

## The Effect of Dimethylethanolamine on Hepatic and Biliary Phospholipid Metabolism

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### ABSTRACT

**AIM:** To investigate the influence of dimethylethanolamine (DMEA), a phosphatidylcholine (PC) precursor, on hepatic and biliary PC metabolism.

**METHOD:** HepG-2 cells, perfused rat liver and patients with bile fistula were exposed to exogenous DMEA.

**RESULTS:** DMEA added to HepG-2 cell culture caused accumulation of phosphatidyl dimethylethanolamine (PDMEA) in the cells. In the presence of C<sup>14</sup>-acetate, the incorporation of C<sup>14</sup>-fatty acids into PDMEA increased, while incorporation into PC decreased as a function of DMEA concentration in the medium. Incorporation into phosphatidylethanolamine (PEA) and phosphatidylserine (PS) was unaffected. Accumulation of PDMEA in the cells continued to increase for 24 h and was maximal in the presence of 0.2 mM DMEA. The concentration of cellular PDMEA and PC were 20 and 35 nmoles per mg protein, respectively. The fatty acid composition of PDMEA resembled that of PC. PDMEA was converted to PC upon removal of DMEA from the medium. When DMEA was added to the perfused rat liver, PDMEA were detected in bile. In the presence of C<sup>14</sup>-acetate, the specific radioactivity of PDMEA was 5 to 10 times higher than that of PC. PDMEA of high specific radioactivity was also isolated from liver microsomes. The fatty acid composition of biliary PDMEA resembled that of microsomal PDMEA in contrast to PC which was enriched with 16:0 and 18:2.

**CONCLUSION:** Exogenous DMEA can modulate bile lipid

composition in HepG-2 cells and perfused rat liver.

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**Key words:** Bile; Phospholipids; Dimethylethanolamine; Cholelithiasis

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### INTRODUCTION

Cholesterol gallstone disease is very common in the Western world. Interactions between the three major biliary components – cholesterol, phospholipids and bile salts - are crucial in the pathogenesis of this disease. The importance of phospholipids in biliary cholesterol secretion, solubilization and precipitation has recently been recognized. Biliary phospholipid concentration as well as composition has been shown to influence bile lithogenicity<sup>[1,2]</sup>. Addition of hydrophobic phospholipids to model and native bile prolongs the nucleation time of the bile. Moreover, increasing the saturation of the Sn-2 acyl chain of PC inhibits biliary cholesterol crystallization<sup>[3]</sup>. Unfortunately, influencing biliary phospholipids in vivo by dietary manipulation is difficult and extremely limited<sup>[4-7]</sup>. Addition of ethanolamine and linoleic acid to the diet of rats and hamsters caused a slight increase in biliary PEA, while cholesterol concentration and the cholesterol saturation index were decreased<sup>[8]</sup>. In humans, addition of PEA (rich in linoleic acid) to the diet was found to increase the percentage of linoleic acid in biliary PC, which is the main phospholipid in bile, without changing PEA or PC concentrations<sup>[7]</sup>.

In the liver, sequential methylation of PEA yields PMMEA, PDMEA, and eventually PC<sup>[9]</sup>. Thus, synthesis of PC in the liver and its subsequent secretion into bile might be expected to increase by providing precursors such as MMEA or DMEA. Modulation of PC synthesis induced by DMEA treatment was previously demonstrated in Chinese hamster ovary cells, LM fibroblasts and rat hepatocytes<sup>[10-12]</sup>.

In the present study we investigated the effects of DMEA on phospholipid metabolism in human hepatoma HepG-2 cells, perfused rat liver model and in humans with a bile fistula.

## MATERIALS AND METHODS

### Materials

Medium-MEM, FCS, glutamine and penicillin-streptomycin solutions: Biological Industries (Beth Haemek, Israel); DMEA: Sigma (St. Louis, MO, USA).

$C^{14}$ -labeled acetate: NEN (Boston, MA, USA); Thin-layer chromatography (TLC): plates (silica gel 60) and all solvents of analytical grade: Merck (Darmstadt, Germany); Scintillation fluid - Ultima Gold: Packard (Meriden, CT, USA).

