

Biliary Lipids, Bile Acids, and Gallbladder Function in the Human Female

EFFECTS OF PREGNANCY AND THE OVULATORY CYCLE

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ABSTRACT To study the events that might lead to an increased risk of cholesterol gallstones, we examined biliary lipid composition and secretion and bile acid composition and kinetics at different stages of pregnancy or ovulation in young, nonobese, healthy women.

Lipid composition and bile acid distribution were determined in duodenal fluid obtained in the fasting state and after stimulation of the gallbladder. Biliary lipid secretion was measured by the marker-perfusion technique. Bile acid kinetics were determined with cholic and chenodeoxycholic acids labeled with carbon-¹³, by measuring the relative abundance of ¹³C in duodenal bile acids for 4–5 d. In a subset of patients we measured gallbladder storage and emptying during the kinetic study.

The phase of the ovulatory cycle had no effects, but there were significant changes during pregnancy. The lithogenic or cholesterol saturation index of fasting hepatic and gallbladder bile increased during the second and third trimesters. The mean secretion rate of biliary lipids was not altered, but in the last two-thirds of pregnancy, cholesterol secretion increased in relation to bile acid and phospholipid secretion. There was a progressive decrease in the percentage of chenodeoxycholic acid and a similar increase in the

percentage of cholic acid. The pool size of each major bile acid increased in the first trimester. Chenodeoxycholic acid and deoxycholic acid pools, but not cholic acid pools, subsequently decreased. The fractional turnover rate of both primary bile acids was slower during pregnancy. The synthesis rate of chenodeoxycholic but not cholic acid decreased in a linear manner during the first 20 wk of pregnancy. The rate of enterohepatic cycling of the bile acid pool was reduced throughout pregnancy.

The volume of the fasting gallbladder and the residual volume after a physiologically stimulated contraction were directly correlated with bile acid pool size. The residual volume was also directly related to total bile acid synthesis.

INTRODUCTION

Epidemiologic studies throughout the world show that cholesterol gallstones occur two to three times as commonly in women as in men (1–7). The increase in prevalence begins at puberty (3) and persists only during the childbearing years (3, 4). Pregnancy (4) and oral contraceptive steroids (8) increase the risk of gallstones in women and estrogen administration increases the risk in both men and women (9, 10). Little is known of the biochemical and physiological mechanisms responsible for the increased risk of gallstone formation in these populations. Biliary bile acids and lipids in pregnant women have not been studied with modern methods.

In this paper, we report biliary lipid composition and secretion and bile acid composition and kinetics in nonobese, healthy pregnant, and nonpregnant women of the same age and weight. In the latter group,

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subjects were studied in different phases of the ovulatory cycle, as confirmed by appropriate serum hormone levels.

There were no effects of the cycle, but major changes were identified during pregnancy. After the first trimester, cholesterol saturation of gallbladder and fasting hepatic bile increased. Although the mean rate of secretion of cholesterol, phospholipid and bile acids was not altered, the rate of secretion of cholesterol relative to that of bile acid and phospholipid was increased, which indicates alteration in coupling of biliary lipids. There were changes in bile acid composition that might explain the increased lithogenicity of bile. Marked alterations in bile acid kinetics occurred that appear to be secondary to altered gallbladder function.

METHODS

Subjects. The volunteer subjects were 23 pregnant and 23 nonpregnant healthy Caucasian women in the same age group (Table I). The mean (\pm SE) age of the pregnant women as 22.7 (\pm 0.8) and of the nonpregnant women (25.1) (\pm 0.7) yr. None had clinical or laboratory evidence of disease of the liver, gastrointestinal tract, thyroid, gallbladder, or lipid metabolism. An oral cholecystogram was done in two nonpregnant subjects and ultrasound examination of the gallbladder was performed in all subjects to exclude gallstones. No subject was obese. The percentage of body fat, calculated from anthropometric measurements and the equation described by

Steinkamp et al. (12) was 22.9 (\pm 1.1) for pregnant subjects and 26.1 (\pm 0.8) for nonpregnant subjects.

Although it was usually not possible to perform serial studies in all phases of the menstrual cycle or in different stages of pregnancy and after pregnancy, several subjects did volunteer for multiple studies. Their results are analyzed separately and also as members of appropriate groups.

31 studies of biliary lipid composition were done on the 23 pregnant women. Four women had multiple studies: two were studied in each trimester and two were studied in both and second and third trimesters. 9 women were studied during the first, 10 during the second, and 12 during the third trimester of pregnancy. 30 studies were done on the 23 nonpregnant subjects. 14 were studied in the follicular phase of their cycle, 3 in midcycle, and 13 in the luteal phase. Two were studied in all three phases and three were studied in two phases. 19 bile acid kinetic studies—7 in the follicular phase, 2 in midcycle, and 10 in the luteal phase—were performed in 15 nonpregnant women. 26 kinetic studies were performed on 18 pregnant subjects. 7 women were studied in the first trimester, 8 in the second trimester, and 11 in the third. Since only a few women returned for sequential studies, it was necessary to compare groups of individuals. The groups did not differ from each other with respect to age, weight, or body fat.

In each nonpregnant subject the phase of the menstrual cycle was determined from the 1st d of the last menstrual period and verified by the serum levels of estradiol, progesterone, and luteinizing hormone (Table I), determined two or three times during each study (13). In the pregnant subjects serum progesterone, estrone, estradiol, and estril concentrations were measured once during each study. All measurements were made by radioimmunoassay by Endocrine Sciences, Tarzana, Calif.

TABLE I
Patient Data*

	No. of studies	Week of pregnancy	Age	Weight	Ideal weight†	Body fat‡	Serum hormones				
							Progesterone	Estrone	Estradiol	Estril	Luteinizing hormone
			yr	kg	%	%					
									ng/dl		mIU/ml
Nonpregnant (n = 23)											
Follicular phase	14	—	23.7 \pm 0.9	56.0 \pm 1.5	103.6 \pm 2.9	26.6 \pm 0.9	0.83 \pm 0.1	—	5.8 \pm 0.6	—	9.9 \pm 2.1
Midcycle	3	—	28.3 \pm 3.2	54.2 \pm 2.6	102.3 \pm 5.8	26.7 \pm 2.5	1.67 \pm 0.7	—	22.0 \pm 5.3	—	27.5 \pm 10.9
Luteal phase	13	—	26.9 \pm 1.0	58.6 \pm 2.7	101.4 \pm 3.0	25.7 \pm 1.4	5.66 \pm 1.7	—	11.7 \pm 1.8	—	13.7 \pm 3.4
Pregnant (n = 23)											
1st trimester	9	9.4 \pm 0.6	23.1 \pm 1.0	56.0 \pm 2.1	—	24.7 \pm 1.9	28.1 \pm 2.4	87 \pm 16	198 \pm 44	118 \pm 15	—
2nd trimester	10	21.2 \pm 1.2	23.7 \pm 1.3	58.5 \pm 3.0	—	22.0 \pm 1.8	58.4 \pm 7.6	443 \pm 73	993 \pm 158	327 \pm 68	—
3rd trimester	12	34.0 \pm 0.7	21.2 \pm 0.8	60.9 \pm 1.3	—	23.0 \pm 1.3	127.2 \pm 14.4	777 \pm 172	1847 \pm 153	728 \pm 91	—

* Figures represent mean \pm SEM.

† Calculated from Desirable Weight Table of Metropolitan Life Insurance Company (11).

‡ Calculated by the anthropometric method of Steinkamp et al. (12).

