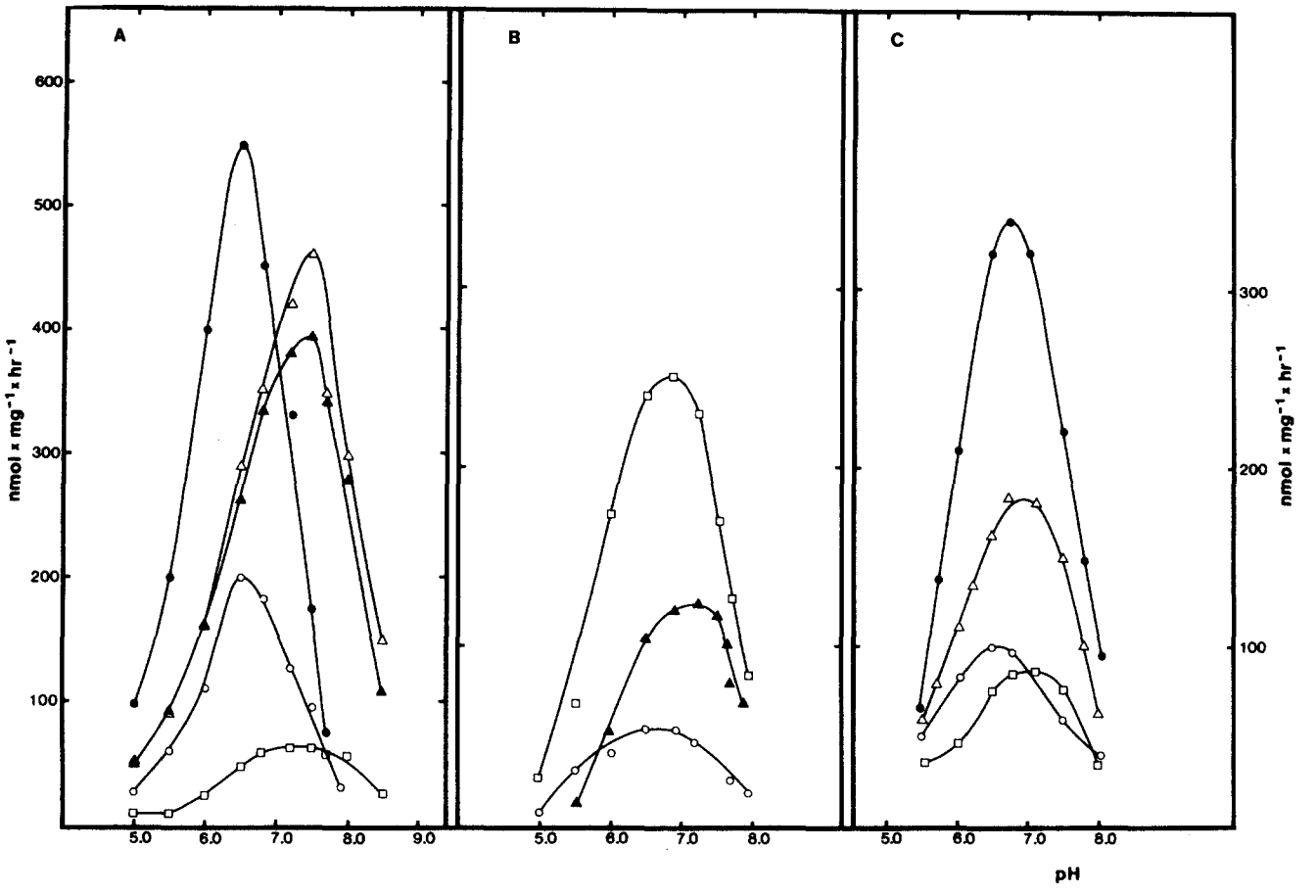


Fig. 5. The pH optima for enzymatic formation of bile acid glucuronides. Reactions (at 0.25 mM for 3α-epimers and 0.4 mM for 3β-epimers) were carried out as described in Methods, except that the buffer was imidazole for pH 5 and 5.5, HEPES for pH 6 to pH 8.5, and Tris for pH 8 to 8.5. Panel A: hydroxyl-linked glucuronides of 3α5β-BA; Panel B: carboxyl-linked glucuronides of 3α5β-BA; Panel C: 3β5β-BA (see Fig. 4B for glucuronide type). (●), C_{24}; (○), C_{23}; (□), C_{22}; (△), C_{21}; (▲), C_{20}.

four glucuronides of 3β acids, two were identified chromatographically (Tables 2 and 3) and spectrally (Table 1) as hydroxyl-linked (C_{24}) or carboxyl-linked (C_{20}), respectively.

