The phosphatidylethanolamine *N*-methyltransferase pathway is quantitatively not essential for biliary phosphatidylcholine secretion

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Abstract The phosphatidylethanolamine *N*-methyltransferase (PEMT) pathway of phosphatidylcholine (PC) biosynthesis is not essential for the highly specific acyl chain composition of biliary PC. We evaluated whether the PEMT pathway is quantitatively important for biliary PC secretion in mice under various experimental conditions. Biliary bile salt and PC secretion were determined in mice in which the gene encoding PEMT was inactivated (*Pemt*$^{-/-}$) and in wild-type mice under basal conditions, during acute metabolic stress (intravenous infusion of the bile salt tauroursodeoxycholate), and during chronic metabolic stress (feeding a taurocholate-containing diet for 1 week). The activity of CTP:phosphocholine cytidylyltransferase, the rate-limiting enzyme of PC biosynthesis via the CDP-choline pathway, and the abundance of multi-drug-resistant protein 2 (Mdr2; encoded by the *Abcb4* gene), the canalicular membrane flippase essential for biliary PC secretion, were determined. Under basal conditions, *Pemt*$^{-/-}$ and wild-type mice exhibited similar biliary secretion rates of bile salt and PC (~145 and ~28 nmol/min/100 g body weight, respectively). During acute or chronic bile salt administration, the biliary PC secretion rates increased similarly in *Pemt*$^{-/-}$ and control mice. Mdr2 mRNA and protein abundance did not differ between *Pemt*$^{-/-}$ and wild-type mice. The cytidylyltransferase activity in hepatic lysates was increased by 20% in *Pemt*$^{-/-}$ mice fed the basal (bile salt-free) diet ($P < 0.05$). We conclude that the biosynthesis of PC via the PEMT pathway is not quantitatively essential for biliary PC secretion under acute or chronic bile salt administration.—Verkade, H. J., R. Havinga, D. J. Shields, H. Wolters, V. W. Bloks, F. Kuipers, D. E. Vance, and L. B. Agellon. **The phosphatidylethanolamine *N*-methyltransferase pathway is quantitatively not essential for biliary phosphatidylcholine secretion. *J. Lipid Res.* 2007.** 48: **2058–2064.**

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The liver secretes large amounts of phosphatidylcholine (PC) into plasma, as a surface component of lipoproteins, and into bile. Previously, we estimated that the hepatic secretion of PC into lipoproteins was as quantitatively important as that into bile (1). Biliary PC secretion accounts for a large amount of PC expenditure, with an estimated daily amount equivalent to the entire PC content of the liver (2) or ~70% of hepatic phospholipid content (3). Bile PC is specific in its acyl chain composition, containing predominantly C16:0 fatty acid at the *sn*-1 position and C18:1 or C18:2 fatty acid at the *sn*-2 position (4, 5). The liver has two pathways available for PC biosynthesis: via the CDP-choline pathway (de novo from choline) or via the phosphatidylethanolamine *N*-methyltransferase (PEMT) pathway (three successive methylations of phosphatidylethanolamine catalyzed by a single enzyme, PEMT). Previously, we demonstrated in PEMT-deficient (*Pemt*$^{-/-}$) mice that the acyl chain specificity of bile PC was independent of its biosynthetic origin (5). However, it is not known whether PEMT is quantitatively essential for biliary PC secretion.

It is unclear whether or not the amount of PC secreted into bile is influenced by the route or rate of hepatic PC biosynthesis. Robins and Armstrong (6) found that supplementation of the diet with choline increased the biliary PC secretion rate in rats. It was suggested that dietary choline increased hepatic PC synthesis, which then became available for biliary secretion. In contrast, however, LeBlanc et al. (7) did not observe an increased biliary PC secretion rate in rats fed a choline-supplemented diet. The availability of the *Pemt*$^{-/-}$ mouse strain uniquely allows us to address the contribution of hepatic PC biosynthesis to the amount of PC secreted into bile. The se-

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cretion of PC into bile is deleterious for the liver and is lethal upon the inhibition of PC biosynthesis via both the PEMT and CDP-choline pathways (through inactivation of the *Pemt* gene and withdrawal of dietary choline, respectively) (8). Prevention of biliary PC secretion, through inactivation of the PC-specific flippase multi-drug resistant protein 2 (Mdr2; encoded by the *Abcb4* gene), enables *Pemt*$^{-/-}$ mice to survive the inhibition of de novo PC synthesis (8, 9). In this study, we addressed whether the PEMT pathway is of quantitative importance for biliary PC secretion, particularly under conditions of metabolic stress, such as acute or chronic bile salt administration. We compared biliary PC secretion rates in *Pemt*$^{-/-}$ and wild-type (control) mice under basal conditions, during acute metabolic stress [intravenous tauroursodeoxycholate (TUDC) infusion], and during chronic metabolic stress (feeding a taurocholate-supplemented diet).

MATERIALS AND METHODS

Animals and diets

Experiments involving the use of animals were performed according to protocols approved by the University of Alberta Health Sciences Animal Welfare Committee and in accordance with the guidelines established by the Canadian Council on Animal Care. *Pemt*$^{-/-}$ and *Pemt*$^{+/+}$ (wild-type) mice were maintained by homozygous breeding, given free access to chow and water, and maintained in a temperature-controlled environment under a reverse 12 h light/dark cycle. The *Pemt*$^{-/-}$ and wild-type mouse colonies have a mixed genetic background of 129/Ola and C57BL/6J. The semisynthetic (control) diet (No. 901387; ICN Biomedicals, Montreal, Quebec, Canada) contained 0.4 wt% choline chloride and 0.05 wt% cholesterol. The major species of fatty acids in the control diet were C16:0 (24%), C18:0 (16%), C18:1 (39%), C18:2 (9%), and C20:4 (0.5%). In another experiment, *Pemt*$^{-/-}$ and wild-type mice were exposed to chronic metabolic stress in the form of feeding the same diet supplemented with sodium taurocholate (0.5 wt%; Sigma-Aldrich Canada, Ltd., Oakville, Ontario, Canada) for 1 week.