The subjects were instructed in a standard diet containing 500 mg/d of cholesterol without other restrictions 1 wk before being studied in the Clinical Research Center, where the same diet was continued.

All subjects volunteered for the study and gave a written informed consent. The study was approved annually by the Human Subject Committee of the University of Colorado School of Medicine.

Procedures. The following tests were done on all subjects: complete blood count, urinalysis, thyroid function tests, serum protein electrophoresis; and the serum concentration of bilirubin, alkaline phosphatase, creatinine, cholesterol, triglyceride, aspartate amino transferase, and alanine amino transferase. There was no difference between the two groups except for the serum lipids. The fasting serum triglyceride concentration was 106 ± 9 mg/dl in the control subjects, 203 ± 23 mg/dl in the second trimester ($P \leq 0.0001$), and 250 ± 27 mg/dl ($P \leq 0.001$) in the third trimester of pregnancy. The serum cholesterol was higher only in the third trimester (control 190 ± 8 mg/dl, third trimester 243 ± 14 , $P < 0.005$).

A triple lumen polyvinyl tube complex was used in all studies. The two aspiration tubes had an internal diameter of 1.58 mm and the infusion tube had an internal diameter of 1.0 mm. The tube complex was positioned in the duodenum by fluoroscopy in nonpregnant subjects so that the proximal collecting orifice was at the level of the ampulla of Vater; the distal collecting orifice was 17 cm and the infusion site was 5 cm distal to the ampulla. In pregnant subjects, the tube was positioned so that aspiration from the proximal opening yielded golden yellow fluid. Its position was confirmed by two methods: first, by finding that after stimulation of the gallbladder the fluid in the proximal tube turned a dark amber to greenish-brown color. Second, after gallbladder stimulation by an amino acid infusion through the infusion tube, the bilirubin concentration in the fluid aspirated from the proximal collection site was always equal to or greater than the bilirubin concentration in fluid aspirated from the distal collection site. The mean bilirubin concentration of the fluid from the proximal collecting tube 19.5 ± 3.2 , whereas that from the distal collection tube was 10.0 ± 1.3 mg/dl ($P < 0.001$). This difference allowed us to conclude that the proximal orifice was not in the stomach, but at or distal to the ampulla of Vater.

Biliary lipid composition. The concentration of biliary lipids was measured in fluid aspirated from the tube opening opposite the ampulla of Vater in the fasting state and in a sample obtained after gallbladder stimulation. The former will be referred to as "fasting hepatic bile", and the latter as "gallbladder bile". The gallbladder was stimulated to contract by a 60-ml bolus into the duodenum of a solution containing glucose 5 g/dl and amino acids 4.3 g/dl (14). Its osmolality was 520 mosmol/liter. The solution contained no phosphorus.

During the secretion of gallbladder bile the aspirate was collected in a container immersed in ice and shielded from light. The bile was then mixed thoroughly, an aliquot taken for analysis and the remainder returned to the patient via the tube at the conclusion of each day's study.

A sample of the darkest bile was centrifuged at 3,800 rpm for 20 min and the sediment was examined microscopically for cholesterol crystals.

In each subject, fasting hepatic bile and gallbladder bile were collected for analysis on days 2 or 3 and the results presented are the means of these several measurements. When the bile acid concentration of fasting hepatic bile was < 1 μ mol/ml, the data were not used (15).

For measurement of bile acid concentration an aliquot of duodenal bile was placed in 10 parts methanol and bile acid

concentration was determined spectrophotometrically with 3- α hydroxysteroid dehydrogenase (16) (Worthington Biochemical Corp., Freehold, N. J.).

Cholesterol concentration was determined by gas-liquid chromatography with 5- β -cholestan-5-ol as internal standard (17). For phospholipid determination, aliquots of bile were added to 20 parts of chloroform: methanol 2:1 (vol/vol) and lipid phosphorus was assayed by the method of Bartlett (18).

The relative molar percentage of each lipid was determined and the lithogenic index calculated according to Thomas and Hofmann (19) and the criteria of Hegardt and Dam (20) and Holzbach et al. (21). Correction for total lipid concentration, as recommended by Carey and Small (15), could not be made because the samples were diluted to an unknown degree by pancreatic juice and possibly by perfusate and gastric juice.

Biliary lipid secretion. Biliary lipid secretion was measured the last day of each study with the techniques and calculations introduced by Grundy and Metzger (22), as modified by Shaffer and Small (23). The amino acid mixture containing bromsulphalein as nonabsorbable marker was infused into the duodenum at the rate of 3.5 ml/min with a Harvard peristaltic pump (Harvard Apparatus Co., Inc., Millis, Mass.). The infusion was begun at 3:00 a.m. and continued until 1:00 p.m. Since the biliary lipid secretion fluctuates widely during the first few hours of such an infusion, collections of duodenal bile for analysis were begun only after 4 h and continued for 6 h. An aliquot of each mixed hourly sample was taken for measurement of bile acid, phospholipid, cholesterol, and bromsulphalein (24). The hourly output of each lipid was calculated by standard equations (22, 23).

Bile acid analysis. A sample of bile obtained from the duodenum after gallbladder stimulation by intraduodenal amino acids was added to methanol and 4 N NaOH with 5- β -cholanolic acid as an internal standard and hydrolyzed as described below. The bile acids were then extracted, methylated with diazomethane (25) or with dimethoxypropane (26), and trimethylsilyl or acetate derivatives (27, 28) were prepared. GLC was performed on 6-ft glass columns at 220°C with 1% HiEff 8BP (Applied Science Laboratories, Inc., State College, Pa.) on 100/120 mesh Gas Chrom Q with helium as the carrier gas at 30 ml/min (29). Bile acid reference standards were those used in previous studies (30).

Bile samples were examined for the presence of bile acid sulfates by the thin-layer chromatographic (TLC) method previously employed (31).

Bile acid kinetics. The day before admission to the Clinical Research Unit, the subjects took capsules containing 25 mg of 24-[13 C]cholic acid (CA)¹ and 24-[13 C]chenodeoxycholic acid (CDCA). The 13 C-labeled bile acids, synthesized by the procedure of Tserng and Klein (32), were supplied by the Argonne Bioanalytical Center and were >99% pure by TLC, GLC, and gas chromatography/mass spectrometry.

Each day for 4 or 5 d the duodenum was intubated and a 3–5-ml sample of bile-rich duodenal fluid (mixed gallbladder bile) was obtained after stimulation of the gallbladder by an intraduodenal amino acid infusion. These samples were used to determine the amount of 13 C tracer present in each primary bile acid expressed as the ratio of 13 C/ 12 C. Values were corrected for the natural abundance of 13 C. An aliquot of bile was hydrolyzed in methanolic NaOH (4 N) at 120°C, 15 lb/in² for 12 h in Parr bombs, and after cooling and acidifying, the bile acids were extracted, methylated with freshly distilled diazomethane, and acetylated by a procedure

¹Abbreviations used in this paper: CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; (FTR, fractional turnover rate.

similar to that described by Roovers et al. (28). The bile acid methyl ester acetates were extracted with diethyl ether and the extract was evaporated to dryness at room temperature under a stream of dry nitrogen. The residue was redissolved in a small amount of acetone to facilitate transfer to a 4.0-ml vial and the solvent was again removed under nitrogen. The vials were sealed with a teflon-lined cap and stored at 4°C until ready for analysis. For analysis the samples were dissolved in acetone and an aliquot removed.