Tissue cultures: Human hepatoma HepG-2 cells were grown in MEM supplemented with 10% FCS, 200 mM glutamine and 100 IU/L penicillin-streptomycin, in a 37°C incubator with 5% CO<sub>2</sub>. Cells were incubated in 35 mm plastic dishes, when cells reached confluency, the growth medium was removed and replaced with 2 mL fresh medium.

HepG-2 cells were incubated for 24 h in the presence of increasing concentrations (0.1 to 0.8 mM) of DMEA. After 24 h the medium was replaced with fresh medium which contained DMEA and [1- $C^{14}$ ] acetate. To measure  $C^{14}$ -acetate incorporation, 2 mL fresh medium containing DMEA and 0.1 mM [1- $C^{14}$ ] acetate (5 mCi/mMole) was added and incubation continued for 3 h.

At the end of the incubation, the medium was removed and the cells washed 3 times with PBS. All incubations were done in triplicates. Results are normalized to mg protein and represent the mean of 3 independent determinations. Variations did not exceed 5%.

### Isolated Perfused Rat Liver

Male Wistar rats (300-360 g) were fed Purina Rodent Chow ad libitum and maintained under a constant light cycle of 12 h. The surgical procedures were performed as described by Corasanti *et al.*<sup>[13]</sup>. Rats were anesthetized with pentobarbitone (50 mg/kg body wt ip) (Ceva, Paris, France), the bile duct was cannulated with PEA - 10 tubing (Clay Adams, Parsippany, NJ), and the portal vein with a 16-gauge Teflon intravenous catheter (BOC Ohmeda AB, Helsingborg, Sweden). The liver was then perfused at a constant flow of 20 mL/min with oxygenated KRB buffer containing (in mM) 120 NaCl, 4.7 KCl, 1.2 KH<sub>2</sub>PO<sub>4</sub>, 1.2 MgSO<sub>4</sub>, 1.8 CaCl<sub>2</sub> and 25 NaHCO<sub>3</sub>. Glucose (5.5 mM) and heparin (200 U/100 mL) were added to the buffer.

After cannulation of the inferior vena cava, the livers were transferred into a heated perfusion chamber and perfused in a recirculating closed system (volume: 200 mL) at a constant flow rate of 40 mL/min with KRB buffer containing 5.5 mM glucose and 1% bovine serum albumin. The KRB buffer was gassed continuously with a humidified mixture of 95% O<sub>2</sub> - 5% CO<sub>2</sub> and maintained at 37±0.5°C. After 10 min of equilibration a bolus of 50 µM of [1- $C^{14}$ ] labeled acetate (2 mCi/mMole) and 0.4 mM DMEA (final concentrations) was added. Taurocholic acid (30 µM) was infused continuously to stimulate bile secretion. The livers were perfused for 2 h. At the end of the experiment the livers were perfused for 15 min with fresh KRB.

Bile samples were collected every 15 min and bile flow was measured gravimetrically in prepared tubes. At the end of the experiments bile samples were pooled. The viability of the perfused liver was assessed throughout the perfusion by monitoring perfusion pressure (7-12 cmH<sub>2</sub>O), determining baseline bile flow, and measuring release of lactate dehydrogenase as well as observation of the general appearance of the liver. Livers and bile samples were frozen at -70°C for lipid analysis. The mean weight of the livers was 10.4±1.0 g. Two 1 g samples of each liver were used for lipid extraction and analysis. The results presented in the tables are the mean value of these determinations.

### Isolation of microsomes

Two 2 g samples of liver were homogenized in PBS. Cell debris and nuclei were removed by centrifugation at 1000 g, mitochondria at 10 000 g and microsomes were sedimented by centrifugation at 100 000 g for 60 min. The microsomes were suspended in a small volume of PBS and used for lipid analysis.