Experimental design

To study biliary secretion rates, the gallbladders of *Pemt*$^{-/-}$ and wild-type mice (fed the control or the chronic metabolic stress diet) were cannulated after distal ligation of the common bile duct under ketamine/xylazine and diazepam anesthesia. During the same procedure, the mice also received a jugular vein catheter. For 30 min, bile was collected in two 15 min fractions, after which the mice were infused with TUDC (43 mM TUDC in phosphate-buffered saline, pH 7.4) through the jugular vein. The TUDC dose administered to each mouse was increased stepwise: 150 nmol/min for 30 min, 300 nmol/min for 30 min, 450 nmol/min for 30 min, and 600 nmol/min for 60 min. The bile salt infusion in mice fed the basal diet was extended for another 60 min at a dose of 750 nmol/min. During the infusion period, the mice were kept under anesthesia and bile was collected in 15 min fractions, except for the 60 min infusion dosages, for which bile was collected in 10 min fractions. Body temperature was maintained by placing the mice in an incubator during the experiment. Bile flow was determined gravimetrically, assuming a density of 1 g/ml bile.

Separate *Pemt*$^{-/-}$ and wild-type mice were used to measure CTP-phosphocholine cytidylyltransferase (CT) activity and hepatic Mdr2 mRNA and protein contents. Mice fed the control or chronic metabolic stress diet were euthanized by cardiac puncture under anesthesia. Immediately thereafter, the liver was excised, divided for the different analyses, frozen in liquid nitrogen, and stored at −70°C until analysis.

Biochemical analyses

The total bile salt and cholesterol concentrations in bile were determined using commercial diagnostic kits based on 3α-hydroxysteroid dehydrogenase for total bile salts and cholesterol oxidase for unesterified cholesterol. PC concentration was determined by a choline oxidase assay for PC (Wako Chemical USA, Inc., Richmond, VA). The results from the inorganic phosphorous and choline concentrations in the bile samples, as determined by the method of Bartlett (10) and the choline oxidase-based assay, respectively, were similar.

For the determination of hepatic CT activity, total membranes were isolated by centrifugation of the liver homogenates at 600 *g* for 10 min to pellet unbroken cells and nuclei. The supernatant was centrifuged at 100,000 *g* for 1 h. The membrane pellet, containing membrane-associated CT, was resuspended in homogenization buffer (11), and the supernatant contained the soluble fraction. CT activity was measured in the homogenate, as well as in soluble and microsomal fractions, in the presence of PC/oleate vesicles, as described previously (11).

Amount of Mdr2 protein in hepatic plasma membranes

Plasma membranes were isolated as described previously (12). Liver tissue (~2 g) pooled from three or four wild-type or *Pemt*$^{-/-}$ mice fed the control diet (i.e., without taurocholate supplementation) or fed for 1 week the same diet supplemented with taurocholate were used. Protein concentrations were determined according to Lowry et al. (13). Relative enrichments of Na^+/K^+-ATPase as a marker enzyme for the plasma membrane fraction (i.e., the specific activity of this enzyme in the isolated plasma membrane preparation divided by its activity in the homogenate) were used to determine the degree of purification of the isolated membranes in the different experimental groups (12).

Plasma membranes equivalent to 7.5 μg of protein were electrophoresed through a 4–15% polyacrylamide gel at 100 V. The proteins were electrophoretically transferred onto a nitrocellulose filter (Amersham, Little Chalfont, UK) by tank blotting. Ponceau S staining was performed to check equal protein transfer. The filters were blocked for 1 h at 4°C in a solution of Tris-buffered saline with 0.1% Tween and 4% skim milk powder, pH 7.4. The blots were incubated with the primary antibody anti-MDR3 (kindly provided by Dr. J. Schepers, Amsterdam) cross-reacting with murine Mdr2 (14) at a 1:1,000 dilution overnight at room temperature and washed; immune complexes were then detected using horseradish peroxidase-conjugated goat anti-mouse IgG2b (Southern Biotechnology Associated, Birmingham, AL) by the ECL Western blotting kit (Amersham). Protein density was determined by scanning the blots using an Image Master VDS system (Pharmacia Biotech, Uppsala, Sweden). Blots were run after loading of equal protein amounts and after correction for plasma membrane enrichments (Na^+K^+-ATPase).

RNA isolation and measurement of mRNA levels by real-time PCR

Total RNA was isolated from ~30 mg of liver tissue by the TRIzol method (Invitrogen, Paisley, UK). RNA was converted to single-stranded cDNA by a reverse transcription procedure with Moloney murine leukemia virus-RT (Roche Diagnostics, Mannheim, Germany) according to the protocol of the manu-

facturer using random primers. cDNA levels were measured by real-time PCR using the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA).

