Cholesterol synthesis in the perfused liver of pregnant hamsters

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Abstract Pregnancy is a risk factor for the development of cholesterol gallstones. In pregnant women, biliary cholesterol saturation and secretion are increased. To investigate whether this was due to increased cholesterol synthesis, we studied hepatic cholesterol synthesis in Syrian Golden hamsters. Female controls and animals 10- to 14-days pregnant were studied. The studies were performed in the in situ perfused hamster liver. Cholesterol synthesis was determined by measuring the incorporation of ^3H_2O added to the perfusate into hepatic, perfusate, and bile cholesterol during a 90-min period. In both pregnant groups, bile flow decreased significantly, but biliary cholesterol concentration increased only in the 14-day pregnant group. The cholesterol synthesis rate averaged (mean ± SD) 172 ± 27, 127 ± 37, and 552 ± 79 nmol • hr^{-1} • g $liver^{-1}$ in controls, 10-day, and 14-day pregnant animals, respectively. The 14-day pregnant animals secreted a markedly higher fraction (47.3 ± 11.3 vs. 11.1 ± 13.4%; $P < 0.01$) of newly synthesized cholesterol into bile but not into perfusate. Chenodeoxycholate, but not cholate, synthesis rate was decreased in both pregnant groups. We conclude from our studies that hepatic cholesterol synthesis increases towards the end of pregnancy in the hamster and that more newly synthesized cholesterol is secreted into bile at that time. This could at least partially explain the increased biliary cholesterol saturation and secretion observed in women in the third trimenon, and explain pregnancy as a risk factor in the development of cholesterol gallstones.—**Reichen, J., G. Karlaganis, and F. Kern, Jr.** Cholesterol synthesis in the perfused liver of pregnant hamsters. *J. Lipid Res.* 1987. **28:** 1046–1052.

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Pregnancy is a risk factor for the development of cholesterol gallstones in women (1). An increase in biliary cholesterol saturation has been observed in the second half of pregnancy (2). This has been ascribed to a change in the coupling of cholesterol to bile acid secretion (2). An increase in fecal sterol excretion has been described in pregnant hamsters (3).

We explored whether cholesterol synthesis is altered during pregnancy. The studies were performed in Syrian Golden hamsters, a species selected because of the similarities of bile acid composition (4) and regulation of sterol synthesis (5) to those of humans. Cholesterol synthesis was studied using the incorporation of ^3H_2O into cholesterol (6, 7) in the in situ perfused hamster liver.

MATERIALS AND METHODS

Animal experiments

Female Golden Syrian Hamsters were obtained from Madoerin Animal Farm, Fuellinsdorf, Switzerland. The animals were kept in temperature- and humidity-controlled animal quarters under a 12-hr light/dark cycle. Three groups of four animals each were studied: a control group consisting of virgin littermates, and pregnant animals at the 10th and 14th day of pregnancy, respectively.

Hamster liver perfusion was carried out in situ using a technique described previously for rat liver (8, 9). The animals were anesthetized with pentobarbital, 50 mg/kg body weight intraperitoneally. The common bile duct was cannulated with a PE10 catheter and the cystic duct was ligated. The animal was then heparinized, the portal vein was cannulated in situ, and perfusion was started. The abdominal inferior vena cava was cut and then the thoracic inferior vena cava was cannulated after a thoracotomy. The abdominal vena cava was then ligated, thus establishing recirculating perfusion. Viability of the preparation was monitored as described before (9).

The perfusion medium consisted of Krebs-Ringer-bicarbonate buffer containing bovine serum albumin (2% w/v) and glucose (0.1% w/v). It was gassed with oxygen -CO_2 95:5 (v/v). The pH was monitored continuously and corrected with $NaHCO_3$ when necessary. Temperature was kept constant at 37°C in a thermostatted cabinet. Taurocholate was infused into the perfusion reservoir at a rate of 0.1 μmol/min to maintain basal bile salt secretion. After an equilibration period permitting the liver to warm up, 1 Ci of ^3H_2O (Amersham International, Amersham,

Buckinghamshire, U.K.) was added to the perfusate reservoir and perfusion was continued for 90 min. Bile was sampled in three 30-min intervals into tared tubes and weighed. At the end of the experiment, the liver and perfusate were removed and processed as described below.