Lipid extraction and analysis of HepG-2 cells: Cells attached to the plates were extracted twice with hexane/isopropanol (3:2, by vol), first with 1.5 mL for 60 min and then with 1 mL for 15 min<sup>[14]</sup>. The amount of the radioactivity in the extracts was determined using a Kontron-Betasmatic counter (Zurich, Switzerland). The hexane/isopropanol extracts were dried with a gentle stream of N<sub>2</sub> and the lipids dissolved in chloroform. Aliquots of the lipid extract were used for TLC. Neutral lipids were separated on Silica gel 60 plates employing hexane/diethyl ether/methanol/acetic acid (90:20:3:2, by vol.) as solvent. Phospholipids (PL) were separated using chloroform/methanol/acetic acid/water (100:20:12:5, by vol.) as solvent. To separate PDMEA butanol/acetic acid/water (90:20:20, by vol.) was used.

TLC plates were dried and subsequently sprayed with 0.02% 2,7-dichlorofluorescein in ethanol. Lipid spots were detected under UV light, scraped into scintillation vials, and suspended in 3.5 mL of scintillation fluid for determination of radioactivity. To isolate cholesterol, the overlapping spot of diacylglycerides and cholesterol on the TLC plates was collected and saponified with 0.5 M methanolic KOH for 30 min at 50°C. Samples were then acidified and extracted with hexane. Aliquots were placed on TLC plates as described above.

To determine the amount of the different phospholipids, the corresponding spots after the separation were collected, saponified with 0.5 M methanolic-KOH at 50°C for 30 min. Fatty acids (FA) were extracted with hexane after acidification. The solvent was removed and the FA methylated with diazomethane according to Schlenk and Gellerman<sup>[15]</sup>. The methyl-esters were separated by GLC on a 30 m PAG column at a temperature range of 185-220°C using a Hewlett-Packard gas chromatograph (series 5790A)+HP-3396 recorder-integrator. For quantitative analysis heptadecanoic acid was added as an internal standard assuming that the response of the detector for all fatty methyl-esters was identical.

Liver and bile lipid extraction and analysis: Liver samples (1 g) were homogenized with 5 mL of PBS using an Ultra-Turax (Janke and Kunkel Ika-Werk, Staufen, Germany). The homogenates were then extracted with chloroform/methanol according to Bligh and Dyer<sup>[16]</sup>. 1 mL of the pooled bile from each experiment and of microsomal suspensions was similarly extracted.

The amount of the radioactivity in the extracts was determined as described in the previous section. Aliquots of the lipid extracts were used for the separation of neutral lipids and PL by TLC and for quantitative analysis as described in the previous section.

Cholesterol was determined directly from the chloroform/methanol extract by an enzymatic colorimetric method using a cholesterol oxidase kit from Boehringer Mannheim GmbH (Ingelheim, Germany).

Protein determination: Cells (after lipid extraction) were incubated overnight at room temperature with 2 mL of 0.2 M NaOH to extract protein. The amount of protein was determined according to the method of Markwell *et al.*<sup>[17]</sup>.

### Human experiments

Patients and study protocol: Seven patients, who had undergone cholecystectomy, choledochotomy and T tube placement for symptomatic gallstone disease, were enrolled in the study. Patients were an average of eight days after the operation (range 5-11 days) and the T-tube had been clamped for 4-6 days to ensure the enterohepatic

circulation prior to the study<sup>[18]</sup>. The patients (five females; average age 73±12 years) were kept on a constant diet (1500 cal/d) of 30% lipids, 20% protein and 50% carbohydrates. In addition, the diet of five patients was enriched with DMEA (DEANOL<sup>®</sup>, Rischiaril, Vecchi & Piam, Genova, Italy) 1.5 g day for two weeks. Two patients served as controls under the same conditions but without DMEA in their diet. Hepatic bile samples were collected by gravity from the T-tube for 30 min at time 0 and after two weeks at the end of the study. The first 5 mL of each bile sample were discarded. In between sampling, the T-tube was kept clamped.

All patients were well nourished and their hepatic, thyroid and renal functions were within normal limits. None of the subjects had recent weight loss (>10% during 6 past months), obesity (body mass index > 30kg/m<sup>2</sup>), significant hypercholesterolemia (cholesterol >250 mg/dL), hypertriglyceridemia (triglycerides >250 mg/dL), diabetes mellitus or cholangitis.