For the PCR amplification studies, an amount of cDNA corresponding to 30 ng of total RNA was amplified using the qPCR core kit (Eurogentec, Seraing, Belgium) essentially according to the protocol of the manufacturer and optimized for amplification of the particular gene using the appropriate forward and reverse primers (Invitrogen) and a template-specific 3′-TAMRA (6-carboxytetramethylrhodamine)/5′-FAM (6-carboxyfluorescein)-labeled double dye oligonucleotide probe (Eurogentec). The primers used for β-actin and Mdr2 mRNA quantitation were identical to those described previously (15). Calibration curves were run in the same experiments. The data obtained were processed using the ABI Sequence Detector software (version 1.6.3; Applied Biosystems). All quantified expression levels were within the linear part of the calibration curves and were calculated using these curves. PCR results were normalized to β-actin mRNA levels.

Calculations and statistics

All values are given as means ± SD for the number of animals indicated. Results obtained in *Pemt*$^{-/-}$ and wild-type mice were analyzed by the Mann-Whitney *U*-test, using SPSS 10.1 software, with $P < 0.05$ as the threshold for significance.

RESULTS

Bile formation in mice under basal physiological conditions

The deficiency of the PEMT pathway did not significantly affect body weight or liver weight (absolute or relative) in mice on the basal diet (**Table 1**). In addition, bile flow and biliary secretion rates of bile salts and phospholipids were similar in wild-type and *Pemt*$^{-/-}$ mice fed the basal diet during the first 30 min after interruption of the enterohepatic circulation by gallbladder cannulation (Table 1). Accordingly, the PC-to-bile salt molar ratio was similar in bile of wild-type and *Pemt*$^{-/-}$ mice (0.19 ± 0.11 and 0.22 ± 0.07, respectively). The cholesterol secretion rate was higher in the latter.

TABLE 1. Liver and bile parameters in wild-type and *Pemt*$^{-/-}$ mice fed the control diet

Parameter	Wild Type	*Pemt*$^{-/-}$
Body weight (g)	23.9 ± 4.8	21.7 ± 1.6
Liver weight (g)	1.0 ± 0.2	0.8 ± 0.1
Relative liver weight (% of body weight)	4.3 ± 1.1	3.8 ± 0.5
Bile secretion		
Bile flow (μl/min/100 g body weight)	4.7 ± 1.7	4.7 ± 1.2
Bile salts (nmol/min/100 g body weight)	141 ± 48	149 ± 72
Phospholipids (nmol/min/100 g body weight)	25 ± 13	31 ± 13
Cholesterol (nmol/min/100 g body weight)	0.3 ± 0.2	0.6 ± 0.1[a]
Phospholipid-to-bile salt ratio	0.19 ± 0.11	0.22 ± 0.07

Pemt$^{-/-}$, phosphatidylethanolamine *N*-methyltransferase-deficient. Wild-type and *Pemt*$^{-/-}$ mice were fed the control diet. Bile was collected for 30 min after cannulation of the gallbladder. Data are means ± SD of four to eight mice per group.

[a] $P < 0.05$, compared with control.

The capacity of biliary PC secretion can be determined by administration of a bile salt in stepwise increasing doses (3). **Figure 1** shows the relationship between the biliary secretion rates of bile salts and of phospholipid or cholesterol in wild-type and *Pemt*$^{-/-}$ mice during the infusion of TUDC in a stepwise increasing dose. Bile salt secretion rates under these conditions were similar between *Pemt*$^{-/-}$ and wild-type mice. At any bile salt secretion rate, *Pemt*$^{-/-}$ mice secreted similar amounts of PC into the bile as wild-type mice. Similar to the results on biliary PC secretion, biliary cholesterol secretion rates were similar in *Pemt*$^{-/-}$ and wild-type mice (Fig. 1).