Apparent Michaelis constants for the BA substrate were determined for all reactions (**Table 4**). For lithocholic acid, K_M = 36 μM (pH 6.5) was obtained, in good agreement with published values for microsomes (9) and the purified 3-hydroxy androgen GT (18). This suggests that the method of introducing BA into an aqueous medium using Brij 58 is enzymologically equivalent to other methods used for this purpose (e.g., 18). Generally, the particular method may be of little consequence if membrane-bound enzymes are measured: the hydrophobic substrate will largely partition into the membrane and its concentration in the lipid phase, rather than that in water, might well be rate-determining for the enzymatic reaction. In that case the observed apparent K_M values would be relatively independent of the method of substrate preparation, as indeed they are for lithocholate (9, 18, and this report), and would be valuable for the purpose of comparisons. In this light, the K_M values reported in Table 4 indicate that short-chain BA could compete successfully with conventional BA and would likely undergo glucuronidation in vivo.

Specificity for 3α- and 3β-epimers

Monohydroxylated BA occur as two epimers, 3α and 3β, the former being prevalent in biological materials. The selectivity of the hydroxyl-specific BA glucuronyltransferase activity towards the 3α and 3β epimers is shown in Fig. 4 (hatched bars). In the case of C_{24}, the activity towards isolithocholic acid was one-third of that toward lithocholic acid, in excellent agreement with reference 18. The discrimination against the 3β-hydroxy epimers was even more evident for C_{20} and C_{21}, where no H-glucuronides were formed from the β epimers. Although no such comparison can be made for C_{22} and C_{23} because of the unknown character of the glucuronide, the data indicate that the stereochemistry of C-3 has a large and consistent effect on the rate of the enzymatic reaction. It is noteworthy that the configuration of C-3 remained

TABLE 4. Apparent Michaelis constants of the bile acid UDP-glucuronyltransferase activity from rat liver microsomes with various bile acids as substrates

BA Substrate	Glucuronide Type	K_M
C_{24} 3α5β	H (5a)	36.4 ± 0.2 (n = 7)
C_{24} 3β5β	H (5b)	79.2 ± 6.1 (n = 6)
C_{23} 3α5β	H (4a)	34.5 ± 1.1 (n = 3)
C_{23} 3α5β	C (4f)	37.1 ± 2.8 (n = 3)
C_{23} 3β5β	U	31.7 ± 1.3 (n = 2)
C_{22} 3α5β	H (3a)	38.3 ± 0.9 (n = 7)
C_{22} 3α5β	C (3f)	97.7 ± 5.1 (n = 7)
C_{22} 3β5β	U	40.1 ± 3.1 (n = 4)
C_{21} 3α5β	H (2a)	79.1 ± 4.7 (n = 6)
C_{20} 3α5β	H (1a)	75.9 ± 4.2 (n = 7)
C_{20} 3α5β	C (1f)	93.8 ± 7.6 (n = 7)
C_{20} 3β5β	C (1h)	72.6 ± 4.1 (n = 4)

The assays were carried out at pH values optimal for each product, as listed in Methods. Michaelis constants were obtained numerically by Lineweaver-Burk analysis; the lines were fitted by a weighted least-square method (46).

H and C, hydroxyl- and carboxyl-linked glucuronide; U, unidentified. The symbols of glucuronides (in parentheses) refer to Fig. 2.

unchanged during the in vitro glucuronidation reactions reported here, unlike in vivo experiments in which a 3β → 3α conversion has been observed (28).

The carboxyl-directed GT activity did not recognize either epimer of C_{24} as a substrate; it did, however, act on short-chain bile acids (except C_{21}). The amounts of C-glucuronide formed are shown in Fig. 4 (dotted bars).

