Effect of ethynylestradiol on biliary excretion of bile acids, phosphatidylcolines, and cholesterol in the bile fistula rat[1]

Fred Kern, Jr.,[2] Håkan Eriksson, Tore Curstedt, and Jan Sjövall

Department of Chemistry, Karolinska Institutet, Stockholm, Sweden

Abstract The effects of ethynylestradiol on endogenous bile acids, their capacity to conjugate and excrete intravenously infused cholic acid, the concentrations of biliary cholesterol and lecithin, and the individual molecular species of phosphatidylcholine have been determined in male and female Sprague–Dawley rats. Endogenous biliary bile acids were analyzed by gas–liquid chromatography–mass spectrometry. Eleven bile acids were identified and several minor bile acids, primarily muricholates, could not be completely characterized. After 5 days of treatment with ethynylestradiol (1 mg/kg per day), the percentage of cholic acid decreased and the percentage of 6β-hydroxylated bile acids, including several monounsaturated species, increased. Ethynylestradiol caused a decrease in bile acid-independent bile flow. Intravenous infusion of cholic acid at a high concentration caused cholestasis in control animals but, after ethynylestradiol treatment, cholestasis developed during the infusion of a much lower concentration of cholate, indicating a lowered threshhold for bile acid-induced cholestasis. In the treated rats, there was a slight increase in excretion of unconjugated endogenous bile acids, and a striking impairment of conjugation of intravenously administered cholic acid. One of the few sex-related differences observed was an increased concentration of biliary phospholipids in untreated male rats. Both phospholipid and cholesterol concentrations in the bile were higher in the treated animals. The molar percentage of cholesterol was always 1–2%, but it was slightly higher in treated animals, especially males. Ethynylestradiol treatment also affected biliary phospholipid by causing a marked increase of phosphatidylcholine species containing palmitic and oleic acid residues and a decrease of species containing stearic and linoleic acid residues. There was no increase in biliary excretion of long chain polyunsaturated species, which might have indicated damage to membranes, in response to ethynylestradiol either alone or with cholic acid infusion. Some of these ethynylestradiol-induced changes in biliary bile acid and lipid excretion are probably peculiar to the rat, but others, such as the increase in molar percentage of cholesterol and cholestasis, may be relevant to disorders in man, especially cholesterol gallstones and idiopathic cholestasis of pregnancy.

Supplementary key words gas–liquid chromatography–mass spectrometry · biliary cholesterol · bile acid conjugation · [24-^{14}C]cholic acid infusion · cholestasis · bile flow

Many investigators have studied the hepatic effects of natural and synthetic estrogens, especially ethynylestradiol, a common estrogenic component of contraceptive steroids. In the rat, estrogens increase liver size and water content, RNA, DNA, and protein content. They decrease the bile salt-independent fraction of bile flow and the maximal transport capacity for bile acids, bilirubin, and bromosulfophthalein (BSP) (1–6). Ethynylestradiol reduces hepatic cytochrome P-450 activity (7) and inhibits microsomal hydroxylation of steroids in vitro (8). It is not known, however, to what extent it affects hydroxylation and conjugation reactions involved in bile acid formation in vivo and whether changes in bile acid metabolism, especially conjugation, are related to the cholestatic effect of ethynylestradiol. In this study, we examined the effect of ethynylestradiol on bile acid metabolism in vivo by analyzing bile acids in bile of both male and female rats given this drug. We also determined the capacity of these rats to conjugate and excrete cholic acid infused intravenously and related the results to the cholestatic effect of ethynylestradiol.

Ethynylestradiol, other estrogens, and contraceptive steroid preparations are also known to alter the composition of biliary lipids in several animal species, including man, so that the bile becomes more saturated with cholesterol (9–11). This is consistent with the clinically important observation that cholesterol gallstones occur more frequently in women during the childbearing age than in men (12, 13) and that contraceptive steroids cause an increase in

[1] Published in part in Advances in Bile Acid Research, 1975. S. Matern, J. Hackenschmidt, P. Back, and W. Gerok, editors. F. K. Schattauer Verlag, Stuttgart, Germany, and in abstract form in *Gastroenterology* **67:** A-25/802, 1974.

[2] Permanent address: University of Colorado Medical Center, 4200 East Ninth Avenue, Denver, CO 80220.

Abbreviations: GLC, gas–liquid chromatography; MS, mass spectrometry; EE, ethynylestradiol, TMS, trimethylsilyl.

the incidence of gallstones (14, 15). The effect of ethynylestradiol on biliary lipid composition in the rat has not been carefully studied. In the experiments reported here we measured the concentrations of cholesterol and lecithin in the bile of treated rats.

The nature of the cellular effects of ethynylestradiol on the liver is unknown. It is possible that this synthetic estrogen damages cell membranes either directly or indirectly, even though such damage is not seen by electron microscopy (8). Injury to membranes might result in the biliary excretion of phosphatidylcholine species with long chain polyunsaturated fatty acids, derived from membranes. The individual molecular species of biliary phosphatidylcholine were, therefore, determined.

These studies provide a fairly comprehensive understanding of the effects of a large dose of ethynylestradiol on bile acids and biliary lipids in the rat.

MATERIALS AND METHODS

Animals

Sprague–Dawley rats, weighing 200–250 g at the beginning of the experiment, were housed in clean animal quarters and allowed free access to pelleted food and water. They were given daily subcutaneous injections of ethynylestradiol (17α-ethynyl-1,3,5-estratriene-3,17β-diol) (1 mg/kg per day) dissolved in 0.1 ml of propylene glycol for 7 days or an equivalent amount of propylene glycol alone. There were 4–6 rats in each group. Several slightly different experimental designs were used.

A polyethylene cannula (PE 10) was inserted into the bile duct proximal to the pancreatic ducts and a similar cannula was placed in either a saphenous or femoral vein of ether-anesthetized rats. All animals were placed in restraining cages. Collection of bile was started immediately after the operation. An intravenous infusion of a solution of glucose (0.28 M) and NaCl (0.078 M) was also begun immediately and maintained at a constant rate (0.03546 ml/min) by means of an infusion pump. After 3–4 hr of biliary drainage, the infusion solution was changed to one containing 30 mM sodium cholate (prepared from recrystallized cholic acid, pure according to thin-layer and gas–liquid chromatography) and [24-^{14}C]cholate (about 50–60 $\times$ 10^4 dpm/ml solution, The Radiochemical Centre, Amersham, sp act 53 mCi/mmol, 99% pure) and 2.0% bovine serum albumin (Sigma Co., St. Louis, MO) in addition to the glucose and NaCl (pH 7.4). This infusion was continued at the same rate for 130 min, delivering 1.06 μmol of cholate/min, or 0.43 μmol/min per 100 g.

Bile was collected in tared test tubes. After the first 10 min, samples were collected and weighed every 20 min for 2 hr. If the bile flow was intermittent in any animal, the data were discarded. Both male and female rats were studied.

In another series of control and treated female rats, 30 mM taurine (Sigma Co.) was included in the infusion with 30 mM sodium cholate. There were no other changes in the protocol.

In a separate series of experiments with female rats, solutions containing different concentrations of sodium cholate were used. Each solution was given intravenously at the rate mentioned above (0.03546 ml/min) for 45 min. In these experiments a tracer amount of [24-^{14}C]cholate was added to the glucose–NaCl solution initially infused in order to label the endogenous cholate. This solution was infused for 20 min and then replaced by the unlabeled glucose–saline solution for 2–3 hr. The bile acid solutions were then administered in the following sequence: 3.75, 7.5, and 15 mM sodium cholate, labeled with [^{14}C]cholate. Bile secreted during the second and third 15-min periods was used for analysis.