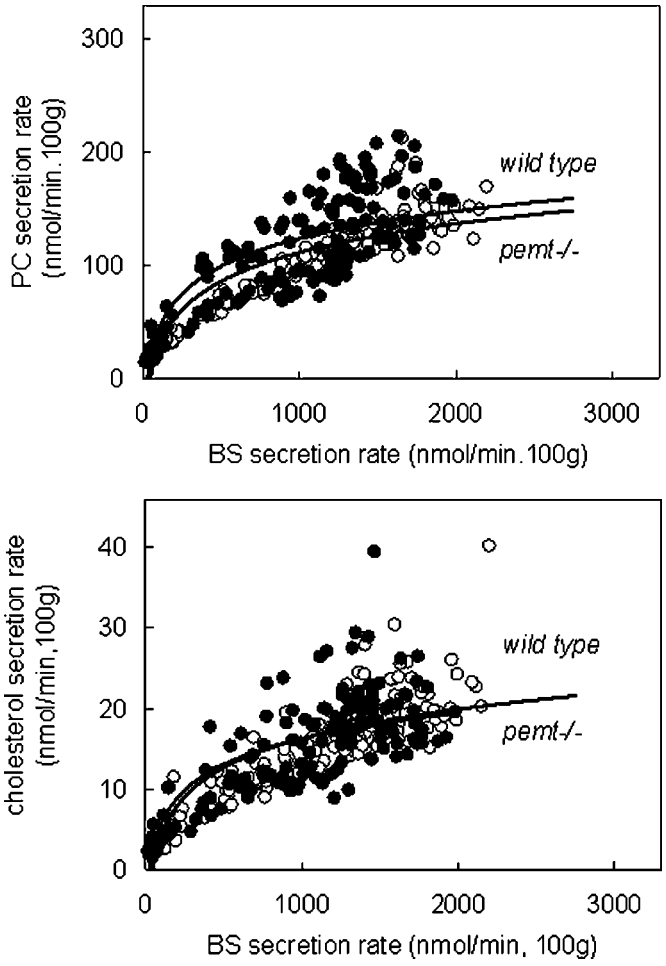

Fig. 1. Biliary lipid secretion during acute metabolic stress. Relationships between biliary secretion rates of bile salts (BS) and phosphatidylcholine (PC; top panel) or cholesterol (bottom panel) in wild-type mice (open symbols) and phosphatidylethanolamine *N*-methyltransferase-deficient mice (*Pemt*$^{-/-}$) (closed symbols) fed the control diet during intravenous infusion of tauroursodeoxycholate (TUDC) in step-wise increasing dosages. Each dot represents an individual bile sample during the course of the experiment. As detailed in Materials and Methods, a maximum of 20 bile samples were collected per mouse. The relationships can be characterized by the following equations: phospholipids, $y = 36.8\text{Ln}(x) - 143.2$, $r^2 = 0.82$ (wild type; n = 8) and $y = 35.2\text{Ln}(x) - 119.7$, $r^2 = 0.66$ (*Pemt*$^{-/-}$; n = 9); cholesterol, $y = 5.2\text{Ln}(x) - 19.9$, $r^2 = 0.70$ (wild type; n = 8) and $y = 4.6\text{Ln}(x) - 15.7$, $r^2 = 0.61$ (*Pemt*$^{-/-}$; n = 9).

Mdr2 P-glycoprotein is known to function as a PC flippase in the bile canalicular membrane, and its expression can be rate-limiting for biliary PC secretion (16). Mdr2 mRNA abundance and Mdr2 protein content in hepatic plasma membrane fractions were similar in *Pemt*$^{-/-}$ and wild-type mice (**Fig. 2**). Bile flow and biliary bile salt secretion rate were also virtually identical in *Pemt*$^{-/-}$ and wild-type mice under basal conditions (Table 1), indicating similar bile salt-independent fractions of the bile flow. CT is the rate-limiting enzyme for the CDP-choline pathway of PC biosynthesis. The CT activity in liver homogenates and in microsomal subfractions was 20% higher in *Pemt*$^{-/-}$ mice compared with controls ($P < 0.05$) (**Table 2**).

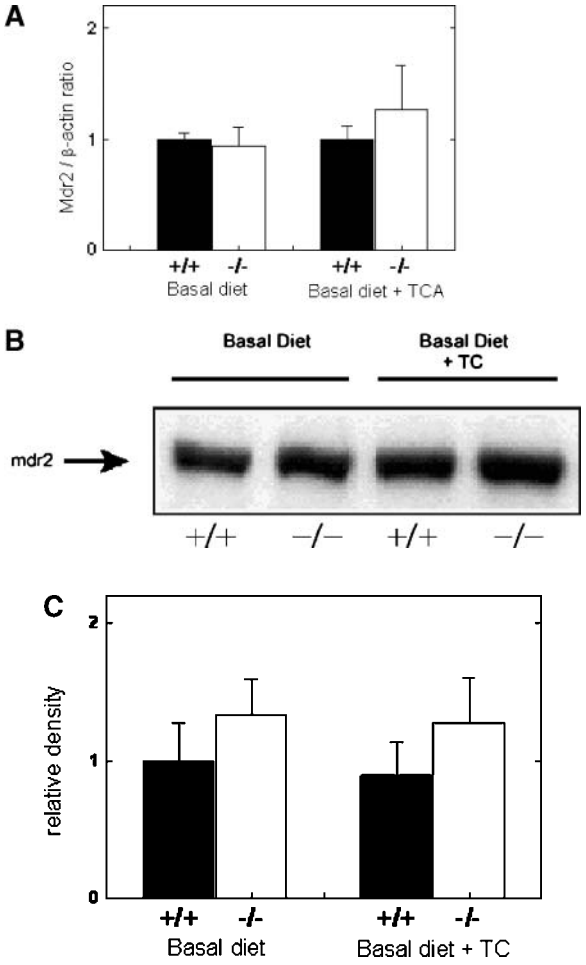

Fig. 2. Hepatic multi-drug-resistant protein 2 (Mdr2) mRNA abundance and Mdr2 protein content in hepatic plasma membrane fractions. A: mRNA abundance of Mdr2 was determined by real-time PCR in liver tissue from wild-type and *Pemt*$^{-/-}$ mice fed the basal (control) diet or for 1 week the same diet supplemented with 0.5% taurocholate (TC; w/w). PCR results were normalized to β-actin mRNA levels. B: Protein immunoblots using an anti-MDR3 antibody (cross-reacting against mouse Mdr2) on plasma membranes from wild-type and *Pemt*$^{-/-}$ mice fed the basal (control) diet or for 1 week the same diet supplemented with 0.5% taurocholate (w/w). Liver tissue was pooled from three or four mice per group. C: Densitometry of Mdr2 protein immunoblots for the four groups, normalized to wild-type mice fed the basal (control) diet (set at 1.0). The results were similar upon loading equal protein amounts or upon correction for plasma membrane enrichments (Na^+K^+-ATPase). Further experimental details are provided in Materials and Methods. The differences between the groups did not reach statistical significance for Mdr2 mRNA (n = 5 per group) or for Mdr2 protein (n = 3 per group). Values shown are means ± SD.