### Bile analysis

Bile lipids were extracted as described by Folch<sup>[19]</sup>. Bile salt concentration was determined enzymatically<sup>[20]</sup>, cholesterol as described by Abell *et al.*<sup>[21]</sup> and phospholipids as described by Bartlett<sup>[22]</sup>. The cholesterol saturation index (CSI) was calculated using the critical tables of Carey<sup>[23]</sup>.

### Statistics

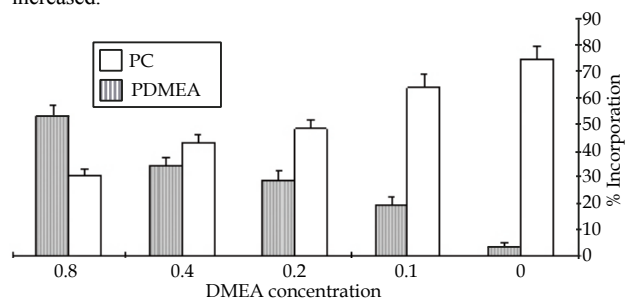
Data is presented as the mean value of independent determinations with their standard deviation. The student t-test was performed on data collected. *P* value <0.05 was considered significant.

Local ethical committee approval was obtained for all in vivo tests (human and animal). Human subjects signed an approved informed consent form before enrollment in the study.

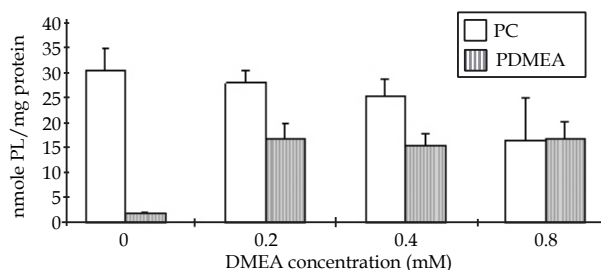
## RESULTS

### HepG-2 cells

**Effect of DMEA concentration:** As the concentration of DMEA in the medium was increased, the incorporation of C<sup>14</sup>-acetate into PC (expressed as % of the total amount of radioactivity incorporated into cellular PL) decreased, while incorporation into PDMEA increased (Figure 1). No change was seen in the incorporation of acetate into PEA and PS (not shown). The addition of DMEA affected the total amount of acetate incorporated into cellular lipids. Figure 2 shows the accumulation of PDMEA and PC in HepG-2 cells. PC concentration in the cells decreased as DMEA in the medium was increased. The accumulation of PDMEA was maximal at 0.2 mM DMEA and did not increase further as DMEA in the medium was increased.



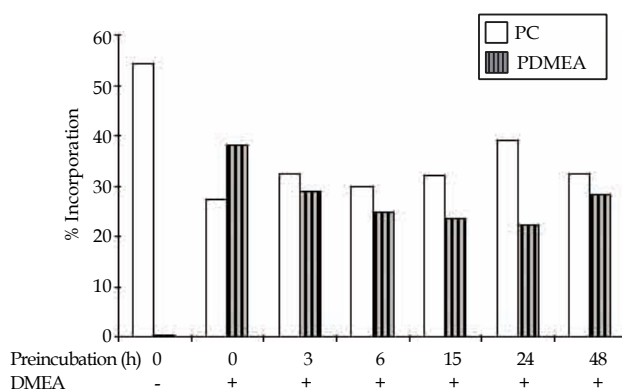
**Figure 1 Incorporation of C<sup>14</sup>-acetate into PC and PDMEA by HepG-2 cells.** Cells were incubated for 24 h in the presence of increasing concentration of DMEA. The medium was then replaced by 2 mL fresh medium containing [1-C<sup>14</sup>]-acetate (0.1 mM-0.5 Ci) and the incubation continued for 3 h: 15-18 nmole/mg protein of acetate were incorporated cellular phospholipids. The results are expressed as % of C<sup>14</sup>-acetate incorporated into cellular PL. All incubations were done in triplicates. The results presented are the mean of 3 independent determinations.