Isotope ratio measurements. The bile acid methyl ester acetate mixtures were analyzed by gas chromatography-mass spectrometry-stable isotope ratio (33–35). The gas chromatography used was a Varian series 1400 (Varian Associates, Palo Alto, Calif.), with a glass column (1 mm × 183 cm) packed with 1% Poly S 179 on 100/120 Mesh Gas Chrom Q (Applied Science Laboratories Inc.). Column oven temperature was maintained isothermally at 265°C; the injector and gas chromatograph outlet temperatures were 285°C. Helium was the carrier gas at a flow rate of 9.0 ml/min. A Biospect quadrupole mass spectrometer (Scientific Research Instruments, Baltimore, Md.) operating in the chemical ionization mode (isobutane reagent gas) was used. The ion source temperature was maintained at 160°C and the source pressure was 0.6 Torr. Under isobutane chemical ionization conditions, the most abundant ions produced for the unlabeled methyl ester acetates of chenodeoxycholate (CDCA) and cholate (CA) are m/z 371 and m/z 429, respectively. A stable isotope ratio meter-multiple ion detector designed and constructed at Argonne National Laboratory was used to quantify the ratio of the ion intensities m/z 372/ m/z 371 for 24-¹³C-labeled and unlabeled CDCA derivatives. Similarly, the ratio of the ion intensities m/z 430/ m/z 429 was quantitated for the 24-¹³C-labeled and unlabeled CA derivatives. From these isotope ratio measurements, the atom percentage of excess of 24[¹³C]CA can be determined as follows (36, 37): Atom % excess = $[R'/(R' + 1)] \times 100$, where R' is the isotope ratio corrected for natural abundance contributions by subtracting the isotope ratio of unlabeled standard from the isotope ratio of the sample. A plot of the natural logarithm of the atom percentage of excess of each sample vs. time yields the first-order decay curve first described by Lindstedt (38). A linear regression least-squares analysis yields the slope and intercept. The slope is equal to the first-order rate constant, i.e., the fraction of the pool that turns over per unit time. From the intercept, the pool size can be derived as follows: $PS = [(D)/(C) (100e^B)] - D$, where PS is the pool size, D is the dose of 24-¹³C-labeled bile acid administered, B is the intercept at time zero, and C is a correction factor due to percentage labeling and instrumental mass discrimination. This factor is determined from the observed atom percentage of a dilute labeled standard divided by the theoretical atom percentage of excess of the standard.

The synthesis rate under steady state conditions will be equal to the fractional turnover rate (FTR) times the pool size.

Deoxycholic acid (DCA) pool size was calculated from its proportion determined by GLC and the pool size of CA and CDCA.

The CDCA and CA pool size measurements, determined as described above, had an average SE of <10% for all studies. The average SE of the slopes (FTR) in >90% of the studies was 13.5%. A high degree of uncertainty (>35% relative SE) was observed in the FTR measurements of ~10% of the studies. These studies were usually in subjects with slowly turning over bile acid pools for which data had been collected for <1 half-life.

The error in the isotope ratio measurements (relative SD 0.2–0.7%) cannot account for the 10–15% error in the bile acid kinetic parameters. This error could be attributed to

deviations from the idealized assumptions that the bile acids exist in single steady state pools with strictly first-order turnover rates.

Gallbladder volume and emptying. Gallbladder function was measured during the period of the bile acid kinetic study in eight pregnant women (11 studies; four in the first trimester, three in the second, and four in the third) and in eight nonpregnant women. Gallbladder volume was measured in the morning after a 12-h overnight fast by a previously described and validated real time ultrasonographic method (39). After two base-line measurements, an intraduodenal infusion of the amino acid mixture used in the biliary lipid secretion study was given at 6 ml/min for 90 min. Gallbladder volume was measured every 5 min for the first 40 min, the period of maximum contraction, and every 10 min during the last 50 min of the infusion. Fasting volume, residual volume after maximal contraction, the rate constant of emptying, and the percentage emptied were calculated.

Statistical methods. Results are expressed as means ± SEM. group means were compared by nonpaired Student's t test. Bivariate regression analyses were performed by the method of least squares. The relationships of the secretion rates of the various biliary lipids were analyzed by linear and nonlinear regression analysis, by fitting the data to the equations: $y = x/(a + bx)$, $y = ax$, and $y = ax + b$, where a and b are constants and y and x are lipid secretion rates (in micromolars per kilogram per hour). In each instance the fit of the data to these expressions was no better than to the linear expression, $y = ax + b$. Only the data from the latter regression analysis are reported. The group regression lines were compared by F statistics according to the "extra sum of squares" principle (40).

RESULTS

Biliary lipid composition. The lithogenic index of gallbladder bile was determined two or three times in each subject. The mean percentage of variance was 10.3 ± 3.3 in all subjects.

The biliary lipid composition and calculated lithogenic index of gallbladder bile were not affected by the phase of the menstrual cycle (Table II). In the several women studied in two or three phases there were no consistent trends. Accordingly, for comparison with data from pregnant women, the results of multiple studies on the same woman in different phases of the cycle were averaged and the averages were used in calculating results for all nonpregnant subjects.

In the nonpregnant women there were no statistically significant correlations between molar percentage of cholesterol or lithogenic index of either fasting hepatic or gallbladder bile and body weight or percentage of body fat. Furthermore, no index of bile saturation with cholesterol correlated significantly with serum concentrations of progesterone, luteinizing hormone, or estradiol.

The mean lithogenic indices of gallbladder bile (Table II) in the second and in the third trimesters of pregnancy were similar, and the mean of the values obtained in the last two trimesters combined (1.16 ± 0.09) was slightly higher than the mean for all control subjects (0.95 ± 0.05 , $P < 0.01$) and higher than in the first tri-

TABLE II
*Biliary Lipid Composition and Lithogenic Index of Fasting Hepatic Bile and Gallbladder Bile**

	Fasting hepatic bile				Gallbladder bile			
	BA	PL	CHOL	LI†	BA	PL	CHOL	LI
	<i>molar %</i>							
Nonpregnant (n = 23)								
Follicular phase (n = 14)	72.4±1.8	18.7±1.3	8.9±0.7	1.47±.10	76.0±1.0	18.3±0.8	5.6±0.4	0.96±.06
Midcycle (n = 3)	74.7±0.27	17.5±0.76	7.9±0.55	1.39±.14	75.5±0.89	18.5±1.5	6.0±0.63	1.02±.15
Luteal phase (n = 13)	74.0±1.2	17.1±0.8	8.9±0.8	1.57±.15	73.0±1.5	20.7±1.4	6.3±0.4	1.01±.08
All subjects (n = 23)§	72.7±1.1	18.4±0.8	8.9±0.6	1.49±.09	74.0±0.9	20.1±0.8	5.9±0.3	0.95±.05
Pregnant (n = 23)								
1st trimester (n = 9)	75.7±1.9	16.2±1.3	7.9±0.73	1.43±.08	76.7±1.7	17.7±1.4	5.6±0.39	0.98±.06
2nd trimester (n = 10)	72.5±1.4	16.7±0.87	10.4±1.2	1.79±.15	77.2±1.2	16.5±0.68	6.3±0.73	1.15±.12
3rd trimester (n = 12)	74.0±2.8	16.4±1.7	9.5±1.3	1.70±.14	75.7±1.2	17.7±0.86	6.7±0.63	1.17±.10

Abbreviations used in this table: BA, bile acids; PL, phospholipids; CHOL, cholesterol.

* Numbers indicate means±SEM.

† Lithogenic index, calculated according to Thomas and Hofmann (19), and according to the criteria of Hegardt and Dam (20) and Holzbach et al. (21).