Lipid extraction and group separation

Approximately 1 g of liver, the whole bile specimen, and 4 ml of perfusate were extracted according to Bligh and Dyer (10) after adding [4-^{14}C]cholesterol (New England Nuclear, Boston, MA), [4-^{14}C]cholesteryl oleate, and tauro-[^{14}C]carbonyl cholate as internal standards. The phases were separated by centrifugation. The lower phase was extracted a second time with an upper phase mixture (10). The upper phases were combined, 30 ml of methanol was added, and the sample was evaporated to dryness in a Rotavapor at 50°C. The lower phase was evaporated in a Rotavapor at room temperature.

Cholesterol and cholesteryl esters were separated by a step gradient on Sep-Pak silica cartridges (Waters Chromatography Division, Schlieren, Switzerland). The cartridges were washed with 10 ml of petroleum ether (bp 40°–60°C). The samples were dissolved in 30 ml of petroleum ether and applied onto the column. A first fraction, containing the cholesteryl esters, was eluted with 10 ml of petroleum ether–diethyl ether 98.5:1.5 (v/v) and evaporated to dryness under nitrogen (11). The column was washed with 10 ml of petroleum ether–diethyl ether 93:7 (v/v). Cholesterol was then eluted with petroleum ether–diethyl ether 25:75 (v/v) and evaporated to dryness under nitrogen at 30°C (12). To validate this approach, the relevant biological materials were spiked with [^{14}C]cholesterol and [^{14}C]cholesteryl oleate. Recoveries were 98.6 ± 5.0 and 92.8 ± 4.1%, respectively (n = 6).

Determination of lipid concentrations

Cholesterol was further purified by thin-layer chromatography on a precoated aluminum sheet (0.2 mm silica gel 60 F254) (Merck AG, Darmstadt, Germany) using a Linomat III sample applicator (CAMAG AG, Muttenz, Switzerland). A modification of the system described by Polokoff, Iwahashi, and Simon (13) consisting of heptane–diisopropyl ether–acetic acid 60:40:3 (v/v) was used. A cholesterol reference spot was applied to either side of the thin-layer chromatography plate; it was visualized with 10% phosphomolydbic acid in ethanol. The cholesterol sample spot was localized using the reference spot, cut out, and eluted with 10 ml of diethyl ether during 30 min in an ultrasonic bath. Half of the sample was concentrated to a volume of 100 μl for assay of ^{14}C and ^{3}H radioactivity.

The other half was evaporated to dryness under nitrogen. Coprostanol (10 nmol for bile and perfusate, 100 nmol for liver homogenate) was added as an internal standard and trimethylsilyl ethers were prepared (14). The residue was taken up in 100 μl of hexane; 0.3 μl was injected by an on-column injector (15). Capillary gas–liquid chromatography was performed on an OV73 column as previously described for bile alcohols (16). The retention times of cholesterol and coprostanol in this system were 10.0 and 7.5 min, respectively. All results were corrected for losses as monitored by the final recovery of the added [^{14}C]cholesterol.

The silica SepPak eluates containing the cholesteryl esters were transferred to Teflon-lined test tubes and 0.5 ml of sodium hydroxide (0.5 M in methanol) was added. Hydrolysis was carried out for 30 min at 110°C (11). The solution was neutralized to pH 7 with 0.5 M hydrochloric acid in methanol and brought to dryness. The liberated cholesterol was extracted by the Bligh-Dyer procedure (10). The lower phase was evaporated at 40°C under a stream of nitrogen and the cholesterol was prepared as described above.

Bile acid separation

The aqueous phase of the Bligh-Dyer extraction was evaporated to dryness in a Rotavapor together with 30 ml of ethanol. To completely eliminate $^{3}H_2O$, bile acids were extracted on SepPak C18 cartridges (17). The methanolic eluate was hydrolyzed using cholylglycine hydrolase (18) and reextracted on SepPak C18 (17). Bile acids were then further purified on 0.2 mm silica gel 60 F254 using isooctane–diisopropylether–acetic acid 50:25:25 (19). The reference bile acids, cholate, chenodeoxycholate, and deoxycholate, were visualized with 10% phosphomolybdic acid in ethanol. Half of the spots corresponding to cholate and chenodeoxycholate were concentrated for assay of ^{14}C and ^{3}H radioactivity; the other half was brought to dryness, methylated, and silylated after addition of coprostanol as an internal standard. Mass was determined by capillary gas–liquid chromatography as previously described (18).

