Occurrence of sulfated 5α-cholanoates in rat bile[1]

H. Eriksson, W. Taylor,[2] and J. Sjövall

Department of Chemistry, Karolinska Institute, Stockholm, Sweden

Abstract Bile acids in bile from male and female rats with cannulated bile ducts have been analyzed by repetitive scanning gas–liquid chromatography–mass spectrometry after initial fractionation of conjugate classes on diethylaminohydroxypropyl Sephadex LH-20. Sex differences were observed in the amounts and types of bile acids in the sulfate fraction. The proportion of total bile acids excreted as sulfates was higher in female (0.9–1.3%) than in male (0.1–0.2%) rats. Most of the sulfated bile acids had a 5α configuration, allochenodeoxycholic acid being the major compound in bile from female rats. This bile acid was also present in the nonsulfate fraction but could not be found in bile from male rats. The results indicate that gas–liquid chromatography–mass spectrometry has to be used to provide sufficient specificity in the bile acid analyses. Thus, compounds from the sulfate fraction having the retention times of cholic and chenodeoxycholic acid derivatives were found to be due to derivatives of the 3β,5α-isomers of these bile acids.

Supplementary key words allo bile acids · sex difference · ion exchange chromatography · gas–liquid chromatography–mass spectrometry

The predominant primary bile acids in most vertebrates are cholic and chenodeoxycholic acids. Deoxycholic acid, the most common secondary bile acid in bile, is formed from cholic acid by bacterial removal of the 7α-hydroxyl group (see 1). The bile acids occur in bile conjugated mainly with taurine or glycine. Since the discovery by Palmer (2) and Palmer and Bolt (3) of sulfated lithocholic acid conjugates in human bile, interest in other sulfated bile acids has increased. For example, large amounts of bile acid sulfates have been found in the urine of patients with cholestatic disease (4–6).

In the course of a study of the effects of ethynylestradiol on bile acid metabolism in the rat, a number of previously unidentified bile acids was found in the sulfate fraction of rat bile. Many of these compounds had the gas–liquid chromatographic–mass spectrometric properties of 5α bile acids and occurred exclusively or predominantly in bile from female rats. This report describes the identification of some of the compounds as well as a detailed analysis of the bile acid pattern of different conjugate groups in rat bile.

MATERIALS AND METHODS

Reference compounds

The substituted 5β-cholanoic acids were the same as those used in previous investigations in this laboratory. We are grateful to Drs. W. H. Elliott and A. Kallner for generous gifts of substituted 5α-cholanoic acids.

Collection of bile

Following a number of screening analyses of bile samples from male and female rats, a comprehensive study was carried out using two male and two female rats of the Sprague-Dawley strain. The rats were kept in restraining cages and had free access to food pellets and water. Collection of bile at 12-hr intervals was started immediately after insertion of the bile duct cannula (at about 9 AM). The samples were stored at −20°C until analyzed.

Analysis of bile

The samples collected over the periods 0–12 hr (mainly representing bile acids from the circulating pool) and 48–60 hr (almost exclusively representing bile acids produced in the liver) were analyzed in detail. Two ml of bile were added drop by drop to 20 ml of ethanol during agitation in an ultrasonic bath. After filtration and evaporation of the solvent, the residue was dissolved in 2 ml of 72% aqueous ethanol. To convert bile salts to bile acids, the solution was passed through a 1-g column of Amberlyst A-15 in the H^+ form followed by 11 ml of 72% aqueous ethanol. The effluent from this column was applied

Abbreviations: GLC–MS, gas–liquid chromatography–mass spectrometry.

[1] The terminology used throughout this paper follows "Recommendations for Bile Salt Nomenclature" drawn up by an ad hoc committee in August, 1975, published in "The Hepatobiliary System". W. Taylor, editor. 1976. Plenum Press, New York. 639–641. A preliminary account of the results of this research was presented at the IVth International Congress of Liver Diseases: "Liver and Bile" in Basel, 1976.

[2] Present address: Physiology Department, The Medical School, The University, Newcastle upon Tyne NE1 7RU, England.

directly to a 0.6-g column of diethylaminohydroxypropyl Sephadex LH-20 (DEAP-LH-20) in the acetate form (7). After elution of neutral compounds, the bile acids were recovered in five separate fractions (unconjugated, glycine conjugated, taurine conjugated, monosulfated, and disulfated) by elution with acetic acid and acetate buffers of increasing pH. The fractions were taken to dryness; glycine- and taurine-conjugated bile acids were hydrolyzed in 15% NaOH in 50% aqueous ethanol at 110°C for 10 hr; sulfate fractions were subjected to solvolysis for 72 hr in acidified ethanol–acetone 1:9 (v/v) (3) and, after evaporation to dryness, to alkaline hydrolysis. The unconjugated and the solvolyzed and hydrolyzed bile acids were extracted with ethyl acetate from the acidified aqueous solution. Methyl esters were prepared with diazomethane in diethyl ether–methanol 9:1 (v/v), and trimethylsilyl ethers by treatment with pyridine – hexamethyldisilazane – trimethylchlorosilane 3:2:1 (by vol) (8).

Gas–liquid chromatographic analyses of these derivatives were carried out using columns (3.5 m × 3.6 mm) filled with 1–2% Hi-Eff 8 BP on Gas-Chrom Q, 80–100 mesh (Applied Science Labs., State College, PA) at 220–230°C, or 1.5% SE-30 on 80–100 mesh Chromosorb W HP. Flame ionization detection was used. Quantitative results were obtained by comparing peak areas (Hi-Eff 8 BP columns) with those given by known amounts of the derivative of cholic acid. Corrections for differences in response, which were small in a few cases tested, were not made.

Gas–liquid chromatography–mass spectrometry was carried out using the same columns as above. A modified LKB 9000 instrument equipped with a magnetic tape recording system was employed (9). Spectra were taken at 22.5 eV by time-programmed repetitive scanning, and data were evaluated on an IBM 1800 computer. A bile acid was considered identified when retention times and mass spectrum were identical with those of an authentic compound. In some cases oxidation to the keto acids was also performed to facilitate identification (10).

RESULTS

At the beginning of this study methods based on the use of Sephadex LH-20 for separation of sulfated and nonsulfated bile acids were employed (11). The GLC–MS analyses indicated that the composition of the sulfate fraction was extremely complex. It was not possible to obtain a reliable quantitation of the bile acids due to the presence of compounds formed from metabolites of steroid hormones during solvolysis and hydrolysis. However, it was evident that the amount of sulfated bile acids was higher in female than in male rats and that bile from female rats contained an isomer of chenodeoxycholic acid (allochenodeoxycholic acid, see below) that was not present in male rats. After development of a method for bile acid analysis based on ion exchange separation of the conjugate classes (7), it became possible to carry out more accurate quantitative determinations. Two rats of each sex were then studied in detail and the results of the preliminary studies were confirmed and extended.