§ There were 23 nonpregnant and 23 pregnant subjects. Some were studied more than once in different phases of the cycle or at different times in pregnancy. Since no differences could be attributed to phase of the cycle, values for individual patients were averaged and the average used in calculating the means for nonpregnant subjects.

mester of pregnancy (0.98 ± 0.06 , $0.05 < P < 0.1$). In three of the five subjects studied serially during pregnancy the lithogenic index was greater in the second than in the third trimester. In the two subjects studied in all three trimesters there was no sequential change. The lithogenic index of fasting hepatic bile was higher in the second and third trimesters of pregnancy combined (1.76 ± 0.10) than in the nonpregnant subjects (1.47 ± 0.08 , $P < 0.04$) and higher than in the first trimester (1.43 ± 0.08 , $P < 0.02$).

In the pregnant subjects, biliary cholesterol saturation was not significantly correlated with body weight

or percentage of body fat; or with the serum concentrations of progesterone, estrone, estradiol, estriol, or total estrogen (estrone, estradiol, and estriol combined).

Cholesterol crystals were not found in any subject.

Biliary lipid secretion. About 20% of subjects developed nausea during the amino acid infusion. Some vomited, causing the study to be terminated.

There were no significant effects of pregnancy or of different phases of the menstrual cycle on the mean hourly rate of secretion of any biliary lipid (Table III). There was no significant correlation between the rate of secretion of any lipid and age, body weight, percent-

TABLE III
*Biliary Lipid Secretion**

	Bile acid	Phospholipid	Cholesterol
	<i>μmol/kg/h</i>		
Nonpregnant controls			
Follicular phase (n = 10)	19.4±1.2	3.40±.27	1.01±.09
Midcycle (n = 3)	19.2±1.9	3.20±.26	.95±.02
Luteal phase (n = 9)	19.4±2.2	3.20±.26	1.03±.08
All subjects (n = 16)	19.1±1.3	3.12±.24	1.01±.07
Pregnant			
1st trimester (n = 8)	19.3±2.3	3.30±.24	1.07±.10
2nd trimester (n = 9)	16.7±1.5	2.91±.29	.90±.11
3rd trimester (n = 9)	19.1±3.0	3.26±.49	1.28±.17
All subjects (n = 18)	18.4±1.5	3.27±.29	1.11±.09

* Figures are mean±SEM. Some subjects were studied in several phases of the cycle or in more than one trimester.

age of body fat, or the serum concentration of any sex hormone measured.

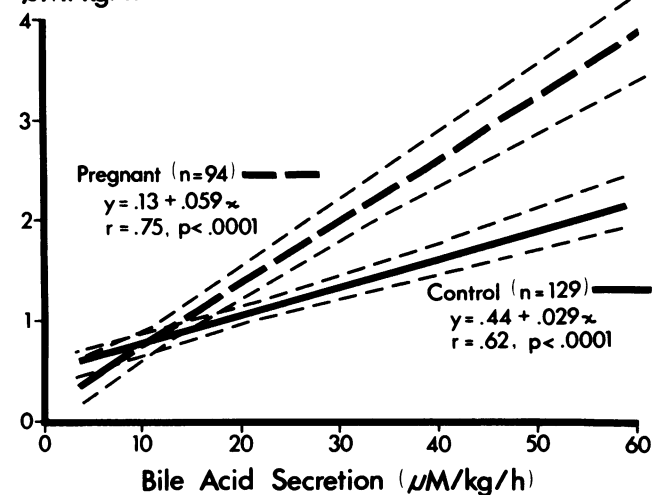
Our method of study, as discussed below, probably accounts for the generally low mean secretion rates in all groups. Although there was no difference in mean secretion rates between groups, there were significant group differences in the relationships of the biliary lipids to each other as calculated from single linear regression analyses. In the second and third trimesters of pregnancy considered separately and together (Fig. 1) the cholesterol secreted per mole of bile acid and per mole of phospholipid was approximately twice as great as in the first trimester or in the nonpregnant subject. The phospholipid secreted per mole of bile acid slightly, but significantly, increased in the second and third trimesters ($0.171 \mu\text{M/kg per h}$ in the pregnant, $0.125 \mu\text{M/kg per h}$ in the control, $F(n1-2, n2-2) = 10.5$, $t = 3.2$, $P < 0.001$). The apparent discrepancy between the findings of similar mean hourly secretion rates and the different relationships between the rates of lipid secretion is explained by the nonuniform distribution of secretion rates with the majority being low and by the nonparallel regression lines shown in Fig. 1. The lines tend to intersect at approximately the level of the mean secretory rates, but their slopes are distinctly and significantly different.

Bile acid distribution. In most subjects there was a small amount of lithocholic acid and urodeoxycholic acid as well as trace amounts of sulfated bile acids. In addition, small quantities of keto bile acids were frequently present. $3\alpha\text{OH}, 12\text{keto-}$, $3\alpha\text{OH}, 7\text{keto-}$, and $3\alpha 12\alpha, \text{diOH}, 7 \text{ ketocholanoic}$ acids were identified by gas chromatography/mass spectrometry. The total of these secondary bile acids varied from 5 to 12% and was the same in pregnant and nonpregnant subjects. These bile acids were disregarded in calculating percentages of the major bile acids. No bile acid was identified in pregnant subjects that was not present in the nonpregnant ones.

There were no changes in the bile acid distribution associated with the ovulatory cycle. During pregnancy, however, there was a progressive decrease in the percentage of CDCA and a corresponding increase in the percentage of CA (Fig. 2A and B). The ratio of CA to CDCA increased significantly in the second and third trimesters (Fig. 3). There was also a decrease in the percentage of DCA as pregnancy progressed ($r = -0.495$, $P < 0.01$). DCA was $26.1 \pm 4.4\%$ of the total in the first trimester, $21.9 \pm 3.8\%$ in the second, and $14.2 \pm 1.9\%$ in the third. The increase in the percentage of CA and the decrease in the percentage of DCA were correlated with the increases in serum progesterone, and total serum estrogen concentrations (CA vs. progesterone, $r = 0.58$, $P < 0.001$; CA vs. total estrogen, $r = 0.61$, $P < 0.001$; DCA vs. progesterone, $r = -0.50$, $P < 0.005$; DCA vs. total estrogen, $r = -0.55$,

Cholesterol Secretion

($\mu\text{M/kg/h}$)



Cholesterol Secretion

($\mu\text{M/kg/h}$)

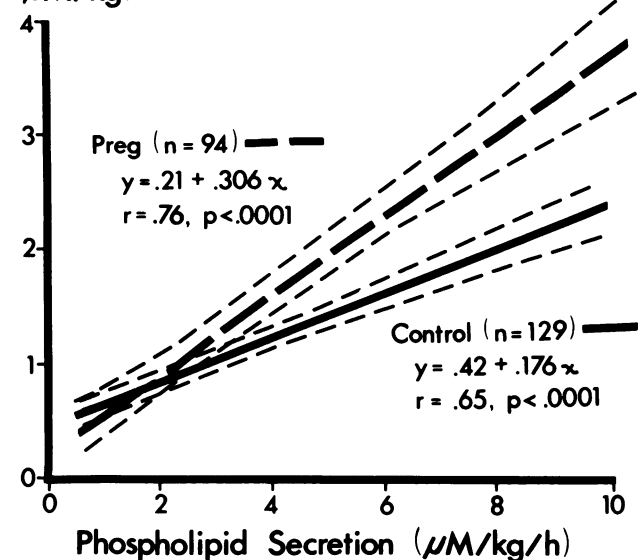


FIGURE 1 The relation of cholesterol secretion to bile acid secretion (A) and to phospholipid secretion (B) in the second and third trimesters of pregnancy combined compared with control subjects. The regression lines were calculated from hourly biliary lipid outputs with the equation, $y = ax + b$. The lines represent the range of actual data, not extrapolations. The differences between the slopes are highly significant ($P \leq 0.0001$). The light dashed lines show the 95% confidence limits.