In further experiments also involving female rats only, either 60, 90, or 120 mM sodium cholate was infused at the same pump rate for 1 hr. These animals were studied under ether anesthesia almost immediately after bile duct and femoral vein cannulation. Each rat was given only one bile salt solution. Each concentration of sodium cholate was given to three control and three treated rats. The infusion solution contained 4 g/100 ml of albumin, which usually prevented hemolysis.

In all experiments, body temperature was maintained at 37–38°C by a heat lamp.

Analysis of bile

Radioactive bile acids. The radioactivity in each sample of bile collected after the beginning of the cholate infusion was assayed in a liquid scintillation spectrometer (Model 2425, Packard Instrument Co., Downers Grove, IL) using an automatic external standard for quench correction. In some experiments two bile samples from each rat, usually the first and last 20-min specimens collected during the cholate infusion, were analyzed for cholic acid conjugates. Unconjugated and glycine-conjugated bile acids were separated from taurine conjugates by solvent extraction as described by Levine (16). Usually 0.1 ml of bile was added to 1 ml of water, acidified with HCl, and then extracted three times with ethyl acetate. The ethyl acetate fraction, containing the glycine-conjugated and unconjugated bile acids, was washed with water until neutral, dried under a stream of

nitrogen, and reconstituted to a known volume. A fraction of this was taken for radioactivity measurement and the remainder was analyzed by thin-layer chromatography (silica gel G plates, Merck, Darmstadt) together with appropriate standards. The plates were developed in ethyl acetate–methanol–acetic acid 70:30:10 (by vol.). After detection of the compounds by iodine vapor the distribution of radioactivity was determined either with a radiochromatogram scanner (Berthold, Karlsruhe, Germany), or by scraping the silicic acid and counting the radioactivity. Agreement between the two techniques was excellent.

Gas–liquid chromatographic analysis of bile acids. (For details see ref. 17, 18.) Aliquots of bile samples collected before cholate infusion were added to 10–20 vol of 95% ethanol during agitation in an ultrasonic bath. After centrifuging and removing the ethanol, the residue was hydrolyzed with 15% NaOH in 50% aqueous ethanol at 110°C for 10 hr. The hydrolysate was acidified and diluted with water and the bile acids were extracted with ethyl acetate. The solvent was removed and the bile acids were methylated with diazomethane and converted to trimethylsilyl ethers (17). The derivatives were analyzed by GLC using glass columns, 3.5 m × 3.6 mm, filled with 1–2% HiEff 8 BP (17) on Gas-Chrom Q 80–100 mesh (Applied Science Labs., State College, PA) at 220–230°C. Care was taken not to use the columns at higher temperatures since separation factors decreased and the columns deteriorated rapidly. Quantitative results were obtained by comparing peak areas with those given by known amounts of reference compounds. In those cases where authentic compounds were not available, a structurally related bile acid derivative was used as reference.

For gas–liquid chromatographic measurement of unconjugated bile acids, an aliquot of bile was added to 10 volumes of water; it was then acidified, sonicated, and extracted into ethyl acetate, as described above. The unconjugated bile acids in the ethyl acetate were methylated with diazomethane and the trimethylsilyl ether derivatives were prepared and analyzed as above.

Identification of bile acids. The methyl ester trimethylsilyl ethers were analyzed for GLC–MS using HiEff 8 BP (as above) and SE-30 (1.5% on Chromosorb W HP, 80–100 mesh) as stationary phases. A modified LKB 9000 instrument (LKB Instruments, Rockville, MD) with a magnetic tape recording system was used (19, 20). Data were evaluated on an IBM 1800 computer (17, 20, 21). A bile acid was considered identified when the retention times and mass spectra were identical with those of authentic compounds (reference compounds were those used in previous studies from this laboratory (22)).

A number of unknown bile acids were found, which were isolated and partially characterized in the following way. A pooled sample of hydrolyzed bile was methylated, and the methyl esters were applied to thin-layer plates (80 μg/cm starting line). The plates were developed in benzene–isopropanol–acetic acid 30:10:1 (by vol.) according to Eneroth (23). The plates were divided into seven zones according to the distribution of compounds as visualized by spraying a strip of the plate with sulfuric acid. The zones were scraped off the plate on to 200 mg Unisil (Clarkson Co., Williamsport, PA) columns prepared in benzene. The columns were eluted with 3 ml each of benzene, 30% ethyl acetate in benzene, ethyl acetate, and 50% methanol in ethyl acetate. All fractions were analyzed by GLC after preparation of trimethylsilyl ethers. The major unknown compounds were found in the ethyl acetate fraction of the zone that contained muricholates (lower mobility than methyl cholate).

The unknown bile acids were analyzed by GLC–MS of the methyl ester trimethylsilyl ether derivatives before and after hydrogenation or reaction with acetone. Hydrogenation was performed in 1 ml of ethanol using 1 mg of palladium on charcoal (24). Acetonides were prepared with $CuSO_4$ as catalyst (24).

Analysis of cholesterol. A known amount of 5β-cholestan-3β-ol, as internal standard, and 50–100 μl of bile were added to 1–2 ml of 70% aqueous ethanol during agitation in an ultrasonic bath. The solution was extracted with hexane, 1–2 ml, and the extracted sterols were converted to trimethylsilyl ethers which were analyzed by GLC on a 1.5% SE-30 column at 240°C. Peak areas were determined by triangulation and cholesterol concentration was calculated from the relationship of its peak area to that of the internal standard. Duplicate analyses were within 10% of each other.

Analysis of phosphatidylcholines. Biliary phospholipids were extracted with chloroform–methanol according to Folch et al. (25) and lipid-soluble phosphorus was determined (26). The phosphatidylcholines were isolated by chromatography on the anion exchanger butylaminohydroxypropyl Sephadex LH-20 in the acetate form (27, 28). Phospholipase C hydrolysis yielded diglycerides which were separated as trimethylsilyl ethers according to number of carbons and degree of unsaturation by reversed-phase chromatography on Lipidex-5000 (28). GLC on 1.5% SE-30 columns of fractions from this chromatography yielded the percentage compositions of individual molecular species of the diglycerides. Trimyristin was used as external standard.

TABLE 1. Relative retention times[a] of TMS ethers of substituted methyl cholanoates found in rat bile

Peak	Rel. Ret. Time	Structure of Bile Acid
1	0.80	3α,7α,12α-trihydroxy-5α-cholanoic acid
2	0.91	3α,6β,7α-trihydroxy-5β-cholanoic acid
3	1.00	3α,7α,12α-trihydroxy-5β-cholanoic acid
4	1.33	3α,7α-dihydroxy-5α-cholanoic acid
5	1.38	3α,6α,7α-trihydroxy-5β-cholanoic acid
6	1.51	3α,7β,12α-trihydroxy-5β-cholanoic acid
7	1.55	3α,12α-dihydroxy-5β-cholanoic acid
8	1.71[b]	3α,7α-dihydroxy-5β-cholanoic acid
9	1.77[b]	3α,6β,7β-trihydroxy-5β-cholanoic acid
III	1.80[c]	Compound III (see Table 2)
I	1.86[b]	Compound I (see Table 2)
10	2.04	3α,6α-dihydroxy-5β-cholanoic acid
II	2.31	Compound II (see Table 2)
11	2.63	3α,7β-dihydroxy-5β-cholanoic acid

A Hi-EFF 8 BP (1.5% on Gas Chrom Q) column (2.7 m × 3.4 mm) was used at 220°C in the GLC–MS instrument.

[a] TMS ether of methyl cholate = 1.00.

[b] Under the conditions of quantitative analysis when a lower temperature and a longer column are used these compounds are separated (see Fig. 1).

[c] Under the conditions of quantitative analysis this compound appears together with β-muricholic acid derivatives.