The bile acids identified and their occurrence in different conjugate fractions are summarized in **Table 1.** As expected, most of the bile acids in both male and female animals were found in the fractions containing taurine and glycine conjugates (about 85–90% and 10–15%, respectively). Unconjugated bile acids constituted 1–1.5% of the total in the 0–12 hr samples but were detected only in trace amounts in the 48–60 hr samples, and significant amounts of bile acids with the chromatographic properties of disulfates were found only in the 48–60 hr sample from female animals where they constituted about 0.3% of the total.

Larger amounts of bile acids were found in the monosulfate fraction of bile from female than from male rats. In the male animals 0.1–0.2% of the total bile acids were found in this fraction and, in the 0–12 hr sample, essentially all of these bile acids were isomers of 3,12-dihydroxycholanoic acid. This is seen from the fragment ion current chromatograms of *m/e* 255 and 345 (**Fig. 1**) which are prominent ions in spectra of the methyl ester trimethylsilyl ethers of these compounds. Spectra of the three isomers, found both in male and female rat bile, are shown in **Fig. 2.** The ion of mass 208 is particularly specific for 3,12-bis-trimethylsiloxycholanoic acid methyl esters (12). These bile acids disappeared with time after the cannulation of the bile duct and were essentially absent from bile in the 48–60 hr sample (**Fig. 3**).

In contrast to bile from male rats, the monosulfate fraction of bile from female rats contained 0.9–1.3% of the total bile acids. The methyl ester trimethylsilyl ether of the major compound gave a spectrum that was very similar to that of a derivative of chenodeoxycholic acid (**Fig. 4**). Both the intensity relationships between different ions and the relative retention time data indicated that the compound was a 5α isomer and comparison with authentic material showed it to be 3α,7α-dihydroxy-5α-cholanoic acid.

TABLE 1. Retention times and amounts of bile acids excreted in different conjugate fractions of bile from a male and a female bile fistula rat

			Bile Acid Excretion, 0–12 hr[b]										Bile Acid Excretion, 48–60 hr[b]									
	HiEff 8BP		U nmol		G μmol		T μmol		MoS nmol		DiS		U		G μmol		T μmol		MoS nmol		DiS nmol	
Bile Acid[a]	t_R[c]	Scan[d]	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
5αB-3α,7α,12α-ol	0.82	18	14	19	0.06	0.07	0.37	0.80	–	25	–	–	–	–	0.06	0.20	0.45	1.25	1	15	–	8
5βB-3α,6β,7α-ol	0.92	26	13	52	0.13	0.13	0.67	3.82	–	17	–	+	+	–	0.26	0.20	4.63	1.50	6	+	+	7
5βB-3α,7α,12α-ol	1.00	30	150	675	2.04	4.36	7.71	31.0	–	40	–	+	+	+	3.91	1.95	26.9	11.8	17	(–)	+	(–)
5αB-3β,7α,12α-ol	1.02	31	(–)	(–)	(–)	(–)	(–)	(–)	–		–	–	–	–	(–)	(–)	(–)	(–)		68		16
5αB-3α,12α-ol	1.20	41	–	–	–	–	–	–	2	20	–	–	–	–	–	–	–	–	–	–	–	–
5αB-3α,7α-ol	1.40	50	–	–	–	–	–	0.74	–	167	–	–	–	–	–	0.17	–	2.03	–	221	–	54
B-tetrol[e]	1.46	54	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	17	–	–	–
5βB-3α,12α-ol	1.60	59	–	–	–	–	2.02	–	9	36	–	–	–	–	–	–	–	–	1	–	–	–
5βB-3α,7α-ol	1.74	65	23	–	0.10	0.40	0.75	4.49	–	67	–	–	–	–	0.73	0.79	7.47	6.52	(–)	(–)	–	–
5αB-3β,7α-ol	1.74	65	(–)	–	(–)	(–)	(–)	(–)	–		–	–	–	–	(–)	(–)	(–)	(–)	6	47	–	–
5βB-3α,6β,7β-ol	1.83	67	27	–	0.32	0.23	1.34	1.70	–	+	–	–	–	–	0.30	0.20	6.57	1.56	–	–	–	–
ΔB-3ξ,6β,7β-ol[e]	1.95	72	30	–	0.32		0.69	0.85	–	–	–	–	–	–	–	–	–	–	–	–	–	–
5βB-3α,6α-ol	2.15	79	–	–	–	–	1.81	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
5αB-3β,12α-ol	2.18	81	–	–	–	–	–	–	23	75	–	–	–	–	–	–	–	–	–	–	–	–
ΔB-3α,6β,7β-ol[e]	2.45	88	–	–	–	–	0.37	0.85	–	–	–	–	–	–	–	–	–	–	–	–	–	–

[a] Abbreviations: B, cholanoic acid; greek letters denote configurations of hydroxyl groups.

[b] U, G, T, MoS, DiS, unconjugated, glycine-conjugated, taurine-conjugated, monosulfated, and disulfated bile acids, respectively. A plus sign indicates presence of bile acid below limits of quantitation; (–) indicates that minor amounts of the bile acid may be present and escape detection. M, male; F, female.

[c] Retention time of methyl ester TMS ether relative to the derivative of cholic acid.

[d] Number of the scan from the analyses of 0–12 hr bile from female rat shown in Fig. 1. In those cases where a bile acid occurs only in the male rat bile samples, scan numbers from these analyses are indicated.

[e] See text.

