Comparative analysis of cholesterol transport in bile from patients with and without cholesterol gallstones

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Abstract Aggregation of cholesterol-phospholipid vesicles in supersaturated biles precedes cholesterol crystal formation. In this study we examined the relationship between the percentage of cholesterol carried by vesicles and/or their composition and the propensity to form cholesterol crystals (nucleation time). Bile (common bile duct, gallbladder and T-tube) was obtained from patients with and without gallstones. Gel filtration chromatography resolved three peaks, a void volume vesicle, a smaller vesicle (identified by electron microscopy and of distinct composition compared to the larger void volume vesicle), and the mixed micelle. The void volume vesicle was present in 11 of 28 abnormal gallbladder biles, but in none of the 10 normal gallbladder biles. Despite this difference, no correlation between the nucleation time of whole bile with either the percentage of cholesterol carried by or cholesterol/phospholipid ratio of the void volume vesicle was found. Nucleation time was, however, found to correlate with the composition of the small-vesicular transport form. No significant difference in the composition or percentage of the small-vesicular form or the combined vesicular forms was found between normal and abnormal gallbladder biles, although the latter nucleated significantly more rapidly. Our results confirm the importance of vesicles in the nucleation process but suggest that other factors, not yet identified, appear to be responsible for the more rapid nucleation seen in abnormal gallbladder biles. —**Pattinson, N. R., K. E. Willis, and C. M. Frampton.** Comparative analysis of cholesterol transport in bile from patients with and without cholesterol gallstones. *J. Lipid Res.* 1991. **32:** 205–214.

Supplementary key words cholesterol • phospholipid • bile acid • vesicles • nucleation

In bile cholesterol is solubilized by mixed micelles consisting of bile acids and phospholipids (1). More recently, the presence of a second cholesterol carrier, phospholipid vesicles, has been identified in human bile (2–11). In dilute hepatic biles these vesicles are quantitatively very important and, consistent with most transport systems, are very stable (5). However, studies examining cholesterol monohydrate crystal nucleation in both gallbladder and concentrated model biles have demonstrated that the sequence of events leading to nucleation involves vesicle aggregation (8, 9, 11–15). The changes leading to vesicular destabilization within the gallbladder are poorly understood. In this study we have used gel filtration chromatography to separate the respective transport components in native bile to determine: *1)* whether the propensity to form stones in gallbladder biles from gallstone patients, compared with non-gallstone formers is due to a difference in the percentage of cholesterol carried by the vesicular phase and/or the lipid (cholesterol/phospholipid) composition of the vesicles; *2)* the compositional factors that favor vesicle formation and an alter vesicular cholesterol/phospholipid ratio; and *3)* whether the in vitro assayed nucleation time is correlated with the distribution of cholesterol between its carriers and/or the composition of the vesicle.

MATERIALS AND METHODS

Bile samples were obtained from 42 consecutive patients undergoing cholecystectomy. During surgery, gallbladder bile samples were aspirated under sterile conditions by puncture of the gallbladder after ligation of the cystic duct. The gallbladder contents were aspirated completely so as to avoid stratification. Twenty-four bile duct samples were obtained by drainage from a catheter introduced into the common bile duct via the cystic duct, prior to operative cholangiography. Thirteen of these were paired with their corresponding gallbladder bile. In addition, biles were obtained from a T-tube placed in the common bile duct. Ten normal gallbladder biles were obtained from patients without biliary tract disease, undergoing abdominal surgery for gastrectomy (4), sigmoid colectomy (5), pyloroplasty, and vagotomy (1). Approval of the protocol used in this investigation was obtained from the ethical committee of our institution. All patients gave

Abbreviation: CSI, cholesterol saturation index.

written informed consent prior to entering the study. The gallbladder was punctured with the 21-gauge needle and the entire biliary contents were removed. Two "normal" gallbladder biles (without stones) were found to contain cholesterol monohydrate crystals under polarizing light microscopy. These two samples were therefore treated as having abnormal lipid chemistry, while two patients with pigment stones, whose samples were devoid of cholesterol crystals, were included with the normals (these samples are indicated in the respective tables). The sterility of all biles (prior to processing) was assured by aerobic and anaerobic culture using standard methods.

Bile was ultracentrifuged for 2–4 h at 100,000 *g* at 22°C to remove endogenous mucus, cellular debris, and cholesterol crystals. The crystal-free supernatant was recovered and labelled for 30 min at 37°C with a micellar solution (20 μl/ml bile) containing [^{3}H]phosphatidylcholine and [^{14}C]cholesterol [The 10-ml stock solution contained 700 μmol taurocholic acid, 25 μmol egg lecithin, 75 μCi dipalmitoyl phosphatidylcholine (choline-methyl-^{3}H) (New England Nuclear, Boston, MA), 20 μCi [4-^{14}C]cholesterol (Amersham Lab., England)]. The radiolabeled lipid equilibrated with the endogenous lipid within 30 min, as indicated by our observation of identical specific activities in each lipid transport form after gel filtration chromatography. Prior to gel filtration and setting up of nucleation experiments, the bile was filtered through a sterile 0.22-μm microdisc filter. The total handling time was 3–6 h.

Column chromatography

The cholesterol transport forms in bile (common bile duct and gallbladder) were separated using gel filtration chromatography (Sepharose CL-4B-200). Two different sized columns were used, 70 × 1.7 cm and 12 × 0.8 cm. Each was equilibrated with phosphate-buffered saline containing 0.04% sodium azide and 6 mM taurocholate. The void volume of each column was determined with Dextran blue, molecular weight 2 × 10^6. Flow rates were 17 ml/h and 9 ml/h for large and small columns, respectively. Aliquots of bile (500 μl) were applied to the large column and fractions of 1.7 ml were collected. Aliquots of bile (100 μl) were applied to the small column and fractions of 300 μl were collected. An aliquot (100 μl) of each fraction was counted in a liquid scintillation counter (Packard Tricarb model 3330) after the addition of 5 ml of scintillation fluid (660 ml Triton X-100, 1330 ml toluene, 11 g PPO and 0.4 g dimethyl POPOP). Transport forms in T-tube bile were also separated using gel filtration chromatography, but in this instance the resin, Ultrogel AcA34 (LKB, Sweden) was used. Conditions were as for the small column above.

