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GALL STONES

Effect of phospholipids and their molecular species on cholesterol solubility and nucleation in human and model biles

Z Halpern, M Moshkowitz, H Laufer, Y Peled, T Gilat

Abstract

Much research in the pathophysiology of gall stones has been devoted to various molecular species of bile salts. Recent findings have shown the importance of phospholipids in biliary pathophysiology. In the present study the addition of increasing doses of egg lecithin to human and model biles progressively prolonged the nucleation time. Concurrently biliary cholesterol was shifted from the vesicular to the non-vesicular carrier(s) while the cholesterol/phospholipid ratio of the remaining vesicles was progressively lowered. Model bile solutions of identical lipid concentration were prepared using phosphatidylcholine, phosphatidylserine, and phosphatidylethanolamine as the only phospholipid. With phosphatidylethanolamine most of the cholesterol was shifted to the vesicular carrier while phosphatidylserine shifted most of the cholesterol to the non-vesicular carrier(s). With phosphatidylcholine the cholesterol was distributed in both carriers. Phosphatidyl choline species composed of various acyl fatty acids in the sn-1 and sn-2 positions were used as the sole phosphlipid in otherwise identical model bile solutions. With palmitic acid in the sn-1 position and arachidonic acid in the sn-2 position most of the cholesterol was found in the non-vesicular carrier. When stearic acid was used in sn-2 position instead of arachidonic acid most of the cholesterol was found in the vesicular carrier. These and other variations in phospholipid molecular species shifted cholesterol among its carriers and also modified the nucleation time of model biles. Most of these effects were also found upon addition of the various phospholipid species to human biles. These findings show the importance of phospholipid species in biliary pathophysiology and may be useful when trying to manipulate cholesterol carriers and solubility in bile.

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Department of Gastroenterology, Ichilov Hospital, Tel Aviv Medical Center and Sackler Faculty of Medicine, Tel Aviv University, Israel
Z Halpern
M Moshkowitz
H Laufer
Y Peled
T Gilat

Correspondence to:
Z Halpern, MD, Department of Gastroenterology, Ichilov Hospital, 6 Weizman St, 64239 Tel Aviv, Israel.

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The aetiology of cholesterol gall stones has not been completely elucidated. Research interest during the last two decades has been focused on the metabolism of biliary lipids and its effect on gall stone formation. It has become evident that a disturbed metabolism of cholesterol and/or bile salts is a major factor in gall stone formation.[1,2] The role of phospholipids in this process is only now beginning to be appreciated.[3–5] It has been reported[6,7] that the addition of minute amounts of phosphatidylcholines normalised the short nucleation time of bile from patients with cholesterol gall stones while having only a minimal effect on the cholesterol saturation index. Similar amounts of bile salts did not prolong the nucleation time.[8] In the present study we have analysed the effect of various phospholipids and different molecular species of lecithin on cholesterol distribution between its carriers in bile and their effect on the nucleation time.

Methods

BILES

Six gall bladder bile samples (Table I, biles 2–7) were obtained at cholecystectomy and one hepatic bile (Table I, bile no 1) from a patient with an indwelling T tube. All patients had cholesterol gall stones. Data on these biles are shown in Table I. All patients gave their informed consent to bile sampling.

MATERIALS

The following synthetic molecular species of phospholipids with identical fatty acid composition (sn-1 palmitoyl sn-2 oleoyl) were used: phosphatidylserine, phosphatidylethanolamine, and phosphatidylcholine. The following molecular species of phosphatidylcholine: sn-1 palmitoyl sn-2 stearoyl, sn-1 palmitoyl sn-2 oleoyl, sn-1 palmitoyl sn-2 arachidonoyl and sn-1 stearoyl sn-2 oleoyl were used. All were purchased from Avanti Polar Lipids, Inc (Birmingham, AL). The synthetic phospho-

