Phenotypic characterization of *Lith* genes that determine susceptibility to cholesterol cholelithiasis in inbred mice: integrated activities of hepatic lipid regulatory enzymes[1]

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Abstract **There is no consensus whether hepatic lipid regulatory enzymes play primary or secondary roles in cholesterol cholelithiasis. We have used inbred mice with *Lith* genes that determine cholesterol gallstone susceptibility to evaluate the question. We studied activities of regulatory enzymes in cholesterol biosynthesis (HMG-CoA reductase), cholesterol esterification (acyl-CoA:cholesterol acyltransferase) and the "neutral" (cholesterol 7α-hydroxylase) and "acidic" (sterol 27-hydroxylase) pathways of bile salt synthesis in strains C57L/J and SWR/J as well as recombinant inbred (AKXL-29) mice, all of which have susceptible *Lith* alleles, and compared them to AKR/J mice with resistant *Lith* alleles. We determined hepatic enzyme activities of male mice before and at frequent intervals during feeding a lithogenic diet (15% dairy fat, 1% cholesterol, 0.5% cholic acid) for 12 weeks. Basal activities on chow show significant genetic variations for HMG-CoA reductase, sterol 27-hydroxylase, and acyl-CoA: cholesterol acyltranferase, but not for cholesterol 7α-hydroxylase. In response to the lithogenic diet, activities of the regulatory enzymes in the two bile salt synthetic pathways are coordinately down-regulated and correlate inversely with prevalence rates of cholesterol crystals and gallstones. Compared with gallstone-resistant mice, significantly higher HMG-CoA reductase activities together with lower activities of both bile salt synthetic enzymes are hallmarks of the enzymatic phenotype in mice with susceptible *Lith* alleles. The most parsimonious explanation for the multiple enzymatic alterations is that the primary *Lith* phenotype induces secondary events to increase availability of cholesterol to supply the sterol to the hepatocyte canalicular membrane for hypersecretion into bile.**—Lammert, F., D. Q-H. Wang, B. Paigen, and M. C. Carey. **Phenotypic characterization of *Lith* genes that determine susceptibility to cholesterol cholelithiasis in inbred mice: integrated activities of hepatic lipid regulatory enzymes. *J. Lipid Res.* 1999.** 40: **2080–2090.**

Supplementary key words gallstones • genetics • HMG-CoA reductase • cholesterol 7α-hydroxylase • sterol 27-hydroxylase • acyl-CoA:cholesterol acyltransferase • microscopy • recombinant inbred mice

Cholesterol gallstone susceptibility among inbred strains of mice is conferred by *Lith* genes (1). We have utilized quantitative trait locus (QTL) analysis (2), a powerful genetic technique, to identify mouse chromosomal regions that contain pathophysiologically relevant *Lith* genes (1, 2). A major *Lith* gene (*Lith1*) was identified first on mouse chromosome 2 (2). Recently, additional QTLs have been localized on mouse chromosomes 5, 7, 11, 17, 19, and X (3, 4). Due to the close homology between human and mouse genomes (5), identification of mouse gallstone genes may elucidate previously unknown but pathophysiologically relevant genetic determinants of cholesterol cholelithiasis in humans. We have demonstrated that, when challenged with a lithogenic diet containing 15% dairy fat, 1% cholesterol, and 0.5% cholic acid, genetically determined cholesterol gallstone formation in inbred mice is characterized by marked cholesterol supersaturation and rapid cholesterol crystallization (6). By acutely interrupting the enterohepatic circulation with construction of a biliary fistula, cholesterol supersaturation of bile in mice with susceptible *Lith* alleles was shown to originate from hepatic hypersecretion of biliary cholesterol in the face of high biliary lipid output rates (7).

Not known is how proteins encoded by *Lith* genes increase hepatic secretion of biliary lipids. We hypothesized that putative candidate genes might be suggested from a detailed knowledge of the regulatory enzymes involved in hepatic cholesterol metabolism both before and during feeding the lithogenic diet. As is well known (8), relative

Abbreviations: Acat, acyl-CoA:cholesterol acyltransferase; C7h, cholesterol 7α-hydroxylase; ChMC, cholesterol monohydrate crystals; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; Hmgr, HMG-CoA reductase; HPLC, high performance liquid chromatography; LC, liquid crystals; S27h, sterol 27-hydroxylase; QTL, quantitative trait locus; RI, recombinant inbred mice No. 29 of AKXL strain set.

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excess cholesterol secretion could result from increased cholesterol synthesis, decreased cholesterol esterification, or decreased cholesterol catabolism to bile salts in the liver. Regulatory enzymes of these pathways are HMG-CoA reductase (Hmgr, EC 1.1.1.3.4) (9), acyl-CoA:cholesterol acyltransferase (Acat, EC 2.3.1.26) (10), as well as cholesterol 7α-hydroxylase (C7h, EC 1.14.13.17) and sterol 27-hydroxylase[4] (S27h, EC 1.14.13.15) for the "neutral" and "acidic" pathways of bile salt synthesis, respectively (12, 13).

It has emerged from numerous studies of patients with gallstones that activities of hepatic lipid regulatory enzymes vary tremendously, being either normal, diminished, or elevated compared with control human subjects (8, 14–17). The lack of consistency in these studies adds to our uncertainty about the relationships, if any, between activities of hepatic lipid regulatory enzymes and cholesterol gallstone formation. In addition, Björkhem, Lund, and Rudling (18) reported different mechanisms for regulation of lipid regulatory enzymes in rats, and they pointed out that controversy is, in part, related to differences between and within outbred strains of laboratory rats. In contrast, inbred strains of mice are genetically homogeneous and homozygous at all genetic loci (5), thus providing an optimal resource for analyzing genetic variation of enzyme activities in gallstone disease (1).

In the present study, we report the enzymatic phenotypes of *Lith* genes with respect to hepatic activities of the major lipid regulatory enzymes Hmgr, C7h, S27h, and Acat in different inbred strains of mice with susceptible and resistant *Lith* alleles. We hypothesize that *Lith* genes may modulate hepatic activities of one or more lipid regulatory enzyme(s) simultaneously to cause inappropriately high cholesterol secretion into bile (7). Furthermore, we used recombinant inbred mice (5) to explore whether observed differences in enzymatic phenotypes can be attributed to *Lith* genes. Our results suggest that the observed genetic differences of hepatic activities of lipid regulatory enzymes are secondary effects of the primary phenotype of *Lith* genes, and are, in part, an integrated response that ensures the continuous supply of hepatic cholesterol for hypersecretion into bile.