Bile formation in mice under chronic metabolic stress

Recently, we found that *Abcb4*$^{-/-}$;*Pemt*$^{-/-}$ mice (lacking both Mdr2 and PEMT) can efficiently adapt to chronic metabolic stress in the form of choline deprivation (9). Enhancing biliary PC secretion is another form of metabolic stress, as indicated by the deleterious effects for the liver of the inhibition of PC biosynthesis via the CDP-choline pathway in *Pemt*$^{-/-}$ mice but not in *Abcb4*$^{-/-}$;*Pemt*$^{-/-}$ mice (8). We investigated whether bile formation differed between wild-type and *Pemt*$^{-/-}$ mice exposed to bile salt feeding for 1 week. Interestingly, no significant differences in the secretion rates of biliary bile salt, PC, or cholesterol were found between *Pemt*$^{-/-}$ and wild-type mice after acute interruption of the enterohepatic circulation (**Table 3**). Both in *Pemt*$^{-/-}$ and wild-type mice, basal bile flow and biliary secretion rates of bile salts, phospholipids, and cholesterol were at least 2- to 4-fold higher during chronic metabolic stress, compared with the condition in which mice were fed the control diet (Table 1). Similar to the observations after acute metabolic stress, the abundance of Mdr2 protein in isolated liver plasma membrane fractions was similar in *Pemt*$^{-/-}$ and wild-type mice (Fig. 2), as were steady-state Mdr2 mRNA values (Mdr2/β-actin ratio, 0.99 ± 0.11 and 1.26 ± 0.39, respectively; NS). Under chronic metabolic stress, CT activity was comparable in *Pemt*$^{-/-}$ and wild-type mice (**Table 4**).

Bile flow and biliary secretion rates of bile salts, phospholipids, and cholesterol were also similar when the mice fed the taurocholate-containing diet were exposed to intravenous TUDC infusion in stepwise increasing dosages (**Fig. 3**). TUDC infusion in mice under chronic metabolic stress approximately doubled the biliary bile salt secretion rates, compared with the rates immediately after interruption of the enterohepatic circulation (during the last hour, the secretion rate of bile salt was 1,050 ± 447 and 905 ± 323 nmol/min/100 g, and that of PC was 146 ± 46 and 135 ± 33 nmol/min/100 g, in *Pemt*$^{-/-}$ and wild-type mice, respectively; NS). Cholesterol secretion

TABLE 2. Cytidylyltransferase activity in livers from wild-type and *Pemt*$^{-/-}$ mice fed the control diet

Variable	Wild Type	*Pemt*$^{-/-}$
	nmol/min/mg protein	
Total lysate	1.61 ± 0.24	1.94 ± 0.19[a]
Microsomes	1.35 ± 0.23	1.65 ± 0.14[a]
Cytosol	0.17 ± 0.06	0.24 ± 0.06

Cytidylyltransferase activity was determined in samples of liver homogenates and subcellular fractions of *Pemt*$^{-/-}$ and wild-type mice, as detailed in Materials and Methods. Each value is based on isolation of samples from five mice per group.

[a] $P < 0.05$, compared with control.

TABLE 3. Hepatic and bile parameters in wild-type and *Pemt*$^{-/-}$ mice fed a taurocholate-supplemented (0.5 wt%) diet for 1 week

Parameter	Wild Type	*Pemt*$^{-/-}$
Body weight (g)	26.2 ± 3.7	23.0 ± 1.1[a]
Liver weight (g)	1.2 ± 0.2	0.9 ± 0.1[b]
Relative liver weight (% of body weight)	4.5 ± 0.5	3.9 ± 0.2[a]
Bile composition		
Bile flow (μl/min/100 g body weight)	11.4 ± 3.9	12.9 ± 3.0
Bile salts (nmol/min/100 g body weight)	442 ± 270	599 ± 332
Phospholipids (nmol/min/100 g body weight)	105 ± 42	107 ± 39
Cholesterol (nmol/min/100 g body weight)	2.4 ± 1.2	2.7 ± 1.2
Phospholipid-to-bile salt ratio	0.26 ± 0.07	0.21 ± 0.11

Wild-type and *Pemt*$^{-/-}$ mice were fed a taurocholate-supplemented (0.5 wt%) diet for 1 week. Bile was collected for 30 min after cannulation of the gallbladder. Data are means ± SD of four to eight mice per group.

[a] $P < 0.05$, compared with controls.

[b] $P < 0.001$, compared with controls.

showed some variability and was not profoundly different between *Pemt*$^{-/-}$ and wild-type mice (Fig. 3).

DISCUSSION

The availability of the *Pemt*$^{-/-}$ mouse strain permitted the determination of the quantitative requirement of PEMT for PC secretion into bile. The present data on bile secretion rates, in combination with literature data, allowed calculation of the actual fractional turnover of liver PC toward bile secretion. Under basal (physiological) conditions, wild-type mice secreted 9 μmol of PC into bile per day (this study). The total amount of PC in the liver of a wild-type mouse was ~11 μmol (this study). These estimates imply that the daily biliary PC secretion equals ~82% of the total PC present in the liver. The biosynthesis rate of PC in rat liver via the CDP-choline pathway is ~0.35 μmol/h/g liver and that via the PEMT pathway is ~0.11 μmol/h/g liver, determined by ^{1}H-NMR (17). Assuming similar hepatic PC biosynthetic rates in rats and mice, this would account for 11 μmol of PC synthesized daily (for an adult mouse of 25 g body weight, 1 g liver). Based on these calculations, we anticipated that an important quantitative role of PEMT for the repletion of biliary PC would manifest as a decreased capacity to secrete PC into bile.

TABLE 4. Cytidylyltransferase activity in livers from wild-type and *Pemt*$^{-/-}$ mice fed a taurocholate-supplemented (0.5 wt%) diet for 1 week

Variable	Wild Type	*Pemt*$^{-/-}$
	nmol/min/mg protein	
Total lysate	1.43 ± 0.15	1.41 ± 0.36
Microsomes	1.27 ± 0.25	1.30 ± 0.40
Cytosol	0.13 ± 0.08	0.07 ± 0.03

Cytidylyltransferase activity was determined in samples of liver homogenates and subcellular fractions of *Pemt*$^{-/-}$ and wild-type mice, as detailed in Materials and Methods. Each value is based on isolation of samples from five mice per group.