TABLE I *Composition of biles*

	CHOL	*PL*	*BS*	*Total lipid*	*CSI*	*Nucleation time*	*% Cholesterol in vesicles*
	— — mole % — —			(g/dl)	%	days	%
1	17·2	21·7	61·1	0·8	461	3	68
2	14·0	22·0	64·0	5·1	210	3	24
3	8·9	18·8	72·3	9·4	137	7	13
4	18·9	23·4	57·8	2·7	298	6	47
5	10·4	27·2	62·4	3·4	154	1	13
6	7·6	18·0	74·4	11·3	117	4	0
7	4·8	15·3	79·9	11·8	83	6	0

CHOL – cholesterol, PL – phospholipid, BS – bile salts, CSI – cholesterol saturation index.

lipids and phosphatidylcholine gave a single spot by thin layer chromatography and were more than 98% pure by gas liquid chromatography of their fatty acid methyl esthers. All other lipids were purchased from Sigma Chemical Co. The egg lecithin used contained five different fatty acids.

METHODS

(1) Model biles were prepared by a modification of the method described by Kibe *et al.*[9 10] Measured aliquots of lipid stock solutions were used to produce a model bile with the desired composition: cholesterol – 10 mol%, bile salt (sodium taurocholate) – 72 mol%, phospholipid (egg yolk lecithin) – 18 mol%, total lipid concentration – 8·0 g/dl.

(2) The handling of native and model biles, cholesterol labelling and chromatographic separation to vesicular and non-vesicular phases were performed as described previously.[5 10 11] The dimensions of the chromatographic column were 10×330 mm and the flow rate was 0·7 ml/min. The percentage of biliary cholesterol carried by the vesicular and non-vesicular fractions was calculated by measuring the area under their respective peaks, as a proportion of the total area under the cholesterol radioactivity curve eluted from the chromatographic column.[10] The use of radioactive markers was previously validated by comparison with endogenous lipids.[11] The fractions of the isolated vesicular and non-vesicular peaks, obtained by chromatographic separation of biles, were pooled for chemical analysis.

(3) Nucleation time was determined as described by Holan *et al*,[12] using a Carl Zeiss microscope equipped with a polarising system. Samples were kept for up to 30 days at 37°C under nitrogen and studied daily in search of cholesterol crystals.

(4) Analytic procedures: bile acid concentration was determined enzymatically.[13] For the analysis of cholesterol and phospholipids the methods of Abbel *et al*[14] and Bartlett[15] were used.

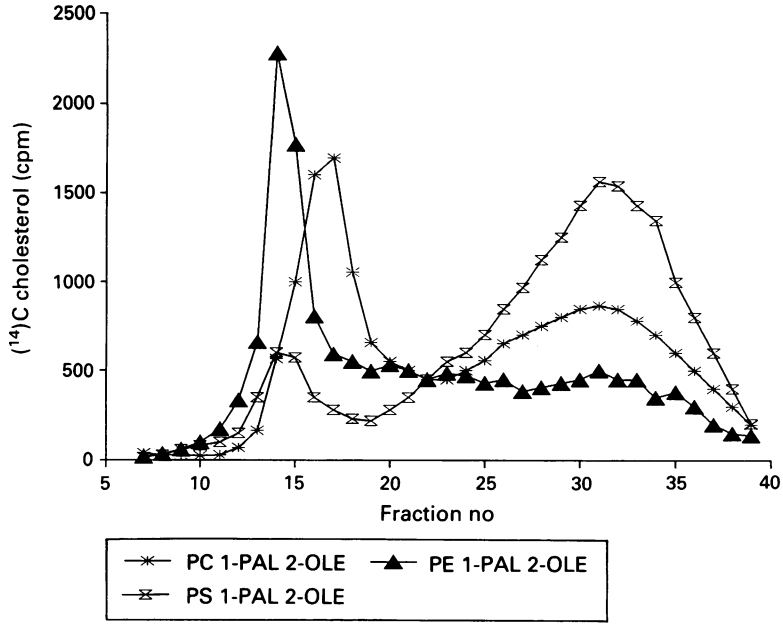


Figure 1: Elution profiles of ^{14}C cholesterol of model biles prepared with different phospholipid head groups. (PC 1-pal 2-ol= phosphatidylcholine sn-1 palmitoyl sn2 oleoyl, PE 1-pal 2-ol= phosphatidylethanolamine sn-1 palmitoyl sn-2 oleoyl, PS – 1-pal 2-ol= phosphatidylserine sn-1 palmitoyl sn-2 oleoyl).