METHODS

Chemicals

For high performance liquid chromatography (HPLC), appropriate reagents were obtained from Fisher Scientific Co. (Pittsburgh, PA). (25*R*)-cholest-5-en-3β,27-diol (27-hydroxycholesterol) used as an HPLC standard was a generous gift from Dr. J. Shoda (Tsukuba, Japan). Additional HPLC standards (cholest-5-en-3β,7α-diol and cholest-5-en-3β,7β-diol) were obtained from Steraloids Inc. (Wilton, NH). Purities were greater than 98% by thin-layer chromatography and HPLC. dl-[3-^{14}C]HMG-CoA and [1-^{14}C]oleoyl-CoA were obtained from DuPont New England Nuclear (Boston, MA). Their radiochemical purities as verified by paper chromatography were greater than 97%. dl-[5-^{3}H]mevalonolactone and [1,2,6,7-^{3}H]cholesteryl oleate, obtained from DuPont New England Nuclear (Boston, MA), were used as internal standards. As determined by thin-layer chromatography, radiochemical purities were 99%. Lovastatin was a generous gift from Dr. E. Falk (Hoechst-Marion-Roussel, Frankfurt, Germany). Oligonucleotide primers for microsatellite markers (MapPairs™) were purchased from Research Genetics (Huntsville, AL). All other chemicals and solvents were American Chemical Society (ACS) or reagent grade quality (Sigma Chemical Co., St. Louis, MO). Water was filtered, ion-exchanged, and glass-distilled (Corning Glass Works, Corning, NY).

Inbred mice and recombinant inbred strains

Four inbred mouse strains with different cholesterol gallstone susceptibilities were studied. They were C57L/J, SWR/J, AKR/J, and AKXL-29 (RI) male mice obtained from The Jackson Laboratory (Bar Harbor, ME). High gallstone susceptibility of male C57L mice is conferred principally by susceptible alleles at the major gallstone locus, *Lith1* (2). Intermediate gallstone susceptibility of SWR mice (2) is due to susceptible alleles at *Lith* loci distinct from *Lith1* (F. Lammert, D. Q-H. Wang, M. C. Carey, and B. Paigen, unpublished observations). AKR mice, which exhibit low gallstone susceptibility, have resistant alleles at both *Lith* loci (2).

AKR and C57L mice are the progenitors of the AKXL recombinant inbred strain set developed at the Jackson Laboratory. Recombinant inbred mice are derived from an outcross between inbred strains followed by inbreeding the F_2 progeny (5).[5] RI mice were deliberately selected for having susceptible C57L alleles at the major QTL locus *Lith1* (2). We confirmed that RI mice carry susceptible alleles at *Lith1* by genotyping microsatellite markers *D2Mit11* and *D2Mit66*, which define the *Lith1* locus (2). Genomic DNA preparation from spleens, PCR amplification of DNA, and agarose separation of PCR products were carried out as described previously (19).

Experimental protocol and timing of enzymatic analyses

Over a 2-week adaptation period, 6-week-old male mice were fed Purina laboratory chow (Mouse Diet 1401, S. Hanky Road, St. Louis, MO). They were then fed for 12 weeks a semisynthetic lithogenic diet (20), which contained (wt/wt) 15% dairy fat, 2% corn oil, 50% sucrose, 20% casein, cellulose, essential vitamins and minerals, supplemented with 1% cholesterol and 0.5% cholic acid. As additional controls, different groups of mice were fed for 2 weeks, *1*) the semisynthetic high-fat diet containing 1% cholesterol, *2*) chow containing 1% cholesterol, or *3*) chow containing 0.5% cholic acid. Food and water were available ad libitum. Body weight gain and food intake of each strain neither differed significantly from data in our preceding study (6) nor from age-matched control mice fed the chow diet (21). Mice were housed in groups of five per cage. The mouse room was illuminated from 6.00 am to 6.00 pm with an automated 12-h light-cycle.

[4] Mitochondrial sterol 27-hydroxylase has been often referred to as 26-hydroxylase in previous work. Because the enzyme hydroxylates C-27, the pro-(*S*)-methyl group at C-25 which is derived from C-3′ of mevalonate (11), the conventional designation for the enzyme is 27-hydroxylase (12). The other terminal carbon, designated as C-26, is not hydroxylated by this or other mitochondrial enzymes. Furthermore, mammalian liver contains little microsomal 26-hydroxylase activity, and this enzyme does not recognize cholesterol as a substrate (12).

[5] Recombinant inbred strains derived from the progenitor strains AKR and C57L are designated AKXL, followed by the strain identification number (1–29) (5). On average, each recombinant inbred strain shares 50% of the genome with both progenitor strains and thus provides a 50% chance of detecting discordance between a specific phenotype and a *Lith* gene.

Cholecystectomy and hepatectomy were performed (6) before (day 0) and at frequent intervals from 3 to 84 days during feeding the lithogenic diet. To exclude alterations of enzyme activities by fasting, mice were not fasted in contrast to our previous experimental design (6). Because activities of mouse Hmgr and C7h are known to exhibit circadian rhythms (22), procedures were performed between 8:00 and 9:00 am to minimize diurnal variations. All procedures were executed according to the Guide for the Care and Use of Laboratory Animals (Department of Health and Human Services, Public Health Service) and the Public Health Service Policy on Humane Care and Use of Laboratory Animals, and all protocols were approved by the Institutional Animal Care and Use Committees of Harvard University and The Jackson Laboratory.

Assays of enzyme activities

For preparation of liver microsomes or mitochondria, frozen mouse livers (stored at −70°C) were thawed and homogenized at 4°C in 100 mm potassium phosphate buffer (pH 7.4), containing 100 mm sucrose, 50 mm KCl, 0.5 mm EDTA, 1 mm glutathione, and 20 μm leupeptin, or in 10 mm potassium phosphate buffer (pH 7.4), containing 250 mm sucrose and 0.5 mm EDTA, respectively. Microsomes and mitochondria were prepared by differential centrifugation and resuspended in potassium phosphate buffer and 100 mm TRIS/HCl (pH 7.7), respectively. Because the same microsomes were utilized for Hmgr, C7h, and Acat determinations, no NaF was added as a phosphatase inhibitor, but it was demonstrated recently that phosphorylation does not play a role in feedback regulation of Hmgr by end-products of mevalonate metabolism (23). Protein concentrations were determined using the Coomassie Blue-binding method with bovine serum albumin as standard (24). All enzyme activities were measured in duplicate and expressed per mg microsomal or mitochondrial protein.

Microsomal activities of Hmgr were determined by measuring the conversion of [^{14}C]HMG-CoA to [^{14}C]mevalonic acid using a radiochemical assay (25). An acidic ion exchanger (Dowex 50 WX4, Boehringer Ingelheim, Heidelberg, Germany) was used for lactonization. HMG-CoA and mevalonolactone were separated by anion exchange chromatography columns (AG 2-X8 resin Bio-Rad Laboratories, Richmond, CA), equilibrated extensively with 1 m propionic acid overnight.[6] Products were quantified by liquid scintillation counting with [^{3}H]mevalonolactone as internal standard. In addition, microsomal activities of Hmgr were determined in the presence of 1 μm lovastatin, which was added in methanol–100 mm NaOH 90:10 (vol/vol) to the reaction mixture, and percent lovastatin-inhibitable of total Hmgr activity was calculated.

