# Intrahepatic biliary cholesterol and phospholipid transport in humans: effect of obesity and cholesterol cholelithiasis[1]

**H. A. Ahmed, R. P. Jazrawi, P. M. Goggin, J. Dormandy, and T. C. Northfield[2]**

Division of Biochemical Medicine, St. George's Hospital Medical School, Cranmer Terrace, London SW17 ORE, United Kingdom

**Abstract** The mode of transport of biliary lipids within the hepatocyte and the role of the bile canalicular membrane (BCM) in biliary lipid secretion are not well understood. We hypothesized that biliary cholesterol and phospholipid are co-transported across the hepatocyte in vesicular form from the endoplasmic reticulum to the bile across the BCM. We obtained wedge liver biopsies and fasting gallbladder bile from 15 cholesterol gallstone patients and 10 control subjects. BCM, basolateral membrane (BLM), and many microsomal vesicular fractions were isolated by centrifugation. One of the vesicular fractions (V3) was enriched in both the microsomal and the BCM marker enzymes and had a high phosphatidylcholine proportion in its phospholipid with a fatty acid pattern similar to biliary phosphatidylcholine. Moreover, its cholesterol content was increased in the obese cholesterol gallstone subjects, who had an increase in cholesterol synthesis, as indicated by the increased activity of the HMG-CoA reductase. The cholesterol content correlated with HMG-CoA reductase activity. A direct correlation was found between cholesterol/phospholipid ratio in V3, BCM, and in bile but not in the BLM. These data are in agreement with the assumption that this vesicular fraction is involved in the transport of cholesterol and phospholipid from the endoplasmic reticulum to the site of secretion in the BCM, and thence to bile, and that this transport is enhanced in obese gallstone patients.—**Ahmed, H. A., R. P. Jazrawi, P. M. Goggin, J. Dormandy, and T. C. Northfield.** Intrahepatic biliary cholesterol and phospholipid transport in humans: effects of obesity and cholesterol cholelithiasis. *J. Lipid Res.* 1995. **36:** 2562-2573.

**Supplementary key words** cholesterol • phospholipid • vesicles • bile • canalicular membrane • cholelithiasis

The mechanism of hepatic biliary lipid transport and secretion into bile and the derangement in pathological states have been extensively studied in recent years. Gregory et al. (1) reported that biliary lipids are derived from a microsomal subpool regulated by the flux of bile acids. They also reported that cholesterol and phospholipid are co-transported from the microsomes to bile. The secretion of cholesterol and phospholipid parallel each other and are uncoupled from bile acid secretion, (2–4). Moreover, the rate of biliary cholesterol synthesis and secretion is increased in obese patients with or without cholesterol gallstone disease, (5, 6). Yousef, Bloxam, and Phillips (7) reported that the average rate of turnover of phospholipid in the bile canalicular membrane (BCM) is much greater than that in the remaining plasma membrane and other cell membrane fractions. They noted that phospholipid in BCM exists in two or more pools, turning over at different rates. In 1983, Coleman et al. (8) reported an increased number of vesicles in the region of the bile canaliculus during active bile acid secretion, which could be interpreted as an accumulation of biliary lipid supply. They also reported that bile acids are secreted into the bile canaliculus separate from and prior to cholesterol and phospholipid. Thus the only lipids to which bile acids in the canaliculus are exposed, and from which biliary lipids could be obtained, are those of the BCM. Lowe, Barnwell, and Coleman (9) suggested that biliary lipids are obtained from microdomains of biliary type lipids in the BCM, which are vesiculated and solubilized by bile acids. They also suggested that biliary type lipid is brought continuously to the membrane via vesicular traffic, which could be inhibited by colchicine. The transcellular vesicular cholesterol transport from the endoplasmic reticulum to the plasma membrane has been demonstrated by Kaplan and Simoni (10) in Chinese hamster ovary cells. They reported that these vesicles had a high cholesterol/protein ratio characteristic of plasma membranes, but a cholesterol/phospholipid ratio resem-

Abbreviations: BCM, bile canalicular membrane; BLM, basolateral membrane; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase; IBW%, ideal body weight per cent.

[1]Parts of this work were published in abstract form: *Clin. Sci.* **72 (Suppl. 16)** 32p (1987) and **74:** 1p (1988).

[2]To whom correspondence should be addressed.