EXPERIMENTAL DESIGN

(1) Addition of egg lecithin to native and model biles. Aliquots of egg lecithin were placed into a glass test tube and flushed to dryness under a stream of nitrogen at 37°C, and subsequently lyophylised for 18 hours (biles 3–7). One millilitre of native or model bile samples were added to the dried lecithin and shaken at 37°C for one hour. After one hour samples of the bile were taken for nucleation studies and chromatographic separation. Solubilisation of egg lecithin by this process was tested with radiolabelled phospholipid. At one hour solubilisation was ≥90% and did not increase after further incubation for up to 24 hours. There was also no difference in solubilisation between 1 μmol/ml and 20 μmol/ml. Mixing experiments adding egg lecithin were performed with seven human biles (six gall bladder, one hepatic, Table I) and three model biles.

(2) The effect of synthetic phospholipids and different molecular species of phosphatidylcholine. Model biles were prepared with the same lipid concentration as above but with different phospholipids. Each experimental data point in Figures 1 and 2 is the average of three experiments in three identical model biles.

Addition of synthetic phosphatidylcholine to native biles was performed as described above.

STATISTICAL ANALYSIS

The Student's paired *t* test was used to compare the nucleation time experiments, the per cent of cholesterol in the vesicular phase and the cholesterol/phospholipid ratios of native biles after the addition of phospholipids.

Results

Figure 5 shows the effect of the addition of egg lecithin on the nucleation time of seven human biles. Increasing amounts of egg lecithin progressively prolonged the nucleation time. After the addition of 1 μmol/ml of lecithin, the nucleation time was lengthened in only one bile and after 2·5 μmol/ml in only two biles. After 5 μmol/ml the nucleation time increased considerably in five of seven biles ($p<0·05$). Similar results were seen in the three model biles. Figure 6 shows the percentage of biliary cholesterol in the vesicular phase as a function of the amount of lecithin added. The addition of lecithin progressively shifted cholesterol to the non vesicular phase. This phenomenon was more marked in the three model biles with few vesicles remaining after the addition of 10 μmol/ml of lecithin. This shift was accompanied by a progressive decrease in the cholesterol/phospholipid ratio of the remaining vesicles. Below a ratio of 0·5 vesicles become unstable in the chromatographic milieu and cannot be detected (unpublished). The cholesterol/phospholipid ratio of the non-vesicular phase remained stable upon the addition of egg lecithin. Three gall bladder biles and three model biles were analysed and the data for the human biles are shown in Figure 3.

In order to investigate the effect of different phospholipids on cholesterol solubility and the