Hepatic activities of C7h were determined by the HPLC-based assay system described in detail by Hylemon et al. (26). Activities of S27h in mitochondria were determined essentially as described by Petrack and Latario (27). To preserve enzyme activities, this necessitates that S27h assays are performed immediately after mitochondrial isolation. Mitochondria (750 μg) were incubated at 37°C for 15 min in 100 mm potassium phosphate buffer (pH 7.4) in the presence of an NADPH-regenerating system (1.2 mm NADPH, 5 mm sodium isocitrate, 0.2 U isocitric-dehydrogenase) and exogenous cholesterol (330 nmol), which was delivered in methyl-β-cyclodextrin (final wt/wt concentration 0.8%). Upon termination of the reaction with 40% (wt/vol) sodium taurocholate, cholesterol oxidase was added to convert 27-hydroxycholesterol to the 3-keto-derivate ((25*R*)-27-hydroxy-cholest-4-en-3-one), and 1.5 μg cholest-5-en-3β,7β-diol was added as recovery standard. Steroid products were extracted with hexane, dried under nitrogen, and dissolved in dodecane containing 5% (vol/vol) CH_3Cl and 5% (vol/vol) 2-propanol. Products were subsequently quantified using normal phase HPLC on a silica column in hexane–2-propanol–glacial acetic acid 96.5:2.5:1 (vol/vol/vol). Acat activities were measured in liver microsomes exactly as described (28). Lipids were extracted from livers and assayed for total and free cholesterol as described previously (21).

Bile and gallbladder phenotypes

Gallbladder bile (bulk bile and bile with mucin gel) of non-fasted male mice was examined immediately after cholecystectomy both macroscopically and by polarizing light microscopy (Photomicroscope III, Carl Zeiss Inc., Thornwood, NY) as described (6). Bile samples were observed using direct and polarizing light for the presence of solid and liquid crystals as well as stones without a cover slip, and then using phase contrast optics following compression with a cover glass. Small, aggregated, and fused liquid crystals (LC) as well as cholesterol monohydrate crystals (ChMC), sandy stones, and gallstones were defined according to our previous criteria (6, 29, 30). Gallbladder volumes were determined gravimetrically (6).

Statistical analysis

Data are expressed as means ± SEM. Differences among strains were assessed for statistical significance by Student's *t*-test or chi-square test. Correlations were tested by calculating Spearman's rank-order correlation coefficient. Statistical significance was defined as a two-tailed probability <0.05.

RESULTS

Bile and gallbladder phenotypes

To correlate enzyme activities with the physical–chemical state of gallbladder bile, we examined bulk and gelled biles by polarizing light microscopy in all mice. Data on cholesterol crystallization sequences and gallstone formation are illustrated schematically in **Fig. 1**.

Non-fasted C57L and AKR mice display crystallization sequences essentially identical to those established previously in a larger number of fasted animals (6). Figure 1 (top panel) shows that in C57L mice, mucin and liquid crystals (LC) are detected within the first week of feeding the lithogenic diet and accumulate rapidly in all mice. By 7 days, solid cholesterol monohydrate crystals (ChMC) appear and their numbers increase appreciably over the next weeks. By 28 days, gallbladder bile contains masses of aggregated ChMC as well as gallstones. After 56 days 75% and after 84 days 100% of C57L mice develop stones.

Figure 1 (second and third panels) displays that, with respect to the cholesterol crystallization sequences in RI and SWR inbred mice with susceptible *Lith* alleles (RI, SWR), the gallbladder bile phenotypes in RI are most similar to those of C57L mice. We observed a layer of mucin gel adherent to the gallbladder wall and liquid crystals in most RI

[6] To validate this assay modification, we compared Hmgr activities in 6 female C57L and AKR mice on a chow diet with values from the female mice in our initial study (2), which used TLC instead of anion exchange chromatography. Activities (C57L, 21 ± 2 pmol/min/mg; AKR, 54 ± 4 pmol/min/mg) do not differ significantly from values reported in ref. 2. In response to lithogenic diet feeding for 42 days, Hmgr activities decrease to 10 ± 2 pmol/min/mg in female AKR mice but remain unchanged (22 ± 3 pmol/min/mg) in female C57L mice (2).

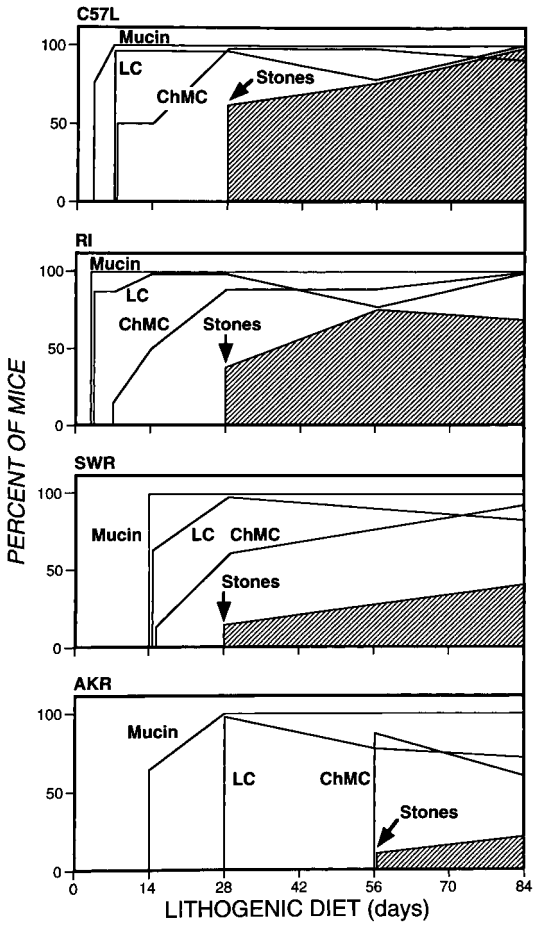

Fig. 1. Percent of non-fasted male mice forming mucin gel, liquid crystals (LC), cholesterol monohydrate crystals (ChMC), and stones as functions of days on the lithogenic diet. LC include small, aggregated, and fused (multilamellar vesicles) varieties; stones include sandy stones and hard stones with round contours (see ref. 6 for definitions and illustrations). C57L mice and RI (recombinant inbred) mice AKXL-29 display high susceptibilities, SWR mice display intermediate, and AKR mice display low susceptibilities to cholesterol gallstone formation. The data for C57L and AKR mice are similar to our recent study of fasted mice (6).

mice by 3 days. We first detected ChMC by 7 days, and these enlarged in size and aggregated subsequently. In similar fashion to C57L mice, gallstones are detected by 28 days, and 75% of RI mice had formed stones by 56 days.

Figure 1 (third panel) depicts that the majority of SWR mice show LC by 14 days and ChMC by 28 days, implying slower development of the physical–chemical phenotype in gallbladder bile compared with C57L and RI mice. Nonetheless, gallstones are first observed at 28 days, and they increase in number to 40% of the mice, during the time course of feeding. Not displayed in Fig. 1 are arc-like and tubular crystals (6, 29, 30), most likely transient metastable forms of hydrating anhydrous cholesterol (29), which appear in a few SWR mice at 84 days, i.e., later than LC and ChMC. This indicates that the gallbladder bile of these mice follows the crystallization pathway characteristic for region C of the crystallization pathways (6, 29). Similarly, anhydrous cholesterol crystals were observed between 3 and 7 days of feeding the lithogenic diet in gallbladder bile from fasted C57L mice (6). Their absence in the present study can be explained by a shift of cholesterol crystallization sequences in the non-fasted state with mouse gallbladder bile lipid compositions falling in region C of the crystallization pathways (6, 29). In AKR mice (Fig. 1, bottom panel), appearances of mucin gel, LC, and ChMC are delayed markedly, and only 10–20% of AKR mice form gallstones by 84 days.