Statistical evaluation. Standard deviations were calculated using Yates correction for small numbers. Data are presented as mean ± 2 standard errors. Differences between means were evaluated by Student's *t* test.

RESULTS

Treated animals lost 5–10% of body weight. Lighter animals were used for controls and heavier ones for treatment so that, at the time of the experiment, their weights were nearly identical.

Identification of bile acids

The bile acids found in the samples studied are listed in **Table 1**. Several of the compounds were present in very small quantities and their presence was disregarded in the subsequent quantitations. GLC analyses of total bile acids in bile from female rats treated with ethynylestradiol are shown in **Fig. 1**.

Two of the major compounds, I and II, shown in Fig. 1 and Table I remain unidentified. The mass spectra of the trimethylsilyl ethers had intense peaks at *m/e* 285 and 195 typical of the 6ξ,7β-bis-trimethylsiloxy structure. Both compounds gave peaks at *m/e* 636, 546, and 456, indicating that they were either tetrahydroxycholanoates or trihydroxycholanoates with one double bond. Compound I showed an ABCD-ring fragment ion at *m/e* 251 and a side chain ion at *m/e* 115, indicating that the substitution

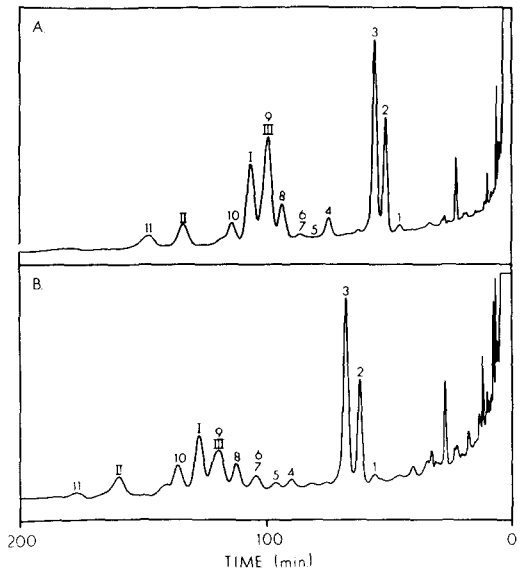

Fig. 1. GLC tracing of total bile acids from two female rats (*A* and *B*) treated with ethynylestradiol. The trimethylsilyl ethers of methyl cholanoates were separated on Hi-Eff 8BP (1.5% on Gas Chrom Q) at 220°C. The numbered peaks are identified in Table 1.

was in the ring system (29). Compound II, on the other hand, gave an ABCD-ring fragment ion of mass 253 and no definite peak at *m/e* 115, indicating that the substitution was in the side chain. Both compounds formed acetonides, indicating a *cis*-glycol structure, and both compounds could be hydrogenated (**Table 2**). Upon hydrogenation, compound II gave β-muricholate. Hydrogenation of compound I, on the other hand, yielded a product the trimethylsilyl ether of which had a retention time shorter than that of β-muricholate, while the mass spectra of the two compounds were identical.

In conclusion, compound I appears to be a bile acid with a 6β,7β-dihydroxy structure and a double bond

TABLE 2. Retention time[a] characteristics of derivatives of bile acids from rat bile showing mass spectrometric properties similar to those of derivatives of β-muricholate

	Relative Retention Times (TMS Ethers)				
	Untreated		Hydrogenated		
Compound	SE-30	Hi-Eff 8BP	SE-30	Hi-Eff 8BP	Acetonide SE-30
β-Muricholate	1.26	1.75	1.26	1.75	1.26
I	1.30	1.83	1.15	1.58	1.22
II	1.37	2.33	1.26	1.75	1.37
III	1.15	1.75	—[b]	—[b]	1.11
IV	1.60	3.09	1.62	3.12	—[b]
V	1.65	3.19	—[b]	—[b]	—[b]
VI	1.75	4.10	—[b]	—[b]	—[b]

[a] TMS ether of methyl cholate = 1.00.

[b] Not known.

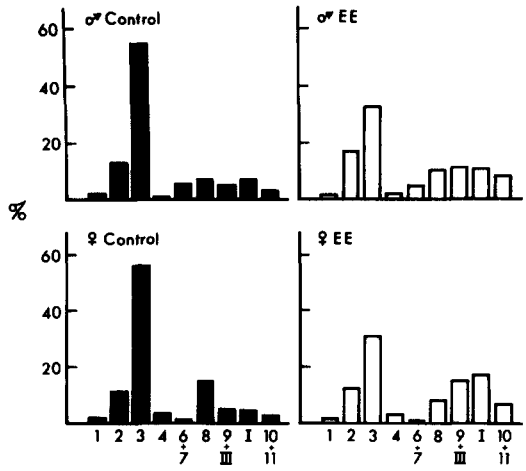

Fig. 2. The mean distribution of total bile acids in male and female rats, treated and control. The numbers refer to the peaks, as shown in Fig. 1 and identified in Table 1.

in the ring system. Compound II appears to be a β-muricholic acid having a double bond in the side chain.

In addition to compounds I and II there were at least four other bile acids showing the typical peaks at *m/e* 285 and 195 in the spectra of the trimethylsilyl ethers (compounds II–VI, Table 2). With the exception of compound IV, these bile acids also appeared to be unsaturated, compounds III and VI in the side chain and compound V in the ring system. Since compound III had a retention time close to that of β-muricholate its presence will influence the apparent quantity of the latter acid. Repetitive scanning GLC with construction of fragment ion current chromatograms of *m/e* 546 (for compound III) and *m/e* 548 for (β-muricholate) indicated that compound III might account for 10–50% of the area of the "β-muricholate" peak in gas chromatograms obtained with a flame ionization detector (see Fig. 1B).

Distribution of bile acids

The bile excreted during the first 20 min following bile duct cannulations was analyzed with respect to composition of total (**Fig. 2**) and free bile acids.

There was a higher concentration of bile acids in one female series (mean 31.1 ± 3.9 mM) than in the males (mean 18.8 ± 0.89 mM). The reason for this is not known, but it is not believed to be a true sex difference since another series of female rats had about the same bile acid concentration (17.8 ± 2.0 mM) as the males. The percentage of chenodeoxycholic acid was higher in the untreated female, 14.7%, than in the untreated male rats, 7.6% ($P < 0.01$). Female rats excreted 3α,7α-dihydroxy-5α-cholanoic acid (allochenodeoxycholic) which constituted about 3% of the total bile acids, whereas only half of the untreated male rats excreted this bile acid (0–2% of the total) ($P < 0.001$). The percentage of compounds 6 and 7 was higher in the male, 5.9%, than in the female controls, 1.0% ($P < 0.001$). There were no other sex-related differences in distribution of the bile acids.

Ethynylestradiol administration resulted in a marked increase in the percentage of β-muricholic acid in both sexes. In the male, it increased from 5.9 to 11.8% ($P < 0.01$) and in the female, from 5.5 to 15.4% ($P < 0.02$). There was no change in percentage of α-muricholic acid. The relative amount of cholic acid decreased considerably in both sexes, from 55% in the untreated to 33% in the treated ($P < 0.001$) rats. Although the concentration of bile acids in the bile of different animals varied much more than the relative amounts of individual bile acids, the proportion of cholic acid was always lower and that of the 6-hydroxylated bile acids higher in treated than in control rats (**Table 3**). Chenodeoxycholic acid showed less marked changes; its contribution to the bile acid pool decreased in female and increased in male rats, but neither change was significant. Compound I increased significantly in EE-treated rats of both sexes.