Chemical analysis

Bile lipid analysis was performed after extraction by chloroform–methanol 2:1 (v/v) (phospholipid analysis) or ethanolic KOH and hexane (cholesterol analysis). Cholesterol was determined enzymatically (Monotest-Boehringer Mannheim) and phospholipids by total phosphorus analysis (16). Bile acids were determined enzymatically using 3α-hydroxysteroid dehydrogenase (17). The cholesterol saturation index (CSI) was calculated using the cholesterol saturation tables derived by Carey (1).

The percentage of biliary cholesterol and phospholipid carried by vesicular and micellar fractions was calculated by measuring the area under the respective radioactivity peaks eluted from the chromatographic column. Using these percentages and having measured the endogenous phospholipid and cholesterol content of the original bile sample, the vesicular and micellar cholesterol/phospholipid ratio was determined. When the small-vesicular zone was present its proportion was calculated by subtracting the micellar zone and void volume vesicular zone (after manual plotting-assuming peak symmetry) from the total radioactivity curve.

Nucleation studies

After ultracentrifugation and filtration (0.22 μm microdisc) bile samples were incubated at 37°C in sterile 1.5-ml screw-top tubes and observed daily under a polarizing microscope for the first appearance of cholesterol monohydrate crystals. Incubation was continued for a total of 21 days.

Electron microscopy

Fractions from each transport component separated by gel filtration were fixed in 2.5% glutaraldehyde–PIPES buffer, pH 7.3 1:1 (v/v). Carbon-coated 200-mesh copper grids were floated on the fixed bile specimens for 20 min, stained in 2% uranyl acetate for 3 min, and air-dried. Observations of the samples were performed on a Phillips CM 12 electron microscope at an operating voltage of 100 kV. Measurements of vesicle size were made on paper enlargements.

Statistical analysis

Statistical analysis involved the use of Pearson's correlation coefficient and paired and unpaired Student *t*-tests, as appropriate, for the comparison of variables between different types of bile samples. The *t*-test comparisons among the three groups were corrected for the multiple comparisons by using the conservative Bonferroni adjustment to the α level (i.e., 0.05/3) to preserve a genuine $\alpha = 0.05$. Similarly, the probabilities associated with the correlation coefficients were corrected for the number of comparisons done. Survival analysis, using the Montel

Cox Statistic, was used to compare nucleation times. This technique is routinely used to compare the times to a predetermined event whether or not the event occurs within the study period. Results are given as the mean ± SEM.

RESULTS

Bile composition

The cholesterol, phospholipid, bile acid, total lipid concentration, and CSI values of abnormal and normal gallbladder and abnormal common bile duct bile are given in **Tables 1-3**. Normal gallbladder bile had a lower CSI (1.21 ± 0.10 vs. 1.39 ± 0.06), and a higher total lipid concentration (8.56 ± 0.86 vs. 6.83 ± 0.44 g/dl) compared to abnormal gallbladder biles, although neither difference was statistically significant. Total lipid concentration inversely correlated with CSI in abnormal gallbladder ($r = -0.47$, n = 31, $P<0.05$) and bile duct biles ($r = -0.59$, n = 22, $P<0.05$), although the relationship was not significant in normal gallbladder bile ($r = -0.31$, n = 10). Hepatic bile from cholesterol gallstone patients had a significantly higher mean CSI value than abnormal gallbladder bile ($P<0.05$; paired and unpaired data).

Separation of cholesterol transport forms/percentage distribution

Gel filtration profiles revealed the existence of three cholesterol-carrying transport forms. In addition to the vesicular peak (eluting at the void volume) and the micellar peak, there was present a third form eluting between the two, or, in the absence of a void volume peak, it appeared as a shoulder adjacent to the micellar peak. Electron microscopy identified it as a unilamellar vesicle (**Fig.**

TABLE 1. Abnormal gallbladder bile

Subject	CH	PL	BA	Conc.	CSI	CH %			CH/PL Ratio		
						Ves	S-Ves	Mic	Ves	S-Ves	Mic
	mM	*mM*	*mM*	*g/dl*							
MA[a]	4.1	8.0	50.1	3.24	1.66	5.4	8.8	85.8	1.86	1.06	0.46
HA	10.8	26.2	105.9	7.65	1.24	0.0	38.0	62.0		0.67	0.34
HU	7.6	23.9	61.3	5.16	1.17	2.4	22.5	75.1	1.72	1.36	0.25
CR	5.6	10.4	46.5	3.30	1.85	27.9	26.4	45.8	1.04	0.88	0.36
GR	15.7	41.4	157.7	11.56	1.08	0.0	26.5	73.5		0.65	0.33
SP	9.2	26.1	80.5	6.32	1.17	0.0	30.8	69.2		0.42	0.32
JO	7.4	22.4	69.7	5.44	1.12	0.0	44.5	55.5		0.42	0.28
VH	18.3	53.6	126.6	11.08	1.13	0.0	14.0	86.0		0.78	0.31
NI	5.0	11.0	47.7	3.38	1.57	0.0	30.4	69.6		0.77	0.39
AV	12.0	19.2	72.4	5.50	1.94	5.7	29.4	64.9	1.40	0.65	0.59
PR	10.4	15.8	76.6	5.39	1.97	0.0	22.5	77.5		0.87	0.62
HD	16.8	30.8	136.0	9.71	1.50	0.0	14.8	85.2		0.68	0.53
WI[a]	12.7	57.2	130.0	11.31	0.77	0.0	7.0	93.0			
AS	5.9	19.4	99.4	6.61	0.90	0.0	10.6	89.4			
MC	9.8	29.4	124.5	8.77	0.99						
OS	9.5	27.4	103.6	7.58	1.10	0.0	22.5	77.5		0.89	0.29
DO	2.6	9.1	38.7	2.70	1.09	0.0	26.9	73.1		0.40	0.27
CK	5.9	20.1	54.7	4.47	1.09	6.0	21.3	72.7	1.83	0.45	0.23
BE	10.6	17.6	78.0	5.60	1.82	0.0	29.1	70.9		0.84	0.54
FI	10.0	25.1	121.1	8.28	1.15	0.0	49.5	50.5		0.40	0.40
HP	6.0	13.2	82.3	5.29	1.31	0.0	23.2	76.8		0.64	0.42
MI	7.2	10.1	53.3	3.68	2.23						
CH	13.2	32.6	88.9	7.40	1.34						
RO	11.2	27.7	99.7	7.63	1.29	0.8	31.7	67.5	1.19	0.57	0.39
FN[a]	12.4	23.4	80.9	6.26	1.67	17.1	5.9	77.0	1.53	0.81	0.45
BL[b]	21.2	46.4	108.1	9.72	1.50	3.4	35.6	61.0	0.50	0.54	0.42
RS[a]	13.1	31.3	155.6	10.57	1.14	0.0	4.8	95.2		0.61	0.41
HO[a]	15.3	36.0	69.0	6.77	1.64	1.0	9.1	89.9	0.35	0.56	0.42
CO[a]	10.7	26.8	115.6	8.17	1.17	4.5	10.8	84.7	0.86	0.57	0.38
ED[a]	11.4	18.7	81.2	5.88	1.85	39.4	0.0	60.6	1.72		0.43
CL[b]	11.3	24.3	97.7	7.11	1.40	3.9	42.1	54.0	1.00	0.60	0.38
Mean	10.5	25.3	90.7	6.83	1.39	4.2	22.8	73.0	1.25	0.68	0.39
± SE	0.8	2.2	5.7	0.44	0.06	1.7	2.4	2.5	0.14	0.04	0.02