**Figure 2 Accumulation of PC and PDMEA as a function of DMEA concentration in the medium.** Cells were incubated as described in the legend to figure 1. PC and PDMEA were isolated and the amount of FA in each lipid determined as described in the methods. Results are expressed as nmole PL/mg protein (560 g FA=1 mole PL). The results presented are the mean of 3 independent determinations.

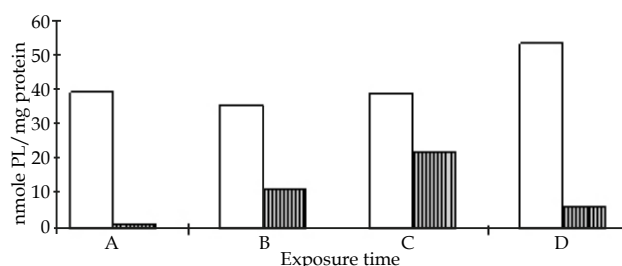
**Effect of incubation time:** To study the effect of DMEA exposure time, HepG-2 cells were exposed to DMEA for 3, 6, 15, 24 and 48 h, after which fresh medium which contained DMEA + [1-<sup>14</sup>C] acetate was added for 3 h.

The percent of acetate incorporated into PDMEA was maximal when cells were incubated with C<sup>14</sup> acetate and DMEA (0.4 mM) for 3 h and did not change significantly upon preincubation with DMEA (Figure 3). Correspondingly, incorporation of acetate into PC was maximally decreased after 3 h incubation with DMEA.



**Figure 3 Effect of time of exposure to DMEA on C14-acetate incorporation.** Cells were exposed to 0.4 mM DMEA as indicated for the preincubation period. Fresh medium containing 0.4 mM DMEA + C<sup>14</sup>-acetate was added following the preincubation period and the incubation continued for additional 3 h. First column: control without DMEA. Results are calculated as in figure 1. The results presented are the mean of 3 independent determinations.

Significant amounts PDMEA accumulated inside the cells after 3 h of exposure to DMEA which increased further when cells were exposed for 24 h (Figure 4).



**Figure 4 Effect of time of exposure to DMEA on PC and PDMEA accumulation.** Cells were exposed to DMEA (0.4 mM) for different time and PC and PDMEA accumulation measured as described in figure 2. Duration of exposure to DMEA: (A) none (control). (B) 3 h. (C) 24 h. (D) 24 h followed by 24 h fresh medium (devoid of DMEA). The results presented are the mean of 3 independent determinations.

After 24 h of incubation with DMEA (with subsequent addition of fresh medium without DMEA), the amount of PDMEA inside cells was markedly reduced while PC increased, suggesting conversion of PDMEA to PC. The methylation of PDMEA was shown by incubating cells with [<sup>3</sup>H-CH<sub>3</sub>]-methionine. After 3 h of incubation 2.5 nmole/mg protein of [<sup>3</sup>H-CH<sub>3</sub>] were incorporated into cellular PC. When DMEA was added together with [<sup>3</sup>H-CH<sub>3</sub>]-methionine for 3 h, 7.9 nmoles were incorporated into PC. After 24 h preincubation with DMEA (followed by 3 h incubation with [<sup>3</sup>H-CH<sub>3</sub>]-methionine) 8.7 nmoles were incorporated. When the cells were allowed to accumulate PDMEA and subsequently incubated in fresh medium (devoid of DMEA), the incorporation of [<sup>3</sup>H-CH<sub>3</sub>]-methionine (after 3 h) was reduced to 6.3 nmoles, which is significantly higher than the control, indicating methylation of PDMEA to PC.

**Fatty Acids composition:** Table 1 shows that the FA compositions of PDMEA did not differ significantly from that of PC in HepG-2 cells grown in the presence of DMEA.

**Table 1** Fatty acid composition of PC and PDMEA of HepG-2 cells grown in the presence of DMEA.

	16:0	16:1	18:0	18:1	18:2	20:3	20:4	22:6
PC	30.1±2.3	8.3±0.8	8.1±1.1	37.0±2.3	1.7±0.1	2.1±0.2	1.6±0.2	1.5±0.3
PDMEA	22.7±1.9	6.0±0.6	10.8±1.3	36.1±2.3	1.9±0.1	3.0±0.3	2.4±0.3	2.7±0.3

PC and PDMEA were isolated from cells grown in the presence of 0.1, 0.2 and 0.4 mM DMEA. FA were obtained by alkaline hydrolysis, extracted into hexane after acidification, methylated with diazomethane and separated by GLC as described in the methods. The results are expressed as % of total FA and represent the average of these determinations.

