A bile alcohol sulfate as a major component in the bile of the small skate (*Raja erinacea*)

G. Karlaganis,[1] S. E. Bradley, J. L. Boyer,* A. K. Batta,† G. Salen,† B. Egestad, and J. Sjövall****

Department of Clinical Pharmacology, University of Berne, Berne, Switzerland; Mount Desert Island Biological Laboratory,* Salsbury Cove, ME and Department of Medicine, Yale University School of Medicine,* New Haven, CT; Division of Gastroenterology, New Jersey Medical School,† Newark, NJ; Department of Physiological Chemistry, Karolinska Institutet,** Stockholm, Sweden

Abstract The nature of bile alcohols and bile acids in gallbladder and hepatic bile from perfused livers of the small skate (*Raja erinacea*) has been investigated. The main bile alcohol sulfate was isolated by thin-layer chromatography and analyzed by fast atom bombardment mass spectrometry and ^{13}C NMR. Following solvolysis and purification on Lipidex-DEAP, the bile alcohol profile was measured by capillary gas-liquid chromatography-electron impact mass spectrometry. Based on these studies and on comparison with authentic scymnol sulfate and scymnol, the main bile alcohol was identified as 5β-cholestane-3α,7α,12α,24ξ,26,27-hexol sulfate. The mean ± SD concentration in gallbladder bile from five different skates was 24.6 ± 8.7 mmol/l. Only 0.1 mmol/l of cholic acid and its conjugates was found in a pool of skate bile. The main bile alcohol sulfate in the bile of the small skate seems to be a metabolic end product, present in a concentration comparable to that of bile salts in mammals.—**Karlaganis, G., S. E. Bradley, J. L. Boyer, A. K. Batta, G. Salen, B. Egestad, and J. Sjövall.** A bile alcohol sulfate as a major component in the bile of the small skate (*Raja erinacea*). *J. Lipid Res.* 1989. **30:** 317–322.

Supplementary key words 5β-cholestane-3α,7α,12α,24ξ,26,27-hexol sulfate • scymnol sulfate • bile alcohols • bile acids • gas-liquid chromatography-mass spectrometry

Active bile salt secretion plays an important role in bile formation of mammals including man, dog, rat, and the guinea pig, presumably by producing an osmotic pressure gradient within the canaliculi promoting the passive movement of water and diffusible solute from sinusoidal blood to the canicular lumen. To what extent such a mechanism may be operative in lower vertebrates is unknown. The bile salts in the Cyclostomes and Elasmobranchs appear to be largely sulfated bile alcohols whose physicochemical properties differ from those of the bile salts commonly found in mammalian bile (1). Just how these differences may affect the contribution of bile salts to bile formation remains obscure. This question cannot be addressed with confidence, however, in the absence of a more precise definition of the identity of the bile salt concerned. Scymnol sulfate (5β-cholestane-3α,7α,12α,24ξ,26,27-hexol 26(or 27) sulfate) appears to be the major bile salt in bile of several species of sharks and rays (2), but its identification has been based largely on chromatographic evidence. In connection with on-going studies of bile formation in the small skate (*Raja erinacea*), definitive identification of the bile salts in the bile of this species was needed. This report presents the results of a study using a combination of chromatographic and spectroscopic techniques.

MATERIALS AND METHODS

Scymnol sulfate was kindly donated from the Haslewood Collection by Dr. A R. Tammar. Scymnol was obtained by solvolysis according to Hirano et al. (3).

All solvents were of reagent grade. Sep-Pak C_{18} cartridges (Waters Associates, Milford, MA) were washed with 10 ml of methanol and 10 ml of water before use. Lipidex-DEAP (diethylaminohydroxypropyl Sephadex LH-20) was from Packard Instrument Co. (Downers Grove, IL) and was washed by stirring from the top at 70°C for 1 hr in 20% aqueous ethanol and then in ethanol. It was stored at −20°C. Immediately before use it was converted into the hydroxide form for isolation of solvolyzed bile alcohols or into the acetate form for isolation of bile acids (4).

Abbreviations: FAB-MS, fast atom bombardment mass spectrometry; GLC-MS, gas-liquid chromatography-mass spectrometry; GLC, gas-liquid chromatography; *m/z*, mass/charge ratio; NMR, nuclear magnetic resonance; R_f, distance moved relative to solvent front; RI, retention index (Kovats index); TLC, thin-layer chromatography; TMS, trimethylsilyl; sfor, single-frequency off-resonance.