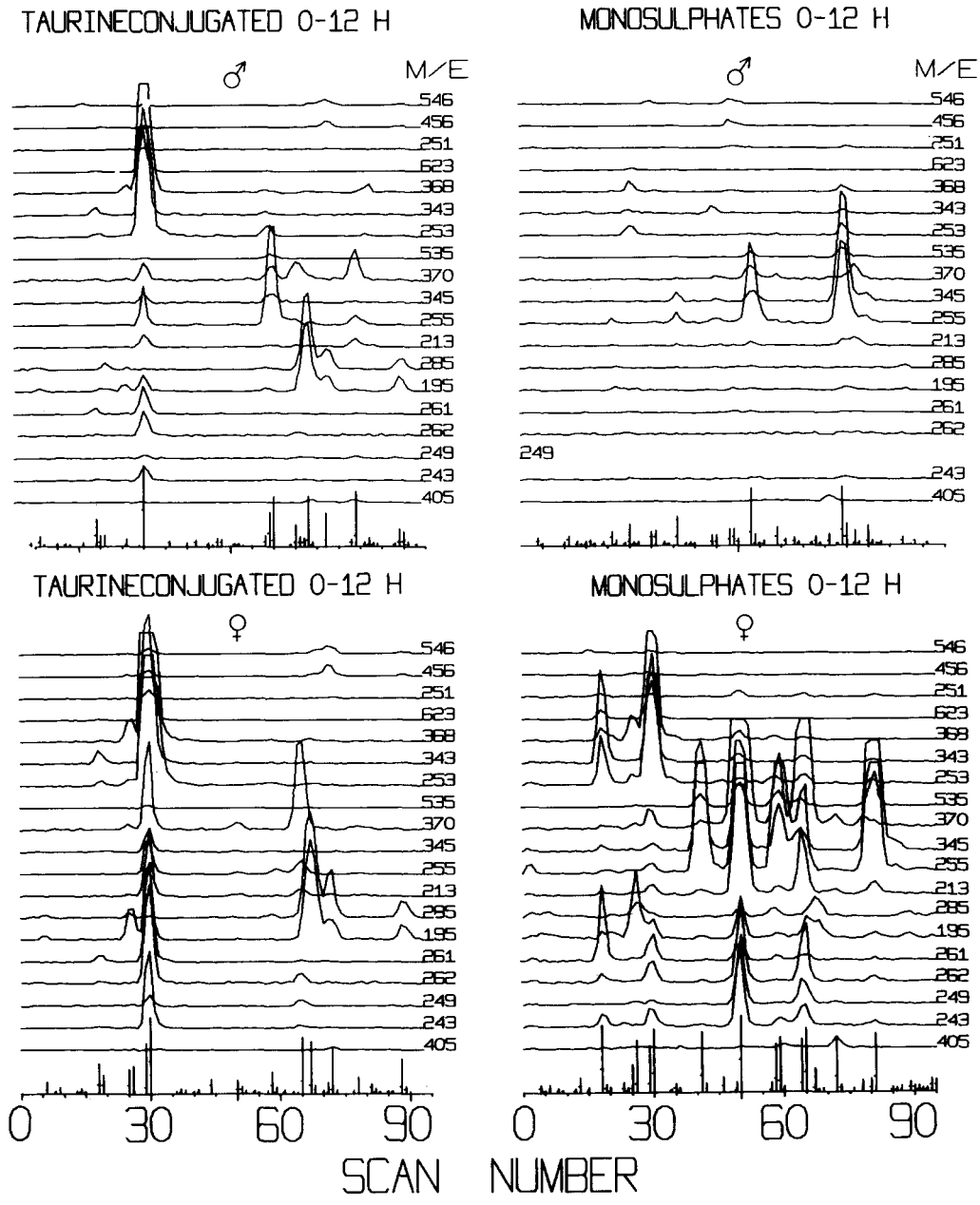

Fig. 1. Fragment ion current chromatograms constructed by the computer from the GLC–MS analyses of methyl ester trimethylsilyl ethers of bile acids isolated from the taurine conjugate (left panels) and monosulfate (right panels) fractions of bile excreted during the first 12 hr after bile duct cannulation of a male (upper panels) and a female (lower panels) rat. The first scan was started 7 min after injection of the sample. The interval between scans was 10.2 sec and was increased by 0.6 sec/min retention time. The samples from male and female rats were analyzed under slightly different conditions and the scan numbers are not exactly the same for corresponding compounds.

This acid constituted 36% of all monosulfated bile acids in the 0–12 hr sample and increased to 63% in the 48–60 hr sample. It was also present in the nonsulfated fractions of glycine- and taurine-conjugated bile acids. Thus, about 1.6% of the taurine-conjugated bile acids in the 0–12 hr samples consisted of 3α,7α-dihydroxy-5α-cholanoic acid and as much as 8.1% in the 48–60 hr samples. Ten to fifteen percent of the total 3α,7α-dihydroxy-5α-cholanoic acid was present in the sulfate fractions. The compound could not be detected in any of the fractions of male rat bile.

The fractions of glycine- and taurine-conjugated bile acids from bile of both male and female rats contained 3α,7α,12α-trihydroxy-5α-cholanoic acid (1–5% of total bile acids) (cf. Figs. 1 and 3). Although