Biliary lipid composition, percentage distribution of cholesterol between its transport forms and their respective compositions for abnormal gallbladder bile; CH, cholesterol; PL, phospholipid; BA, bile acid; Ves, vesicle; S-Ves, small vesicle; Mic, micelle; CSI, cholesterol saturation index.

[a]These bile samples were chromatographed on a small (12 × 0.8 cm) column of Sepharose CL-4B-200. All others were chromatographed on large (70 × 1.7 cm) columns, pre-equilibrated, and eluted with phosphate-buffered saline containing 6 mM taurocholate.

[b]Biles without stones but containing cholesterol monohydrate crystals.

TABLE 2. Common bile duct bile

Subject	CH	PL	BA	Conc.	CSI	CH %			CH/PL Ratio		
						Ves	S-Ves	Mic	Ves	S-Ves	Mic
	mM	*mM*	*mM*	*g/dl*							
MA	2.0	5.1	36.6	2.27	1.32	40.4	17.8	41.8	0.74	0.49	0.25
HA[a]	4.0	10.2	43.8	3.10	1.38	0.0	37.1	62.9		0.70	0.31
HU	2.2	4.9	12.9	1.10	2.13	42.0	0.0	58.0	0.99		0.32
CR	4.4	8.1	20.3	1.79	2.33	60.3	0.0	39.7	1.48		0.28
GR	7.3	15.7	55.0	4.19	1.59	26.0	0.0	74.0	0.71		0.41
SP	6.8	14.2	49.0	3.77	1.67	0.0	12.5	87.5		0.62	0.47
JO	2.7	7.6	47.4	3.02	1.20	0.0	0.0	100.0			0.36
VH	7.8	31.4	73.6	6.35	0.92	15.0	0.0	85.0	0.41		0.23
NI	3.0	5.4	20.1	1.52	2.39	57.2	0.0	42.8	0.86		0.37
AV	4.8	5.7	51.3	3.15	2.36	59.5	0.0	40.5	1.45		0.54
PR	3.7		28.5			42.5	0.0	57.5			
HD	7.0		62.0			0.0	5.8	94.2			
WI	3.4	7.5	15.0	1.45	2.27	43.4	0.0	56.6			
PE	2.7	8.0	35.0	2.57	1.20	14.3	5.1	80.6	0.71	0.45	0.28
BR	0.9	1.7	5.3	0.43	5.19	53.3	0.0	46.7	1.15		0.34
FE	2.6	12.4	38.2	2.94	0.84	0.0	0.0	100.0			0.21
LY	2.7	8.1	34.9	2.45	1.29						
HE	4.7	8.6	36.1	2.62	1.99	0.0	35.6	64.4		0.88	0.46
BR	4.1	9.6	47.6	3.24	1.48	0.0	26.7	73.3		0.85	0.36
FO[a]	5.1	10.4	29.4	2.45	1.95	22.4	34.8	42.8	1.29	0.72	0.31
PH	1.0	2.8	15.6	1.02	1.64						
LI[a]	3.6	9.4	37.5	2.71	1.41	0.0	43.3	56.7		0.72	0.30
BL	2.3	6.5	28.8	2.74	1.42	53.1	0.0	46.9	0.57		0.25
HT[a]	5.5	10.5	39.5	2.96	1.87	36.1	13.8	50.1	0.70	0.87	0.40
Mean	3.9	9.3	36.0	2.63	1.81	25.7	10.6	63.7	0.92	0.70	0.34
± SE	0.4	1.3	3.3	0.27	0.19	5.1	3.2	4.3	0.10	0.05	0.02

Biliary lipid composition, percentage distribution of cholesterol between its transport forms, and their respective compositions for common bile duct bile; CH, cholesterol; PL, phospholipid; BA, bile acid; Ves, vesicle; S-Ves, small vesicle; Mic, micelle; CSI, cholesterol saturation index.

[a]Biles were chromatographed on a small (12 × 0.8 cm) column of Sepharose CL-4B-200; all others were chromatographed on large (70 × 1.7 cm) columns, pre-equilibrated, and eluted with phosphate-buffered saline containing 6 mM taurocholate.

1) with a mean diameter of 39.2 ± 2.32 nm. This we termed the small-vesicular form. The mean diameter of the void volume vesicle, as determined by electron microscopy, was 69.9 ± 2.29 nm.