$P < 0.005$). The progressive decrease in CDCA did not correlate significantly with the increase in hormone concentration.

Bile acid kinetics. There were no statistically significant differences in kinetic measurements between

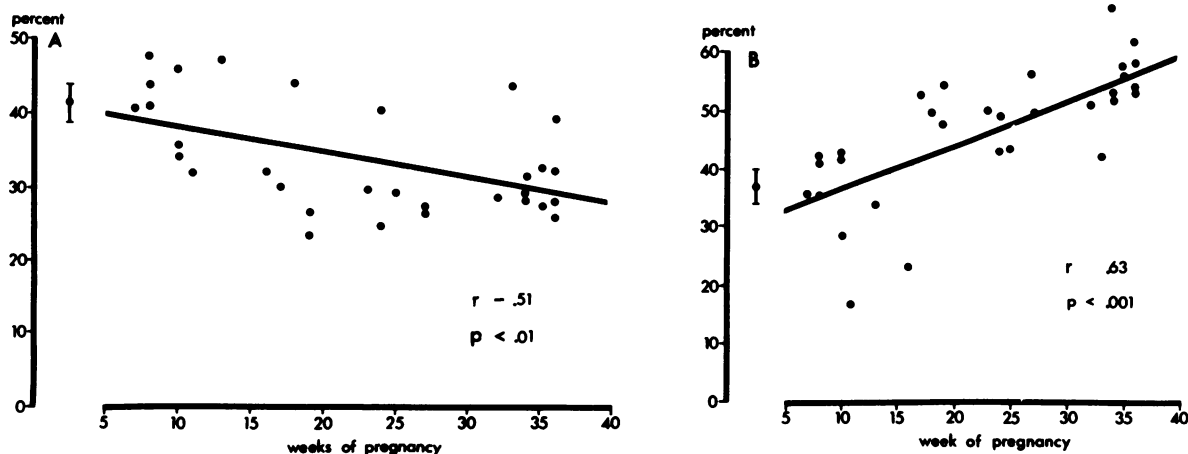


FIGURE 2 The percentage of the total bile acid pool that is CDCA (A) decreases and the percentage that is CA (B) increases progressively during pregnancy. The mean \pm SEM (\bar{x}) for the controls are shown on the left.

patients in different phases of the ovulatory cycle (Table IV, Figs. 4, and 5). Accordingly, all data from nonpregnant control subjects were combined for comparison with pregnant subjects. Data from nonpregnant subjects studied more than once were averaged and the mean value used for calculating the group mean.

There was an increase in the total bile acid pool during pregnancy (all pregnant $110.1 \pm 10.6 \mu\text{M/kg}$; nonpregnant $72.4 \pm 4.9 \mu\text{M/kg}$; $P < 0.004$). In the first trimester the mean pool size of each component bile acid was almost twice as large as in the control, nonpregnant group (for CA, $P < 0.05$; for CDCA, $P < 0.02$; for DCA, $P < 0.02$). As pregnancy progressed the CDCA and DCA pool sizes decreased and were at control levels by the third trimester. The CA pool, on the other hand, maintained an elevated level throughout

pregnancy (compared with control, second trimester, $P < 0.001$; third trimester $P < 0.03$). The pool size of individual bile acids in the several subjects studied serially during pregnancy did not show any definite trend except that the size of the DCA pool decreased in four of five subjects.

The mean FTR of each primary bile acid was slower during pregnancy than in the nonpregnant controls. This was especially marked in each trimester for CA (compared with controls, first trimester, $P < 0.05$; second trimester, $0.05 < P < 0.1$; third trimester, $P < 0.05$), but there was no progressive change during pregnancy. The mean FTR of CDCA was significantly different from controls only in the second trimester (0.328 and 0.151/d in controls and pregnant, respectively, $P < 0.001$).

The mean daily synthesis rate of CA increased $\sim 35\%$ above the mean of the control subjects in early pregnancy (P , NS) and remained constant throughout pregnancy (Fig. 4). The mean daily synthesis rate of CDCA did not change appreciably in the first trimester. In the second trimester it decreased to 50% of the first trimester rate (55% of the control rate) and then increased again to the nonpregnant value in the third trimester.

The FTR and synthesis rate of CDCA, but not CA, decreased progressively and significantly during the first half of pregnancy (Fig. 5A & C). There was no serial change during the second half as all kinetic measures fluctuated widely (Fig. 5B & D). The synthesis rate of CDCA increased as the CDCA pool size increased in pregnant subjects ($r = 0.58$, $P < 0.005$) but the synthesis rate of CA was not significantly correlated with its pool size.

The number of enterohepatic cycles per hour of the bile acid pool (calculated from the hourly bile acid

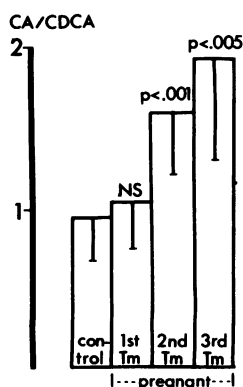


FIGURE 3 The ratio of CA to CDCA in the bile is significantly greater during the second and third trimesters of pregnancy than in the control subjects. There was no change in the first trimester. The means and SEM are shown. The numbers of subjects in each group are those shown in Table I.

TABLE IV
Bile Acid Kinetics*

	Pool size				Synthesis rate		Fractional turnover rate		Entero-hepatic cycles
	CDCA	CA	DCA	Total	CDCA	CA	CDCA	CA	
	$\mu\text{mol/kg}$				$\mu\text{mol/kg/d}$		d^{-1}		$No./d$
Nonpregnant									
Controls (n = 15)†	28.8±1.7	26.8±2.4	16.7±2.2	72.4±4.9	8.87±0.99	10.7±1.0	0.328±0.044	0.454±0.054	6.57±0.44
Follicular phase (n = 7)	29.9±2.5	26.7±3.5	20.0±2.2	75.4±6.0	7.20±0.9	8.9±1.1	0.253±0.037	0.366±0.061	6.05±0.53
Midcycle (n = 2)	24.0±6.7	25.5±9.9	22.9±7.8	71.9±8.8	5.70±0.9	10.5±3.6	0.247±0.031	0.417±0.023	6.30±0.30
Luteal phase (n = 10)	27.5±2.3	26.1±3.8	15.2±3.1	68.7±6.7	9.20±1.4	11.9±1.6	0.361±0.063	0.518±0.076	7.45±0.61
Pregnant (n = 18)†									
1st trimester (n = 7)	46.6±6.7	48.4±9.7	33.5±6.7	128.5±17.6	9.80±1.7	13.3±2.0	0.244±0.045	0.306±0.047	3.87±0.58
2nd trimester (n = 8)	33.0±2.9	48.9±3.8	24.8±5.2	106.7± 6.6	4.90±0.5	12.8±2.1	0.151±0.015	0.284±0.064	3.90±0.56
3rd trimester (n = 11)	30.0±5.3	51.8±8.4	14.3±4.2	96.1±16.3	8.90±2.3	13.3±3.2	0.309±0.050	0.275±0.054	4.56±0.82

* Values are means±SEM.