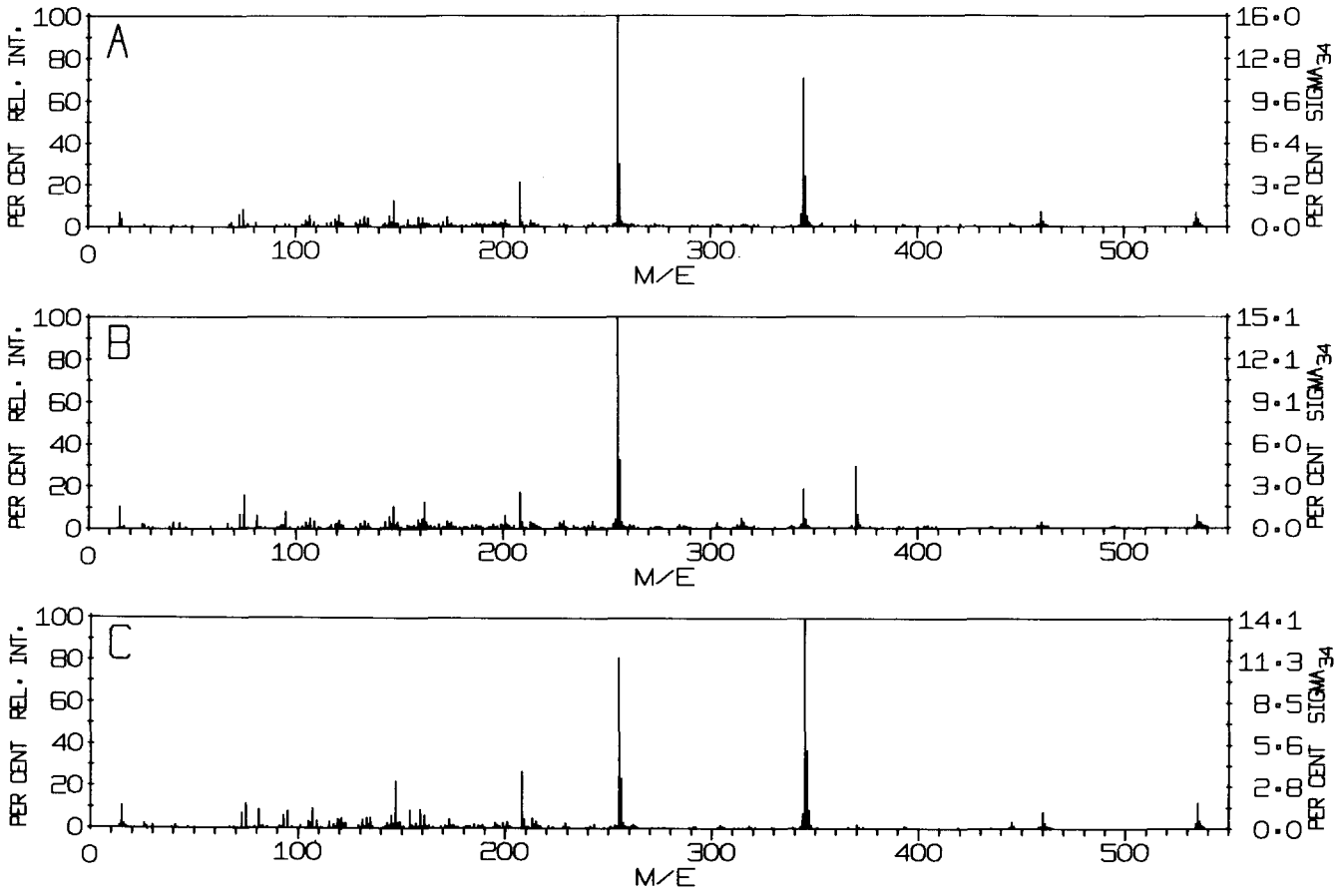

Fig. 2. Mass spectra of the methyl ester trimethylsilyl ether derivatives of three isomers of 3,12-dihydroxycholanoic acid present in the monosulfate fraction of bile collected 0–12 hr after bile duct cannulation of a female rat. *A*, derivative of 3α,12α-dihydroxy-5α-cholanoic acid; *B*. derivative of deoxycholic acid; *C*, derivative of 3β,12α-dihydroxy-5α-cholanoic acid.

present in the monosulfate fraction, this bile acid (like other trihydroxy bile acids) constituted only a small percentage of sulfated bile acids.

The monosulfate fraction of bile collected between 48 and 60 hr contained bile acids, the derivatives of which had essentially the same retention times as those of cholic and chenodeoxycholic acid on Hi-Eff 8 BP columns. The mass spectra were also similar but intensity relationships indicated that the compounds might be 5α isomers (**Figs. 3** and **5**). Oxidation with chromic acid yielded 3,7-diketo- and 3,7,12-triketo-5α-cholanoic acids as the major products. The methyl ester trimethylsilyl ether of 3β,7α-dihydroxy-5α-cholanoic acid was found to have the same retention time and mass spectrum as the derivative of the dihydroxy acid from the monosulfate fractions. The retention time and the mass spectral intensity relationships for the derivative of the trihydroxycholanoate were those expected for the derivative of 3β,7α,12α-trihydroxy-5α-cholanoic acid. However, the authentic compound was not available for direct comparison.

The monosulfate fraction from the 48–60 hr sample from male rats contained a number of unknown bile acids, several of which appeared to be unsaturated or tetrahydroxylated (see Fig. 3 scan nos. 54 and 82). Thus, several compounds gave intense peaks at *m/e* 546 corresponding to a methyl cholanoate skeleton with two trimethylsiloxy groups and two double bonds. The mass spectrum of the TMS ether of one of these compounds (scan no 54, Fig. 3) showed several similarities to the spectrum of the trimethylsilyl ether of methyl 2β,3β,7α,12α-tetrahydroxy-5β-cholanoate (kindly donated by Professor G. A. D. Haslewood) and might therefore arise from the derivative of a 2-hydroxylated bile acid.

The taurine and glycine conjugate fractions from bile of both male and female rats contained unsaturated bile acids structurally related to β-muricholic acid (13, 14). These compounds (see Fig. 1, scan nos. 72 and 88) were found only in bile collected between 0 and 12 hr, indicating that an intact enterohepatic circulation is required for their formation.