Fig. 2 shows four representative elution profiles of bile samples applied to the smaller gel filtration column. Five of the 28 abnormal and 2 of the 10 normal gallbladder biles exhibited profiles similar to that shown in Fig. 2A;

TABLE 3. Normal gallbladder bile

Subject	CH	PL	BA	Conc.	CSI	CH %			CH/PL Ratio		
						Ves	S-Ves	Mic	Ves	S-Ves	Mic
	mM	*mM*	*mM*	*g/dl*							
SM	7.8	19.9	71.9	5.37	1.30	0.0	30.7	69.3		0.45	0.37
LO	13.7	41.4	146.1	10.91	0.98	0.0	10.0	90.0		0.55	0.32
ES	11.6	18.1	78.7	5.72	1.91	0.0	22.5	77.5		0.82	0.60
MU	14.8	26.5	117.0	8.37	1.59	0.0	31.5	68.5		0.73	0.50
ME	9.3	26.6	99.3	7.30	1.09	0.0	21.0	79.0		0.52	0.32
WO	3.7	15.8	61.8	4.40	0.83	0.0	15.3	84.7		0.56	0.22
MC[a]	15.3	40.2	133.6	10.27	1.14	0.0	32.6	67.4		0.56	0.33
PA	17.2	53.8	145.8	11.99	1.00	0.0	27.9	72.1		0.49	0.28
DE	14.6	33.8	144.5	10.28	1.21	0.0	33.8	66.2		0.72	0.36
MO[a]	16.3	54.1	126.3	11.02	1.01	0.0	12.7	87.3		0.69	0.28
Mean	12.4	33.0	112.5	8.56	1.21	0.0	23.8	76.2		0.61	0.36
± SE	1.3	4.4	10.3	0.86	0.10	0.0	2.8	2.8		0.04	0.03

Biliary lipid composition, percentage distribution of cholesterol between its transport forms, and their respective compositions for normal gallbladder bile. Biles were chromatographed on a large (70 × 1.7 cm) column of Sepharose CL-4B-200, pre-equilibrated, and eluted with phosphate-buffered saline containing 6 mM taurocholate; CH, cholesterol; PL, phospholipid; BA, bile acid; Ves, vesicle; S-Ves, small vesicle; Mic, micelle; CSI, cholesterol saturation index.

[a]Pigment stones.

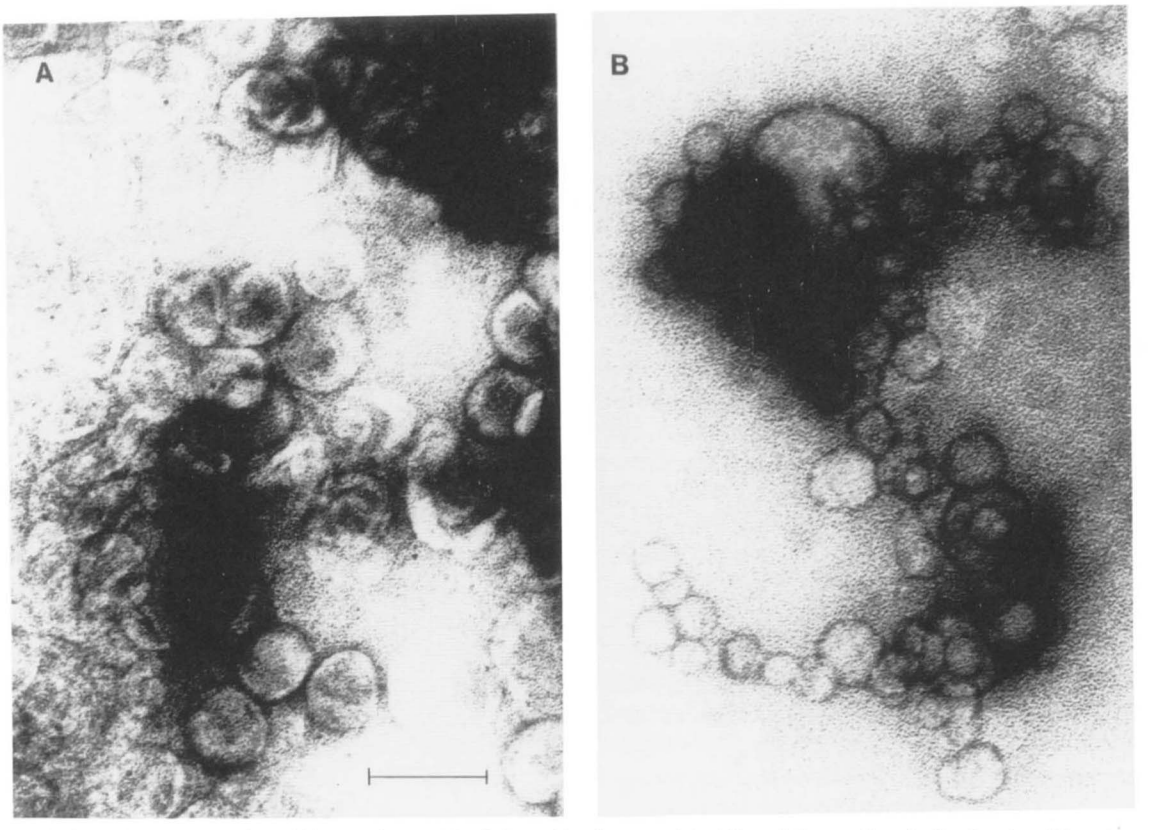

Fig. 1. Negatively stained preparation (2% uranyl acetate) of the void volume vesicle (A) and the small-vesicular fraction (B) separated by gel filtration. The bar in the lower right corner represents 100 nm.