Detailed data on fasting gallbladder volumes in C57L and AKR mice from previous experiments have been reported elsewhere (6). However, gallbladder sizes in non-fasted RI mice increase significantly from 8 ± 1 μl before feeding the lithogenic diet (day 0) to 10 ± 2 μl by 3 days and 19 ± 3 μl by 7 days, and these volumes do not differ significantly from those of C57L mice. After 7 days, there are no further increases in gallbladder volumes of RI mice, but values are higher compared with AKR (5–9 μl) and SWR mice (4–14 μl).

Enzymatic phenotypes: hepatic activities of lipid regulatory enzymes

The hepatic activities of Hmgr, C7h, S27h, and Acat were determined on chow diet (day 0) and at frequent intervals (3, 7, 14, 28, 56, and 84 days) during feeding the lithogenic diet. These data are summarized in **Table 1** as means ± SEM.

Basal Hmgr activities on chow diet do not differ significantly between gallstone-resistant AKR and gallstone-susceptible C57L mice (Table 1). In contrast, both SWR and RI mice display significantly lower basal Hmgr activities. Whereas there are no significant differences for basal C7h activities, hepatic S27h activities in mice on chow diet show marked genetic variation among the inbred strains. Gallstone-resistant AKR mice display significantly higher basal S27h activities (329 ± 18 pmol/min/mg mitochondrial protein) than gallstone-susceptible RI mice (189 ± 11 pmol/min/mg) and C57L mice (215 ± 40 pmol/min/mg), with SWR mice giving intermediate values (261 ± 16 pmol/min/mg) (Table 1). Basal Acat activities on chow diet, which range from 0.21 to 0.34 nmol/min/mg (Table 1), are significantly higher in gallstone-susceptible C57L mice compared with gallstone-resistant AKR mice.

Figure 2 demonstrates selected aspects of the data to illustrate the significant differences between inbred strains with susceptible and resistant *Lith* alleles during lithogenic diet feeding. In the individual panels of Fig. 2, strains are rank-ordered from left to right by increasing degrees of gallstone susceptibility (AKR < SWR < RI < C57L). In response to the lithogenic diet, gallstone-resistant AKR mice decrease their Hmgr activities progressively with time to 48 pmol/min/mg by 28 days and 24 pmol/min/mg by 84 days (Table 1). However, the activities do not decline in mice with intermediate and high cholesterol gallstone susceptibilities (SWR, RI, C57L). Figure 2 (top panels) illustrates responses of Hmgr activities at 28 and 84 days of feeding the lithogenic diet as % of basal activities on chow diet (day 0). Hmgr activities of AKR mice fall significantly

TABLE 1. Hepatic activities of lipid regulatory enzymes and hepatic cholesterol contents

Lithogenic Diet	Mouse Strain	Hmgr	C7h	S27h	Acat	Total Cholesterol	Esterified/ Free Ratio
day							
0	AKR	65 ± 5	14 ± 3	329 ± 18	0.21 ± 0.05	8 ± 2	11.8 ± 1.0
	SWR	40 ± 4	13 ± 2	261 ± 16	0.28 ± 0.07	11 ± 1	6.8 ± 0.7
	RI	33 ± 1	12 ± 3	189 ± 11	0.23 ± 0.02	13 ± 2	7.4 ± 1.5
	C57L	60 ± 11	10 ± 2	215 ± 40	0.34 ± 0.02	7 ± 1	9.7 ± 0.4
3	AKR	56 ± 7	6 ± 1[a]	178 ± 23[b]	0.85 ± 0.03[b]	24 ± 2[a]	9.1 ± 1.8
	RI	43 ± 6	4 ± 1[a]	116 ± 19[b]	1.24 ± 0.13[b]	28 ± 5[a]	9.5 ± 1.5
	C57L	73 ± 12	5 ± 1[a]	87 ± 9[b]	1.13 ± 0.07[b]	24 ± 1[b]	7.0 ± 0.5
7	AKR	45 ± 7[a]	7 ± 2	171 ± 23[b]	1.04 ± 0.07[b]	24 ± 3[a]	7.0 ± 0.8
	RI	45 ± 6	2 ± 1[b]	117 ± 16[b]	1.47 ± 0.07[b]	29 ± 1[b]	9.4 ± 1.2
	C57L	75 ± 9	4 ± 1[b]	58 ± 7[b]	1.01 ± 0.03[b]	26 ± 2[b]	7.8 ± 1.0
14	AKR	45 ± 7[a]	9 ± 1	167 ± 19[b]	1.12 ± 0.07[b]	33 ± 2[b]	9.0 ± 1.3
	RI	51 ± 5	2 ± 1[b]	101 ± 11[b]	1.44 ± 0.13[b]	22 ± 1[a]	10.9 ± 0.7
	C57L	78 ± 11	4 ± 1[b]	61 ± 9[b]	1.17 ± 0.16[b]	27 ± 3[b]	9.9 ± 0.2
28	AKR	48 ± 5[a]	9 ± 3	196 ± 18[b]	1.20 ± 0.12[b]	37 ± 5[a]	6.8 ± 1.8
	SWR	51 ± 5[a]	8 ± 3	162 ± 15[b]	1.25 ± 0.12[b]	39 ± 4[b]	9.9 ± 1.6
	RI	45 ± 8	2 ± 1[b]	73 ± 10[b]	1.72 ± 0.11[b]	25 ± 2[b]	11.1 ± 1.6
	C57L	73 ± 8	2 ± 1[b]	118 ± 23[a]	1.54 ± 0.17[b]	35 ± 2[b]	7.7 ± 0.6
56	AKR	19 ± 3[b]	6 ± 2[a]	168 ± 21[b]	1.33 ± 0.08[b]	27 ± 1[a]	9.0 ± 1.3
	RI	30 ± 4	2 ± 1[b]	47 ± 5[b]	1.42 ± 0.12[b]	24 ± 2[a]	11.0 ± 0.5
	C57L	54 ± 8	3 ± 1[b]	35 ± 6[b]	1.46 ± 0.09[b]	28 ± 2[a]	8.3 ± 0.5
84	AKR	24 ± 6[b]	10 ± 2	163 ± 15[b]	1.20 ± 0.11[b]	34 ± 5[b]	9.5 ± 1.1
	SWR	47 ± 11	3 ± 1[b]	71 ± 14[b]	1.65 ± 0.09[b]	39 ± 2[b]	9.0 ± 1.0
	RI	36 ± 3	2 ± 1[b]	52 ± 14[b]	1.79 ± 0.07[b]	26 ± 4[a]	8.8 ± 1.3
	C57L	62 ± 8	2 ± 1[b]	24 ± 4[b]	1.58 ± 0.07[b]	26 ± 2[b]	7.6 ± 0.7

Values are expressed in pmol/min/mg microsomal protein (Hmgr, C7h), pmol/min/mg mitochondrial protein (S27h), nmol/min/mg microsomal protein (Acat) as means ± SEM ($n \geq 6$) of mg/g liver (total cholesterol) as means ± SEM ($n \geq 3$).