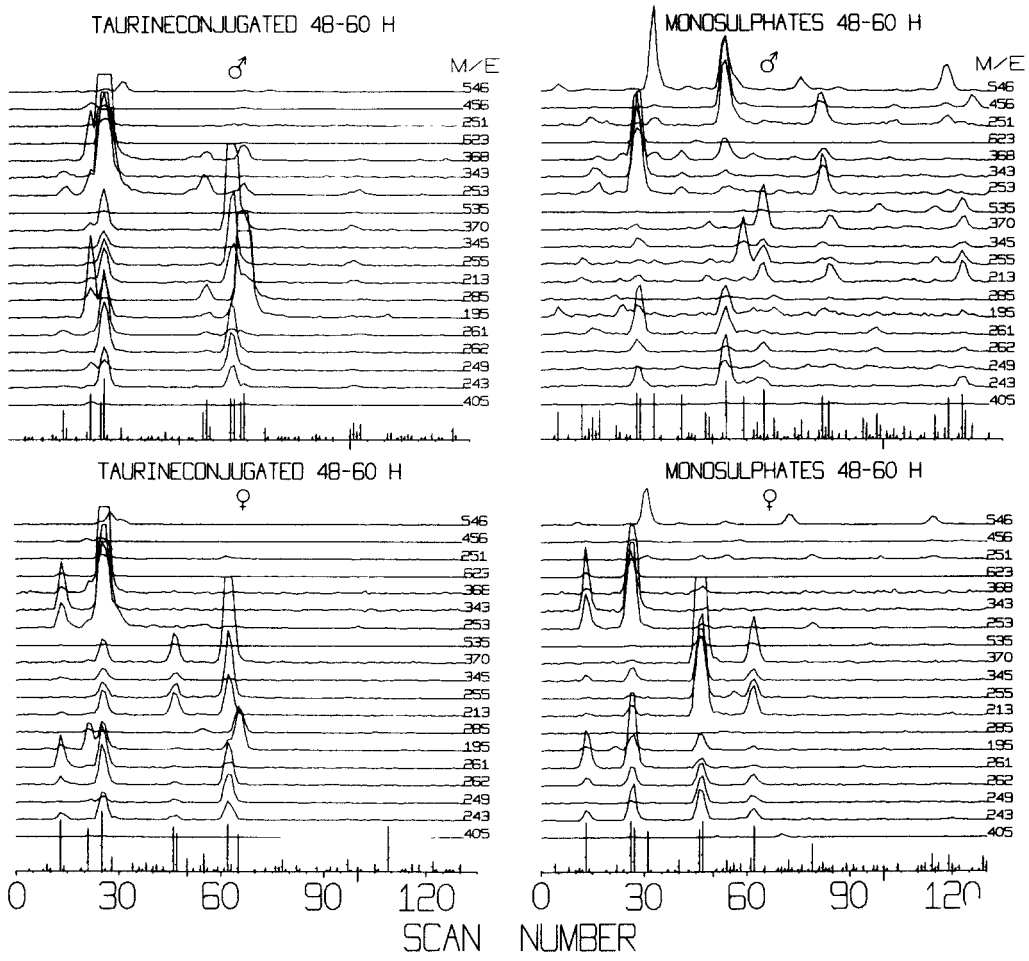

Fig. 3. Fragment ion current chromatograms constructed by the computer from the GLC–MS analyses of methyl ester trimethylsilyl ethers of bile acids isolated from the taurine conjugate (left panels) and monosulfate (right panels) fractions of bile excreted 48–60 hr after bile duct cannulation of a male (upper panels) and a female (lower panels) rat. The repetitive scan programs were about the same as in Fig. 1.

DISCUSSION

A sex difference in the bile acid composition of Wistar rat bile has been reported by Yousef, Kakis, and Fischer (15). Bile collected for 5 hr following bile-duct cannulation was analyzed, and female rats were found to excrete less β-muricholic, deoxycholic, and hyodeoxycholic acids than male rats. This difference was also seen in the present study. However, in an investigation of larger groups of Sprague-Dawley rats there was no sex difference in the excretion of β-muricholic and hyodeoxycholic acids (14). Instead, more chenodeoxycholic acid was excreted by female than male rats, consistent with a report by Beher, Casazza, and Lin (16). The bile acid composition of the samples analyzed in the present study (and that of Kern et al. (14)) was quite different from that reported by Yousef et al. (15) and by Subbiah, Kuksis, and Mookerja (17). In particular, the content of deoxycholic and hyodeoxycholic acids was much lower in our samples. This could be due to the use of different rat strains and diets, and to differences in the methods used to establish specificity of the gas–liquid chromatographic analyses. In view of the complex influence of hepatic, intestinal, dietary, and hormonal factors on the composition of the bile acid pool, it may not be meaningful to compare results from studies carried out in different laboratories. Furthermore, since chenodeoxycholic, β-muricholic, and hyodeoxycholic acids are partly interconvertible during the enterohepatic circulation, the relative amounts of these compounds may be expected to vary depending on the composition of the intestinal flora.

The two most striking sex differences in bile acid composition were the higher percentages of sulfated and allo bile acids in the female rats. This is compatible with the higher activities of sulfotransferase (18) and 5α-reductase (19–21) in female rat