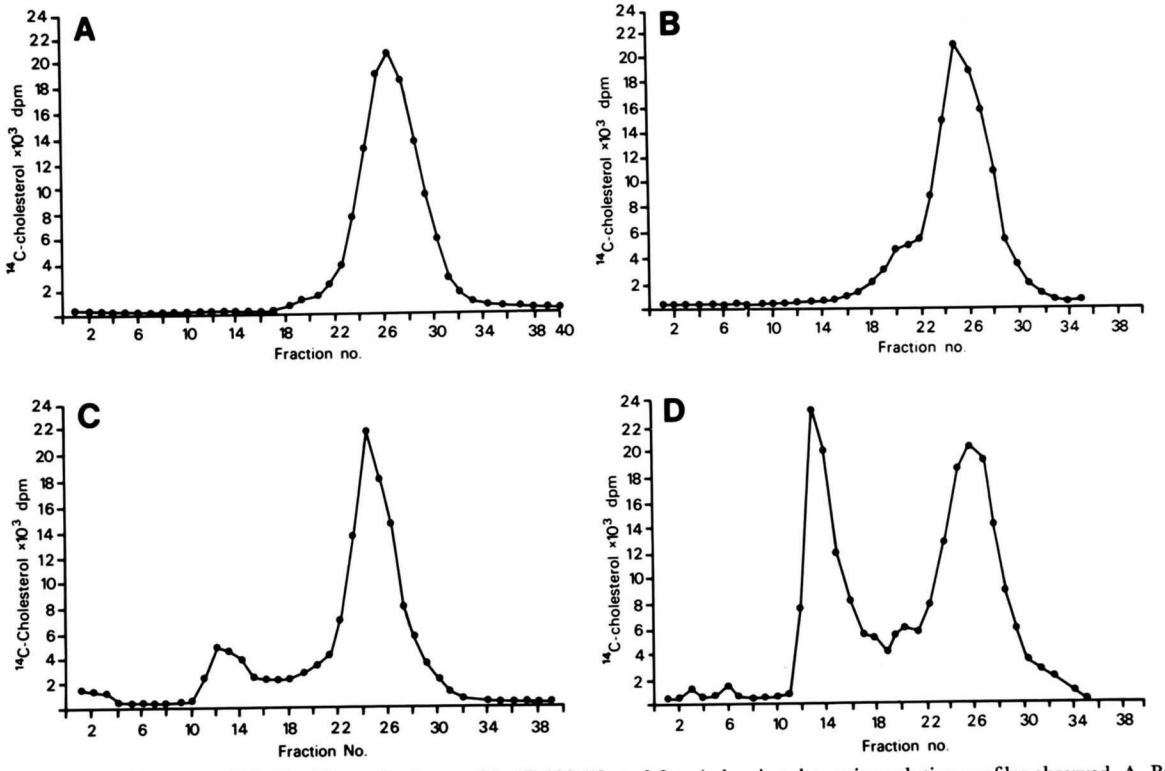

Fig. 2. Gel filtration of human gallbladder bile on Sepharose CL-4B-200 (12 × 0.8 cm) showing the various elution profiles observed. A. Bile containing only a micellar peak. B. Bile containing predominantly micelles with an small-vesicular shoulder. C. Bile containing micelles with an small-vesicular shoulder and a minor void volume vesicular peak. D. Bile containing a distinct void volume vesicular peak.

12 of 28 abnormal and 8 of 10 normal gallbladder biles, similar to Fig. 2B; 10 of 28 abnormal similar to Fig. 2C; and one of 28 abnormal gallbladder biles similar to Fig. 2D. Of the bile duct biles 9 of 21 eluted with a profile similar to (D) with the remaining biles having patterns similar to each of (A) (4 of 21), (B) (4 of 21), or (C) (4 of 21).

Elution of gallbladder bile samples from the larger gel filtration column more completely resolved the small-vesicular cholesterol peak, although the relative proportions remained unchanged. **Fig. 3A** and **3B** shows the elution profile of two gallbladder biles and Fig. 3C that of one bile duct sample.

The vesicular peak eluting at the void volume represented a significant proportion of total biliary cholesterol (25.7 ± 5.1%) in bile duct samples (Table 2). However, it made up only 4.2 ± 1.7% of the total cholesterol in abnormal biles, being present in only 12 of the 28 samples (Table 1) and in normal gallbladder biles no void volume vesicular peak was found. The percentage of small-vesicular cholesterol in abnormal gallbladder bile was 22.8 ± 2.4% compared with 10.6 ± 3.2% for common bile duct bile ($P<0.01$). No difference in the percentage of small-vesicular cholesterol was found in gallbladder biles between patients with and without stones.

The percentage of void volume vesicular cholesterol was positively correlated with CSI for both bile duct (r = 0.56, n = 20, $P<0.05$) and abnormal gallbladder biles (r = 0.49, n = 28, $P<0.05$). The percentage of small-vesicular cholesterol, however, did not correlate with CSI ($r = -0.08$) in abnormal gallbladder biles, nor did the sum of the percentages of vesicular cholesterol, i.e., void volume and small vesicles ($r = 0.26$, n = 28). The relationship between percentage small-vesicular cholesterol and CSI failed to reach significance ($r = 0.39$, n = 10) in normal gallbladder biles.

Composition of the transport forms: cholesterol/phospholipid ratio

The void volume vesicular form of abnormal gallbladder bile had a cholesterol/phospholipid ratio (1.25 ± 0.14) that was distinct from both the small-vesicular (0.68 ± 0.04, $P<0.01$, n = 11) and the micellar fraction (0.39 ± 0.02, $P<0.01$, n = 25).

The mean cholesterol/phospholipid ratio of the bile duct void volume vesicular form, although higher (0.92 ± 0.10), was not statistically different from that of the small-vesicle (0.70 ± 0.05), although both were significantly different from the micelle (0.34 ± 0.02, $P<0.01$, n = 9). While the mean void volume vesicular cholesterol/phospholipid ratio was higher in abnormal gallbladder than in common bile duct biles, the difference did not reach statistical significance. No difference in the small-vesicular composition was found between gallbladder and common bile duct bile for either the paired or non-paired data. Nor was there a difference in the small vesicular composition between abnormal and normal biles.