[1]To whom correspondence should be addressed at: Federal Office for Environmental Protection, Hallwylstrasse 4, CH-3003 Berne, Switzerland.

Collection of bile samples

Samples of bile were obtained from the gallbladders and hepatic ducts of isolated perfused livers of male small skates (*Raja erinacea*) obtained by trawl in waters of Southwest Harbor, ME, and maintained in large tanks with rapidly exchanging 15°C sea water at the Mount Desert Island Biological Laboratory (5). Bile was aspirated from the gallbladder and a cannula was inserted in the cystic duct after ligation of the common bile duct for collection of hepatic duct bile. All samples were frozen immediately and kept frozen until analysis. Gallbladder bile was used for isolation and analysis if not otherwise stated. Bile samples were obtained from different skates. In addition, a pool of 18 ml of bile collected from several gallbladders was used for isolation of the major bile alcohol.

Isolation of the major bile alcohol sulfate

Bile (1 ml) was diluted with 1 ml of methanol and applied on a preparative thin-layer plate (20 × 20 cm) containing 2.0-mm-thick silica gel 60 without fluoresence indicator (Merck AG, Darmstadt, Germany) using a Linomat III sample applicator (CAMAG AG, Muttenz, Switzerland). The plate was developed in ethyl acetate–methanol–acetic acid–water 65:20:10:5 (by vol) (6). A small part at the side of the plate was cut off with a glass cutter and bile alcohols were visualized with 10% phosphomolybdic acid in ethanol. One major spot appeared with an R_f value of about 0.43, identical to that given by the reference scymnol sulfate. The band corresponding to this spot was scraped into 100 ml of methanol and held for 5 min in an ultrasonic bath. The methanolic solution was evaporated and the residue was dissolved in 10 ml of water and centrifuged at 27000 *g*. The supernatant was divided and passed through four Sep-Pak C_{18} cartridges. Each cartridge was washed with 10 ml of water and eluted with 10 ml of methanol, which was evaporated. The residue was dissolved in 2 ml of water and lyophilized, yielding 6.46 mg of a white powder per ml of bile.

Fast atom bombardment mass spectrometry

Samples, about 6 μg, of scymnol sulfate and the bile alcohol sulfate from skate bile (isolated by TLC) were applied in 5 μl of 70% aqueous methanol under a stream of nitrogen to the FAB target coated with the glycerol matrix. Negative ion FAB spectra were recorded by a VG 11-250 data system using a VG 7070E instrument (VG Analytical, Manchester, U.K.) equipped with a FAB source and an Ion Tech atom gun (7) operating with xenon at 8 kev. The scan rate was 10 s × decade^{-1} and the resolution 1000.

^{13}C Nuclear magnetic resonance spectroscopy

The ^{13}C NMR spectrum was recorded at 100 MHz using a Fourier-Transform-equipped Varian XL-400 spectrometer. The isolated major bile alcohol sulfate (8 mg) was dissolved in a mixture of 0.7 ml chloroform-d_1 and 0.1 ml methanol-d_4 and was analyzed in a tube (5 mm o.d.) at probe ambient temperature of 21°C. Chemical shifts are expressed in parts per million (ppm) relative to tetramethylsilane as internal standard. The ^{13}C NMR spectrum was recorded in both the proton noise - decoupling mode and in a single-frequency off-resonance (sfor) proton decoupling mode.