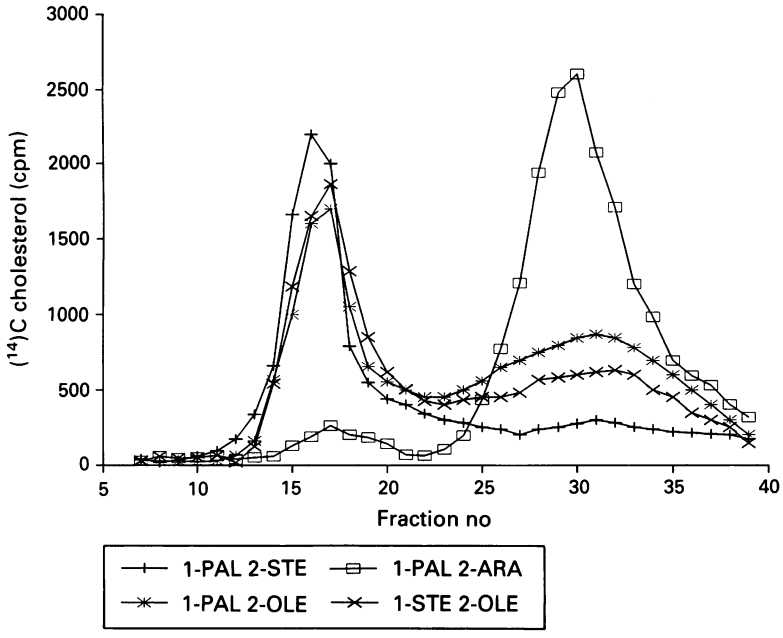


Figure 2: Elution profiles of ^{14}C cholesterol of model biles prepared with different molecular species of lecithin. (1-pal 2-ste=sn-1 palmitoyl sn-2 stearoyl, 1-pal 2-ole= sn-1 palmitoyl sn-2 oleoyl, 1-pal 2-arc=sn-1 palmitoyl sn-2 arachidonoyl, 1-ste 2-ole=sn-1 stearoyl sn-2 oleoyl).

nucleation time we chose three synthetic phospholipids with an identical fatty acid composition but different head groups (see methods). The elution profile of ^{14}C cholesterol of the three lipid mixtures prepared with equal amounts of the different phospholipids is shown in Figure 1. With phosphatidylethanolamine most of the cholesterol (85%) was in the vesicular fraction, while with phosphatidylserine most of the cholesterol was in the non-vesicular fractions (80%). With phosphatidylcholine cholesterol was almost evenly distributed (55% in vesicles) between both carriers. The nucleation time of these model biles was also markedly affected by the variation in phospholipid species (Table II). Figure 2 shows the elution pattern of four different model biles prepared with different molecular species of phosphatidylcholine (see methods). With a saturated fatty acid (palmitic acid) in the sn-1 position and the polyunsaturated arachidonic acid in sn-2 position

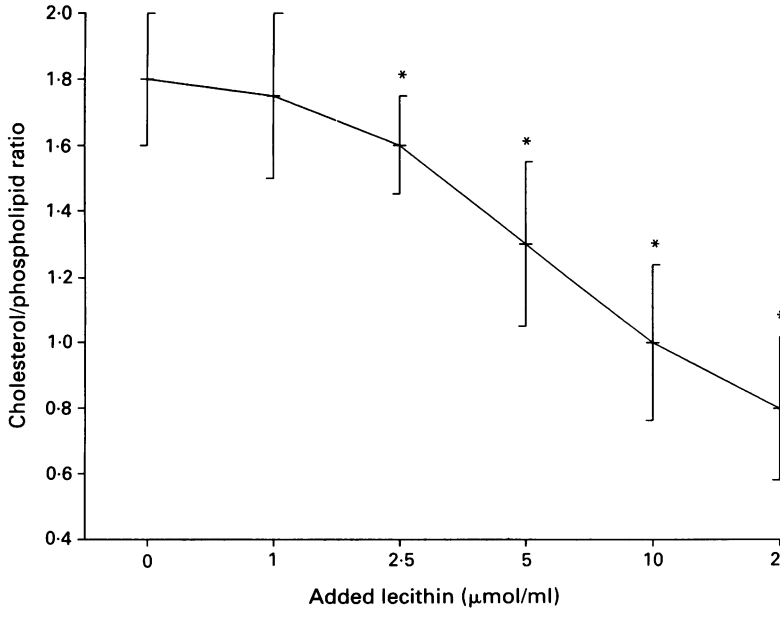
TABLE II *Nucleation time of model biles prepared with different phospholipids*