Unconjugated bile acids were present in a relatively high concentration in the bile from the male rats. They constituted about 10% of the total bile acid in untreated and about 15% in treated male rats. The reason for the high concentration of unconjugated bile acids is not known; in other studies unconjugated bile acids constituted a much lower percentage. The major compounds in this fraction were cholic acid and 6-hydroxylated trihydroxy bile acids. The quantitative analyses of this fraction were performed using an SE-30 column, which does not separate all individual bile acids identified by means of GLC–MS on the Hi-Eff 8BP column. Thus, three groups of bile acids were determined: *1*) cholic + α-muricholic acids,

TABLE 3. Major changes in bile acid composition caused by ethynylestradiol

	Control	EE-treated	*P*
	Percent		
Cholic acid			
Male[a]	55.1 ± 2.6[b]	33.3 ± 1.6	<0.001
Female	54.5 ± 3.4	32.6 ± 5.0	<0.01
6-Hydroxylated bile acids			
Male	28.8 ± 1.8	48.8 ± 2.0	<0.001
Female	31.2 ± 2.2	49.9 ± 2.8	<0.01

[a] There were six male and five female animals.
[b] Mean ± 1 SEM.

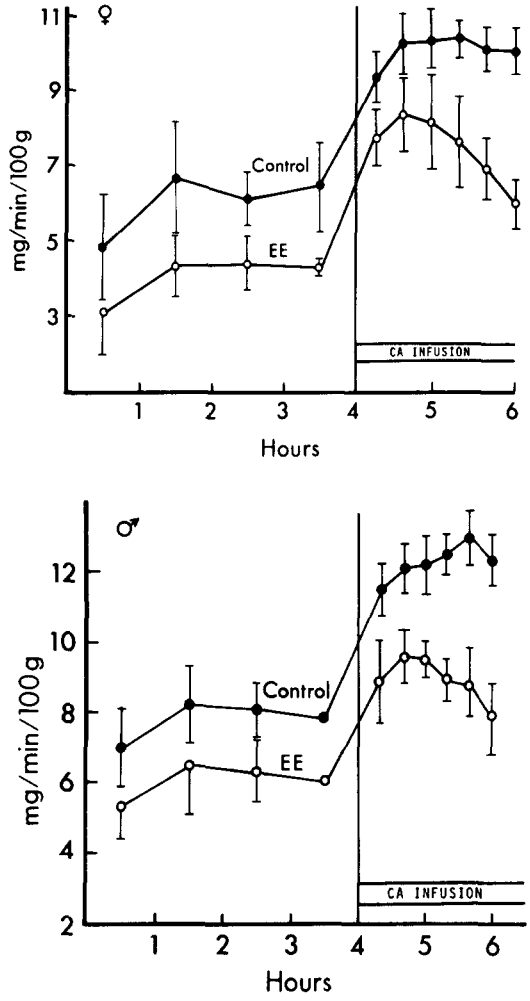

Fig. 3. Bile flow in female (*A*) and male (*B*) rats, showing the cholestatic effect of ethynylestradiol (EE) and the cholestatic effect of cholic acid (CA) infusion (0.43 μmol/min per 100 g) in both groups. Note the decrease in bile flow in the EE-treated animals, beginning about 40 min after the start of the CA infusions.

2) β-muricholic acid + compounds I and II, and *3*) compounds IV and V. The relationships between these groups were 0.56 ± 0.02: 0.37 ± 0.02: 0.07 ± 0.01 in control male rats which changed to 0.42 ± 0.03: 0.47 ± 0.02: 0.1 ± 0.01 when EE was given. No unconjugated dihydroxy bile acids were seen. The female rats excreted much less unconjugated bile acids: about 0.1% of the total bile acids in untreated and 0.5–1% in the treated animals. The ratios for these groups of bile acids described above were not calculated in females because of the low concentrations of the unconjugated bile acids. The main compound in all female rats was cholic acid.

Bile flow

The bile flow was measured for 4 hr following bile duct cannulation. Treatment with ethynylestradiol resulted in a decreased bile flow in both sexes (**Fig. 3**). When cholate was infused at a constant rate, the bile flow increased in all groups of animals and reached a constant level in the control rats in less than one hour. In treated rats of both sexes maximum bile flow was achieved after about 40 min of infusion. The flow then decreased steadily until the end of the experiment, suggesting that bile salt had induced cholestasis.

As the amount of cholate infused was increased, there was an initial linear increase in bile flow that was essentially the same in control and treated rats (**Fig. 4B**). Above a certain concentration of cholate, however, the rate of bile flow abruptly decreased. The concentration at which this bile salt-induced cholestasis occurred was very much lower in the treated than in the control animals.

Cholate excretion during infusion

The biliary cholic acid excretion was calculated from the volume and radioactivity of the bile and the specific activity of the cholate infused. When 1.06

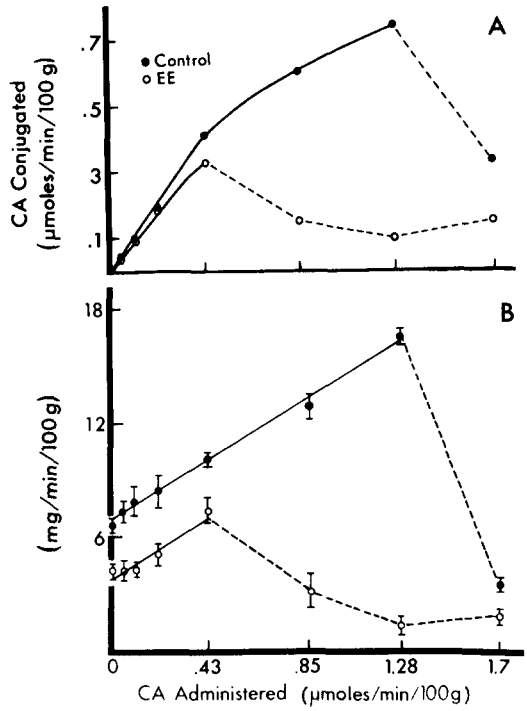

Fig. 4. *A*. The amount of biliary [^{14}C]cholic acid conjugated plotted against the amount administered in control (● —— ●) and EE-treated (○ —— ○) animals. The dashed lines connect the points obtained after cholestasis occurred. *B*, Bile flow in control and ethynylestradiol (EE) treated rats during infusion with increasing concentrations of cholic acid (CA). The data shown are the means ± 2 SEM. The solid lines represent the best fits to the data during the increasing bile flow. The dashed lines connect the data points obtained after bile flow diminished, which occurred with a much lower concentration of CA in the treated than in the control rats.

TABLE 4. Distribution of [^{14}C]cholate

	After 40 Min of Cholate Infusion[a]				After 120 Min of Cholate Infusion[b]			
	No Taurine[c]		Taurine Included[d]		No Taurine		Taurine Included	
	C	EE	C	EE	C	EE	C	EE
	%		%		%		%	
TC	91.7	75.8	90.9	75.9	82.5	54.3	90.0	79.4
GC	0.7	-0-	0.9	1.2	8.6	17.5	1.0	1.7
Unconjugated cholate	7.6	24.2	8.2	22.9	8.8	28.2	9.0	18.9
P[e]	<0.001		<0.001		<0.001		<0.01	

[a] Bile collected 30–50 min after cholate infusion started at 1.06 μmol/min (30 mM).
[b] Bile collected 110–130 min after cholate infusion started.
[c] 4–6 female rats in each group.
[d] 30 mM taurine included with cholate in infusion which is described in detail in text.
[e] Significance of differences in percentage of unconjugated cholate excreted by control and EE-treated animals.
C—Control EE-treated with ethynylestradiol.
TC—Taurocholate; GC—glycocholate.

μmol of cholate was infused per min, the treated males excreted 91.9 ± 1.1% of the [^{14}C]cholate each 20-min period compared to 95.4 ± 1.3% excreted by the male controls ($P < 0.01$). The treated females excreted only 83.5 ± 9.2% vs 96.2 ± 2.2% for the controls ($P < 0.02$). Since ethynylestradiol caused a decreased bile flow, the mean concentration of biliary cholate was significantly higher in treated animals of both sexes ($P < 0.001$). In all animals it was higher in the bile than in the infused solution.