Analysis of bile alcohols after solvolysis

Bile (0.05 to 0.5 ml) was diluted with 5 ml of water and passed through a Sep-Pak C_{18} cartridge, followed by 10 ml of water. Bile alcohols were eluted with 10 ml of methanol. The methanolic solution was concentrated to 0.1 ml and 5 ml of tetrahydrofuran acidified with 5 μl of 4 M aqueous sulfuric acid was added for solvolysis (8). Following incubation for 1 hr at 50°C, 1 ml of methanol was added and the solution was passed through Lipidex-DEAP in OH^- form; the column (80 × 4 mm) was packed in methanol and washed with 3 ml of tetrahydrofuran–methanol 5:1 (v/v), prior to application of the sample. The column was washed with 6 ml of the latter solvent and the liberated bile alcohols were recovered in the combined effluents. Coprostanol (50 nmol) was added as internal standard. TMS ethers were prepared, the residue was dissolved in hexane and analyzed by capillary GLC using a 15-m persilanized glass capillary column (9) coated with OV-73 (10). Analyses by GLC–MS were carried out on the VG 7070E instrument with an electron impact ion source at 70 eV and a Dani 3800 gas chromatograph. A fused silica capillary column coated with cross-linked methyl silicone (Quadrex Corp., New Haven, CT) extended into the ion source and was used at 250°C with an all-glass falling needle injection system.

Analysis of bile acids

One hundred microliters of bile was diluted with 50 ml of water and bile acids were extracted, solvolyzed, subjected to alkaline hydrolysis, and isolated on Lipidex-DEAP as previously described for urine (11). Coprostanol was then added as internal standard, TMS ethers were prepared and analyzed by capillary GLC and capillary GLC–MS on a Finnigan 1020 instrument using a 20-m glass capillary column covered with barium carbonate (12) and coated with polyethyleneglycol 20000 (13).

RESULTS

Structure of the major bile alcohol

The high mass region of the negative ion FAB spectrum (**Fig. 1**) of the isolated major bile alcohol sulfate showed a base peak at *m/z* 547. This corresponds to M-1$^-$ of a

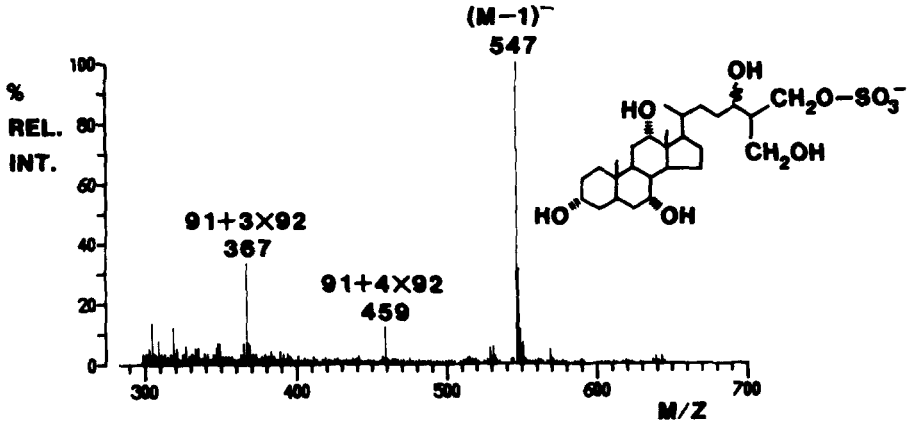

Fig. 1. High mass end of the negative ion FAB spectrum of the isolated main bile alcohol sulfate from skate bile. Peaks at *m/z* 367 and 459 arise from the glycerol matrix.

cholestanehexol sulfate. Reference scymnol sulfate gave an identical spectrum. The FAB spectrum of the crude bile extract was very similar to the spectrum shown in Fig. 1. An additional small peak at *m/z* 531 might arise from small amounts of a cholestanepentol sulfate in the crude extract. The peak height of *m/z* 531 was 9.3% of *m/z* 547.

The ^{13}C NMR chemical shift data of the isolated major bile alcohol sulfate are given in **Table 1**. The multiplicity of each peak is given in the parentheses. Carbons 1–20 were assigned using methyl cholate as the model compound and 5β-cholane-3α,7α,24- and -3α,12α,24-triols were used to assign carbons 21–24 (14). Carbons 25–27 were assigned using 5β-cholestane-3α,7α,26-triol as the

TABLE 1. ^{13}C NMR chemical shifts for 5β-cholestane-3α,7α,12α,24ξ,26,27-hexol sulfate isolated from skate bile and for some reference compounds