† Some subjects were studied more than once.

secretion rate [12] divided by the pool size) was not affected by the ovulatory cycle, but there were fewer cycles per hour (and per day) in the pregnant subjects ($P < 0.001$). Since there was no difference among patients in different stages of pregnancy (Table IV) data from all pregnant subjects were combined.

Gallbladder function and bile acid kinetics. The measurements of gallbladder volume and emptying

and the bile acid kinetics on a subset of the patients are shown in Table V. In other large groups of subjects studied by us, the fasting and residual gallbladder volumes were larger and the emptying rate slower in pregnant than in nonpregnant subjects (30, 31), but such differences were not present in this particular

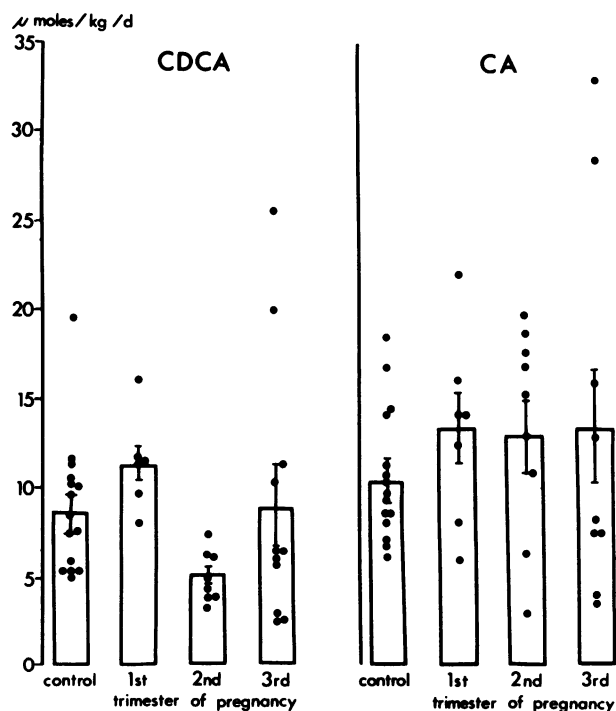


FIGURE 4 Synthesis rates of CDCA and CA in controls and pregnant subjects. In the second trimester the synthesis of CDCA decreased compared with the controls ($P < 0.001$) and compared with the first trimester rate ($P < 0.0001$). The variability of the data is especially marked in the third trimester.

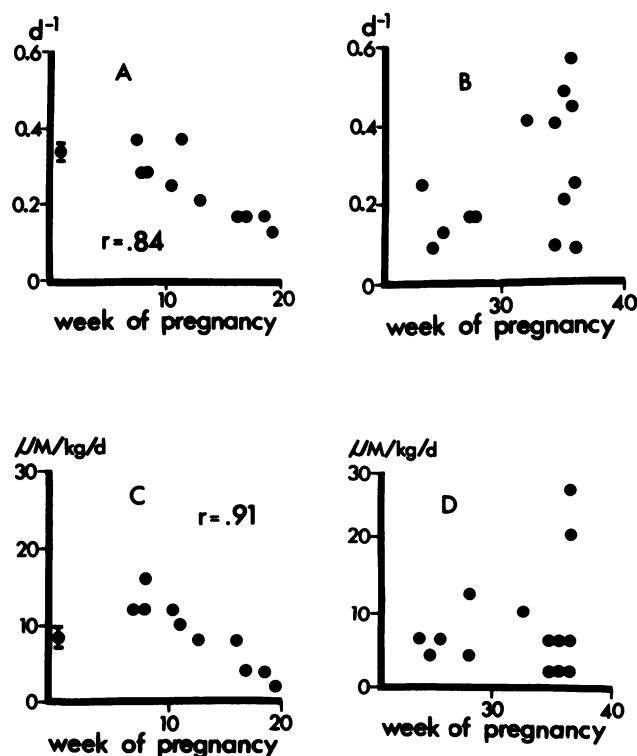


FIGURE 5 CDCA kinetics plotted against the week of pregnancy. Pregnancy is divided into halves to emphasize the striking differences between the first and second half. There were progressive decreases in the FTR (A), and the synthesis rate (C) in the first 20 wk. The earliest studies were performed during week 8. Panels B and D show the lack of any time-related change in the second half of pregnancy.

TABLE V
Sex Hormone Levels, Indices of Gallbladder Function, and Bile Acid Kinetics in Nonpregnant and Pregnant Subjects

Group	Subject	Serum hormones							Bile acid kinetics						
		Phase of cycle			Indices of gallbladder function				Bile acid pool			Synthesis		FTR	
		Proges- terone	Estradiol		FV	RV	b	Emptied	Total	Ca	CDCA	CA	CDCA	CA	CDCA
		ng/ml	ng/dl		ml	ml	min ⁻¹	%	μM/kg			μM/kg/d		d ⁻¹	
Nonpregnant (n = 8)	AS	FP	0.4	5.2	22.7	9.4	.015	58.6	93.9	48.3	37.4	6.83	7.17	.141	.192
	DV	FP	0.5	2.6	16.2	2.0	.037	85.3	70.1	26.6	26.9	8.60	5.70	.324	.213
	PG	FP	0.9	4.7	18.9	5.0	.049	73.5	89.1	33.4	34.8	14.47	10.19	.432	.291
	JR	LP	3.3	6.0	14.3	5.0	.063	65.0	109.2	41.3	32.6	16.94	19.51	.411	.596
	JW	LP	13.4	19.0	11.7	4.3	.055	63.2	47.7	11.6	26.0	9.54	11.71	.823	.733
	LM	LP	15.2	10.0	28.5	9.7	.036	66.0	55.7	26.9	27.7	18.50	7.50	.689	.271
	NM	LP	5.1	5.0	14.3	2.1	.058	85.0	59.9	21.2	28.3	8.57	10.49	.404	.370
	SF	LP	5.7	11.7	21.1	11.0	.018	47.9	97.1	40.9	33.4	10.25	9.65	.250	.289
	Mean				18.5	6.1	0.041	68.1	77.8	31.3	29.6	11.7	10.2	0.434	.370
	±SEM				±1.9	±1.2	±0.006	±4.5	±7.95	±4.3	±2.4	±1.5	±1.5	±0.083	±0.072
Pregnant (n = 8)*	NC	8	20.3	509	25.6	11.2	.014	57.1	—	54.4	—	5.9	—	.108	—
	GB	8	16.3	250	13.4	5.4	.016	60.0	90.2	36.6	43.9	12.4	11.4	.340	.261
	EB	10	30.1	340	21.4	9.2	.018	57.0	143.3	47.9	49.5	14.3	11.6	.298	.234
	CS	11	33.3	392	14.3	7.3	.012	49.0	96.6	27.4	25.7	8.2	9.0	.298	.352
	PR	17	45.5	1325	18.2	6.2	.037	65.0	94.4	52.8	24.9	10.9	3.7	.206	.150
	TM	18	36.2	1366	16.9	1.6	.028	90.0	97.7	59.7	31.8	6.2	4.8	.110	.149
	CS	25	66.5	2970	34.6	14.7	.023	58.0	141.5	60.0	43.4	12.9	6.0	.216	.139
	DW	27	49.0	2049	31.6	12.9	.019	59.0	211.8	96.6	65.4	28.3	11.2	.293	.171
	PR	34	143.5	2664	12.6	2.5	.042	80.0	70.4	41.3	26.4	3.4	2.3	.082	.086
	CS	36	127.8	4422	21.0	9.1	.019	56.7	138.9	77.7	44.3	13.3	19.8	.172	.447
	CS	36	103.3	4014	24.3	13.9	.017	42.8	105.5	52.4	45.5	32.7	25.4	.625	.589
	Mean				21.3	7.6	0.022	61.3	119.0	55.2	40.1	13.5	10.5	0.250	0.255
	±SEM				±2.2	±1.3	±0.003	±4.0	±13.1	±5.8	±3.9	±2.8	±2.3	±0.051	±0.052
	P†				NS	NS	<0.02	NS	<0.02	<0.01	<0.05	NS	NS	NS	NS

Abbreviations used in this table: FV, fasting volume; RV, residual volume after maximal contraction; b, rate constant of emptying; FP, follicular phase; LP, luteal phase.