#### Isolated perfused rat liver

To study the effect of DMEA perfusion on lipid composition of bile secreted by the isolated rat liver DMEA (40 mM) and [1-<sup>14</sup>C] acetate (50 μM-2 μCi/μmole) were added to the perfusate. Bile secretion became stable after 30 min. with an average rate of 1.8 μg/min/g liver, and remained constant for the rest of the perfusion period. Upon addition of DMEA to the perfusate, small amounts of PDMEA were detected in bile (Table 2). Approximately 25% of radioactivity incorporated into biliary PL was contained in PDMEA. Thus, the specific radioactivity of PDMEA was considerably higher than that of PC. Small amounts of C<sup>14</sup>-labeled PEA and PS were also detected in the bile (not shown). Under our experimental conditions, PDMEA was not detected in liver PL. However, when microsomes were prepared from these livers, considerable amounts of C<sup>14</sup>-labeled PDMEA were detected. The specific radioactivity of the microsomal PL was considerably lower than that of biliary PL; the specific radioactivity of microsomal PDMEA was considerably higher than that of microsomal PC (Table 2).

**Table 2** PDMEA synthesis and secretion into bile by the perfused rat liver.

addition to perfusate	Biliary Phospholipids				Microsomal Phospholipids				
	Radioactivity Total	% of PL	nmole	dpm/nmole	Radioactivity Total	% of PL	nmole	dpm/nmole	
	dpm				dpm				
	×10 <sup>3</sup>				×10 <sup>3</sup>				
	PC	PDMEA	PC	PDMEA	PC	PDMEA	PC	PDMEA	
(+)DMEA	25.0	69.7	25.3	188.6	6.4	92	1001.5	23.9	200.3
(-)DMEA	68.8	71.6	22.7	178.3	9.4	274.8	1689.9	40.2	231.5
(-)DMEA	70.0	83.0	3.5	253.7	0.1	238.6			

Livers were perfused for 2 h with 200 mL KRB containing 50 M acetate (2 μCi/μmole) and DMEA 40 mM when indicated. PL was isolated from bile, total liver and liver microsomes. FA were isolated and separated by GLC as described in the methods. μg FA were converted to nmole PL, assuming that 560 μg FA=1 μmole of PL. Each line represents 1 experiment.

The FA composition of biliary PDMEA was different from that of biliary PC but resembled that of microsomal PDMEA (Table 3).

**Table 3** Comparison of FA composition of microsomal and biliary PC and PDMEA.

SAMPLE	FA 16:0	18:0	18:1	18:2	20:4	22:6
Bile PC	32.6±1.1	4.2±2.3	3.7±0.4	34.4±2	17.5±3.2	3.1±1.3
Bile PDMEA	35.3±4.5	11.1±4.1	8.7±2.5	28.4±1.5	8.2±2	2.4±2.4
Microsomal PC	18.3±1.7	22.9±1.1	4.1±0.6	17.8±0.3	29.1±1.1	1.5±0.3
Microsomal PDMEA	32.3±2.8	10.9±2.9	7.1±1.4	26±0.5	17.4±0.6	3.1±0.9

FA was isolated from liver microsomes and bile as described in the methods.

Biliary PC in comparison to microsomal PC was enriched in 16:0 and 18:2 fatty acids.

#### Human studies

Enrichment of the diet with 1.5 g a day of DMEA for two weeks to five patients did not cause appearance of PDMEA in the excreted bile of the subjects. Bile lipid composition is presented in Table 4. The bile composition (cholesterol, bile salts, phospholipids) was unaffected by dietary addition of DMEA.