Abbreviations: Hmgr, HMG-CoA reductase; C7h, cholesterol 7α-hydroxylase; S27h, sterol 27-hydroxylase; Acat, acyl-CoA:cholesterol acyltransferase.

[a] $P < 0.05$, probability that enzyme activities and cholesterol contents are significantly different from day 0.

[b] $P < 0.01$, probability that enzyme activities and cholesterol contents are significantly different from day 0.

to 70% of basal at day 28, and below 40% of basal at day 84, whereas Hmgr activities in strains SWR, RI, and C57L remain at, or even significantly above (SWR at 28 days), basal levels.

Under various Hmgr assay conditions, enzymatic production of acetoacetate by possible contaminating HMG-CoA lyases has been observed (E. Falk, Hoechst-Marion-Roussel, Frankfurt, personal communications 1998/99). To exclude the possibility that Hmgr activities may be apparent values due to the presence of a lyase producing acetoacetate from HMG-CoA, we measured Hmgr in the presence and absence of the Hmgr inhibitor lovastatin (1 μm) in 5 male gallstone-susceptible C57L and resistant AKR mice after 2 weeks of feeding the lithogenic diet (see Methods). Percent lovastatin-inhibitable of total (apparent) Hmgr activities do not differ significantly between C57L and AKR mice (76.0 ± 7.5% and 85.0 ± 4.8%, respectively), indicating that the elevation of enzymatic activities in gallstone-susceptible mice is due to Hmgr.

As listed in Table 1, C7h activities decrease significantly to 4–6 pmol/min/mg by 3 days of challenge with the lithogenic diet. By 7 days, gallstone-susceptible RI and C57L mice are characterized by low C7h activities, which are close to the detection limit of the HPLC-based assay (26). In RI and C57L mice, C7h activities do not differ from each other and remain significantly lower than in AKR mice throughout the feeding period. In contrast, as illustrated in Fig. 2 (middle left panel), C7h activities in SWR mice, which show slower development of the gallbladder bile phenotype compared with RI and C57L mice (Fig. 1), do not differ from AKR mice at 28 days. Figure 2 (middle right panel) shows that by 84 days, all mice with higher cholesterol gallstone susceptibilities (SWR, RI, C57L) exhibit significantly lower C7h activities (15–22% of basal) than gallstone-resistant AKR mice (74% of basal).

Figure 2 (bottom left panel) displays that after lithogenic diet feeding for 28 days, all mice down-regulate S27h activities significantly to 39–60% of basal levels. Whereas gallstone-resistant AKR mice show a maximum reduction of S27h activities to 163 ± 15 pmol/min/mg by 84 days, strains SWR, RI, and C57L are characterized by marked decreases of S27h activities to 24–71 pmol/min/mg (Table 1). Figure 2 (bottom right panel) illustrates that by 84 days, S27h activities in SWR, RI, and C57L mice correspond to 11–28% of basal levels. As described above for Hmgr and C7h, the responses of S27h activities to the lithogenic diet in RI mice closely parallel the changes in C57L mice.

Acat activities increase rapidly to greater than 1.00 nmol/min/mg within the first week of feeding the lithogenic diet (Table 1). After 28 and 84 days, Acat activities in all strains are increased more than 4-fold, with appre-

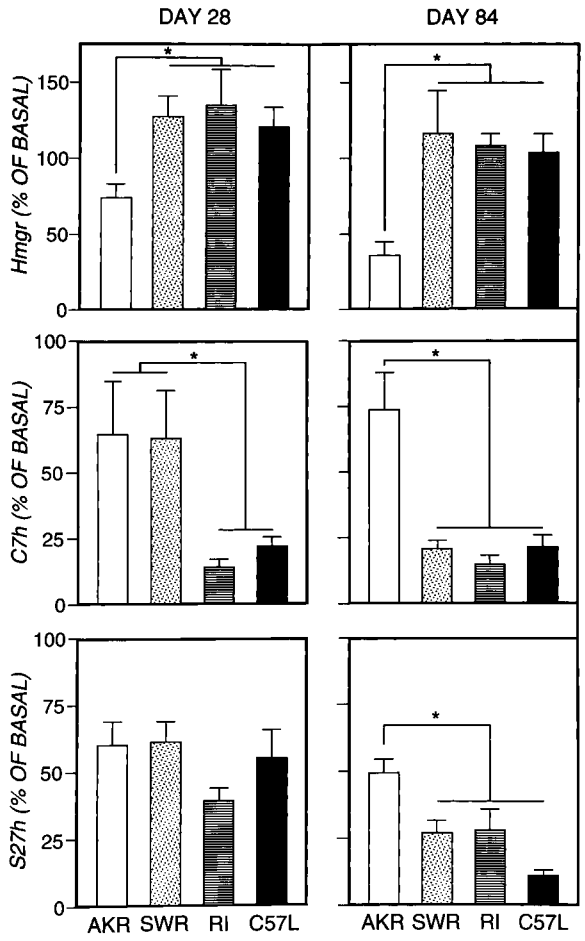

Fig. 2. Hepatic activities of HMG-CoA reductase (Hmgr), cholesterol 7α-hydroxylase (C7h), and sterol 27-hydroxylase (S27h) in male inbred mice on days 28 and 84 of feeding the lithogenic diet. Activities are expressed as % of basal values on chow diet (±SEM). In all panels, the strains are ordered from left to right by increasing degree of cholesterol gallstone susceptibility [AKR < SWR < RI (recombinant inbred mice AKXL-29) < C57L, see Fig. 1]. Hmgr activities decrease significantly ($P < 0.05$) in gallstone-resistant AKR mice by 28 and 84 days, but SWR, RI, and C57L mice do not down-regulate Hmgr activities at either time point (top panels). By 84 days, down-regulation of C7h activities (middle panels) and S27h activities (bottom panels) is significantly ($P < 0.05$) more pronounced in SWR, RI, and C57L mice, compared with gallstone-resistant AKR mice.

ciably higher absolute activities in gallstone-susceptible mice (SWR, RI, C57L) and small differences in basal activities distorting relative comparisons (data not displayed).