In paired bile duct biles the void volume vesicular cholesterol/phospholipid ratio was correlated to CSI

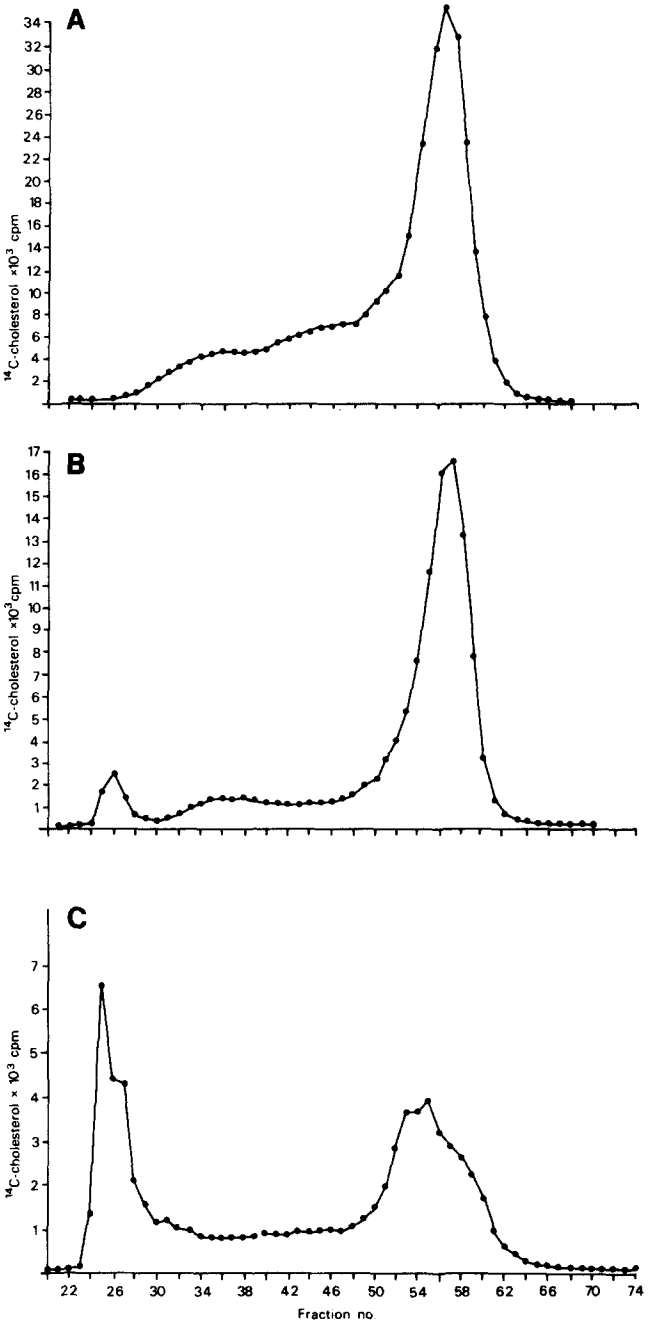

Fig. 3. Gel filtration of human bile on Sepharose CL-4B-200 (70 × 2.4 cm) preequilibrated and eluted with phosphate-buffered saline containing 6 mM taurocholate. A. Gallbladder bile devoid of a void volume vesicular peak but a distinct small-vesicular shoulder leading up to the micellar peak. B. Gallbladder bile with a void volume vesicular peak, a distinct small-vesicular shoulder, and the micelle. C. Bile duct bile containing a well-defined, void volume vesicular peak, a broad small-vesicular zone, and the micelle.

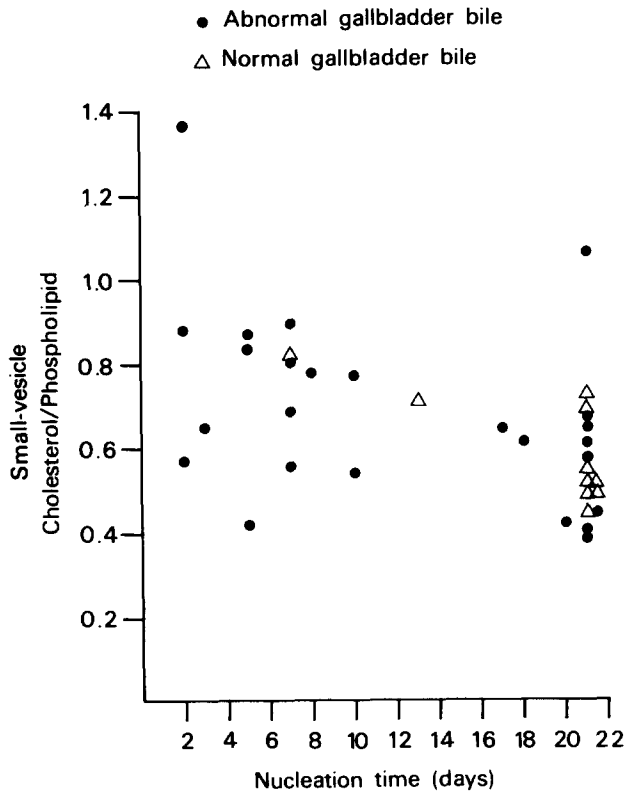

Fig. 4. Correlation between the cholesterol/phospholipid ratio of the small-vesicle and nucleation time of 25 abnormal gallbladder biles ($r = -0.42$, $P<0.05$); and 10 normal gallbladder biles ($r = -0.72$, $P<0.01$).

($r = 0.83$, n = 7, $P<0.05$) and to the cholesterol/phospholipid ratio of whole bile ($r = 0.82$, n = 7, $P<0.05$). When all bile duct biles were included, the cholesterol/phospholipid ratio of the vesicle was positively correlated to the cholesterol/phospholipid ratio of whole bile ($r = 0.78$, n = 12, $P<0.01$) but not significantly correlated to CSI, although a similar trend was observed ($r = 0.53$, n = 12). Neither of these variables was found to be significantly related to the cholesterol/phospholipid ratio of the void volume vesicles in abnormal gallbladder bile although there was a significant negative correlation with total lipid concentration ($r = -0.65$, n = 12, $P<0.05$).

Normal gallbladder bile revealed only small-vesicular cholesterol. The cholesterol/phospholipid ratio of the small vesicle was found to be proportional to CSI ($r = 0.66$, n = 10, $P<0.05$), the cholesterol/phospholipid ratio of whole bile ($r = 0.70$, n = 10, $P<0.05$), and the molar percentage of cholesterol ($r = 0.61$, n = 10, $P<0.05$). No correlation with respect to any of the above was found in abnormal gallbladder biles.