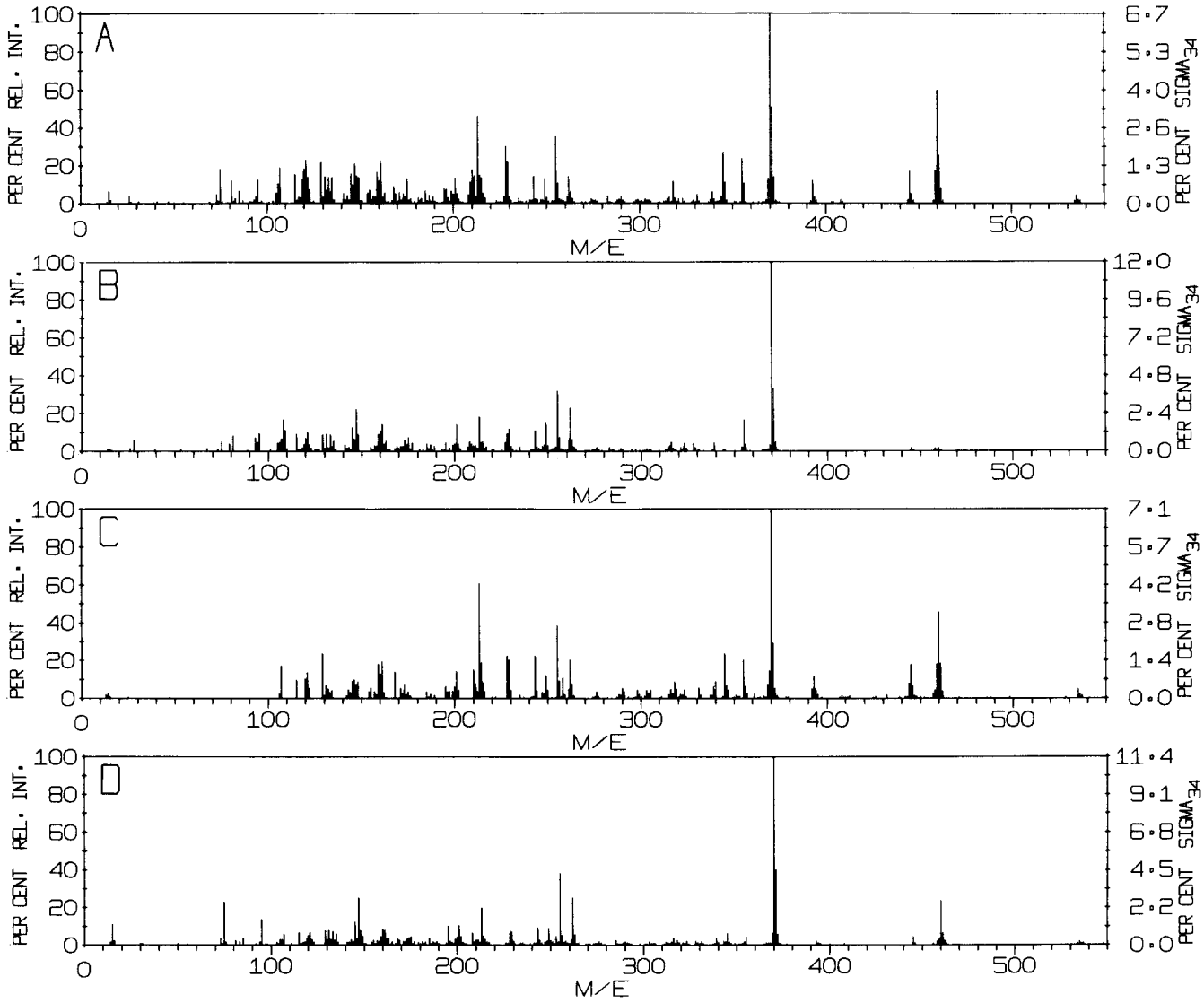

Fig. 4. Mass spectra of the methyl ester trimethylsilyl ether derivatives of three isomers of 3,7-dihydroxycholanoic acid. *A*. derivative of 3α,7α-dihydroxy-5α-cholanoic acid (from monosulfate fraction, 48–60 hr bile sample, female rat); *B*, derivative of chenodeoxycholic acid (from glycine conjugate fraction, 48–60 hr bile sample, male rat); *C*, derivative of 3β,7α-dihydroxy-5α-cholanoic acid (from monosulfate fraction, 48–60 hr bile sample, female rat); *D*, mixture of the derivatives of chenodeoxycholic and 3β,7α-dihydroxy-5α-cholanoic acids (from monosulfate fraction, 0–12 hr bile sample, female rat).

liver, previously demonstrated in vitro using C_{19}, C_{21}, and C_{27} steroids as substrates.

The sulfurylation of bile acids appears to show a marked selectivity towards allo bile acids. Predominant sulfurylation of 5α-cholanoates has also been observed in bile from cats and rabbits (22). This is in agreement with a previous study of monohydroxycholanoates in rats where 3α,5α and 3β,5α isomers were excreted predominantly in the sulfate fraction in contrast to 3β,5β and 3α,5β isomers (11). The sulfate fraction of the bile acid pool from male rats (0–12 hr sample) contained only secondary bile acids, 3β,12α-dihydroxy-5α-cholanoic acid being the major compound. This isomer of allodeoxycholic acid was also the major secondary bile acid in the sulfate fraction from female rats. It might be formed by 7-dehydroxylation of allocholic acid (23) or by bacterial isomerization of deoxycholic acid (24) combined with oxidoreduction at C-3. The latter reaction most likely occurs in the intestine since only small amounts of 3β epimer are formed when bile acids with a 3-oxo-5α structure are reduced by liver preparations (25) whereas steroids with a 3α,5α configuration are extensively epimerized in the intestinal tract (26). The apparently quantitative sulfurylation of allo bile acids with a 3β-hydroxy group explains why these compounds have not previously been found in rat bile.

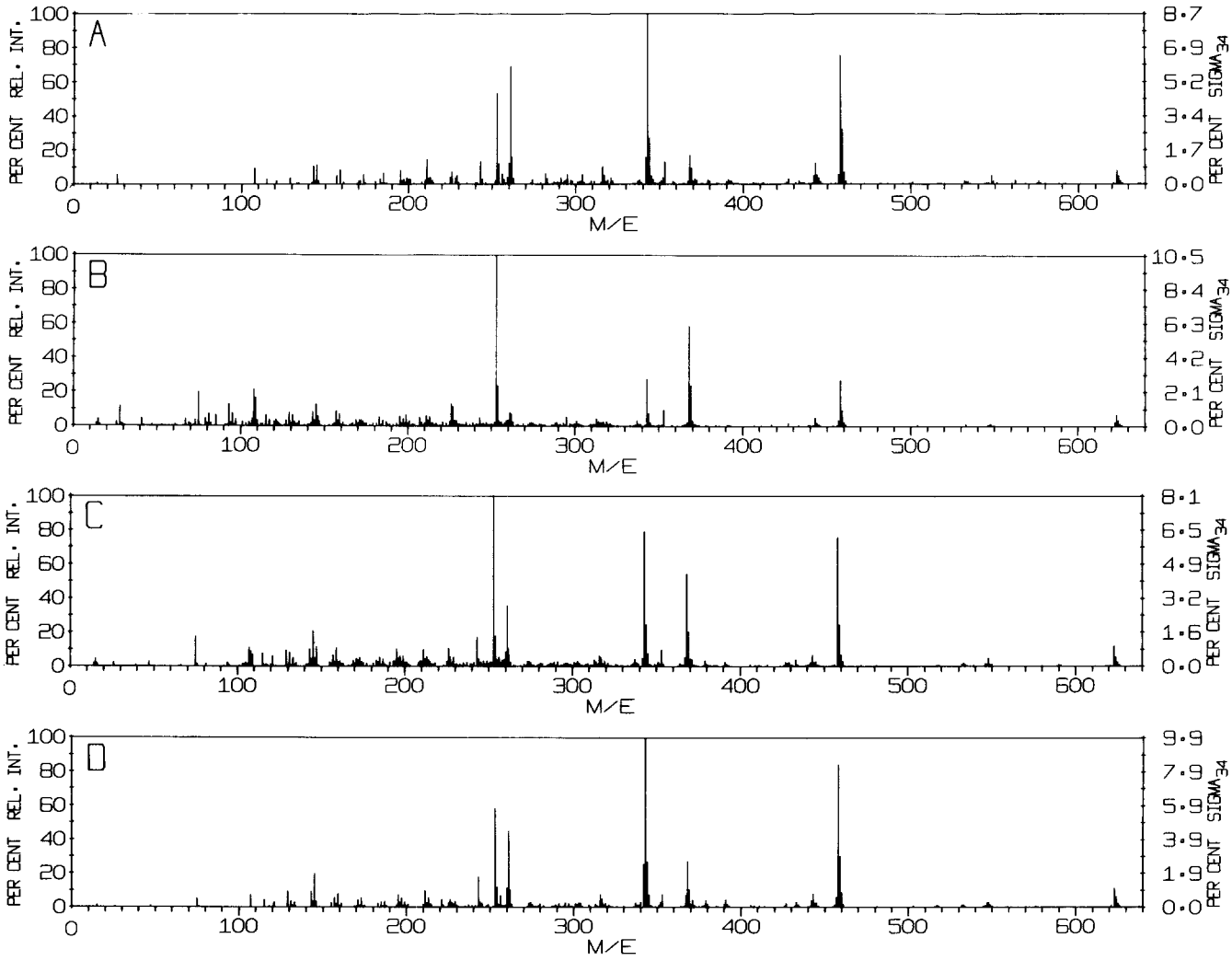

Fig. 5. Mass spectra of methyl ester trimethylsilyl ether derivatives of 3,7,12-trihydroxycholanoic acids. *A*, derivative of 3α,7α,12α-trihydroxy-5α-cholanoic acid (from taurine conjugate fraction, 48–60 hr bile sample, female rat); *B*, derivative of cholic acid (from unconjugated fration, 48–60 hr bile sample, male rat); *C*, mixture of the derivatives of cholic and 3β,7α,12α-trihydroxy-5α-cholanoic acids (from monosulfate fraction, 48–60 hr bile sample, male rat); *D*, derivative of 3β,7α,12α-trihydroxy-5α-cholanoic acid (from monosulfate fraction, 48–60 hr bile sample, female rat).