DISCUSSION

Bile acid glucuronides have been found in various biological fluids. C_{24} glucuronides have been isolated from human urine, serum, and bile, both in normal and pathological states (19–24, 44). These compounds are glucuronidated on one of the hydroxyl groups of the steroid moiety; no poly-glucuronidation has been detected so far (19–24). Cholic and chenodeoxycholic acid 3-O-glucuronides are found in normal human urine (21), but deoxycholic and lithocholic acid glucuronides are largely absent. In part, this may be explained by the C-6 hydroxylation and concomitant C-6 glucuronidation of deoxycholic and lithocholic acid in vivo (21). Thus, material entering the reaction as lithocholate leaves as the glucuronide of a dihydroxy BA and is no longer found in the monohydroxylated fraction; similarly, deoxycholate is converted to a trihydroxy-BA glucuronide.

BA glucuronidation is of special importance in relation to liver disease and cholestasis. First, the relative concentration of BA glucuronides in plasma and urine is increased in cholestasis, suggesting that BA glucuronide formation is increased. Second, it has been assumed that BA glucuronidation, in analogy to glucuronidation of bilirubin and many xenobiotics, promotes urinary excretion and thus BA elimination in the presence of hepatic secretory failure. Third, it has also been assumed that glucuronidation promotes detoxification of BA and decreases the toxicity of BA retained in blood and tissues in cholestasis. Although recent findings suggest that these assumptions may constitute an oversimplification (25), they nevertheless form a useful framework for understanding of the physiology of BA glucuronidation.

Short-chain bile acids which are prevalent in the developing organism and are present to a lesser degree in the adult (26, 27, 29, 31), are secreted in part as the glucuronide (30). Short-chain bile acids and their glucuronides may in certain circumstances modulate bile flow (30 and unpublished results). A second problem of potential interest stems from the fact that bile acids are end-products of sterol metabolism. Some short-chain BA might be metabolically derived from steroid hormones, e.g., progesterone; their analysis is relevant to the understanding of hormone turnover and balance.

In all of the glucuronides mentioned above, the glucuronic acid moiety is linked to a steroidal hydroxyl group, typically in the 3 position (Fig. 3A). The finding of a carboxyl-linked BA glucuronide (Fig. 3B) was therefore unexpected. The only known example of a steroidal acid glucuronidated at the carboxyl group was detected in metabolic studies of deoxycorticosterone degraded to the glucuronide of 3-oxo-androst-4-en-17-carboxylic acid (45). However, in view of the capability of rat liver microsomes to catalyze the formation of BA carboxylic glucuronides (as demonstrated in this study), it appears likely that these compounds might occur naturally. The apparent Michaelis constants for their formation are similar to those found for the conventional hydroxyl-linked glucuronides, suggesting that the carboxyl-directed glucuronidation activity can successfully compete for substrates.

It is striking that the formation of the carboxyl glucuronide is limited to certain short-chain bile acids; no derivative of this type of lithocholic acid was found. No ready explanation of this fact can be offered at this time, especially since there is no obvious correlation between side chain length and site of glucuronidation (Fig. 4).

Although the identity of the glucuronides of 3β-C_{22} and 3β-C_{23} has not been rigorously determined, their R_f values in TLC suggest that they are of the carboxyl type. If so, an interesting regularity emerges: whereas every 3α-BA tested is a better substrate for H-glucuronidation than the corresponding 3β-BA, the latter are generally superior as substrates for C-glucuronidation (with the exception of C_{22}). This is reflected in both the V_{max} (Fig. 4) and the K_M values (Table 4).

It is tempting to speculate about the enzymes involved in the formation of hydroxyl and carboxyl derivatives of BA. Mechanistically it does not appear likely that a single enzyme could catalyze both reactions. If the enzyme that

forms BA carboxylic glucuronides is in fact a separate moiety, it will be interesting to study its possible relationship with other acyl-specific glucuronyltransferases, especially with that specific for bilirubin. In the case of the hydroxyl-directed activity, the sharp bimodal distribution of pH optima (Fig. 5A) could also be indicative of the involvement of more than one enzyme. Answering these questions will require the purification and separation of the enzymes involved.

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