Carbon	Skate Bile Alcohol Sulfate	5β-Cholestane-3α,7α,26-triol (25R + 25S)	5β-Cholane-3α,7α,24-triol	5β-Cholane-3α,12α,24-triol	Methyl Cholate
1	35.51 (T)[a]	35.34	35.8	35.7	35.7
2	30.06 (T)	30.62	31.3	30.5	30.6
3	71.76 (D)	71.82	72.3	71.4	71.6
4	39.40 (T)	39.71	39.7	36.3	39.6
5	41.87 (D)	41.51	42.1	42.7	42.0
6	34.71 (T)	34.59	35.4	27.7	35.1
7	68.53 (D)	68.40	68.8	26.6	68.0
8	39.63 (D)	39.34	40.0	36.3	39.9
9	26.62 (D)	32.84	33.3	33.9	26.7
10	34.96 (S)	35.01	35.5	34.5	35.1
11	28.24 (T)	20.60	20.9	29.0	28.5
12	73.26 (D)	39.71	39.9	73.1	72.5
13	46.56 (S)	42.57	43.0	46.9	46.8
14	41.69 (D)	50.41	50.9	48.4	42.0
15	23.42 (T)	23.67	24.0	24.2	23.5
16	27.81 (T)	28.30	28.5	28.5	28.0
17	47.23 (D)	56.07	56.5	47.6	47.4
18	12.62 (Q)	11.76	11.9	12.9	12.8
19	22.61 (Q)	22.80	23.0	23.4	22.8
20	35.83 (D)	35.74	35.9	35.9	35.7
21	17.59 (Q)	18.66	18.6	18.0	17.6
22	32.17 (T)	36.11	32.3	32.1	31.3
23	29.89 (T)	23.33	29.5	29.5	31.2
24	71.54 (D)	33.55	62.9	63.8	174.5
25	46.00 (D)	35.74			
26	65.44 (T)	68.20			
27	61.15 (T)	16.53			

[a]The multiplicity of peaks in the sfor spectrum is expressed as S (singlet), D (doublet), T (triplet), and Q (quartet).

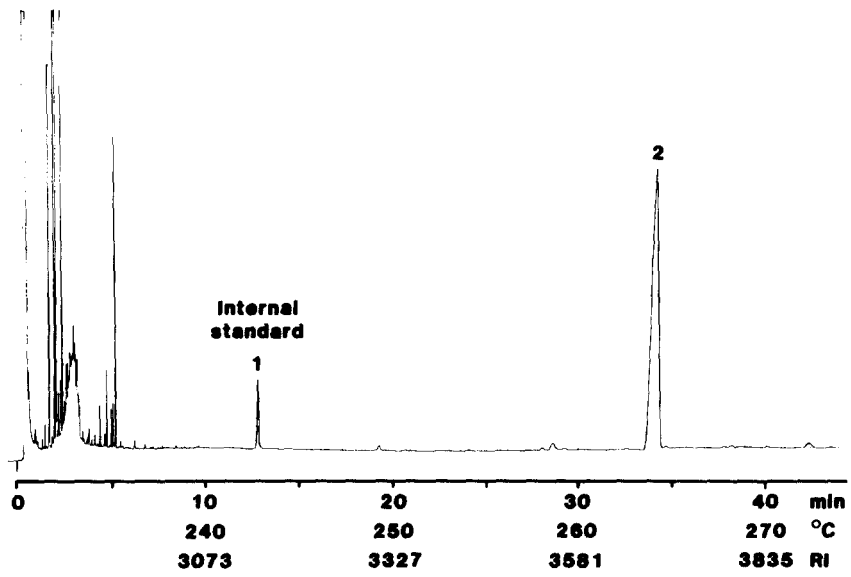

Fig. 2. Capillary GLC analysis of the TMS ethers of bile alcohols from a pool of 18 ml of bile collected from several skate gall bladders. Retention times, temperatures, and retention indices are indicated. Peak no. 1: coprostanol (internal standard); peak no. 2: scymnol (5β-cholestane-3α,7α,12α,24ξ,26,27-hexol).

model compound (15) and the effects of substituents at C-24 and C-27 were taken into consideration (14, 16). The resonances due to carbons 5 and 14 (two doublets at 41.87 and 41.69) could not be correctly assigned and may be reversed. All other resonances could be assigned with the help of the model compounds. Table 1 gives the ^{13}C NMR shifts of 5β-cholestane-3α,7α,26-triol (25R + 25S), 5β-cholane-3α,7α,24-triol, 3α,12α,24-triol, methyl cholate, and the major bile alcohol sulfate isolated from skate bile.