^{14}C and $^{3}H_2O$ radioactivity was determined by liquid scintillation counting using Lumagel (Lumac/3M, Schaensberg, Netherlands) as a scintillator in a Tricarb 2660 liquid scintillation counter (Packard Instruments International, Zurich, Switzerland). Appropriate background subtraction was made and quenching was corrected by the external standard ratio technique. Unless otherwise stated, all samples contained counts of > 5× background. Specific activity of water was determined on an aliquot of the perfusate that had been appropriately diluted.

Data analysis

Cholesterol synthesis rate was calculated according to Dietschy and his colleagues (7, 20, 21). Cholate and chenodeoxycholate synthesis rates were calculated in an analogous fashion from the specific activity of newly synthesized cholesterol. Unless otherwise stated, all results are expressd as mean ± 1 standard deviation. Group means were compared for statistical significance by analysis of variance followed by Student's *t*-test (22) when the former reached significance; $P < 0.05$ was considered statistically significant.

TABLE 1. Body and organ weights for female hamsters

Group	Body	Liver	Uterus
	g	*g*	*g*
Control	107 ± 17[a]	4.39 ± 1.04	
10-Day pregnant	140 ± 13	6.58 ± 1.18	7.25 ± 1.94
14-Day pregnant	151 ± 17	6.34 ± 0.41	20.58 ± 4.29
Significance			
Control vs. 10-day pregnant	< 0.02	< 0.05	
Control vs. 14-day pregnant	< 0.02	< 0.02	
10-Day pregnant vs. 14-day pregnant	> 0.35	> 0.70	< 0.002

[a]Values are given as means ± SD, n = four animals per group.

RESULTS

Organ weights in the three animal groups are given in **Table 1.** Both body and liver weights were significantly increased in the pregnant animals as compared to controls, but there was no difference between the two pregnant groups. Uterus weight, as expected, was increased more in the 14-day pregnant animals.

All perfusions met the viability criteria referred to in the Methods section. There was no difference between the three groups with respect to perfusion pressure or perfusate flow. They averaged 10.0 ± 1.2 cm of H_2O and 2.81 ± 1.00 ml • min^{-1} • g $liver^{-1}$, respectively.

Bile flow and bile composition are reported in **Table 2.** Bile flow was markedly decreased in both pregnant groups as compared to controls, but there was no statistically significant difference between the 10- and 14-day pregnant animals. Biliary cholesterol concentration was increased in the 14-day pregnant hamsters only, the difference being significant compared to both control and 10-day pregnant hamsters (Table 2). By contrast, cholesterol output was significantly decreased in the 10-day pregnant hamsters only, being comparable in near-term pregnant and control animals (Table 2).

Cholesterol and cholesteryl ester contents of liver and perfusate are given in **Table 3.** None of the differences reached statistical significance by analysis of variance.

The hepatic cholesterol synthesis rates in the three groups averaged 172 ± 27, 127 ± 37, and 552 ± 79 nmol • hr^{-1} • g $liver^{-1}$ in the control, 10-day pregnant, and 14-day pregnant groups, respectively (**Fig. 1**). ANOVA showed a significant treatment effect at the $P = 0.001$ level; the differences between the groups are shown in Fig. 1. The specific activities of cholesterol and cholesteryl esters are given in **Table 4.** The specific activity of cholesteryl esters could not be calculated on all the specimens because of a low incorporation of tritiated water. Therefore, no further statistical analysis could be performed on these data.

Control animals secreted 21.2 ± 19.6 nmol/30 min of newly synthesized cholesterol into bile. Ten-day pregnant animals secreted 28.3 ± 15.5 nmol/30 min, not statistically different from controls, but different from the 14-day pregnant animals ($P < 0.002$) which excreted 163.9 ± 80.7 nmol/30 min. This difference was also statistically significant compared to controls ($P < 0.002$). Thus, the percentage of newly synthesized cholesterol secreted into bile was 11.1 ± 13.4, 31.1 ± 20.6, and 47.3 ± 11.3% in the three groups, respectively. Only the difference between control and 14-day pregnant animals reached statistical significance ($P < 0.01$). The corresponding values for perfusate/liver were 0.7 ± 0.6, 0.8 ± 0.7, and 0.4 ± 0.4%, respectively, ANOVA failing to demonstrate any effect of pregnancy.