Because the lithogenic diet contains a mixture of dietary components which each may independently alter one or all of the measured enzyme activities, three additional diets were fed for 2 weeks to the mice that are most (C57L) or least (AKR) susceptible to gallstone formation. Control diets included *1*) the semisynthetic high-fat diet containing 1% cholesterol, *2*) chow containing 1% cholesterol, or *3*) chow containing 0.5% cholic acid. **Table 2** summarizes the activities of hepatic Hmgr, C7h, S27h, and Acat activities in AKR and C57L mice on the three different control diets. With all dietary

TABLE 2. Hepatic activities of lipid regulatory enzymes on control diets

Mouse Strain	Diet (14 Days)	Hmgr	C7h	S27h	Acat
AKR	Fat +1% Cholesterol	16 ± 2[a]	8 ± 1[b]	112 ± 23[a]	0.23 ± 0.04
	1% Cholesterol	16 ± 4[a]	13 ± 1	178 ± 7[a]	0.42 ± 0.02[a]
	0.5% Cholic acid	17 ± 2[a]	2 ± 1[a]	62 ± 18[a]	0.21 ± 0.03
C57L	Fat + 1% Cholesterol	47 ± 2	3 ± 1[a]	59 ± 13[a]	0.68 ± 0.14[b]
	1% Cholesterol	37 ± 3	9 ± 1	97 ± 7[b]	0.46 ± 0.03[a]
	0.5% Cholic acid	61 ± 11	1 ± 1[a]	84 ± 14[b]	0.31 ± 0.08

Values are expressed in pmol/min/mg microsomal protein (Hmgr, C7h), pmol/min/mg mitochondrial protein (S27h), nmol/min/mg microsomal protein (Acat) as means ± SEM (n ⩾ 3). Abbreviations: see Table 1.

[a] $P < 0.01$, probability that enzyme activities are significantly different from day 0 (Table 1).

[b] $P < 0.05$, probability that enzyme activities are significantly different from day 0 (Table 1).

regimens, Hmgr and Acat activities are appreciably higher and C7h and S27h activities are markedly lower in C57L compared with AKR mice. Compared with hepatic enzyme activities in both strains on chow (Table 1), C7h and S27h activities decrease significantly upon feeding a fat plus cholesterol diet. On the same diet, down-regulation of Hmgr is significantly more pronounced in AKR than in C57L mice. When a cholesterol diet is fed, the changes in enzymic activities are similar to feeding a fat plus cholesterol diet except for C7h activities, which are identical to those on chow. Feeding 0.5% cholic acid significantly decreases activities of both regulatory enzymes in bile salt synthesis; however, Acat activities in both strains and Hmgr activities in C57L mice are similar to the values on chow.

Relationships between hepatic lipid regulatory enzymes

The next step in the analysis involved the possible relationships between the different *Lith* phenotypes examined. **Figure 3** (left panel) plots C7h activities versus S27h activities for all strains before and during challenge with the lithogenic diet. In response to the lithogenic diet, C7h and S27h activities for each strain shifted progressively to lower values, i.e., to the lower left of the diagram. Taken together, a marked positive correlation between C7h and S27h activities was observed in all activities ($r = 0.82$, $P < 0.01$).

Figure 3 (right panel) displays C7h activities plotted versus Acat activities in the same format. In response to the lithogenic diet, activities shift to lower C7h and higher Acat activities. This shift to the lower right hand corner of the diagram results in a significant negative correlation between C7h and Acat activities ($r = -0.85$, $P < 0.01$). As expected, a similar negative correlation ($r = -0.70$, $P < 0.01$) is observed between S27h and Acat activities (data not shown).

No correlation is observed in the scattergrams between hepatic Hmgr and S27h/C7h or Acat activities (data not shown). This is presumably because strains SWR, RI, and C57L do not down-regulate Hmgr activities (Table 1), as depicted in Fig. 2 (top right panel). When individual strains

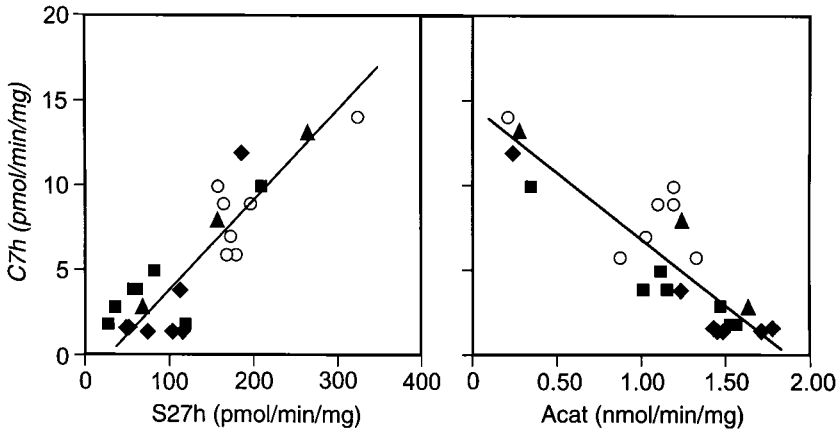

Fig. 3. Activities of cholesterol 7α-hydroxylase (C7h) plotted versus sterol 27-hydroxylase activities (S27h; left panel) and acyl-CoA:cholesterol acyltransferase activities (Acat; right panel). Symbols: ■, C57L mice; ◆, RI mice; ▲, SWR mice; ○, AKR mice. C7h and S27h activities are correlated positively ($r = 0.82$; $P < 0.01$, left panel); C7h and Acat activities are correlated negatively ($r = -0.85$, $P < 0.01$; right panel).

are analyzed, Hmgr activities are positively correlated with S27h activities in AKR mice ($r = 0.83$, $P < 0.05$), but no further correlations are detected.

Relationships between gallbladder and enzymatic phenotypes

For the following analysis of correlations, the percentages of mice forming LC, ChMC, and stones at different time points of lithogenic diet feeding were tested statistically against the mean hepatic enzyme activities measured in the same animal groups. Acat activities correlate positively with prevalences of LC ($r = 0.68$, $P < 0.01$), ChMC ($r = 0.83$, $P < 0.01$), and gallstones ($r = 0.74$, $P < 0.01$) in all strains. Hmgr activities correlate inversely with prevalences of ChMC and gallstones ($r = -0.81$ and -0.76, respectively, $P < 0.05$) in AKR mice, but not in SWR, RI, and C57L mice.

In all strains, C7h activities show inverse correlations with prevalences of LC ($r = -0.65$, $P < 0.01$), ChMC ($r = -0.69$, $P < 0.01$), and gallstones ($r = -0.55$, $P < 0.01$). Correlations between S27h activities and gallbladder phenotypes parallel those seen for C7h activities: LC ($r =$