The sulfate fraction from bile of female rats contained a number of primary bile acids that were present both in the bile acid pool (0–12 hr sample) and in the fistula bile collected after 48 hr. The major bile acid was identified as allochenodeoxycholic acid. This acid was also found in the taurine conjugate fraction. Its formation was markedly sex-specific since it could not be detected in bile from the male rats. This is probably the reason why it has escaped detection in previous studies of rat bile where male rats have usually been investigated. In contrast, allocholic acid has previously been isolated from rat bile (27) and was present in bile from both male and female rats.

The reason for the more pronounced sex difference in the production of allochenodeoxycholic acid than of allocholic acid is not clear. Both compounds can be formed from 5α-cholestan-3β-ol (27, 28) and allocholic acid is the major metabolite of allochenodeoxycholic acid (29). The biosynthesis of 5β-bile acids involves the microsomal formation of 7α-hydroxy- and 7α,12α-dihydroxy-4-cholesten-3-one, which are 5β-reduced in the soluble fraction (30). However, these intermediates may also be 5α-reduced in the endoplasmic reticulum, and 5α-reductase activity is higher in female than in male rats (21). The results of Björkhem and Einarsson indicate (Fig. 1 in ref. 21) that 7α-hydroxy-4-cholesten-3-one is a better substrate than 7α,12α-dihydroxy-4-cholesten-3-one which would favor formation of allochenodeoxycholic acid via this pathway. Allocholic acid, on the other hand, is the major metabolite of 5α-cholestan-3β-ol

(27) and might therefore be formed from exogenous cholestanol in equal amounts in both sexes. Thus, it is possible that the main part of allochenodeoxycholic acid arises via 5α-reduction in the endoplasmic reticulum whereas cholestanol may be the major precursor of allocholic acid. This would explain the greater sex difference in excretion of allochenodeoxycholic acid than of allocholic acid.

Although formation of allochenodeoxycholic acid in female rats may be explained by 5α-reduction of normal intermediates in the major pathway for bile acid biosynthesis, it is also possible that it is formed via the pathways in which 3β-hydroxy-5-cholenoic (31), 3β,7α-dihydroxy-5-cholenoic (32) and 7α-hydroxy-3-keto-4-cholenoic (33) acids are intermediates. These compounds have been shown to give rise to chenodeoxycholic acid in the bile fistula rat but all studies by Usui and Yamasaki (32), Ikawa et al. (33), and by Mitropoulos and Myant (31) were carried out using male rats, and the possible formation of sulfated bile acids was not investigated. The reduction of 3-keto-4-cholenoic acid shows a marked sex difference: all four isomers of 3-hydroxycholanoic acid are formed in the male rat (34) whereas 3α-hydroxy-5α-cholanoic acid (excreted as a sulfate) is the only isomer formed in the female rat (11). From these results allochenodeoxycholic acid might be expected to be formed in larger amounts from 3-keto-4-cholenoic acid precursors in female than in male rats.

The sulfate fraction from bile collected 48 hr after bile duct cannulation of female rats contained practically no 5β bile acids. The gas–liquid chromatographic peaks with retention times similar to those of the derivative of cholic and chenodeoxycholic acids were found to be due to the derivatives of the 3β,5α isomers for which 5α-cholestan-3β-ol (35) or endogenous 3-keto-Δ^4 steroids are potential precursors. Since steroids with a 3β-hydroxy-5α-structure, presumably formed in the microsomes (19, 21), are efficiently oxidoreduced to the 3α epimers (36, 37), the relatively high percentage of 3β-hydroxy-5α-bile acids in the sulfate fraction may be due to a high rate of sulfurylation of the 3β-hydroxy group. This would prevent oxidoreduction, particularly in the female rat liver (38).

The technical assistance of Mrs. K. Brown and Mr. R. Reimendal is gratefully acknowledged. This work was supported by grants from the Swedish Medical Research Council (grant no. 13X-219) and the World Health Organization, W.T. thanks the Wellcome Trust for a travel grant.

Manuscripts received 29 November 1976 and in revised form 19 August 1977; accepted 12 September 1977.

REFERENCES

1. Bergström, S., H. Danielsson, and B. Samuelsson. 1960. Formation and metabolism of bile acids. *In* Lipide Metabolism. K. Bloch, editor. Wiley & Sons, New York. 291–336.
2. Palmer, R. H. 1967. The formation of bile acid sulfates: A new pathway of bile acid metabolism in humans. *Proc. Nat. Acad. Sci.* **58:** 1047–1050.
3. Palmer, R. H., and M. G. Bolt. 1971. Bile acid sulfates: I. Synthesis of lithocholic acid sulfates and their identification in human bile. *J. Lipid Res.* **12:** 671–679.
4. Stiehl, A. 1974. Bile salt sulphates in cholestasis. *Eur. J. Clin. Invest.* **4:** 59–63.
5. Makino, I., K. Shinozaki, S. Nakagawa, and K. Mashimo. 1974. Measurement of sulfated and nonsulfated bile acids in human serum and urine. *J. Lipid Res.* **15:** 132–138.
6. Norman, A., B. Strandvik, and Ö. Ojamäe. 1974. Urinary bile acid conjugates in extrahepatic biliary atresia. *Acta Paediat. Scand.* **63:** 97–102.
7. 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 chromatography–mass spectrometry. *J. Lipid Res.* **18:** 339–362.
8. Makita, M., and W. W. Wells. 1963. Quantitative analysis of fecal bile acids by gas–liquid chromatography. *Anal. Biochem.* **5:** 523–530.
9. Reimendal, R., and J. Sjövall. 1972. Analysis of steroids by offline computerized gas chromatography–mass spectrometry. *Anal. Chem.* **44:** 21–29.
10. Eneroth, P., B. Gordon, R. Ryhage, and J. Sjövall. 1966. Identification of mono- and dihydroxy bile acids in human feces by gas–liquid chromatography and mass spectrometry. *J. Lipid Res.* **7:** 511–523.
11. Cronholm, T., I. Makino, and J. Sjövall. 1972. Steroid metabolism in rats given [1-$^2H_2$]ethanol. Oxidoreduction of isomeric 3-hydroxycholanoic acids and reduction of 3-oxo-4-cholenoic acid. *Eur. J. Biochem.* **26:** 251–258.
12. Sjövall, J., P. Eneroth, and R. Ryhage. 1971. Mass spectra of bile acids. *In* The Bile Acids, Vol. 1. P. P. Nair and D. Kritchevsky, editors. Plenum Press, New York. 209–248.
13. Danielsson, H. 1973. Effect of biliary obstruction on formation and metabolism of bile acids in rat. *Steroids.* **22:** 567–579.
14. Kern, F., Jr., H. Eriksson, T. Curstedt, and J. Sjövall. 1977. Effect of ethynyl estradiol on biliary excretion of bile acids, phosphatidylcholines, and cholesterol in the bile fistula rat. *J. Lipid Res.* **18:** 623–634.
15. Yousef, I. M., G. Kakis, and M. M. Fischer. 1972. Bile acid metabolism in mammals. III. Sex difference in the bile acid composition of rat bile. *Can. J. Biochem.* **50:** 402–408.
16. Beher, W. T., K. K. Casazza, and G. J. Lin. 1971. Effects of age and sex on rat bile acid metabolism. *Proc. Soc. Exp. Biol. Med.* **138:** 645–650.
17. Subbiah, M. T., A. Kuksis, and S. Mookerja. 1969. Secretion of bile salts by intact and isolated rat livers. *Can. J. Biochem.* **47:** 847–854.
18. Wengle, B. 1963. Studies on ester sulphates. Sulphate