Nucleation time

Gallbladder bile from cholesterol gallstone patients nucleated significantly more rapidly than biles from both the nongallstone patients ($P<0.01$) and the abnormal common bile duct ($P<0.01$). The mean nucleation times were 12.84 ± 1.35 (n = 31), 18.80 ± 1.53 (n = 10), and 16.54 ± 1.51 (n = 24) for each bile sample, respectively.

No correlation was found between the void volume vesicular cholesterol/phospholipid ratio of either abnormal bile duct or gallbladder biles and nucleation time. However, the cholesterol/phospholipid ratio of the small-vesicular form was found to be inversely correlated to nucleation time in both abnormal gallbladder ($r = -0.42$, n = 25, $P<0.05$) and normal gallbladder ($r = -0.72$, n = 10, $P<0.01$) biles (although only two of the ten normal gallbladder biles nucleated over the 21 days of observation) (**Fig. 4**).

Effect of concentration per se

Dilute T-tube bile was concentrated on average 4.4-fold in an ultrafiltration cell (M_r cutoff 10,000) (**Table 4**).

TABLE 4. Effect of concentration per se on cholesterol transport in T-tube bile

Bile	n	Conc.	CSI	CH %		CH/PL		
				Vesicle	Micelle	Vesicle	Micelle	Whole Bile
		g/dl						
Dilute	20							
Mean		1.83	2.20	42.0	58.0	1.06	0.35	0.46
± SE		0.20	0.30	8.7	8.7	0.26	0.07	0.04
Conc.	20							
Mean		7.87	1.63	34.7	65.2	1.32	0.34	0.47
± SE		0.77	0.18	9.9	9.9	0.20	0.03	0.05
P[a]		<0.01	<0.01	<0.05	<0.05	NS	NS	NS

The effect of concentration T-tube bile, to levels similar to those found in the gallbladder, on the percentage distribution of cholesterol between its transport forms and their respective compositions. Biles were chromatographed on a column of Ultrogel AcA 34, pre-equilibrated, and eluted with phosphate-buffered saline containing 6 mM taurocholate. T-tube bile was concentrated using an Amicon concentrating cell with a molecular weight exclusion of 10,000; CH, cholesterol; PL, phospholipid.

[a]Paired *t*-test; dilute versus concentrated.

Using the gel filtration resin Ultrogel-AcA 34, two cholesterol peaks were eluted in both dilute and concentrated T-tube biles: a distinct vesicular peak, eluting at the void volume, and a micellar peak. The proportion of vesicular cholesterol decreased from 42.0 ± 8.7% in dilute T-tube bile to 34.7 ± 9.6% in concentrated T-tube bile (Table 4). The percentage of vesicular cholesterol was found to be inversely proportional to total lipid concentration for both dilute T-tube bile ($r = -0.80$, n = 17, $P<0.01$) and concentrated T-tube bile ($r = -0.84$, n = 16, $P<0.01$).

Although in concentrated T-tube bile the mean cholesterol/phospholipid ratio of the vesicle was higher than in the dilute biles, no correlation between vesicle composition and total lipid concentration was found.

DISCUSSION

Phospholipid vesicles, particularly in dilute supersaturated model and native biles, comprise a transport form for biliary cholesterol in addition to the mixed micelle (2–11). However, recent evidence would indicate that the rapid cholesterol monohydrate crystal formation seen in concentrated model biles or abnormal gallbladder biles occurs via vesicle aggregation (8, 9, 11–15). Thus, as the bile is concentrated in the abnormal gallbladder, the stability of the vesicle is somehow changed. This would suggest that perhaps a change in either the percentage and/or composition of the vesicle may foreshadow the dramatic changes in nucleation time.

Obtaining this information requires the separation of the respective biliary components and their assay. To do this cleanly and without change in either their relative amounts or composition, certain critical considerations need to be made. To date two procedures have been successfully used to separate the various biliary transport forms, i.e., ultracentrifugation (5, 6, 9) and gel filtration (3, 4, 12–15, 18). Provided one has sufficient original bile, the former is possibly least likely to result in artifacts. Its major limitation is the time needed to separate the components (48 h) particularly when dealing with extremely metastable abnormal gallbladder biles. Gel filtration, on the other hand, can be performed much more rapidly, although its major shortcoming is the potential disruption and change in composition of transport components using inappropriate bile acid species and concentrations in the eluting buffer (4, 9, 14, 15). Unless one knows the exact bile salt composition of any given bile, and hence the critical micelle concentration, the choice of bile acid species and concentration used in the buffer is at best a calculated guess. Ideally, the gel filtration column should be pre-equilibrated and eluted with the monomeric components of the bile to be separated (3). However, this is impractical when dealing with relatively small volumes of gallbladder and common duct bile.

In view of the above considerations, we chose for the purpose of this study phosphate-buffered saline containing 6 mM taurocholic acid, pH 7.4, as our eluting buffer. We did this for the following reasons. *1*) The critical micelle concentration of taurocholate is between 5 and 7.5 mM, depending on ionic strength and other physicochemical variables. *2*) We found the profile and composition of lipid components in T-tube bile after gel filtration in phosphate-buffered saline containing 6 mM taurocholate to be identical to that obtained by equilibrating the column with the monomeric components of the same bile obtained by ultrafiltration. In addition, rechromatography of the respective components resulted in homogeneous peaks of identical composition. *3*) By adding increasing amounts of exogenous bile acid to T-tube bile and then performing gel filtration, we were able to show, as previously reported (15), a progressive decrease in the percentage of cholesterol carried by the vesicle and a concomitant increase in the vesicles cholesterol/phospholipid ratio (**Table 5**). If, in fact, our choice of buffer concentration was inappropriate, such a change would have been masked. Again, rechromatography of the vesicle resolved a single peak of identical composition.