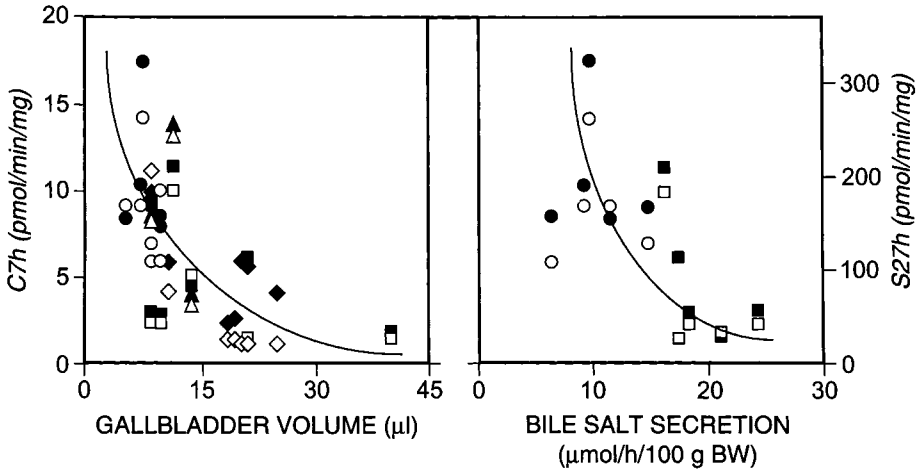

Fig. 4. To assess perturbing effects of large and presumably hypomotile gallbladders on enterohepatic circulation of bile salts, activities of cholesterol 7α-hydroxylase (C7h; open symbols refer to left y-axis) and sterol 27-hydroxylase (S27h; closed symbols refer to right y-axis) are plotted versus gallbladder volumes (left panel) and bile salt secretion rates (right panel, data from ref. 7, D. Q-H. Wang, F. Lammert, B. Paigen, and M. C. Carey, accompanying paper) in corresponding animal groups. Activities are expressed in pmol/min per mg microsomal or mitochondrial protein, volumes in µL, and secretion rates in µmol/h per 100 g body weight (BW). Scales for C7h and S27h were drawn using the regression equation S27h (pmol/min/mg) = 28.56 + 17.54 × C7h (pmol/min/mg), which was derived from data in Table 1 and is displayed in Fig. 3 (left panel). Symbols (white for C7h; black for S27h): □/■, C57L mice; ◇/◆, RI mice; △/▲, SWR mice; ○/●, AKR mice. C7h and S27h activities are correlated negatively with gallbladder volumes ($r = -0.77$, $P < 0.05$; $r = -0.64$, $P < 0.01$) and bile salt secretion rates ($r = -0.62$, $P < 0.05$; $r = -0.71$, $P < 0.05$).

-0.56, $P < 0.01$), ChMC ($r = -0.71$, $P < 0.01$), and gallstones ($r = -0.62$, $P < 0.01$).

Because a normal functioning gallbladder plays a crucial role in the recycling frequency of the bile salt pool, we assessed perturbing effects of large and presumably hypomotile gallbladders (6) on enterohepatic circulation of bile salts, as inferred from C7h activities. **Figure 4** (left panel) plots hepatic C7h and S27h activities versus gallbladder volumes of the mice. C7h and S27h activities are high and gallbladder volumes are small at the beginning of the experiment, and shift to lower activities and larger volumes with duration of lithogenic diet feeding, resulting in an inverse curvilinear relationship ($r = -0.77$, $P < 0.05$ for C7h; $r = -0.64$, $P < 0.01$ for S27h). Figure 4 (right panel) displays the similar inverse relationship between C7h and S27h activities and bile salt secretion rates (from ref. 7, D. Q-H. Wang, F. Lammert, B. Paigen, M. C. Carey, accompanying paper) for corresponding gallstone-susceptible C57L and resistant AKR mice ($r = -0.62$, $P < 0.05$ for C7h; $r = -0.71$, $P < 0.05$ for S27h).

Relationships between hepatic cholesterol concentrations and other phenotypes

The two right-hand columns in Table 1 show total hepatic cholesterol concentrations and the ratio of esterified to free cholesterol in each mouse group. Upon feeding the lithogenic diet, total hepatic cholesterol increased significantly in all mice but concentrations are appreciably lower in gallstone-susceptible C57L and RI mice compared to resistant AKR mice. No significant differences of free and esterified cholesterol are detected among the strains, with 7–12% of total cholesterol being free. As anticipated, cholesterol concentrations are positively correlated with Acat activities ($r = 0.52$, $P < 0.01$). Interestingly, liver cholesterol concentrations are also correlated positively and significantly with the percent of mice forming LC and ChMC in gallbladder bile at different points of lithogenic diet feeding ($r = 0.49$ and 0.43, respectively, $P < 0.05$).

DISCUSSION

The aim of the present study was to define the phenotypes of hepatic lipid regulatory enzymes in inbred mice with susceptible and resistant *Lith* alleles. Our observations focused on phenotypic strain differences that were apparent before or upon feeding the lithogenic diet. Hepatic cholesterol, originating either from biosynthesis via Hmgr or from lipoproteins, can be sorted to the hepatocyte canalicular membrane for secretion into bile, but it is also the major substrate for bile salt synthesis via C7h and/or S27h as well as for Acat, the enzyme responsible for cholesterol esterification. In mice, cholesteryl esters are predominantly stored or used for VLDL assembly and secretion (21).

The current study demonstrates that genetically determined gallstone formation in inbred mice is associated with derangements of all major regulatory enzymes in hepatic cholesterol metabolism. Significantly increased cholesterol biosynthesis via Hmgr and decreased cholesterol catabolism via C7h and S27h are the hallmarks of the enzymatic phenotype in the susceptible mice (Fig. 2). The common denominator of these alterations is that they are all additive in increasing the intracellular availability of cholesterol molecules for secretion into bile. The concept of *Lith* genes modulating hepatic cholesterol metabolism is strengthened first by the similarity of enzymatic phenotypes in SWR, RI, and C57L mice, all of which have susceptible *Lith* alleles (Table 1), and second by the similarity of enzymatic phenotypes irrespective of the dietary regimen (Table 2). Although different dietary lipid compositions modulate the hepatic activities of lipid regulatory enzymes, genetic differences are obvious, with higher Hmgr/Acat and lower C7h/S27h activities in gallstone-susceptible compared with resistant mice.

Over the past decade the concept has emerged that hepatic catabolism of cholesterol to bile salts can be initiated either via hydroxylation of the steroid nucleus by C7h, resulting in "neutral" sterols as bile salt intermediates ("neutral" pathway), or via side-chain oxidation by S27h, with "acidic" sterols as bile salt intermediates ("acidic" pathway) (12, 13). Although decreased catabolism of cholesterol to bile salts via the "acidic" pathway could theoretically have an important influence on intrahepatic pools and thereafter biliary secretion of cholesterol, S27h activities have not been assayed systematically in cholesterol gallstone patients or animal models of cholesterol cholelithiasis. The present work demonstrates the high genetic variation of hepatic S27h activities among inbred strains of mice with different cholesterol gallstone susceptibilities, even prior to feeding the lithogenic diet (Table 1). With exogenous cholesterol delivered in methyl-β-cyclodextrin, specific activities of S27h appear to be approximately 2- to 3-fold higher in all mice strains compared to rats (27), which might be due to species differences in contribution of the "acidic" and the "neutral" pathway to total bile salt synthesis. Under all dietary conditions, inbred mice with highest gallstone susceptibilities (C57L, RI) show lowest hepatic S27h activities (Fig. 2). As expected for mice fed cholic acid (31, 32), all inbred strains of mice display decreases in hepatic activities of both regulatory enzymes in bile salt synthesis (Table 2), whereas feeding cholesterol did not increase activities of both enzymes in the current study. Albeit caution is needed regarding the relevance of cholic acid-containing diets to humans, feeding cholic acid has been shown to make the mouse bile salt pool more like that of humans. After hepatic conjugation with taurine, cholate replaces most tauro-β-muricholate in bile, which promotes intestinal absorption, biliary secretion, and phase separation of cholesterol (33). Furthermore, using acute biliary fistulae, measurements of total and circulating bile salt pool sizes in susceptible (C57L) and resistant (AKR) mice did not show significant increases in either strain (7, accompanying paper), which is probably due to the profound down-regulation of bile salt synthesis. The strong positive correlation between hepatic C7h and S27h activities (Fig. 3) could not be anticipated as both

enzymes differ in substrate specificity, tissue expression, and half-life (34, 35). However, our results are in agreement with recent studies that show coordinate down-regulation of both C7h and S27h by bile salts at transcriptional and post-transcriptional levels in rats (34) and in rat hepatocyte cultures (35, 36).