* 11 studies were performed on eight subjects. CS was studied at 11, 25, and 36 wk. PR was studied at 17 and 34 wk.

† Significance of difference between means of the nonpregnant and pregnant groups (Student's *t* test).

small group of subjects. In the combined group of pregnant and nonpregnant subjects the total bile acid pool correlated positively with fasting and residual gallbladder volumes (Figs. 6A and B) and negatively, though weakly, with the rate constant of emptying ($r = 0.41$, $P < 0.05$). The pool size of each primary bile acid, but not of DCA, correlated significantly with both fasting and residual volumes (Table VI). When the subject groups were examined separately, the total pool size correlated significantly with gallbladder volumes in the pregnant group only. Total bile acid synthesis correlated directly with residual gallbladder volume and indirectly with the percentage emptied (Figs. 6C and D). The synthesis rate of CA, but not CDCA, directly correlated with the fasting volume and the residual volume (Table VI).

Serum lipids and bile acid kinetics. There was a significant relationship between serum triglyceride level and the synthesis rate of CDCA in the pregnant subjects only. As the serum triglyceride increased, the

synthesis of CDCA decreased ($r = -0.60$, $P < 0.005$), but according to multiple linear regression analysis this relationship was not independent of the duration of pregnancy. No significant relationships were found between CA synthesis or other kinetic measurements and either serum lipid level.

DISCUSSION

Evaluation of techniques. Serial studies of the same individuals during different phases of the ovulatory cycle and during and after pregnancy would have provided ideal data for this study, but we were unable to persuade most of these healthy volunteer subjects to cooperate to that extent. Therefore, we compared groups of subjects and in spite of large individual variations in many measures, identified important effects of pregnancy. Other, more subtle, differences secondary to the cycle or to pregnancy may have been missed.

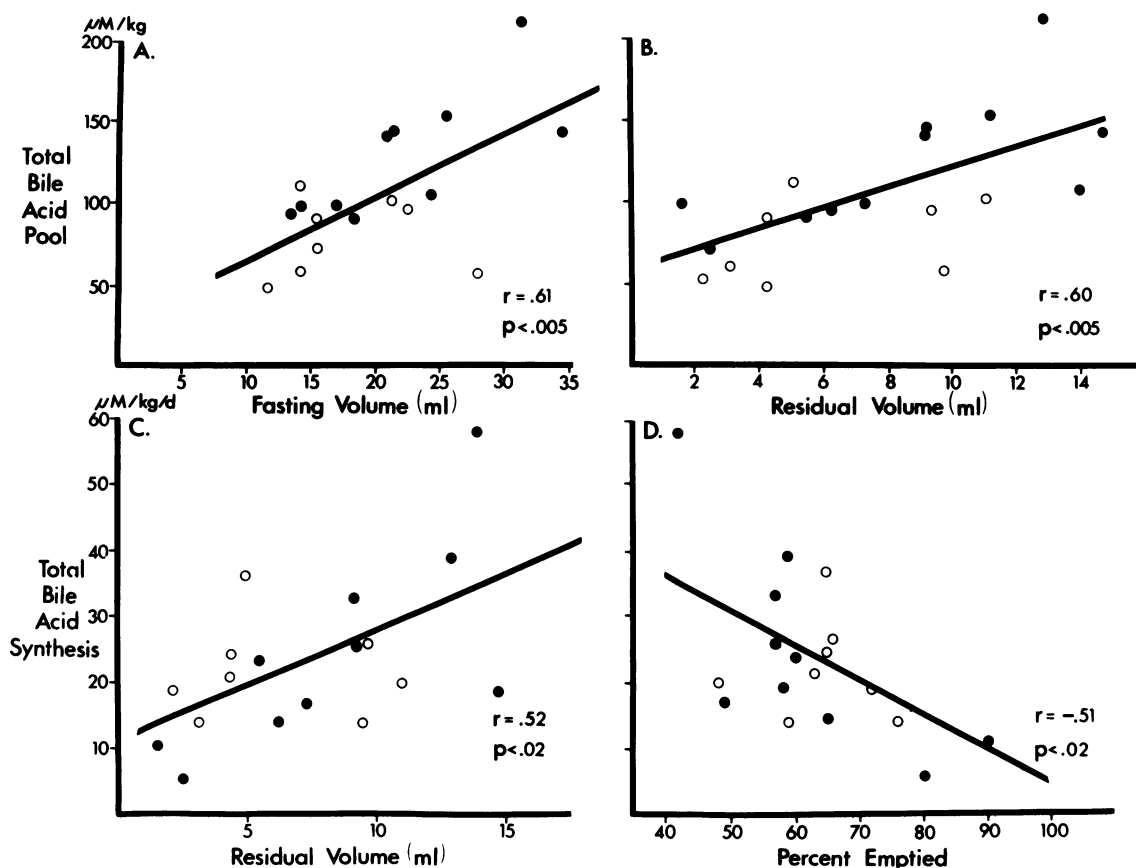


FIGURE 6 The relationship between certain indices of gallbladder function and the total bile acid pool and synthesis rate. Filled circles represent pregnant subjects and open circles non-pregnant ones. The correlation coefficients and *P* values shown are for all studies, pregnant and nonpregnant. For pregnant subjects alone, all four relationships are significant: total bile acid pool vs. fasting volume, $r = 0.78$, $P < 0.005$ (A); pool vs. residual volume, $r = 0.63$, $P < 0.025$ (B); bile acid synthesis vs. residual volume, $r = 0.68$, $P < 0.02$ (C) and synthesis vs. percentage emptied, $r = -0.67$, $P < 0.025$ (D). Since the week of pregnancy did not correlate significantly with any index of gallbladder function, bile acid pool size, or synthesis rate, it can be concluded that the relationships shown are independent of the duration of pregnancy. The correlation coefficients for the nonpregnant subjects alone were not significant.

The mean percentage of variance in the several measurements of lithogenic index of gallbladder bile during each study (10.3 ± 3.3) is a measure of both

analytic and biologic variation and is similar in magnitude to the variation reported by Whiting et al. (41).

The mean secretion rates of biliary lipids in all of our subjects are considerably lower than those reported by Grundy and associates (42, 43) and by other laboratories (44), but they are only slightly less than those reported by Shaffer and Small (23). Most investigators have stimulated biliary lipid secretion by a continuous infusion of a liquid formula of dextrose, fat, and protein, (22, 42–44), but we, like Shaffer and Small (23), used a mixture of essential amino acids instead of the liquid formula. In a separate study of five individuals we measured biliary lipid secretion, gallbladder emptying, and small bowel transit during infusions of amino acids and a complex liquid formula containing 40% fat, similar to the one used by Grundy and Metzger (22). With the liquid formula infusion, gallbladder emptying and small bowel transit were stimu-

TABLE VI
Correlation of Pool Size and Synthesis Rate of Primary Bile Acids to Fasting and Residual Gallbladder Volume*

	Pool ($\mu\text{M/kg}$)		Synthesis ($\mu\text{M/kg/d}$)	
	CDCA	CA	CDCA	CA
Fasting volume	0.60†	0.61†	NS	0.48§
Residual volume	0.61†	0.53	NS	0.56

* $n = 16$. The figures given are correlation coefficients.