Bile acid synthesis rates are reported in **Table 5.** ANOVA failed to show an effect of pregnancy on cholic acid synthesis rate while an effect at the $P < 0.001$ level on chenodeoxycholate synthesis rate was seen. Further analysis showed

TABLE 2. Bile flow and cholesterol in bile

Group	Bile Flow	[Cholesterol]	Cholesterol Excretion
	$\mu l/min^{-1}g^{-1}$	*nM*	$nmol \cdot min^{-1}g^{-1}$
Control	1.06 ± 0.29	0.241 ± 0.118	0.263 ± 0.084
10-Day pregnant	0.49 ± 0.12	0.204 ± 0.071	0.095 ± 0.025
14-Day pregnant	0.40 ± 0.06	0.893 ± 0.411	0.330 ± 0.138
Significance			
Control vs. 10-day pregnant	< 0.02	> 0.60	< 0.02
Control vs. 14-day pregnant	< 0.005	< 0.025	> 0.30
10-Day pregnant vs. 14-day pregnant	> 0.25	< 0.02	< 0.02

The means of the three bile collection periods are given ± 1 standard deviation (n = four animals per group).

TABLE 3. Cholesterol and cholesteryl ester content of liver and perfusate in three groups of hamsters

	Cholesterol		Cholesteryl Esters	
	Liver	Perfusate	Liver	Perfusate
	μmol/g	*nmol*	*μmol/g*	*nmol*
Controls	1.359 ± 0.381[a]	0.539 ± 0.340	0.297 ± 0.395	0.221 ± 0.097
10-Day pregnant	0.918 ± 0.113	0.696 ± 0.674	0.385 ± 0.133	0.147 ± 0.152
14-Day pregnant	1.040 ± 0.147	1.811 ± 0.942	0.104 ± 0.202	0.251 ± 0.097
Significance				
Control vs. 10-day pregnant	NS[b]	NS	NS	NS
Control vs. 14-day pregnant	NS	NS	NS	NS
10-Day pregnant vs. 14-day pregnant	NS	NS	NS	NS

[a]Means ± SD, n = four animals per group.
[b]Not significant by ANOVA.

a statistically significant decrease by 50% and 76% decrease compared to controls after 10 days ($P < 0.025$) and 14 days ($P < 0.001$) of pregnancy, respectively. The difference between the two pregnant groups reached significance at the $P < 0.01$ level.

DISCUSSION

Our study demonstrates a threefold increase of hepatic cholesterol synthesis in 14- but not 10-day pregnant hamsters, pregnancy lasting 15 days in this species. At that time more newly synthesized cholesterol was excreted into bile but not into perfusate. In contrast to the changes in cholesterol synthesis which were restricted to late pregnancy, both pregnant groups had reduced chenodeoxycholate synthesis rates as compared to female control animals. Similarly, bile flow was reduced in both pregnant groups.

Use of tritiated water as a precursor to measure cholesterol synthesis is the method of choice since it avoids problems associated with precursor pools and precursor uptake (21). The initially described method used digitonin precipitation to isolate the sterols (7). Tedious precautions were necessary to avoid nonspecific binding of tritiated water to these precipitates (7, 21). We have avoided these difficulties by substituting solid phase extraction on SepPak silica columns followed by thin-layer chromatography. Our method has the advantage of complete elimination of tritiated water not associated with the sterols and of being more rapid than the digitonin precipitation method. Fractionated elution of the lipids from the SepPak cartridge (11, 12) achieved rapid and quantitative separation of cholesterol from cholesteryl esters.