	Nucleation time (days)
Phospholipid species:	
Phosphatidylserine sn-1 palmitoyl sn-2 oleoyl	20
Phosphatidylethanolamine sn-1 palmitoyl sn-2 oleoyl	6
Phosphatidylcholine sn-1 palmitoyl sn-2 oleoyl	5
Phosphatidylcholine molecular species:	
Phosphatidylcholine sn-1 palmitoyl sn-2 stearoyl	20
Phosphatidylcholine sn-1 palmitoyl sn-2 oleoyl	6
Phosphatidylcholine sn-1 palmitoyl sn-2 arachidonoyl	5
Phosphatidylcholine sn-1 stearoyl sn-2 oleoyl	4

most of the cholesterol was in the non-vesicular phase. When (under the same conditions) the sn-2 position was occupied by a saturated fatty acid (stearic acid) almost all biliary cholesterol was found in the vesicular fraction. When the fatty acid in the sn-2 position was monounsaturated (oleic acid) cholesterol was present in both carriers though more in the vesicular fraction. Results were similar when the saturated fatty acid in sn-1 position (palmitic acid) was replaced by another saturated fatty acid (stearic acid). The nucleation time of the model biles prepared with the different phospholipids are shown in Table II. Model biles prepared with phosphatidylserine had a longer nucleation time (20 days) than those prepared with phosphatidylethanolamine or phosphatidylcholine having the same fatty acid composition. Model biles prepared with phosphatidylcholine, containing different fatty acids, had the following nucleation times: when both fatty acids (sn-1 and sn-2) were saturated (palmitic and stearic) the nucleation time was prolonged (20 days) in comparison with other phosphatidylcholines containing one unsaturated fatty acid in the sn-2 position.

Different phospholipids and different molecular forms of phosphatidylcholine were also added to three human biles. The results for one hepatic bile are shown in Figures 4 and 7. Addition of phosphatidylethanolamine caused an almost complete shift of cholesterol to the vesicular phase. The addition of phosphatidylcholines containing various fatty acids (see methods), produced the following results: Palmitic stearic phosphatidylcholine shifted cholesterol almost completely to the vesicular phase whereas after the addition of other phosphatidylcholines biliary cholesterol remained in the non-vesicular phase. The results in the three native biles (two gall bladder and one hepatic) were similar.

*Figure 3: Effect of egg lecithin addition on the cholesterol/phospholipid ratio of the vesicular phase in three human (Table I, biles 3–5) (*p<0·05) biles.*



Discussion

The results of this study show that the amount of phospholipids and their molecular species in bile have a decisive influence on cholesterol carriers, on the distribution of cholesterol among the carriers and on the nucleation time of bile. Jungst *et al* were the first to show that the addition of small amounts of phosphatidylcholine to rapidly nucleating biles (from patients with cholesterol gall stones) prolonged and normalised their nucleation time.[6] The addition of similar amounts of bile salts with an equal