**Table 4** Human bile lipids composition.

	No. of patients	PL mM	CHL mM	BS mM
Before DMEA	7	11.8±3.5	4.2±1.6	27.3±7.2
After DMEA	5	9±3.5	3.8±1	26.1±11.7
without DMEA	2	14.3±0.2	3.4±1.1	29.6±5.4

Seven gallstone patients who had bile fistula were kept on a constant diet that was enriched with DMEA 1.5 g day for two weeks. Two patients served as controls under the same conditions but without DMEA in their diet. Bile lipids were extracted as described in the methods.

## DISCUSSION

In the present study, the effects of DMEA on phospholipid metabolism and secretion in HepG-2 cells, the perfused rat liver and human bile were investigated. DMEA inhibited H<sub>3</sub>-choline

Incubation of HepG-2 cells with DMEA in the presence of C<sup>14</sup>-acetate resulted in accumulation of newly synthesized C<sup>14</sup>-labeled PL-PDMEA. In the presence of increasing concentrations of DMEA, the synthesis of C<sup>14</sup>-PDMEA increased, while the synthesis of C<sup>14</sup>-labeled PC decreased. The total incorporation of C<sup>14</sup>-acetate into PL was not affected by the addition of DMEA, suggesting the preferential incorporation of the newly synthesized C<sup>14</sup>-FA into PDMEA. PDMEA accumulation and labeling followed a different time course. Whereas C<sup>14</sup>-FA incorporation increased up to 0.8 mM DMEA, PDMEA accumulation was maximal at 0.2 mM and did not change when the concentration of DMEA in the medium was increased. Furthermore, C<sup>14</sup>-FA incorporation was maximal after 3 h incubation with DMEA; accumulation of PDMEA peaked at 24 h. When DMEA was removed, cellular PDMEA was converted to PC as indicated by an increase in PC concentration and the simultaneous stimulation of [<sup>3</sup>H-CH<sub>3</sub>]-Methionine incorporation into PC. In contrast, changes in phospholipid content and composition in liver plasma membranes were demonstrated after more extended infusion period of DMEA (0.3 mg/kg/min for 15 h) in a previous study<sup>[24]</sup>.

DMEA can be incorporated into PDMEA by base-exchange with PEA or after activation and CDP-DMEA formation. The finding that PDMEA accumulation is accompanied by a decrease of PC suggests the occurrence of a competition between choline and DMEA for the cytidyltransferase which synthesizes CDP-choline or CDP-DMEA and possibly also their further interaction with diacylglycerol.

The accumulation of PDMEA in the cells indicates that PDMEA synthesis is faster than its methylation to PC. In preliminary

experiments we found accumulation of both PMMEA and PDMEA when MMEA was added to the medium (data not shown), indicating the sequential methylation of these phospholipids. The occurrence and physiological importance of the N-methyltransferase in the liver has been documented by many studies of Vance and his coworkers<sup>[9]</sup>.

Sundler and Akesson<sup>[25]</sup> demonstrated the accumulation of PMMEA and PDMEA when rat hepatocytes were exposed to MMEA and DMEA. Ethanolamine inhibited the formation of PMMEA, probably as a result of competition at the level of CDP-ester formation. MMEA and DMEA stimulated the incorporation of [ $C^{14}$ -CH<sub>3</sub>]-labeled methionine into PC. Interestingly, Vance<sup>[26]</sup> showed that the accumulation of PMMEA in rat hepatocytes was toxic to the cells and inhibited VLDL secretion. In these studies DMEA inhibited H<sup>3</sup>-choline incorporation into PC probably due to competition for cytidyltransferase. Katyal and Lombardi<sup>[27]</sup> showed the occurrence of PMMEA and PDMEA in livers of rats fed with MMEA. The fatty acids composition of PMMEA and PDMEA was similar but differed from that of PC and PEA; 18:0, 20:4 and 22:6 were the predominant fatty acids of these phosphatides. Alvaro *et al.*<sup>[28]</sup> studied the effect of *in-vivo* IV infusion of  $C^{14}$ -DMEA on hepatic PL composition. The  $C^{14}$ -PC synthesized from  $C^{14}$ -DMEA was selectively enriched by 3 molecular species: Sn-1 stearoyl/Sn-2 arachidonoyl Sn-1 stearoyl/Sn-2 linoleyl and Sn-1 stearoyl/Sn-2 docosaheranoyl. Although small amounts of PDMEA were detected in the liver,  $C^{14}$ -PDMEA was not found in the bile.