Conjugation of infused cholate

During infusion of 1.06 μmol × min^{-1} significantly more unconjugated cholic acid was excreted by treated rats. The bile secreted during the early part of the cholate infusion contained approximately the same amount of unconjugated cholate as the bile secreted at the end of the infusion, but there was an increase in glycine-conjugated and a corresponding decrease in taurine-conjugated cholate. When 30 mM taurine was included in the infusion, the glycine-conjugated cholate decreased to a very small amount, but there was no significant change in the percentage of unconjugated cholate (**Table 4, Fig. 5**), indicating that the failure of conjugation was not due to taurine depletion.

When the amount of cholate conjugated is plotted against the amount administered (Fig. 4*A*), there is a linear increase up to 0.43 μmol/min per 100 g, in both groups, followed in the controls by a nonlinear, slower increase up to the concentration (1.7 μmol/min per 100 g), where flow rate diminished. In the treated animals, the total amount of substrate conjugated was markedly diminished when the infusion rate was above 0.43 μmol/min per 100 g.

Cholesterol and phosphatidylcholines

The concentration of cholesterol was higher in bile from rats given ethynylestradiol but, since bile flow was lower in the treated animals, cholesterol output was similar in all rats. The cholesterol concentration stayed relatively constant throughout the pre-cholate period, but when cholate was infused, it increased slightly in both groups (**Fig. 6B**). Biliary cholesterol concentration was not sex dependent but, because bile flow in the males was somewhat greater than in the females of this particular series, cholesterol output was slightly higher in the males.

The concentration of total phosphatidylcholines, measured as lipid soluble phosphorus, was consis-

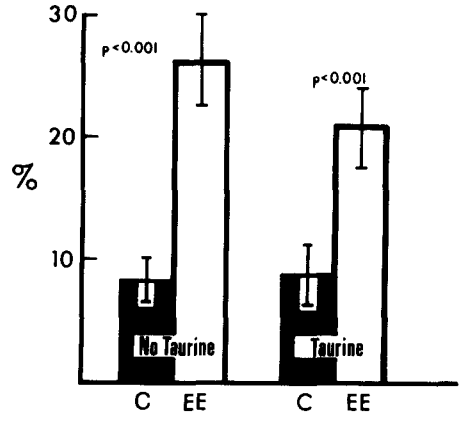

Fig. 5. Mean (<2 SEM) percentage of administered [^{14}C]cholic acid excreted in the bile without conjugation, both with and without taurine, in control (C) and treated (EE) rats.

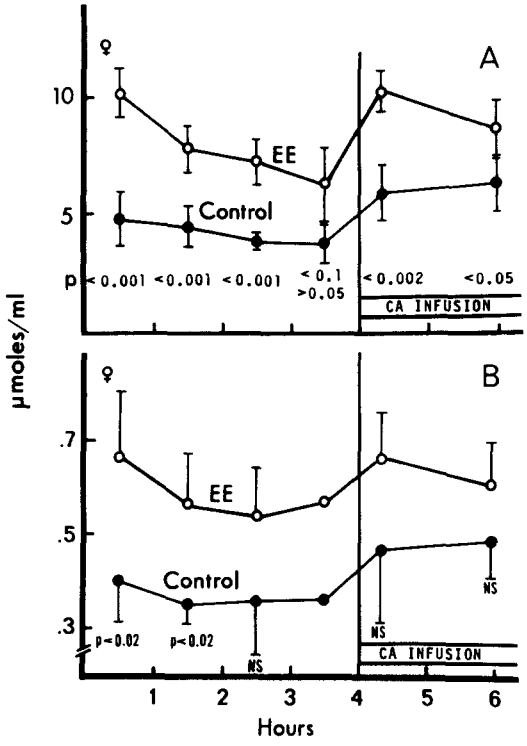

Fig. 6. *A*, Biliary phospholipid concentration in control and ethynylestradiol (EE) treated rats before and during cholic acid (CA) infusion, 0.43 μmol/min per 100 g. Means ± 2 SEM. *B*, Biliary cholesterol concentration in control and ethynylestradiol (EE) treated rats before and during cholic acid infusion, 0.43 μmol/min per 100 g. Means ± 2 SEM.

tently higher in male control rats than in females ($P < 0.001$). In both sexes it was higher in animals treated with ethynylestradiol than in the controls, and was almost identical in treated males and females. Since bile flow was lower in the treated rats, total phospholipid secretion was not affected by ethynylestradiol. After beginning the cholate infusion, there was always an abrupt increase in concentration (Fig. 6*A*) and total output of phospholipid.

Analysis of individual molecular species was carried out on bile from the female rats whose bile acid data are shown in Fig. 2. The results are shown in **Fig. 7**. The most striking changes after treatment with ethynylestradiol were an increased percentage of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine ($P < 0.001$) and a decreased percentage of 1-stearolyl-2-linoleoyl-*sn*-glycero-3-phosphocholine ($P < 0.001$). Significant changes in the distribution of phosphatidylcholines during the 4-hr period prior to cholate infusion were not seen, nor did cholate infusion affect their distribution.

The relationship between cholesterol and phospholipid secretion was remarkably constant in all groups of rats, both during the cholate infusion and during the preinfusion period (**Fig. 8**). For every μmol increase in phospholipid secretion, cholesterol secretion increased 0.0385 μmol. Extrapolation to zero phospholipid secretion shows an apparent phospholipid-independent cholesterol secretion, but such extrapolation may not be justified.

The molar percentage of each lipid in the bile obtained during the first 20 min after bile duct cannulation was calculated. The previously noted high concentration of bile acids in this series of female rats and the higher phospholipid concentration in the males are again noted (**Table 5**). EE treatment caused a decrease in the molar percent of bile acids, a significant increase in the molar percent of phospholipid ($P < 0.05$), and a slight increase in molar percent of cholesterol, especially in the males ($0.1 < P > 0.05$).

DISCUSSION

Endogenous bile acids

Ethynylestradiol, in the high doses given in this study, caused a marked increase in the relative amount of 6-hydroxylated bile acids. This may be analogous to the increase of β-muricholic acid seen after administration of the known cholestatic drugs, α-naphtyl isothiocyanate or methyltestosterone (30, 31) and after ligation of the bile duct (32–34). A number of partially identified, monounsaturated, 6-hydroxylated bile acids closely related to β-muricholic acid were also detected. One of these compounds is probably identical with an unidentified bile acid described by Danielsson (34). These acids also increased upon treatment with ethynylestradiol, as did hyodeoxycholic acid, which is formed from β-muricholic acid during the enterohepatic circulation (35). In contrast, α-muricholic acid remained unaffected by the ethynylestradiol treatment. This bile acid is formed by 6β-

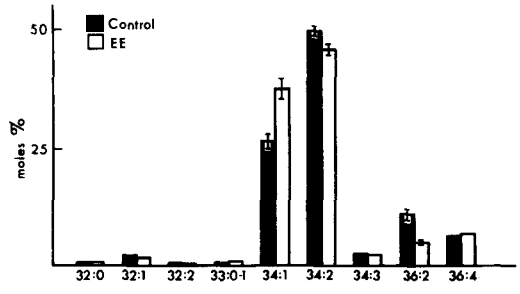

Fig. 7. Phospholipid species in bile collected during the first hr of biliary drainage. Ethynylestradiol (EE) caused a significant increase in 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (34:1) and a significant decrease in 1-stearolyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (36:2).