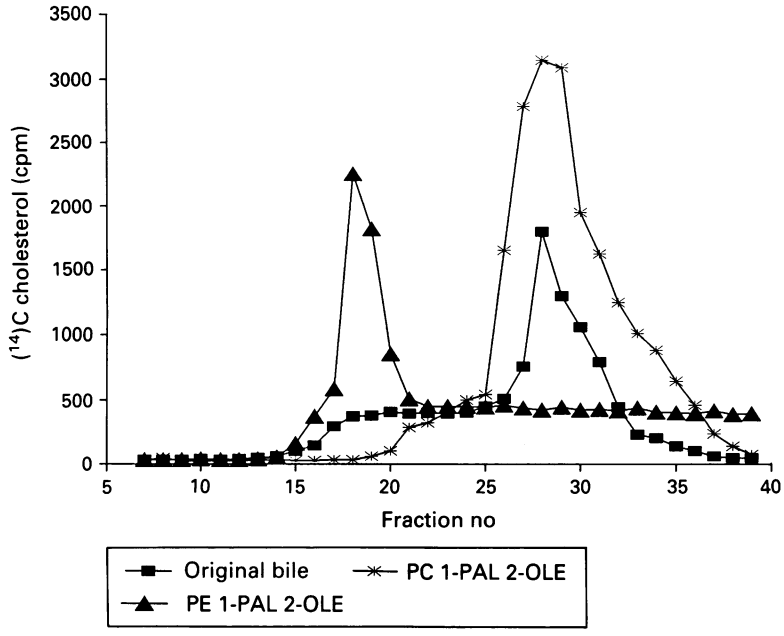
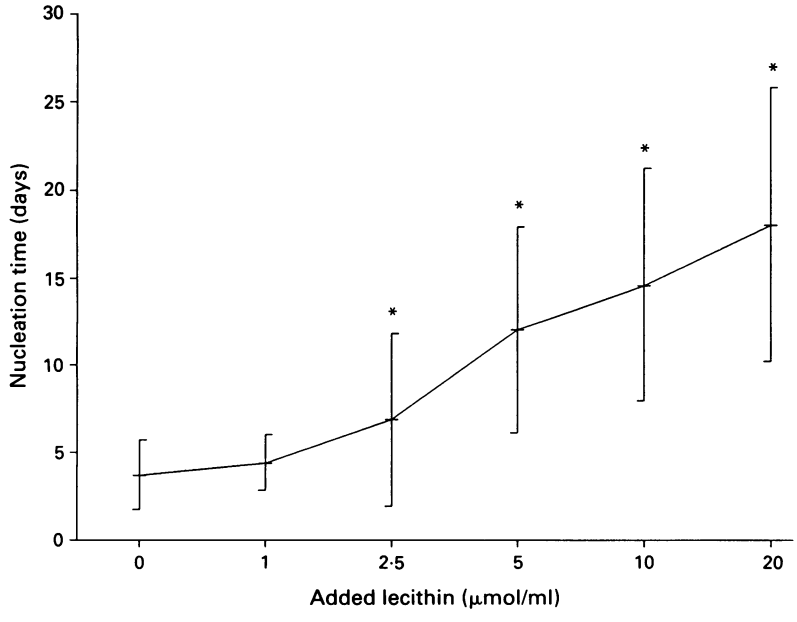


Figure 4: Elution profile of ^{14}C cholesterol of an hepatic bile before and after the addition of 5 μmol of different phospholipids per ml of bile. Original bile composition: Chol – 3·2 mM, BS – 18·5 mM, PL – 6·2 mM.

effect on the cholesterol saturation index had no effect on the nucleation time.[8]

Our data show similarities and differences when compared with those of Jungst *et al.*[6] They added 1·25 and 2·5 μmol of lecithin/ml bile while we added 5 μmol/ml bile, yet the prolongation in the nucleation time of bile was much more marked in their experiments. The nucleation time was prolonged from 1·5 days to 12·2 and 13·2 days after the addition 2·5 μmol of synthetic lecithins. In our experiments the nucleation time was considerably less prolonged (Fig 5). The difference can be explained by the molecular species of lecithins used. We used egg lecithin which is a mixture of lecithins containing mostly unsaturated fatty acids in the sn-2 position. They used synthetic dipalmitoyl and distearoyl lecithins. As seen in Table II these disaturated synthetic lecithins prolonged the nucleation time to 20 days as compared with four to six days with the monosaturated lecithins.

Figure 5: Effect of the addition of egg lecithin on the nucleation time of seven human biles ($p<0·05$).*



Our data show that the addition of lecithin shifts biliary cholesterol from the vesicular to the non-vesicular phase. When a similar shift is caused by the addition of bile salts or free fatty acids to bile the result is a shortening of the nucleation time.[16] Phospholipids, however, prolong the nucleation time. The explanation is seen in Figure 3. The addition of egg lecithin reduced the cholesterol/phospholipid ratio of the remaining vesicles. An increase in the cholesterol/phospholipids ratio has a very potent nucleating effect and shortens the nucleation time of model and human biles,[17,18] whereas a decrease in the cholesterol/phospholipids ratio has an opposite effect. Thus the addition of phosphatidylcholine prolonged the nucleation time by two mechanisms: (A) shift of biliary cholesterol to the more stable carrier (non-vesicular) and (B) a decrease in the cholesterol/phospholipids ratio of the metastable carrier (vesicles).