The finding of a decreased bile flow in pregnant hamsters (Table 2) is in agreement with the data reported by Reyes and Kern (4). These authors ascribed the cholestasis to a

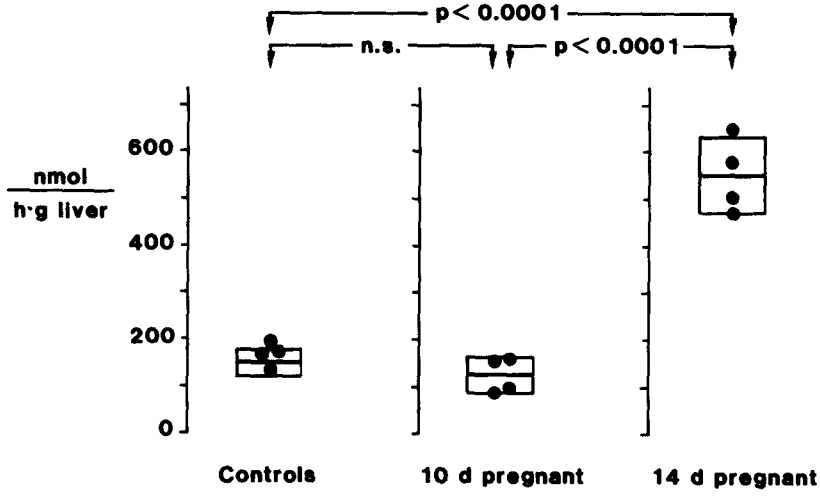

Fig. 1. Cholesterol synthesis rate in the liver of control, 10-day, and 14-day pregnant hamsters. Hepatic cholesterol synthesis was significantly increased at 14 but not at 10 days of pregnancy.

TABLE 4. Specific activity of cholesterol and cholesteryl ester in hamsters

	Cholesterol			Cholesteryl Ester	
	Liver	Bile[a]	Perfusate	Liver	Perfusate
			dpm/nmol		
Control	58.9 ± 28.6	43.8 ± 37.1	35.5 ± 25.9	2.6 ± 1.4[b]	0.4, 2.9[c]
10-Day pregnant	36.3 ± 9.1	60.9 ± 29.0	26.9 ± 16.5	0.5, 1.4	ND[d]
14-Day pregnant	148.6 ± 28.1	388.4 ± 102.9	64.6 ± 62.8	4.6 ± 2.2[b]	6.9 ± 2.8
Significance					
Control vs. 10-day pregnant	> 0.15	> 0.20	NS[e]	ND[f]	
Control vs. 14-day pregnant	< 0.005	< 0.001			
10-Day pregnant vs. 14-day pregnant	< 0.0002	< 0.001			

Values given as means ± 1 standard deviation; n = four animals per group.
[a]Reported only for the last collection period.
[b]n = 3.
[c]n = 2 only, because of a low ^3H incorporation rate.
[d]Radioactivity too low in all samples.
[e]No significant difference by ANOVA.
[f]ANOVA not performed because of missing data (see also b-d).

decrease in the so-called bile salt-independent fraction of bile. Cholesterol concentration in bile was significantly increased toward the end of pregnancy, again in agreement with the earlier report (4). The decrease in bile flow, however, had the effect that cholesterol secretion rate was diminished in 10-day pregnant hamsters and comparable to controls in the 14-day pregnant group (Table 2).

Cholesterol synthesis was increased threefold at the end of pregnancy in the hamster (Fig. 1). The literature is scant and controversial with respect to the effects of pregnancy on cholesterologenesis. Thus, Feingold et al. (23) using a ^3H_2O-incorporation method similar to ours in intact rats observed a 2.5-fold increase in hepatic cholesterol synthesis, a finding comparable to our results. The same authors reported a similar effect of pregnancy on cholesterologenesis in Sanguines Fusciolli monkeys (8). By contrast, Leoni and coworkers (24) found no change of cholesterol synthesis in pregnancy in rats, and quite to the contrary, reported a marked drop just prior to delivery. These authors used [^{14}C]acetate to label newly synthesized cholesterol, but did not provide proof that the pool and uptake of C2 fragments are unaltered by pregnancy.

The percentage of newly synthesized cholesterol excreted into bile in control hamsters is similar to that described by Turley and Dietschy (20) in rats. The ratio of the specific activity of cholesterol in bile and in the liver was close to unity, again a finding similar to that observed in rats (25, 26). The percentage of newly synthesized cholesterol appearing in bile increased threefold in 14-day pregnant animals (Table 4).