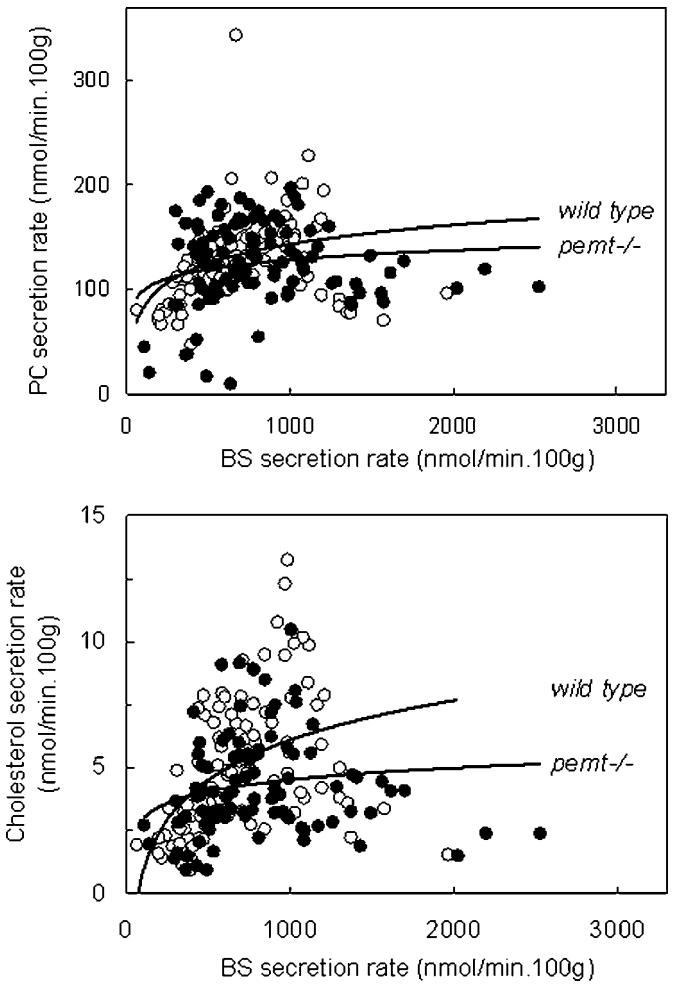

Fig. 3. Biliary lipid secretion during acute metabolic stress in mice pretreated with a taurocholate-containing diet. Relationships between biliary secretion rates of bile salts (BS) and of phosphatidylcholine (PC; top panel) or cholesterol (bottom panel) in wild-type mice (open symbols) and *Pemt*$^{-/-}$ mice (closed symbols) fed the challenge diet (taurocholate 0.5%, w/w) for 1 week during intravenous infusion of tauroursodeoxycholate in step-wise increasing dosages. The relationships can be characterized by the following equations: phospholipids, $y = 26.5\text{Ln}(x) - 39.7$, $r^2 = 0.13$ (wild type; n = 7) and $y = 12.9\text{Ln}(x) + 39.2$, $r^2 = 0.03$ (*Pemt*$^{-/-}$; n = 7); cholesterol, $y = 2.27\text{Ln}(x) - 9.5$, $r^2 = 0.22$ (wild type; n = 7) and $y = 0.67\text{Ln}(x) - 0.09$, $r^2 = 0.03$ (*Pemt*$^{-/-}$; n = 7).

Interruption of the enterohepatic circulation of wild-type and *Pemt*$^{-/-}$ mice on a basal diet showed that the biliary secretion rates of bile salts and PC were very similar. This observation suggests that the CDP-choline pathway of PC biosynthesis (i.e., the only pathway for PC biosynthesis available in *Pemt*$^{-/-}$ mice) can provide sufficient PC for biliary secretion under basal conditions. As demonstrated by the increased enzyme activity of CT, the rate-limiting enzyme for the CDP-choline pathway, the liver of *Pemt*$^{-/-}$ mice does undergo metabolic adaptation to maintain PC homeostasis (Table 2).

Immediately after interruption of the enterohepatic circulation, the biliary cholesterol secretion was higher in *Pemt*$^{-/-}$ mice fed the basal diet compared with controls

(Table 1), but not after feeding the bile salt-containing diet (Table 3). The TUDC infusion after either diet did not indicate that the capacity to secrete cholesterol differed between the genotypes (Figs. 1, 3). These observations together suggest, in our opinion, that the observed difference (Table 1) does not reflect a physiologically relevant population difference between the genotypes.