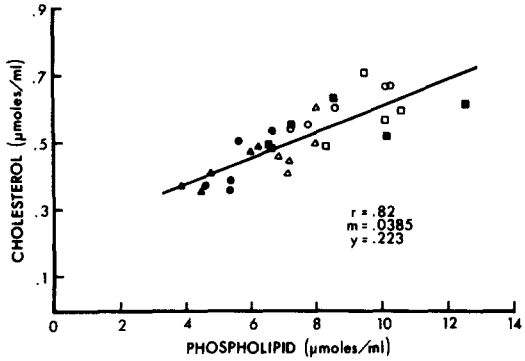

Fig. 8. Relationship between the concentration of biliary cholesterol and that of biliary phospholipid excreted. Data plotted are the mean phospholipid and cholesterol concentrations for each of three hourly preinfusion bile samples and two samples obtained during cholic acid infusion (0.43 μmol/min per 100 g). There were 4–6 animals in each group. ▲ Female controls, △ and ● male controls; ○ female treated, □ ■ male treated.

hydroxylation of chenodeoxycholic acid, the concentration of which also remained unchanged. If the increased proportion of acids of the β-muricholic type was due to enhancement of 6β-hydroxylation caused by an increased concentration of chenodeoxycholic acid in the liver (33), α-muricholic acid would also be expected to increase, as is the case in rats fed chenodeoxycholic acid (36). The possibility therefore exists that the β-muricholic acids were formed via some other metabolic pathway, e.g., by early cleavage of the cholesterol side chain (37–41). Such a sequence could also explain the appearance of ursodeoxycholic acid (42).

The increased proportion of 6β-hydroxylated bile acids contrasts with the finding of decreased 6β-hydroxylation of steroid hormones in microsomal preparations of liver from rats given large doses of ethynylestradiol (7). However, the in vivo effect may represent a sum of influences on several reactions and the net result is determined by the relative effect of ethynylestradiol on these reactions.

Ethynylestradiol also caused a significant decrease in excretion of cholic acid, an effect recently noted by Watanabe (43).

Bile flow and conjugation and excretion of cholate

The cholestasis caused by EE has been shown to be due to a decrease in the bile salt-independent canalicular bile secretion (4) that is thought to be dependent upon membrane-bound Na^+,K^+-ATPase (44). Simon, Sutherland, and Accatino (45) have recently shown that EE reduces the Na^+,K^+-ATPase activity in a liver plasma membrane fraction rich in canalicular membranes. EE also reduces the bile salt-dependent portion of bile flow when the concentration of cholate presented to the liver was excessive. In the EE-treated rats, as the amount of cholate infused and subsequently secreted into the bile was increased, the rate of bile flow increased, as it did in the untreated rats, until the infusion rate was 0.43 μmol/min per 100 g. Above that rate, bile flow stopped almost completely. Similar bile salt-induced cholestasis occurred in the control rats, but at an infusion rate of about 1.7 μmol/min per 100 g. Others have reported a cholestatic effect of high concentrations of bile acids (46) and also of other organic anions, including bilirubin (47), BSP (48, 49), indocyanine green (48, 50, 51), and Rose Bengal (52). The mechanism of this effect is not known for certain but, in the case of Rose Bengal, inhibition of Na^+,K^+-ATPase of hepatic canalicular membranes has been reported (53). Witzleben (54) found that cholic acid overload caused changes in the appearance of hepatic mitochondria, endoplasmic reticulum, and liposomes. The amount of cholic acid employed in his experiments was almost twice as great as that we used in any experimental group.

The finding that EE lowers the threshhold for this anticholeretic effect of cholic acid could be of clinical significance in the syndrome of pruritus gravidarum, and the more severe idiopathic cholestasis of pregnancy, which occurs in the last trimester of pregnancy when the liver is exposed to high concentrations of sex hormones. Approximately 4 mg/kg per day of estrogens and progesterone are synthesized from cholesterol in the third trimester of pregnancy in man (55). When pruritus gravidarum occurs, the serum bile acids are markedly elevated (56). It seems possible that an anticholeretic effect of bile acids might be added to the cholestatic effect of estrogens (57). Although naturally occurring estrogens are metabolized differently than ethynylestradiol (58), this syndrome is reproduced by the administration of small doses of contraceptive steroids to certain patients who had cholestasis during pregnancy (59).

Ethynylestradiol clearly impaired the capacity of the liver to conjugate cholic acid. This defect was

TABLE 5. Distribution of biliary lipids

	Bile Acid		Phospholipid		Cholesterol	
	μmol/ml	%	μmol/ml	%	μmol/ml	%
Female						
C	31.1	85.8	4.8	12.3	.402	1.1
EE	45.2	80.7	10.2	18.1	.665	1.2
Male						
C	18.7	71.3	7.1	27.2	.406	1.6
EE	16.2	60.2	10.1	37.7	.567	2.1

C—Control; EE—treated with ethynylestradiol.

apparent in endogenous bile acids and became more marked as the load of cholate presented to the liver was increased. A similar finding was recently reported by Reimold et al (57). Conjugation of bile acids with taurine or glycine occurs in two stages: first, synthesis of bile-acyl-S-CoA and second, transfer of the acyl group to taurine or glycine. It requires enzymes in hepatic microsomes which are further stimulated by proteins, apparently enzymes, contained in a heterogeneous subcellular fraction, termed the "L fraction" by Schersten (59) and Schersten et al. (60). Induced impairment of this activity is consistent with its impairment of other microsomal functions, such as steroid hydroxylations and bile acid synthesis. It seems, however, as reported by others (61, 62), that conjugation of cholic acid is not necessary for its excretion into bile and, further, the biliary excretion of unconjugated cholate does not appear harmful.

Cholesterol and phospholipid secretion

The consistently increased concentration of phospholipids in the bile of male rats is a sex-related difference in biliary lipids not previously reported. After EE administration, biliary phospholipid concentration was the same in both sexes, but the output was higher in the male because of the greater bile flow in that group. In both sexes, the phospholipid output increased when cholic acid was given. The concentration also usually increased, but the magnitude of the increase was small. There was a decrease in phospholipid concentration during the preinfusion period when the enterohepatic circulation was broken and the endogenous bile acid pool was being depleted. These changes in concentration were more pronounced in the EE-treated rats than in the control animals and may, in part, reflect the decreased bile water in the treated group. There was no clear-cut relationship between phospholipid concentration and the amount of cholate infused. Phospholipid synthesis and secretion are dependent upon bile acid secretion in man and in other species (63, 64), but in these short-term experiments in rats, phospholipid secretion was not affected by bile acid secretion to the extent anticipated.

Cholesterol concentration in the bile was invariably higher in the treated animals, but cholesterol output was not significantly different from the control. It is of interest, however, that in both sexes, especially the males, EE caused a slight increase in molar percent of biliary cholesterol. Although cholesterol concentration of rat bile is very low, well below saturation, the molar percentage increases in response to estrogens, as it does in other species that have much higher bile cholesterol concentrations. In man and the rhesus monkey, estrogens can cause the secretion of bile supersaturated with cholesterol (9, 10).

Cholate infusion (0.43 μmol/min per 100 g) caused little or no change in the composition of individual molecular species of phosphatidylcholines. Ethynylestradiol treatment resulted in a marked increase of the species containing palmitic and oleic acid residues and a decrease of species containing stearic and linoleic acid residues. This change is nonspecific since it is also seen when glucose or fructose is given to bile fistula rats oxidizing ethanol (65). In the latter case, it may be due to an increased synthesis of palmitate and an increased rate of desaturation of oleate. The rates of these processes after treatment with ethynylestradiol are not known. However, it is clear that ethynylestradiol, either alone or with cholate, does not cause a nonspecific damage to membrane structures resulting in an increased biliary excretion of long chain polyunsaturated species of phosphatidylcholines.