Very little newly synthesized cholesterol was incorporated into cholesteryl esters (Table 4). Hepatic cholesterol and cholesteryl ester content were not modified in pregnancy in agreement with the literature (24). This is in contradistinction to the effects of female sex hormones on hepatic cholesterol esterification (27). Hepatic cholesterol esterification was not measured directly in our experiments, however.

Serum cholesterol concentration is increased in pregnancy in women (3, 24, 28, 29). Neither cholesterol nor cholesteryl ester secretion into perfusate was affected by

TABLE 5. Bile acid synthesis rate in three groups of hamsters

	Synthesis Rate	
	Cholate	Chenodeoxycholate
	nmol · hr^{-1} · g liver^{-1}	
Control	95 ± 17	46 ± 11
10-Day pregnant	112 ± 60	23 ± 6
14-Day pregnant	100 ± 12	11 ± 2
Significance		
Control vs. 10-day pregnant	NS[a]	$P < 0.025$
Control vs. 14-day pregnant	NS	$P < 0.001$
10-Day pregnant vs. 14-day pregnant	NS	$P < 0.02$

Values given as means ± SD, n = three animals per group.
[a]Not significant by ANOVA.

pregnancy in our experiments (Tables 3 and 4). This most likely represents a perfusion artefact due to the absence of lipoproteins as acceptor molecules in the perfusate (30).

Chenodeoxycholate, but not cholate, synthesis rate was markedly decreased in both pregnant groups; this effect was significantly more important in the 14-day pregnant hamsters (Table 5). Instead of using the specific activity of water, the specific activity of newly synthesized cholesterol was used to calculate bile acid synthesis. This appears justified since *1)* cholesterol is the preferred precursor for cholate synthesis (31); and *2)* our values for cholate and chenodeoxycholate synthesis in controls (Table 5) are close to the 76 and 47 nmol • hr^{-1} • g $liver^{-1}$ which can be calculated from the data reported in the hamster by Beher et al. (32).

The differential effect of pregnancy on cholate and chenodeoxycholate synthesis rate is in agreement with data obtained in pregnant women by isotope dilution (2). Which hormone is responsible for this effect is unknown at present. Total bile acid synthesis is decreased by estrogens in male rats (33) while a combination of progesterone and estrogens is required to actually decrease bile salt output (34).

Use of the perfused liver permits elimination of non-hepatic events. All the livers reported in the present communication were viable by preset criteria including blood flow and transaminase and potassium release (8, 9), the only exception being a decrease in bile flow. The latter finding does not invalidate the preparation, however, since the same phenomenon has been observed in intact hamsters (4). The perfused rat liver has been successfully used to measure hepatic cholesterologenesis (25, 30). Further support for the validity of our experimental approach is the rate of cholesterol synthesis which is only marginally lower than that reported in intact hamsters (5).

Which hormonal effects are responsible for the changes observed in the present experiments has not been investigated. Pharmacological doses of progesterone increase biliary cholesterol secretion while ethinyl estradiol has the opposite effect and is able to counterbalance the progesterone effects (34, 35). Estrogens are cholestatic (36); ethinyl estradiol treatment causes increased susceptibility of the liver to the cholestatic effects of a high dose of cholic acid (37). This is unlikely to have contributed to the cholestasis observed in the present experiments since the taurocholate infusion used was well below its secretory maximum and selected to mimic normal bile salt secretion rates (4). The interruption of the enterohepatic circulation is unlikely to have confounded interpretation of the present experiments. Thus, enterohepatic circulation was interrupted for 90 min only, while much longer treatments have been used to alter hepatic cholesterol synthesis (27, 34, 35, 38). We cannot rule out a small effect due to absence of feed-back regulation by lipoproteins in the perfusate (39, 40), however.

In conclusion, our study has shown a marked increase in hepatic cholesterol synthesis towards the end of pregnancy in hamsters. This is associated with a marked increase of the percentage of newly synthesized cholesterol appearing in bile. Chenodeoxycholate synthesis is markedly reduced, while cholate synthesis is unaffected in pregnancy. Thus, increased cholesterol synthesis could be a contributing factor to the propensity of multiparous women to develop gallstones.

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