Alexander and Portman (37) first reported lower cholate synthesis in gallstone-susceptible inbred mice, using the isotope-dilution technique. In our studies, lower activities of bile salt synthesizing enzymes were correlated with higher hepatic secretion rates of bile salts (Fig. 4) (data from ref. 7, D. Q-H. Wang, F. Lammert, B. Paigen, and M. C. Carey, accompanying paper) and are thus consistent with increased enterohepatic return of bile salts to the liver. It appears that the increased enterohepatic bile salt flux in gallstone-susceptible mice may be due, in part, to hypomotile gallbladders impaired by excess cholesterol molecules in bile (6), a condition known to perturb the enterohepatic circulation by increasing diversion of hepatic bile salt secretion to the small intestine (38, 39). This possibility is supported in this work, first by the strong inverse correlation between hepatic C7h/S27h activities and gallbladder size, a marker of gallbladder hypomotility (Fig. 4) (38, 39), and second by the increase of hydrophobic bile salts such as taurodeoxycholate, an indicator for increased diversion of bile salts to the anaerobic flora of the large intestine (38), from 2–5% to 5–10% in gallbladder bile of gallstone-susceptible mice during the early stages of lithogenic diet feeding (6, 7). Furthermore, on the lithogenic diet, the more hydrophobic bile salt pool composition amplifies the suppression of bile salt synthesis in gallstone-susceptible mice, as has been shown for the rat (34, 40).

In contrast to C7h and S27h activities, absolute activities of Hmgr correlated neither with gallstone prevalences nor with hepatic secretion rates of biliary cholesterol (7). The genetic variation in Hmgr activities among inbred mice (Tables 1 and 2) is not only consistent with previous studies (22, 31) but also with the multiple roles of the mevalonate pathway, which is regulated transcriptionally and post-transcriptionally by several sterol and non-sterol metabolites of mevalonate (9). In the present study, the responses of Hmgr to the different dietary challenges differ between gallstone-susceptible and gallstone-resistant mice, confirming that inbred mice with susceptible *Lith* alleles resist down-regulation of Hmgr by cholesterol (also see footnote 6 for females) (2). We excluded that this effect is due to HMG-CoA lyase, which can artifactually increase measured Hmgr activities; after 2 weeks of lithogenic diet feeding, 76–85% of total Hmgr activities could be inhibited by a specific Hmgr inhibitor lovastatin and there were no significant differences between gallstone-susceptible and resistant mice. The situation in mice with gallstone-susceptible *Lith* alleles is reminiscent of observations in hypercholesterolemic patients (41) as well as in moderately obese gallstone patients (15), who lack strong feedback regulation of cholesterol synthesis, as inferred from sterol synthesis rates in blood mononuclear cells. Also attributable to preserved high Hmgr activities is that we did not find any coordinate coupling between Hmgr and C7h or S27h activities in gallstone-susceptible mice, which has been described in rats under a variety of pathophysiological and pharmacological conditions (18). However, this is consistent with previous studies on mRNA levels of these lipid regulatory enzymes in mice fed high fat and high cholesterol diets with or without cholic acid (42, 43).

A decrease of hepatic Hmgr activities is not sufficient to prevent cholesterol gallstone formation in certain strains of inbred mice (6, 31), and Hmgr down-regulation in AKR mice on the lithogenic diet was slower and less pronounced compared to mice fed cholesterol or cholic acid alone (Table 2). Endogenously and/or exogenously derived fatty acids, in particular oleic acid, which is the major component of the 15% dairy fat in the lithogenic diet, could also directly increase hepatic Hmgr activities via stimulation of cholesterogenesis and hepatic secretion of triglyceride-rich VLDL (21, 44). In fact, the lithogenic diet increases VLDL levels markedly in gallstone-susceptible strains C57L and SWR in contrast to strain AKR (2, 21), which is consistent with higher Hmgr activities in C57L and SWR mice (Table 1) for VLDL assembly. High Acat activities in gallstone-susceptible strains (Table 1) provide cholesteryl esters, which are incorporated in the core of VLDL (21). The surface coat of VLDL serves as an important source for HDL-cholesterol, from which biliary cholesterol may mainly originate (45, 46). Therefore, inappropriately high VLDL secretion in gallstone-susceptible inbred mice fed the lithogenic diet (2, 21) might indirectly contribute to hepatic hypersecretion of biliary cholesterol, at least if other homeostatic controls are constant. In a recent study, liver cholesteryl ester content was indeed directly correlated with biliary cholesterol concentration (47), and this observation is supported in the present study by the positive correlation of hepatic liver cholesterol concentrations and prevalence rates of ChMC, which indicate cholesterol hypersecretion into bile (6).

Inbred mice with susceptible *Lith* alleles display not only alterations of hepatic lipid regulatory enzymes but also of the cytosolic sterol carrier protein 2 (48), which is believed to play a role in transport of cholesterol within hepatocytes (49), but maps to mouse chromosome 4 (50). As assessed by steady-state hepatic mRNA levels, sterol carrier protein 2 gene expression is enhanced during cholesterol gallstone formation in susceptible mice, whereas gallstone-resistant mice exhibit no increase of mRNA levels (48). High gene expression of sterol carrier protein 2 appears to indicate high rates of intracellular cholesterol trafficking, perhaps mostly that newly synthesized (49), which is consistent with increased availability of intracellular cholesterol in hepatocytes of mice with susceptible *Lith* alleles (1).

In conclusion, this study demonstrates that *Lith* genes modulate hepatic activities of all major lipid regulatory enzymes. The QTL mapping data for *Lith* genes (3, 4) exclude pathophysiologically relevant defects in structural genes of hepatic lipid regulatory enzymes and cytosolic transfer proteins (21, 50). Although unknown proteins

encoded by *Lith* genes could theoretically regulate each lipid regulatory enzyme separately (1), it is unlikely that *Lith* genes induce cholesterol hypersecretion in gallstone-susceptible mice by modulating hepatic activities of all enzymes individually. The most parsimonious explanation is that *Lith* genes may cause hypersecretion of biliary cholesterol primarily (7) and that the multiple alterations of cholesterol metabolism (1) and trafficking (48) could represent secondary events that increase intracellular cholesterol availability and supply the sterol to the hepatocyte canalicular membrane for secretion into bile. The enzymatic phenotypes of *Lith* genes described in the present study highlight the continuing importance of acquiring new insights into the molecular mechanisms of biliary lipid secretion and their roles in cholesterol gallstone pathogenesis.

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