In conclusion, ethynylestradiol in the large doses used in these studies significantly affects bile acid metabolism, bile flow, and the excretion of biliary lipids. Synthetic estrogens given as contraceptive steroids produce similar changes in biliary lipids in man (66). It is tempting to speculate that the increased incidence of cholesterol cholelithiasis in women, especially those who have been pregnant and those taking contraceptive steroids, may be caused by such estrogen effects. The changes in biliary lipid composition, the alterations in bile acid metabolism, and the cholestasis are probably mediated through common effects on hepatic subcellular membranes that regulate the composition and secretion of bile.

The technical assistance of Mrs. Kathleen Brown is gratefully acknowledged. This work was supported by grants from the Swedish Medical Research Council (13x-219) and the World Health Organization.

Manuscript received 19 July 1976 and accepted 28 April 1977.

REFERENCES

1. Campbell, R. M., and H. W. Kosterlitz. 1949. Some effects of pregnancy and lactation on the liver. *J. Endocrinol.* **6:** 171.
2. Song, C. S., and A. Kappas. 1968. The influence of estrogens, progestins, and pregnancy on the liver. *In* Vitamins and Hormones—Advances in Research and Applications. R. S. Harris, I. G. Wool, J. A. Loraine, and K. V. Thimann, editors. Academic Press, New York, N. Y. 147–195.
3. Kreek, M. J., R. E. Peterson, M. H. Sleisenger, and G. H. Jeffries. 1967. Influence of ethinyl estradiol-

induced cholestasis on bile flow and biliary excretion of estradiol and bromosulfophthalein by the rat. *J. Clin. Invest.* **46:** 1080. (Abstract)
4. Gumucio, J. J., and V. D. Valdivieso. 1971. Studies on the mechanism of the ethynylestradiol impairment of bile flow and bile salt excretion in the rat. *Gastroenterology* **61:** 339–344.
5. Harkavy, M., and N. B. Javitt. 1969. Effect of ethynyl estradiol on hepatic excretory function of the rat. *In* Metabolic Effects of Gonadal Hormones and Contraceptive Steroids. H. A. Salhanick, D. M. Kipnis, and R. L. VandWiele, editors. Plenum Press, New York. 11–18.
6. Heikel, T. A. J., and G. H. Lathe. 1970. The effect of oral contraceptive steroids on bile secretion and bilirubin T_m in rats. *Br. J. Pharmac.* **38:** 593–601.
7. Mackinnon, A. M., F. R. Simon. 1975. The pharmacological reversal of cholestasis-induced reduction in hepatic P-450. *Biochem. Pharmacol.* **24:** 748–749.
8. Einarsson, K., J. L. E. Ericsson, J. A. Gustafsson, J. Sjövall, and E. Zietz. 1974. Effects of ethinylestradiol and norethisterone on liver microsomal metabolism of steroids in male and female rats. *Biochim. Biophys. Acta.* **369:** 278–293.
9. Pertsemlidis, D., D. Panveliwalla, and E. H. Ahrens, Jr. 1974. Effects of clofibrate and of an estrogen–progestin combination on fasting biliary lipids and cholic acid kinetics in man. *Gastroenterology.* **66:** 565–573.
10. Lynn, J., L. Williams, J. O'Brien, et al. 1973. Effects of estrogen upon bile. Implications with respect to gallstone formation. *Ann. Surg.* **178:** 514–522.
11. Bennion, L. J., R. L. Ginsberg, M. B. Garnick, and P. H. Bennett. 1976. Effects of oral contraceptives on the gallbladder bile of normal women. *N. Engl. J. Med.* **294:** 189–192.
12. Nilsson, S. 1966. Gallbladder disease and sex hormones—a statistical study. *Acta Chir. Scand.* **132:** 275–279.
13. Sampliner, R. E., P. H. Bennett, L. J. Comess, et al. 1970. Gallbladder disease in Pima Indians: Demonstration of high prevalence and early onset by cholecystography. *N. Engl. J. Med.* **283:** 1358–1364.
14. Boston Collaborative Drug Surveillance Programme: Oral contraceptives and venous thromboembolic disease, surgically confirmed gallbladder disease and breast tumours. 1973. *Lancet.* **1:** 1399–1404.
15. Boston Collaborative Drug Surveillance Program: Surgically confirmed gallbladder disease, venous thromboembolism and breast tumors in relation to postmenopausal estrogen therapy. 1974. *N. Engl. J. Med.* **290:** 15–18.
16. Levin, S. J., C. G. Johnston, and A. J. Boyle. 1961. Spectrophotometric determination of several bile acids as conjugates. Extraction with ethyl acetate. *Anal. Chem.* **33:** 1407–1411.
17. Back, P., J. Sjövall, and K. Sjövall. 1974. Monohydroxy bile acids in plasma in intrahepatic cholestasis of pregnancy. Identification by computerized gas chromatography–mass spectrometry. *Med. Biol.* **52:** 31–38.
18. Makita, M., and W. W. Wells. 1963. Quantitative analysis of fecal bile acids by gas–liquid chromatography. *Anal. Biochem.* **5:** 523–530.
19. Jansson, P. A., S. Melkersson, R. Ryhage, and S. Wikström. 1970. Mass spectral data processing. 2. Computer used for processing of mass spectra recorded on magnetic tape. *Arkiv. Kemi.* **31:** 565–578.
20. Reimendal, R., and J. Sjövall. 1972. Analysis of steroids by offline computerized gas chromatography–mass spectrometry. *Anal. Chem.* **44:** 21–29.
21. Reimendal, R., and J. Sjövall. 1973. Computer evaluation of gas chromatographic–mass spectrometric analyses of steroids from biological materials. *Anal. Chem.* **45:** 1083–1089.
22. 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.
23. Eneroth, P. 1963. Thin-layer chromatography of bile acids. *J. Lipid Res.* **4:** 11–16.
24. Gustafsson, J. A., and J. Sjövall. 1968. Steroids in germfree and conventional rats. 6. Identification of 15α- and 21-hydroxylated C_{21} steroids in faeces from germfree rats. *Eur. J. Biochem.* **6:** 236–247.
25. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **226:** 497–509.
26. Bartlett, B. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* **234:** 466–468.
27. Almé, B., and E. Nyström. 1971. Preparation of lipophilic ion exchangers from chlorohydroxypropylated Sephadex and cellulose. *J. Chromatogr.* **59:** 45–52.
28. Curstedt, T., and J. Sjövall. 1974. Analysis of molecular species of ^{2}H-labelled phosphatidylcholines by liquid–gel chromatography and gas chromatography–mass spectrometry. *Biochim. Biophys. Acta.* **360:** 24–37.
29. Sjövall, J., P. Eneroth, and R. Ryhage. 1971. Mass spectra of bile acids. *In* The Bile Acids: Chemistry, Physiology and Metabolism. P. P. Nair and D. Kritchevsky, editors. Plenum Press, New York. Vol. 1. 209–248.
30. Schaffner, F., H. H. Scharnbeck, F. Hutterer, H. Denk, H. A. Greim, and H. Popper. 1973. Mechanism of cholestasis. VII. α-Naphthylisothiocyanate-induced jaundice. *Lab. Invest.* **28:** 321–331.
31. Czygan, P., H. Greim, D. Trulzsch, F. Hutterer, F. Schaffner, and H. Popper. 1972. Alteration in hepatic bile salt patterns in two models of experimental intrahepatic cholestasis. *In* Bile Acids in Human Disease. P. Back and W. Gerok, editors. F. K. Schattauer Verlag, Stuttgart-New York. 135–137.
32. Mahowald, T. A., J. T. Matschiner, S. L. Hsia, E. A. Doisy, Jr., W. H. Elliott, and E. A. Doisy. 1957. Bile acids. III. Acid I; the principal bile acid in urine of surgically jaundiced rats. *J. Biol. Chem.* **225:** 795–802.
33. Greim, H., D. Trulzsch, J. Roboz, K. Dressler, P. Czygan, F. Hutterer, F. Schaffner, and H. Popper. 1972. Mechanism of cholestasis. 5. Bile acids in normal rat livers and in those after bile duct ligation. *Gastroenterology.* **63:** 837–845.
34. Danielsson, H. 1973. Effect of biliary obstruction on formation and metabolism of bile acids in rat. *Steroids.* **22:** 567–577.
35. Van Heijenoort, Y., E. Sacquet, and M. Riottot. 1974. Degradation bactérienne de l'acide β-muricholique chez le rat. *C. R. Acad. Sci. Ser. D (Paris)* **278:** 1067–1070.
36. Danielsson, H., and G. Johansson. 1974. Effects of long-term feeding of chenodeoxycholic acid on bio-