The perfused rat liver system described permits the simultaneous measurement of PL metabolism in the liver and the secretion of PL and their metabolites into bile. When  $C^{14}$ -acetate and DMEA were added to the perfusate,  $C^{14}$ -labeled PC and PDMEA were detected in the bile and liver microsomes. Approximately 5% PDMEA, of total PL, contained 25% of the newly synthesized FA present in biliary PL in the bile. Thus, the specific radioactivity of PDMEA was 6 to 10 fold higher than that of PC.

Although we could not detect PDMEA in the total liver PL,  $C^{14}$ -PDMEA was isolated from liver microsomes, the specific radioactivity of which was 5 to 8 fold higher than that of PC. These results show the preferential incorporation of the newly synthesized FA from  $C^{14}$ -acetate into PDMEA and suggest the de-novo synthesis of PDMEA by the CDP-DMEA pathway in the intact liver, as we have suggested for cultured HepG-2 cells. In contrast to PC, the FA composition of biliary and microsomal FA was similar containing mainly 16:0, 18:2 and 20:4. Biliary PC, as expected, contained mainly 16:0 and 18:2, suggesting the preferential secretion of these species by the mdr-2 P-glycoprotein system present in the bile canaliculi<sup>[29]</sup>. PDMEA in contrast does not seem to be selectively secreted by this system. In this perspective, Yasumiba *et al.*<sup>[30]</sup> studied male Spargue-Dawley rats, which were infused with DMEA, and analyzed their liver membrane and bile composition. They showed an increase in biliary PL secretion and canalicular membrane fluidity without a change of its canalicular transporter by DMEA.

To assess a possible clinical relevance of the above findings, humans with a bile fistula were fed with DMEA. Despite the influence of DMEA in HepG-2 cells and perfused rat liver, when 1.5 g DMEA was given orally to patients for two weeks, PDMEA was not detected in their bile and bile lipid composition remained unchanged. This may reflect a different metabolic pathway in humans. However, we cannot rule out the possibility that the dose was too small to show an effect or that PDMEA concentration in the bile was too small for detection by our methods. Furthermore, the sample size may have been too small for drawing decisive conclusions.

DMAE, an analogue of diethylaminoethanol, is a precursor

of choline, which in turn facilitates optimization acetylcholine production in the brain. Acetylcholine is a primary neurotransmitter involved in learning and memory. Recent scientific interest has risen with the observation that DMAE plays an important role not only in the hepatobiliary, but also in the neurologic system as an antioxidant with the ability to scavenge specific free radical species<sup>[31]</sup>. Moreover, Dubois and colleagues<sup>[32]</sup> have reported on its effect in patients with prodromal Alzheimer's disease during six months of treatment. Unfortunately, they found no statistically significant difference in cognitive function with this treatment. The authors claim that the absence of drug efficacy in this population may be due to study design flaws such as insufficient powering to assess changes in cognition over time, a relatively short duration of treatment and the lack of validated clinical trial measures designed to assess the prodromal Alzheimer's disease population. This is very similar to our findings in which the effects of DMEA *in vitro* could not be reproduced *in vivo*, most probably due to study design difficulty and a lack of an accepted treatment regimen with this compound.

A limitation of this study is the small number of patients recruited with bile fistula. This is unfortunate but was unavoidable as patients with T-tube drainage of the bile system with benign, stone-related disease, are rare.

## CONCLUSIONS

Exogenous DMEA can modulate bile lipid composition in HepG-2 cells and perfused rat liver. Further work is needed to elucidate if a correct treatment regimen exists for humans and to assess its influence on bile composition. It is still premature to think of DMEA as a putative safe and effective oral treatment for cholelithiasis but more work needs to be done regarding proper dosage regimens to that effect. Further studies are necessary to determine the clinical applicability of these findings, such as the effect on cholesterol gallstones or other hepatobiliary disorders in humans.

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