To determine the versatility of the metabolic adaptations, an acute metabolic stress was imposed in the form of the administration of TUDC in stepwise increasing doses. Bile salt secretion rates under these conditions increased similarly in *Pemt*$^{-/-}$ and wild-type mice fed the basal diet (~15-fold). Even at high bile salt secretion rates, *Pemt*$^{-/-}$ mice secreted similar amounts of PC in the bile as wild-type mice. Also, after the TUDC infusions, the hepatic PC contents were similar in the two genotypes (data not shown). Apparently, the metabolic adaptations under basal conditions in *Pemt*$^{-/-}$ mice are sufficiently versatile to provide an adequate amount of PC when the demand acutely exceeds the basal PC supply by a factor of seven. Moreover, the PEMT pathway does not seem to be required for repletion of the pool of PC for bile secretion under chronic stress (i.e., dietary bile salt feeding for 1 week) (18, 19). In the taurocholate experiment, the biliary PC secretion rate obtained immediately after interruption of the enterohepatic circulation (Table 3) was in the same range as the maximal secretion rate upon TUDC infusion in *Pemt*$^{-/-}$ mice fed the basal diet (Fig. 1). TUDC infusion did not further increase PC secretion in either of the two genotypes of taurocholate-fed mice. Based on these observations, we conclude that the PEMT pathway does not regulate the amount of PC secretion in mice under conditions of acute or chronic bile salt administration. Previously, we demonstrated that chronic metabolic stress in *Pemt*$^{-/-}$ mice induces a wide range of compensatory, PC-conserving processes both intrahepatically and at the interorgan level (9). Because VLDL secretion is a major pathway for PC secretion from the liver, it is reasonable to assume that a decreased loss of PC to lipoprotein secretion would maintain the versatility to cope with demands to supply PC for biliary secretion.

The amount of PC secreted into bile is influenced by several factors (20): *1*) the intracanalicular bile salt concentration (21, 22); *2*) the hydrophobicity of the intracanalicular bile salts (23); *3*) the magnitude of the bile salt-independent fraction of the bile flow (24); *4*) the concentration of hydrophilic organic anions (25); *5*) the abundance of Mdr2 at the bile canalicular membrane (3, 16, 26); and *6*) the lipid composition of the bile canalicular membrane (27–30). Taurocholate feeding did not induce detectable changes in Mdr2 mRNA or protein levels (Fig. 2). Despite the fact that Mdr2 is a farnesoid X receptor target, our present results are consistent with previous mouse studies (including our own) showing that Mdr2 mRNA levels do not change or are only slightly stimulated by bile salt feeding (31–33). Theoretically, one could have hypothesized that PC biosynthesis via the PEMT pathway is an additional, independent factor regulating the amount of PC secreted into bile. Our present results, however, indicate that the PEMT pathway is quantitatively not essential for biliary PC secretion.

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REFERENCES

1. Noga, A. A., and D. E. Vance. 2003. Insights into the requirement of phosphatidylcholine synthesis for liver function in mice. *J. Lipid Res.* **44:** 1998–2005.
2. Walkey, C. J., L. Yu, L. B. Agellon, and D. E. Vance. 1998. Biochemical and evolutionary significance of phospholipid methylation. *J. Biol. Chem.* **273:** 27043–27046.
3. Oude Elferink, R. P., R. Ottenhoff, M. van Wijland, J. J. Smit, A. H. Schinkel, and A. K. Groen. 1995. Regulation of biliary lipid secretion by mdr2 P-glycoprotein in the mouse. *J. Clin. Invest.* **95:** 31–38.
4. Hay, D. W., M. J. Cahalane, N. Timofeyeva, and M. C. Carey. 1993. Molecular species of lecithins in human gallbladder bile. *J. Lipid Res.* **34:** 759–768.
5. Agellon, L. B., C. J. Walkey, D. E. Vance, F. Kuipers, and H. J. Verkade. 1999. The unique acyl chain specificity of biliary phosphatidylcholines in mice is independent of their biosynthetic origin in the liver. *Hepatology.* **30:** 725–729.
6. Robins, S. J., and M. J. Armstrong. 1976. Biliary lecithin secretion. II. Effects of dietary choline and biliary lecithin synthesis. *Gastroenterolgy.* **70:** 397–402.
7. LeBlanc, M. J., V. Gavino, A. Perea, I. M. Yousef, E. Levy, and B. Tuchweber. 1998. The role of dietary choline in the beneficial effects of lecithin on the secretion of biliary lipids in rats. *Biochim. Biophys. Acta.* **1393:** 223–234.
8. Li, Z., L. B. Agellon, and D. E. Vance. 2005. Phosphatidylcholine homeostasis and liver failure. *J. Biol. Chem.* **280:** 37798–37802.
9. Li, Z., L. B. Agellon, T. M. Allen, M. Umeda, L. Jewell, A. Mason, and D. E. Vance. 2006. The ratio of phosphatidylcholine to phosphatidylethanolamine influences membrane integrity and steatohepatitis. *Cell Metab.* **3:** 321–331.
10. Bartlett, G. R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* **234:** 466–468.
11. Yao, Z. M., H. Jamil, and D. E. Vance. 1990. Choline deficiency causes translocation of CTP:phosphocholine cytidylyltransferase from cytosol to endoplasmic reticulum in rat liver. *J. Biol. Chem.* **265:** 4326–4331.
12. Koopen, N. R., H. Wolters, P. Voshol, B. Stieger, R. J. Vonk, P. J. Meier, F. Kuipers, and B. Hagenbuch. 1999. Decreased Na^+-dependent taurocholate uptake and low expression of the sinusoidal Na^+-taurocholate cotransporting protein (Ntcp) in livers of mdr2 P-glycoprotein-deficient mice. *J. Hepatol.* **30:** 14–21.
13. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
14. Kok, T., V. W. Bloks, H. Wolters, R. Havinga, P. L. M. Jansen, B. Staels, and F. Kuipers. 2003. Peroxisome proliferator-activated receptor alpha (PPAR alpha)-mediated regulation of multidrug resistance 2 (Mdr2) expression and function in mice. *Biochem. J.* **369:** 539–547.
15. Plosch, T., T. Kok, V. W. Bloks, M. J. Smit, R. Havinga, G. Chimini, A. K. Groen, and F. Kuiper. 2002. Increased hepatobiliary and fecal cholesterol excretion upon activation of the liver X receptor is independent of ABCA1. *J. Biol. Chem.* **277:** 33870–33877.
16. Smit, J. J., A. H. Schinkel, R. P. Oude Elferink, A. K. Groen, E. Wagenaar, L. van Deemter, C. A. Mol, R. Ottenhoff, N. M. van der Lugt, M. A. van Roon, et al. 1993. Homozygous disruption of the murine mdr2 P-glycoprotein gene leads to a complete absence of phospholipid from bile and to liver disease. *Cell.* **75:** 451–462.
17. Reo, N. V., M. Adinehzadeh, and B. D. Foy. 2002. Kinetic analyses of liver phosphatidylcholine and phosphatidylethanolamine bio-