conjugation in extracts of foetal and juvenile rat liver. *Acta Soc. Med. Upsal.* **68:** 154–166.
19. Schriefers, H. 1967. Factors regulating the metabolism of steroids. *Vitam. Horm.* **25:** 271–314.
20. Shefer, S., S. Hauser, and E. H. Mosbach. 1966. Studies on the biosynthesis of 5α-cholestan-3β-ol. Cholestenone 5α-reductase of rat liver. *J. Biol. Chem.* **241:** 946–952.
21. Björkhem, I., and K. Einarsson. 1970. Formation and metabolism of 7α-hydroxy-5α-cholestan-3-one and 7α,12α-dihydroxy-5α-cholestan-3-one in rat liver. *Eur. J. Biochem.* **13:** 174–179.
22. Taylor, W. 1976. Nonsulfated and sulfated bile acids in the gallbladder bile of male and female rabbits and cats. *In* The Liver. Quantitative Aspects of Structure and Function. R. Preisig, J. Bircher, and G. Paumgartner, editors. Editio Cantor, Aulendorf. 240–248.
23. Kallner, A. 1967. Metabolism of allocholic, allochenodeoxycholic and allolithocholic acid in the rat. *Ark. Kemi.* **26:** 567–575.
24. Kallner, A. 1967. The transformation of deoxycholic acid into allodeoxycholic acid in the rat. *Acta Chem. Scand.* **21:** 87–92.
25. Kallner, A. 1967. On the reduction of 3-keto bile acids in vitro. *Ark. Kemi.* **26:** 553–565.
26. Eriksson, H., J-Å. Gustafsson, and J. Sjövall. 1969. Steroids in germfree and conventional rats. Free steroids in faeces from conventional rats. *Eur. J. Biochem.* **9:** 286–290.
27. Karavolas, H. J., W. H. Elliott, S. L. Hsia, E. A. Doisy, Jr., J. T. Matschiner, S. A. Thayer, and E. A. Doisy. 1965. Bile acids. 22. Allocholic acid, a metabolite of 5α-cholestan-3β-ol in the rat. *J. Biol. Chem.* **240:** 1568–1572.
28. Ziller, S. A., E. A. Doisy, Jr., and W. H. Elliott. 1968. Bile acids. 25. Allochenodeoxycholic acid, a metabolite of 5α-cholestan-3β-ol in the hyperthyroid rat. *J. Biol. Chem.* **243:** 5280–5288.
29. Mui, M. M., and W. H. Elliott. 1971. Bile acids. 32. Allocholic acid, a metabolite of allochenodeoxycholic acid in bile fistula rats. *J. Biol. Chem.* **246:** 302–304.
30. Danielsson, H. 1976. Bile acid metabolism and its control. *In* The Hepatobiliary System. Fundamental and Pathological Mechanisms. W. Taylor, editor. Plenum Press, New York. 389–407.
31. Mitropoulos, K. A., and N. B. Myant. 1967. The formation of lithocholic acid, chenodeoxycholic acid, and other bile acids from 3β-hydroxy-chol-5-enoic acid in vitro and in vivo. *Biochim. Biophys. Acta.* **144:** 430–439.
32. Usui, T., and K. Yamasaki. 1964. Metabolic studies of bile acids. 45. The transformation of 3β,7α-dihydroxychol-5-enic-24-^{14}C acid to chenodeoxycholic acid in the rat. The significance of the C-7α-hydroxyl group in bile acid formation. *Steroids.* **3:** 147–161.
33. Ikawa, S., Y. Ayaki, M. Ogura, and K. Yamasaki. 1972. The metabolism in vivo and in vitro of 3-oxo-7α-hydroxychol-4-enoic acid-24-^{14}C as an intermediate of chenodeoxycholic acid biogenesis. *J. Biochem.* **71:** 579–587.
34. Ogura, M., and K. Yamasaki. 1967. Non-stereospecific reduction of 3-oxochol-4-enoic-24-^{14}C acid in the rat. *Steroids.* **9:** 607–622.
35. Noll, B. W., S. A. Ziller, Jr., E. A. Doisy, Jr., and W. H. Elliott. 1973. Bile acids. 37. Identification of the 3β isomers of allocholic and allochenodeoxycholic acids as metabolites of 5α-cholestanol in the rat. *J. Lipid Res.* **14:** 229–234.
36. Björkhem, I., and J. Gustafsson. 1971. On the conversion of cholestanol into allocholic acid in rat liver. *Eur. J. Biochem.* **18:** 207–213.
37. Noll, B. W., E. A. Doisy, Jr., and W. H. Elliott. 1973. Bile acids. 39. Metabolism of 5α-cholestane-3β,26-diol and 5α-cholestane-3β,7α,26-triol in the rat with a bile fistula. *J. Lipid Res.* **14:** 391–399.
38. Baillie, T. A., H. Eriksson, J. E. Herz, and J. Sjövall. 1975. Specific deuterium labelling and computerized gas chromatography–mass spectrometry in studies on the metabolism in vivo of a steroid sulphate in the rat. *Eur. J. Biochem.* **55:** 157–165.