The GLC profile of the solvolyzed and derivatized bile alcohols showed one main peak with a retention index of 3690 (**Fig. 2**). The TMS ether of scymnol had the same retention index. The electron impact mass spectrum recorded during the elution of the peak obtained from bile (**Fig. 3**) showed a series of significant peaks at *m/z* 681, 591, 501, 411, and 321 formed by loss of 219 mass units (the three terminal carbons with substituents) and consecutive losses of trimethylsilanol from the molecular ion. In addition, peaks were found at *m/z* 720 (M-2×90), 630 (M-3×90), 540 (M-4×90), 451 (M-4×90-89), 361 (M-5×90-89) compatible with a molecular weight of 900. Another series of small peaks

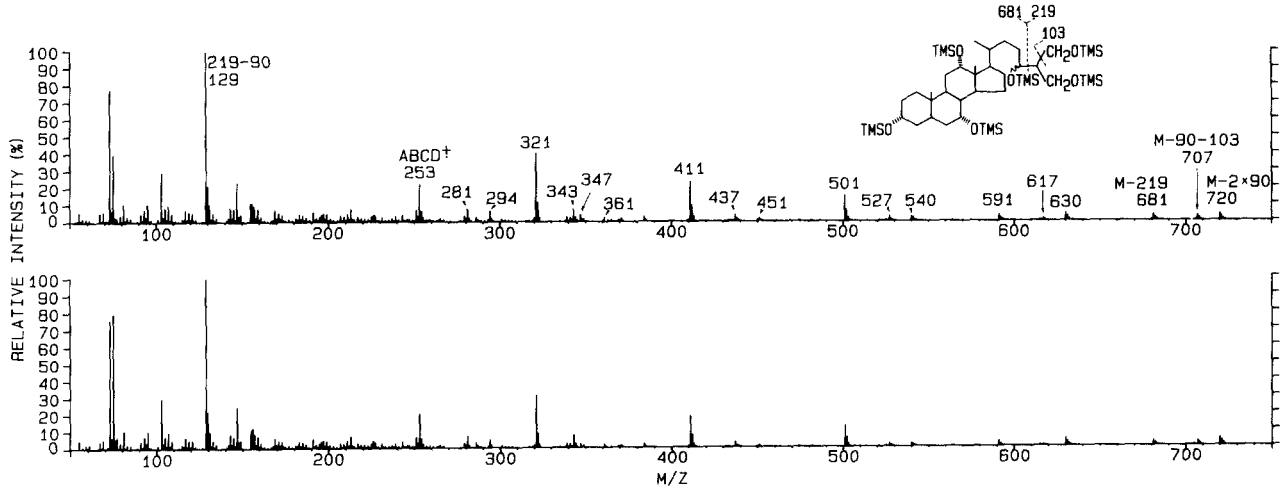

Fig. 3. Electron impact mass spectrum of the TMS ethers of scymnol, 5β-cholestane-3α,7α,12α,24ξ,26,27-hexol (upper spectrum), and the bile alcohol isolated from the bile of the small skate (*Raja erinacea*) (lower spectrum).

were found at *m/z* 707 (M-103-90), 617 (M-103-2×90), 527 (M-103-3×90), 437 (M-103-4×90), and 347 (M-103-5×90). Peaks at *m/z* 253 and 343 indicated three hydroxyl groups in the ring system. The spectrum was identical to that given by the TMS ether of scymnol (Fig. 3).

Biliary concentration of bile alcohols

The pool of 18 ml of gallbladder bile was collected from skates maintained in captivity for periods up to 7 days. The pool had a dry weight of 53.8 mg per g of bile and a concentration of 5β-cholestane-3α,7α,12α,24ξ,26,27-hexol of 16.7 mmol/l as measured by capillary GLC. The yield of bile alcohol after preparative TLC corresponded to a concentration of 11.9 mmol/l. The difference can be explained by losses in the elution of the silica from the preparative TLC plate. The skate bile alcohol profile consists of only one major bile alcohol. In contrast to the bile alcohol profile in humans (11, 17), the GLC profile of bile alcohols in the skate (Fig. 2) consists of one major peak constituting 96% of the total peak area. This is consistent with the FAB mass spectrum of the crude bile extract. The mean concentration of 5β-cholestane-3α,7α,12α,24ξ,26,27-hexol in gallbladder bile from five different skates was 24.6 ± SD 8.7 mmol/l.