synthesis using (13)C NMR spectroscopy. *Biochim. Biophys. Acta.* **1580:** 171–188.
18. Li, Q., S. Yokoyama, and L. B. Agellon. 2002. Active taurocholic acid flux through hepatoma cells increases the cellular pool of unesterified cholesterol derived from lipoproteins. *Biochim. Biophys. Acta.* **1580:** 22–30.
19. Elzinga, B. M., R. Havinga, J. F. Baller, H. Wolters, V. Bloks, A. R. Mensenkamp, F. Kuipers, and H. J. Verkade. 2002. The role of transhepatic bile salt flux in the control of hepatic secretion of triacylglycerol-rich lipoproteins in vivo in rodents. *Biochim. Biophys. Acta.* **1573:** 9–20.
20. Verkade, H. J. 2000. Inhibition of biliary phospholipid and cholesterol secretion by organic anions affects bile canalicular membrane composition and fluidity (Editorial). *J. Gastroenterol.* **35:** 481–485.
21. Kay, R. E., and C. Entenman. 1961. Stimulation of taurocholic acid synthesis and biliary excretion of lipids. *Am. J. Physiol.* **200:** 855–859.
22. Verkade, H. J., R. Havinga, A. Gerding, R. J. Vonk, and F. Kuipers. 1993. Mechanism of bile acid-induced biliary lipid secretion in the rat: effect of conjugated bilirubin. *Am. J. Physiol.* **264:** G462–G469.
23. Coleman, R., and K. Rahman. 1992. Lipid flow in bile formation. *Biochim. Biophys. Acta.* **1125:** 113–133.
24. Verkade, H. J., H. Wolters, A. Gerding, R. Havinga, V. Fidler, R. J. Vonk, and F. Kuipers. 1993. Mechanism of biliary lipid secretion in the rat: a role for bile acid-independent bile flow? *Hepatology.* **17:** 1074–1080.
25. Verkade, H. J., M. J. Wolbers, R. Havinga, D. R. Uges, R. J. Vonk, and F. Kuipers. 1990. The uncoupling of biliary lipid from bile acid secretion by organic anions in the rat. *Gastroenterology.* **99:** 1485–1492.
26. Smith, A. J., J. L. Timmermans-Hereijgers, B. Roelofsen, K. W. Wirtz, W. J. van Blitterswijk, J. J. Smit, A. H. Schinkel, and P. Borst. 1994. The human MDR3 P-glycoprotein promotes translocation of phosphatidylcholine through the plasma membrane of fibroblasts from transgenic mice. *FEBS Lett.* **354:** 263–266.
27. Barnwell, S. G., B. Tuchweber, and I. M. Yousef. 1987. Biliary lipid secretion in the rat during infusion of increasing doses of unconjugated bile acids. *Biochim. Biophys. Acta.* **922:** 221–233.
28. Polichetti, E., N. Diaconescu, P. L. Delaporte, L. Malli, H. Portugal, A. M. Pauli, H. Lafont, B. Tuchweber, I. Yousef, and F. Chanussot. 1996. Cholesterol-lowering effect of soyabean lecithin in normolipidaemic rats by stimulation of biliary lipid secretion. *Br. J. Nutr.* **75:** 471–481.
29. Rioux, F., A. Perea, I. M. Yousef, E. Levy, L. Malli, M. C. Carrillo, and B. Tuchweber. 1994. Short-term feeding of a diet enriched in phospholipids increases bile formation and the bile acid transport maximum in rats. *Biochim. Biophys. Acta.* **1214:** 193–202.
30. Yousef, I. M., S. Barnwell, F. Gratton, B. Tuchweber, A. Weber, and C. C. Roy. 1987. Liver cell membrane solubilization may control maximum secretory rate of cholic acid in the rat. *Am. J. Physiol.* **252:** G84–G91.
31. Fickert, P., G. Zollner, A. Fuchsbichler, C. Stumptner, C. Pojer, R. Zenz, F. Lammert, B. Stieger, P. J. Meier, K. Zatloukal, et al. 2001. Effects of ursodeoxycholic and cholic acid feeding on hepatocellular transporter expression in mouse liver. *Gastroenterology.* **121:** 170–183.
32. Frijters, C. M., R. Ottenhoff, M. J. van Wijland, C. M. van Nieuwkerk, A. K. Groen, and R. P. Oude Elferink. 1997. Regulation of mdr2 P-glycoprotein expression by bile salts. *Biochem. J.* **321:** 389–395.
33. Wolters, H., B. M. Elzinga, J. F. Baller, R. Boverhof, M. Schwarz, B. Stieger, H. J. Verkade, and F. Kuipers. 2002. Effects of bile salt flux variations on the expression of hepatic bile salt transporters in vivo in mice. *J. Hepatol.* **37:** 556–563.