A second consideration is the choice of gel filtration resin. Previous studies have used Sephacryl S-300 (4, 12–15, 18). In this study we used Sepharose CL-4B-200 which has an exclusion limit approximately 7 times larger than Sephacryl-300. Thus, we were able to resolve two vesicular fractions, with distinct cholesterol/phospholipid ratios in both hepatic and gallbladder biles of cholesterol gallstone patients. Using Sephacryl-300 it is unlikely that the two peaks would have resolved sufficiently to be distinguishable. Likewise, using AcA 34, we separated only one vesicular fraction in our T-tube bile samples, i.e., the small-vesicular peak was also excluded and appeared in the void volume. Thus, in comparing our results to those of other workers it should be understood that the vesicular peak they refer to corresponds to the sum of the void volume vesicular and small-vesicular peaks resolved in

TABLE 5. Effect of exogenous bile acid on biliary cholesterol transport

	CH %		CH/PL	
	Vesicle	Micelle	Vesicle	Micelle
Control	52	48	1.03	0.29
+ 50 mM TCH	14	86	1.96	0.39
+ 100 mM TCH	0	100	0.00	0.44

To validate our gel filtration conditions, we examined the effect of adding exogenous taurocholate to T-tube bile on percentage cholesterol distribution between its carriers and their respective compositions. Cholesterol transport components were separated on a column of Ultrogel AcA 34, pre-equilibrated, and eluted with phosphate-buffered saline containing 6 mM taurocholate; TCH, taurocholate; CH, cholesterol; PL, phospholipid.

this study. The finding of two vesicular fractions in bile is not new. In a recent publication Ulloa, Garrido, and Nervi (6) using density gradient ultracentrifugation isolated two homogenous vesicular carriers from human T-tube bile. Although one cannot directly compare the two studies, the void volume vesicle and small-vesicle isolated in this study have a size distribution and composition similar to the two respective fractions separated by Ulloa et al. (6) at a mean density of 1.028 and 1.053 g/ml.

While 12 of 28 abnormal gallbladder biles resolved a void volume vesicular peak, none were found in patients without gallstones. Similarly, Schriever and Jungst (12) found vesicles in only 3 of 14 normal gallbladder biles compared with 18 of 19 biles from gallstone patients. This difference might suggest that the void volume vesicles represent an unstable aggregated multilamellar vesicle. However, no correlation was found between its percentage and cholesterol nucleation time. Further, larger percentage of cholesterol was carried in this form in common duct bile, yet these biles nucleated more slowly than gallbladder biles. Thus, as the bile is concentrated in the gallbladder, there is a shift from larger void volume vesicles to smaller ones. Although the total vesicular percentage was slightly higher in the gallstone patients than non-stone patients, no significant difference was found. Nor did we find a correlation between the percentage of cholesterol carried by vesicles and nucleation time.

Previous work on model biles found a correlation between a high cholesterol/phospholipid ratio of biliary vesicles and a rapid nucleation time (10, 11). In addition, the degree of cholesterol saturation of the vesicles was found to be directly related to the total lipid concentration (10, 11). We therefore anticipated an increase in the cholesterol/phospholipid ratio of vesicles between hepatic and gallbladder biles, and perhaps between vesicles in gallbladder biles from stone compared with non-stone patients. However, although the mean cholesterol/phospholipid ratio of the gallbladder void volume vesicle, when present, was higher than that of the bile duct, the difference was not statistically significant. In the only other comparative studies published to date, Peled et al. (14) isolated vesicles with a cholesterol/phospholipid ratio of 1.29 $\pm$ 0.74 (SD) from gallbladder and 1.47 $\pm$ 0.31 from bile duct, while Lee et al. (9) found levels of 0.52 $\pm$ 0.15 and 0.91 $\pm$ 0.07 in gallbladder and common bile duct samples, respectively. The low cholesterol saturation of the gallbladder vesicle in the latter study may relate to the time taken to separate vesicles from rapidly nucleating abnormal gallbladder bile samples (ultracentrifugation for 48 h). During this period cholesterol from the supersaturated vesicles could have already nucleated. However, in both these studies the cholesterol saturation of the vesicle was higher in the common bile duct samples compared with their paired gallbladder sample. Thus, in contrast to the results found in model bile, no correlation between the total lipid concentration and cholesterol/phospholipid ratio of the vesicle was found, although there was a significant difference in nucleation time between hepatic and gallbladder biles. Similarly, no correlation between the cholesterol/phospholipid ratio of the vesicle and total lipid concentration was found in our T-tube studies.

As above, no difference in the cholesterol/phospholipid ratio of the small-vesicular peak between hepatic and gallbladder bile from abnormal, nor between normal and abnormal, gallbladder biles was found. However, the composition of the small-vesicle, for abnormal and normal gallbladder biles, was found to be inversely correlated to nucleation time despite having a lower cholesterol/phospholipid ratio than the void volume vesicle. Considerably higher ratios were needed in normal gallbladder bile, however, for nucleation to occur.

In summary, we have shown that the lipid composition of bile can influence both the proportion of cholesterol carried in vesicles and their composition. Although we found a difference between abnormal and normal gallbladder biles with regard the void volume vesicle, its presence was not related to in vitro-assayed nucleation time. Nucleation time was correlated with the degree of small-vesicular cholesterol saturation, although considerably higher cholesterol/phospholipid ratios were needed to precipitate nucleation in normal biles. No difference in the percentage or composition of the small vesicle was found between normal and abnormal biles nor was there a difference in the combined vesicular fractions between the two populations. It would appear that other biliary factors influence vesicular stability.

Factors capable of both inhibiting and promoting nucleation have recently been identified in native bile (19–26). Among the factors that have been shown to inhibit nucleation are crude biliary proteins (sensitive to pronase) (19) and, more specifically, apolipoproteins A-I and A-II (20). Those known to promote nucleation are mucin (22–24), soluble glycoprotein (25, 26), and calcium (10). Their respective effects appear to be mediated by direct interaction with the vesicle itself. Thus, while a necessary prerequisite for cholesterol nucleation is the presence of cholesterol-rich vesicles, their stability may ultimately be determined by the balance between the presence or absence of nucleation promotors or inhibitors.

The authors wish to thank the New Zealand Medical Research Council for financial support, Mr. P. Bagshaw and Associate Professor J. Baxter for the provision of bile samples, and Mr. T. Day for performing the electron microscopy studies.

Manuscript received 17 November 1989, in revised form 7 June 1990, and in re-revised form 10 October 1990.

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