Not only the total amount of phospholipids but also the molecular species has profound influences on cholesterol carriers and nucleation in bile. In order to study and characterise these differences lipid mixtures were prepared using only one species of phospholipids Some of these mixtures were definitely unphysiologic. Further studies will be needed to study the effects of various mixtures of phospholipids, as found in native bile. Using different head groups and keeping the acyl chains constant produced the following results. Phosphatidylethanolamine shifted most of the cholesterol to the vesicular phase while phosphatidylserine moved it to the non-vesicular fraction. With phosphatidylcholine, which is the major biliary phospholipid, cholesterol could be found in both carriers depending, however, on the acyl chains. With a saturated fatty acid in the sn-1 position (palmitic acid) and the polyunsaturated arachidonic acid in the sn-2 position most of the cholesterol was in the non-vesicular phase. When under the same conditions a saturated fatty acid (stearic) replaced the arachidonic acid in the sn-2 position almost all biliary cholesterol was in the vesicular fraction. This disaturated phosphatidylcholine is, however, non-physiologic in human biles. Vesicles composed of phosphatidylethanolamine or palmitoylstearoyl phosphatidylcholine may be more rigid and this may contribute to their stability. When the fatty acid in the sn-2 position was monounsaturated oleic acid, cholesterol was present in both carriers though more in the vesicular fraction. Similar results were found when the palmitic acid in the sn-1 position was replaced by another saturated fatty acid (stearic acid). As in the vast majority of human biles the sn-1 fatty acid is palmitic acid and physiologic variations occur mainly in the sn-2 position, some of our data may reflect events in vivo.[19,20] These findings in model biles were reproduced in human biles. The nucleation time of model biles was also markedly affected by varying the added phospholipid species. It was markedly prolonged only when both acyl groups of phosphatidylcholine were saturated (palmitic stearic). Other phosphatidylcholine species such as palmitate oleate, palmitate arachidonate and stearate oleate produced shorter nucleation time. When the acyl group composition was constant and the head

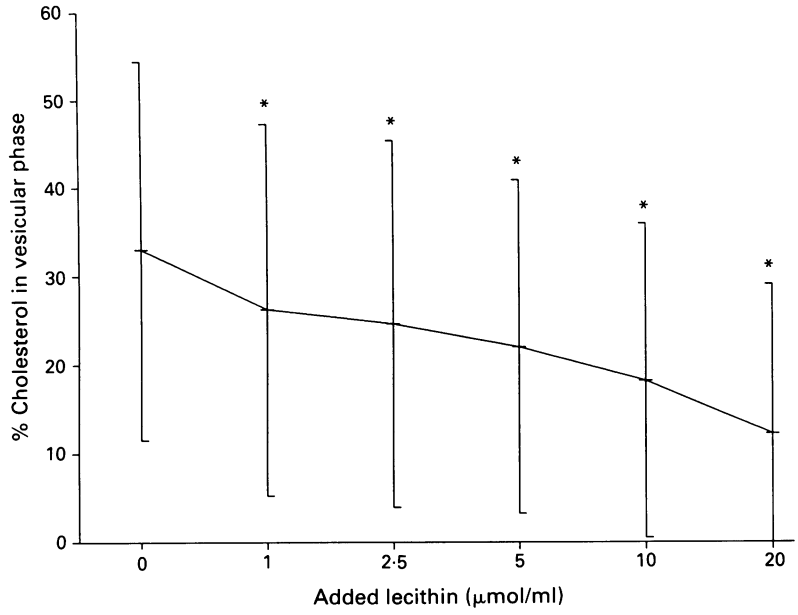


*Figure 6: Effect of the addition of egg lecithin on cholesterol distribution between the vesicular and non-vesicular phases in human biles (Table I, biles 1–5) (*p<0·05).*