Biliary concentration of bile acids

Bile acids in the gallbladder bile pool were analyzed by capillary GLC. Only 0.10 mmol/l of cholic acid could be found. The identity of cholic acid could be confirmed by capillary GLC-MS. The methyl ester TMS ether derivatives of extracted and of authentic cholic acid had identical retention times (24 min) and mass spectra. The base peak ion m/z 253 was used for quantification.

DISCUSSION

The negative ion FAB mass spectrum (Fig. 1), the ^{13}C NMR spectrum (Table 1), and the electron impact mass spectrum (Fig. 3) are consistent with a structure of 5β-cholestane-3α,7α,12α,24ξ,26,27-hexol sulfate for the main bile alcohol in the bile of the small skate (*Raja erinacea*). The retention time and mass spectrum of the TMS ether of the bile alcohol were identical to those given by the reference scymnol TMS ether. The position of the sulfate moiety is not known. In 1898 Hammarsten (18) isolated an alcohol from the bile of the shark *Scymnus borealis* which he called "scymnol"; later this proved to be an anhydro-artefact. Scymnol sulfate is the 26 (or 27) sulfate of 5β-cholestane-3α,7α,12α,24ξ,26,27-hexol (1, 19). On biological grounds and because of the R_f value identical with that of reference scymnol sulfate, one may assume that scymnol sulfate from the skate also carries the sulfate group at a terminal hydroxyl group. Scymnol has been described in different Elasmobranchs, such as *Somniosus microcephalus* (18), *Centrophorus sp.* (20), *Dasyatis akajei* (21, 22), and *Mustelus manazo* (23).

Scymnol is known to be an inefficient precursor of cholic acid in the bile of the rat (24). Since a small amount of cholic acid was found in bile from *Raja erinacea* in the present study and has also been found in small amounts of the bile of the shark (23), it is likely that side chain degradation of bile alcohols to form cholic acid is a minor pathway in these fishes. Thus scymnol is likely to be an end product of cholesterol metabolism in elasmobranch fishes. Administration of scymnol to elasmobranchs under conditions where bile may be collected in the free-swimming state would be necessary to determine this directly.

This work was supported by the Swiss National Science Foundation (grant nos. 3.999.80, 3.926.0.83, and 3.996.0.84) and by the Swedish Medical Research Council (grant no. 13X-219). We would like to thank Dr. H. Ichimiya and Mr. T. Weber for technical assistance, Mrs. M. Dick for secretarial help, Ms. M. Kappeler and G. Green for artwork, and Dr. F. Bachmann (Burgdorf College of Engineering, Switzerland) and R. Reimendal for graphics software for plotting mass spectra.

Manuscript received 8 December 1987 and in revised form 26 August 1988.

REFERENCES

1. Haslewood, G. A. D. 1978. The biological importance of bile salts. *In* Frontiers of Biology. A. Neuberger and E. L. Tatum, editors. North-Holland Publishing Company, London/New York. Volume 47.
2. Tammar, A. R. 1974. Bile salts in fishes. *In* Chemical Zoology. M. Florkin and B. T. Sheer, editors. Vol. VIII. 595–611.
3. Hirano, Y., H. Miyazaki, S. Higashidate, and F. Nakayama. 1987. Analysis of 3-sulfated and nonsulfated bile acids by one-step solvolysis and high performance liquid chromatography. *J. Lipid Res.* **28:** 1524–1529.
4. Almé, B., A. Bremmelgaard, J. Sjövall, and P. Thomassen. 1977. Analysis of metabolic profiles of bile acids in urine using a lipophilic anion exchanger and computerized gas–liquid chromatography–mass spectrometry. *J. Lipid Res.* **18:** 339–362.
5. Reed, J. S., N. D. Smith, and J. L. Boyer. 1982. Hemodynamic effects on oxygen consumption and bile flow in isolated skate liver. *Am. J. Physiol.* **242:** 6313–6318.
6. Sasaki, T. 1965. Stero-bile acids and bile alcohols. LXXVI. Thin-layer chromatography of conjugated bile acids and alcohols. *Hiroshima J. Med. Sci.* **14:** 85–92.
7. Egestad, B., P. Petterson, S. Skrede, and J. Sjövall. 1985. Fast atom bombardment mass spectrometry in the diagnosis of cerebrotendinous xanthomatosis. *Scand. J. Clin. Lab. Invest.* **45:** 443–446.
8. Axelson, M., B. L. Sahlberg, and J. Sjövall. 1981. Analysis of profiles of conjugated steroids in urine by ion-exchange