synthesis and metabolism of bile acids in the rat. *Gastroenterology.* **67:** 126–134.
37. Usui, T., and K. Yamasaki, 1964. Metabolic studies of bile acids. XLV. 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.
38. Mitropoulos, K. A., and N. B. Myant. 1967. The formation of lithocholic acid, chenodeoxycholic acid and α- and β-muricholic acids from cholesterol incubated with rat liver mitochondria. *Biochem. J.* **103:** 472–479.
39. Mitropoulos, K. A., and N. B. Myant. 1967. The formation of lithocholic acid, chenodeoxycholic acid and other bile acids from 3β-hydroxychol-5-enoic acid in vitro and in vivo. *Biochim Biophys. Acta.* **144:** 430–439.
40. Ayaki, Y., and K. Yamasaki. 1970. In vitro conversion of 7α-hydroxycholesterol to some natural C_{24}-bile acids with special reference to chenodeoxycholic acid biogenesis. *J. Biochem. (Tokyo)* **68:** 341–346.
41. Ayaki, Y., and K. Yamasaki. 1972. Identification of 3β,7α-dihydroxychol-5-enoic acid in fistula bile of the rat given cholesterol-4-^{14}C and DL-mevalonate-2-^{14}C. *J. Biochem. (Tokyo)* **71:** 85–89.
42. Yamasaki, K., S. Ikawa, D. Kinoshita, T. Usui, and F. Nakada. 1967. Enzymatic 7β-hydroxylation of 3β-hydroxychol-5-enoic acid. *Yonago Acta Med.* **11:** 159–164.
43. Watanabe, H. 1975. Effect of 17α-ethinylestradiol on biliary excretion of bile acids. *Biochim. Biophys. Acta.* **399:** 79–84.
44. Erlinger, S. 1972. Physiology of bile flow. *In* Progress in Liver Disease, Volume IV. H. Popper and F. Schaffner, editors. Grune and Stratton, New York. 63–82.
45. Simon, F. R., E. Sutherland, and L. Accatino. 1976. The effect of cholestasis produced by ethinyl estradiol on bile acid binding and (Na-K)ATPase activity in rat liver surface membranes. *Clin Res.* **24:** 105A. (Abstract)
46. Fisher, M. M., R. Magnusson, and K. Miyai. 1971. Bile acid metabolism in mammals. I. Bile acid-induced intrahepatic cholestasis. *Lab. Invest.* **21:** 88–91.
47. Witzleben, C. L. 1971. Bilirubin as a cholestatic agent—Physiologic and morphologic observations. *Amer. J. Pathol.* **62:** 181–187.
48. Groszmann, R. J., B. Kotelanski, J. Kendler, et al. 1969. Effect of sulfobromophthalein and indocyanine green on bile excretion. *Proc. Soc. Exp. Biol. Med.* **132:** 712–714.
49. Priestly, B. G., and G. L. Plaa. 1970. Reduced bile flow after sulfobromophthalein administration in the rat. *Proc. Soc. Exp. Biol. Med.* **135:** 373–376.
50. Klaassen, C. D., and G. L. Plaa. 1969. Plasma disappearance and biliary excretion of indocyanine green in rats, rabbits, and dogs. *Toxicol. Appl. Pharmacol.* **15:** 374–384.
51. Horak, W., G. Grabner, and G. Paumgartner. 1973. Inhibition of bile salt-independent bile formation by indocyanine green. *Gastroenterology.* **64:** 1005–1012.
52. Dhumeaux, D., S. Erlinger, J. P. Benhamou, and R. Fauvert. 1970. Effects of rose bengal on bile secretion in the rabbit: inhibition of a bile salt-independent fraction. *Gut.* **11:** 134–140.
53. Leperche, Y., A. Launay, and P. Oudéa. 1972. Effects of phenobarbital and rose bengal on the ATPases of plasma membranes of rat and rabbit liver. *Gut.* **13:** 920–925.
54. Witzleben, C. L. 1972. Hepatic ultrastructural effects of cholic acid overload. *Exp. Mol. Pathol.* **16:** 47–53.
55. Beling, C. G. 1971. Estrogens. *In* Endocrinology of Pregnancy. F. Fuchs and A. Klopper, editors. Harper & Row, New York, 32–65.
56. Sjövall, K., and J. Sjövall. 1967. Serum bile acid levels in pregnancy with pruritus. Bile acids and steroids 158. *Biochim. Biophys. Acta.* **13:** 207–211.
57. Reimold, W. V., M. Henniges, M. Holtermann, and R. Kattermann. 1975. Influence of estradiol and ethinylestradiol on bile acid metabolism of the liver and bile acid output into bile in rats. *In* Advances in Bile Acid Research. S. Matern, J. Hackenschmidt, P. Back, and W. Gerok, editors. F. K. Schattauer Verlag, Stuttgart, Germany. 173–180.
58. Despopoulos, A. 1971. Hepatic and renal excretory metabolism of bile salts: A background for understanding steroid-induced cholestasis. *J. Pharmacol. Exp. Ther.* **176:** 273–283.
59. Scherstén, T. 1967. The synthesis of taurocholic and glycocholic acids by preparations of human liver. I. Distribution of activity between subcellular fractions. *Biochim. Biophys. Acta.* **141:** 144–154.
60. Scherstén, T., P. Björntorp, P. Ekdahl, and S. Björkerud. 1967. The synthesis of taurocholic and glycocholic acids by preparations of human liver. II. An analysis of the stimulating effect of the L fraction. *Biochim. Biophys. Acta.* **141:** 155–163.
61. O'Maillie, E. R. L., T. G. Richards, and A. H. Short. 1967. The influence of conjugation of cholic acid on its uptake and secretion: hepatic extraction of taurocholate and cholate in the dog. *J. Physiol. (London)* **189:** 377–350.
62. Hoffman, N. E., J. H. Iser, and R. A. Smallwood. 1975. Hepatic bile acid transport: effect of conjugation and position of hydroxyl groups. *Amer. J. Physiol.* **229:** 298–302.
63. Nilsson, S. 1970. Synthesis and secretion of biliary phospholipids in man. *Acta Chir. Scand.* Supplement **405:** 1–38.
64. Swell, L., C. C. Bell, Jr., and C. Entenman. 1968. Bile acids and lipid metabolism. III. Influence of bile acids on phospholipids in liver and bile of the isolated perfused dog liver. *Biochim. Biophys. Acta.* **164:** 278–284.
65. Curstedt, T., and J. Sjövall. 1974. Biosynthetic pathways and turnover of individual biliary phosphatidylcholines during metabolism of [1,1-$^2H_2$]ethanol. *Biochim. Biophys. Acta.* **369:** 173–195.
66. Bennion, L. J., R. L. Ginsberg, M. B. Garnick, and P. H. Bennett. 1976. Effects of oral contraceptive on the gallbladder of normal women. *N. Engl. J. Med.* **294:** 189–192.