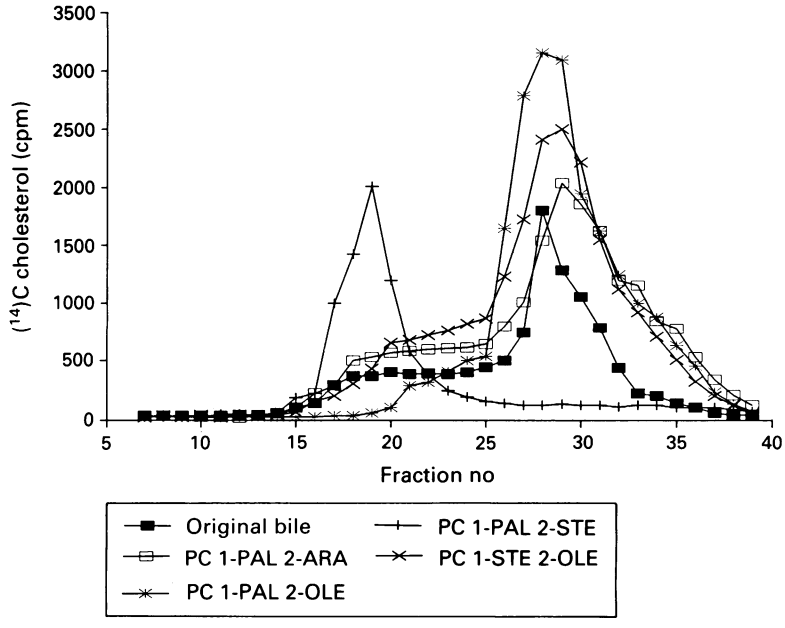
group was varied the nucleation time was short with phosphatidylcholine and phosphatidylethanolamine and longer with phosphatidylserine. It is noteworthy that with phosphatidylserine most of the cholesterol was in the more stable (non-vesicular carrier). One possible interpretation is that the nucleation time can be prolonged under two different circumstances: (1) When most of the cholesterol is in the more stable carrier(s) (the non vesicular); (2) When the metastable carrier is stabilised by a more rigid membrane (disaturated phosphatidylcholine) as well as a lower cholesterol/phospholipids ratio (which always occurs when most of the biliary lipid is found in the vesicles).

We added 5 μmol/ml of a single phospholipid to human biles which contained an average of 21 μmol/ml of various native phospholipids. This moderate change in composition led to major changes in nucleation and cholesterol distribution among the carriers. This raises the possibility of future therapeutic manipulation. Most of the data are new and thus cannot be compared with findings by other investigations. Recent evidence suggests that phospholipids are of major importance in biliary pathophysiology. Biliary cholesterol is probably secreted as phospholipid vesicles[21,22] and phospholipids are probably the main cholesterol carriers in bile.[5,23] A reduction of biliary phospholipids by dietary legumes was associated with an increased cholesterol saturation of bile.[24]

The composition of phospholipids in human hepatic and gall bladder bile has been studied by a number of investigators during recent years.[25–33] More than 90% of phospholipids in human bile are species of phosphatidylcholine but the presence of small amounts of other phospholipids has been observed.[25–27] Seventy to 80% of biliary phosphatidylcholine is sn-1 palmitoyl with either sn-2 oleoyl (18:1) or linoleoyl (18:2). The third most abundant phosphatidylcholine species is sn-1 palmitoyl sn-2 arachidonyl (20:4). Subjects with cholesterol gall stones were reported to have alterations in the molecular species of phosphatidylcholine in bile, although the results of various studies are conflicting.[28–33] In a recent study Cohen *et al* working with model solutions showed a non-even distribution of different molecular species of phosphatidylcholine between the vesicular and micellar carriers.[34] Saturated fatty acids were more abundant in the vesicular phase and unsaturated ones were more abundant in the micellar phase.

In conclusion we have shown that the addition of egg lecithin to biles prolongs the nucleation time, shifts cholesterol from the vesicular phase to the non-vesicular phase and reduces the cholesterol/phospholipids ratio of the remaining vesicles. Different phospholipids and various molecular species of phosphatidylcholine markedly affect cholesterol carriers and solubility in bile. The present findings may contribute to our understanding of cholesterol solubility in bile and may be of use in our attempts to manipulate it.

Figure 7: Elution profile of the same hepatic bile as in Figure 4 after the addition of 5 μmol of different species of phosphatidylcholine per ml of bile.



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