separation and gas chromatography–mass spectrometry. *J. Chromatogr.* **224:** 355–370.
9. Grob, K., G. Grob, and K. Grob, Jr. 1979. Deactivation of glass capillaries by persilylation. *J. High Resolution Chromatogr. Chromatogr. Commun.* **2:** 677–678.
10. Karlaganis, G., A. Nemeth, B. Hammarskjöld, B. Strandvik, and J. Sjövall. 1982. Urinary excretion of bile alcohols in normal children and patients with α1-antitrypsin deficiency during development of liver disease. *Eur. J. Clin. Invest.* **12:** 399–405.
11. Délèze, G., I. Björkhem, and G. Karlaganis. 1986. Bile acids and bile alcohols in two patients with Zellweger (cerebro-hepato-renal) syndrome. *J. Pediatr. Gastroenterol. Nutr.* **5:** 701–710.
12. Grob, K., G. Grob, and K. Grob, Jr. 1977. The barium carbonate procedure for the preparation of glass capillary columns; further informations and developments. *Chromatographia.* **10:** 181–187.
13. Karlaganis, G., and G. Paumgartner. 1978. Analysis of bile acids in serum and bile by capillary gas–liquid chromatography. *J. Lipid Res.* **19:** 771–774.
14. Blunt, J. W., and J. B. Stothers. 1977. ^{13}C NMR spectra of steroids—a survey and commentary. *Org. Magn. Reson.* **9:** 439–464.
15. Batta, A. K., T. H. Williams, G. Salen, D. N. Greeley, and S. Shefer. 1980. Differentiation between the 25R- and the 25S-isomers of 5β-cholestane-3α,7α-26-triol by ^{13}C NMR spectroscopy. *J. Lipid Res.* **21:** 130–135.
16. Dayal, B., G. Salen, V. Toome, and G. S. Tint. 1986. Configuration at C-25 in 5β-cholestane-3α,7α,12α,25,26-pentol excreted by patients with cerebrotendinous xanthomatosis: circular dichroism and ^{13}C NMR studies. *J. Lipid Res.* **27:** 1328–1332.
17. Kuroki, S., K. Shimazu, M. Kuwabara, M. Une, K. Kihira, T. Kuramoto, and T. Hoshita. 1985. Identification of bile alcohols in human bile. *J. Lipid Res.* **26:** 230–240.
18. Hammarsten, O. 1898. Über eine neue Gruppe gepaarter Gallensäuren. *Hoppe-Seyler's Z. Physiol. Chem.* **24:** 322–350.
19. Bridgwater, R. J., T. Briggs, and G. A. D. Haslewood. 1961. Comparative studies of "bile salts." 14. Isolation from shark bile and partial synthesis of scymnol. *Biochem. J.* **82:** 285–290.
20. Haslewood, G. A. D. 1967. Bile salt evolution. *J. Lipid Res.* **8:** 535–550.
21. Ashikari, H. 1939. Über die Galle des "Akajei"-Fisches (*Dasyatis akajei*) und die Konstitution des Scymnols. *J. Biochem.* **29:** 319–324.
22. Sasaki, T. 1966. Stero-bile acids and bile alcohols. LXXXXII. Comparative studies on the bile salts of fishes by thin layer chromatography. *J. Biochem. (Tokyo).* **60:** 56–62.
23. Kouchi, M. 1964. Stero-bile acids and bile sterols. LXX. Studies on the bile of shark, *Mustelus manazo. Hiroshima J. Med. Sci.* **13:** 341–350.
24. Danielsson, H., A. Kallner, R. J. Bridgwater, T. Briggs, and G. A. D. Haslewood. 1962. On the extent of the conversion of scymnol to cholic acid in the rat. *Acta Chem. Scand.* **16:** 1765–1769.