† $P < 0.01$.

§ $P < 0.05$.

|| $P < 0.03$.

lated more effectively and there was a 50–100% greater rate of secretion of each lipid (45). Therefore, our biliary lipid secretion rates and those of others who used an amino acid infusion do not represent maximum rates of secretion.

Effects of the ovulatory cycle. The stage of ovulatory cycle did not seem to affect either biliary lipid or bile acid composition, lipid secretion, or bile acid kinetics. Comparable studies in man are very limited and conflicting. In two of three studies there was no difference in biliary lipid composition in different phases of the cycle (41, 46, 47).

Effects of pregnancy. Biliary lipid composition and secretion and bile acid kinetics have not been studied previously in pregnant women. The only modern report of biliary bile acid composition in pregnancy, published after our study was in progress, showed an increase in percentage of cholic acid in four healthy, pregnant women (48). In a sterol balance study, an increase in fecal acidic sterol excretion in the second trimester was the only statistically significant change (49).

The study of bile acid kinetics would not have been possible without the use of bile acids labeled with the stable isotope of carbon, ^{13}C . The use of stable isotopes in the study of bile acid kinetics has been previously validated (50, 51).

In the last two-thirds of pregnancy the bile was slightly but significantly more saturated with cholesterol than it was in either the first third of pregnancy or in the nonpregnant women, which suggests that pregnancy does indeed increase the risk of gallstones. Cholesterol crystals were not found in any subject, regardless of the degree of supersaturation of gallbladder bile. It is possible that crystals might have been found had we examined the bile after storage at 37°C, as described by Sedaghat and Grundy (52), but this is unlikely because crystals are almost never found in lithogenic bile of individuals without gallstones (52, 53).

Pregnancy did not affect the mean hourly rate of secretion of any biliary lipid during a continuous intraduodenal infusion of amino acids. The regression lines in Fig. 1, however, show that when the bile acid or phospholipid secretion rates were high, the cholesterol secretion rate was greater in the second and third trimesters of pregnancy than in the nonpregnant subjects, which suggests a difference in hepatic coupling of biliary lipids, consistent with the secretion of more lithogenic bile. When bile acid and phospholipid secretion rates were low, as most were in this study, cholesterol secretion was similar in all groups of subjects. The low mean rate of secretion of bile acids probably reflects the submaximal stimulation of the enterohepatic circulation.

There was an increase in total bile acid pool size in pregnancy as early as the first trimester and a decrease in the percentage of CDCA and an increase in the per-

centage of CA. The FTR of CA was decreased throughout pregnancy but that of CDCA in the second trimester only. The mean synthesis rate of CA was increased 35% throughout pregnancy (P , NS). The synthesis of CDCA was not significantly increased. It was significantly decreased in the second trimester. The rate of enterohepatic cycling of the bile acid pool was decreased throughout pregnancy.

We elected to express our results for bile acid pool sizes and synthesis rates in micromoles per kilogram of actual body weight, instead of per kilogram of nonpregnant body weight. Had we used the latter, the differences attributed to pregnancy would have been greater, especially in the late stage of pregnancy.

Mechanism of changes in bile acid kinetics. When bile acid FTR is increased secondary to increased fecal excretion, synthesis is increased and pool size is variably affected. On the other hand, if bile acids are temporarily sequestered within the enterohepatic circulation, either in the gallbladder or small intestine, less will return to the liver and the synthesis rate and pool size will increase until a new steady state is reached (54–56). We suggest that this sequence is at least partially responsible for the increased bile acid pool size in pregnancy. As the fasting and residual gallbladder volume increased, the pool size increased (Fig. 6).

Other studies provide support for a similar relationship between gallbladder volume and bile acid pool size in patients with celiac-sprue (57, 58) and in man and dog after truncal vagotomy (55, 59). Gallbladder volume increases, emptying after meals slows and the bile acid pool increases. On the other hand, after cholecystectomy (when there is no bile storage) bile acids circulate continuously and the pool usually decreases (60–62). The bile acid pool can also be modified by altering intestinal transit. It is temporarily increased by drug-induced retardation of transit to the ileum (56), and is decreased by shortening transit time (63). Slowed intestinal transit in pregnancy was recently reported (64).

Proposed hormonal effects. Although pregnancy is accompanied by many complex hormonal and metabolic changes, it is likely that the elevated serum levels of progesterone, a smooth muscle relaxant, is responsible for the larger gallbladder volume, impaired emptying and slowed intestinal transit in pregnancy. Pregnancy and the administration of progesterone impair gallbladder emptying after a fatty meal and after cholecystokinin in animals (65–67). Fasting volume and residual volume of the gallbladder after a physiologically induced contraction increase progressively with increasing progesterone levels to 80 ng/ml ($r = 0.74$, $P < 0.001$) (Everson, Lawson, McKinley, Johnson, and Kern, paper submitted).

As pregnancy progresses there are increases in the fasting gallbladder volume, residual gallbladder

volume, and average hourly volume throughout the day (68), findings consistent with a progressive increase in the reservoir capacity of the gallbladder, but the bile acid pool size does not continue to enlarge after the first trimester. Since several studies suggest that in the latter part of pregnancy gallbladder bile is dilute (69, 70), the large gallbladder in later pregnancy may not store more bile acid than in early pregnancy. The pool would then be unaltered and the steady state achieved in the first trimester would persist unchanged.

Different responses of individual bile acids in pregnancy. Bennion et al. (71–73) have shown an important relationship between female sex hormones and the ratio of CDCA to CA, similar to the one occurring in pregnancy. They found that in women with active ovarian function the percentage of CDCA and/or the CDCA pool size was smaller than in men or in women without active ovarian function. Contraceptive steroid administration also caused a decrease in percentage of CDCA in the bile acid pool in one group of subjects studied by Bennion et al. (74). In general, both endogenous and exogenous female sex hormones seem to be associated with a relative increase in CA and a decrease in CDCA. This could be due to hormonal modulation of specific enzymes that regulate bile acid biosynthesis. The alternate possibility, that hormones affect intestinal absorption of each primary bile acid differently, seems remote.

Proposed explanation for increased lithogenicity of bile. The administration of CDCA to gallstone patients for the dissolution of gallstones converts the bile acid pool to a predominance of CDCA and decreases the cholesterol saturation of bile (75). Evidence suggests that the individual primary bile acids exercise different effects upon the coupling of phospholipid and cholesterol to bile acids during their secretion into bile (76). The slight but significant increase in lithogenicity of bile of these non-obese pregnant women might be due to the change in bile acid composition.

In summary, we interpret the changes reported as follows: early in pregnancy increased serum progesterone causes gallbladder emptying to be delayed and incomplete, and may also slow intestinal transit, thereby decreasing the rate of return of bile acids to the liver. This decreased rate of return (possibly coupled with an estrogen-induced increased responsiveness of the liver) stimulates the synthesis of both bile acids and the pool expands until a new steady state is reached. As pregnancy progresses the synthesis of CDCA, but not CA decreases, possibly because of hormonal or metabolic effects on hepatic enzymes. The relative decrease in CDCA in the bile might affect coupling of bile acids to phospholipid and cholesterol so that the biliary cholesterol saturation increases.

When the cholesterol saturation is high enough and the conditions are appropriate for nucleation and precipitation of cholesterol crystals, the gallbladder, which empties incompletely, tends to retain the crystals and stone formation begins.

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