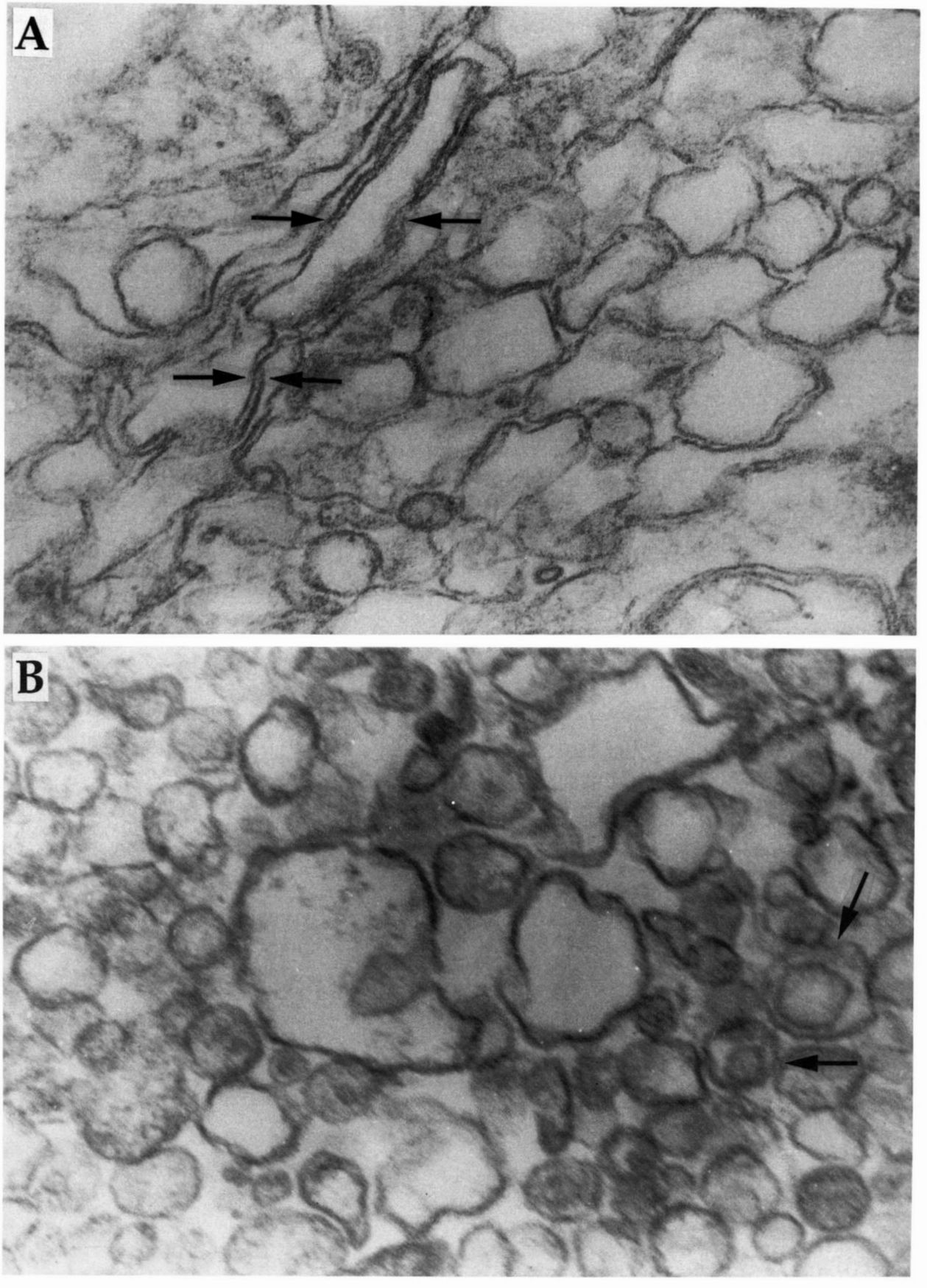

**Fig. 1.** A: Electron micrograph (100,000×) of the bile-canalicular membrane-rich fraction. Many Y-shaped figures are seen due to the tight junction arrangement (arrow). B: Electron micrograph (100,000×) of the microsomal vesicular fraction (V3) showing variable sized mono- and multilamellar vesicles, and the preparation is morphologically very different from BCM preparation. Marker enzymes are shown in Tables 1 and 2.

ratio resembling that in the endoplasmic reticulum. Non-vesicular, carrier protein-mediated transporters for phospholipid have also been suggested. Wirtz and Zilversmit (11) observed that rat liver cytosol stimulates phospholipid exchange between membranes, and subsequently demonstrated that hepatocytes are enriched in a transfer protein specific for phosphatidylcholine (12). They also reported that purified phosphatidylcholine transfer protein catalyzes both intermembrane exchange and net transfer of phosphatidylcholine, but not of other phospholipid classes (13). The protein displays high affinities for *sn*-1 palmitoyl phosphatidylcholine species, the major species in bile. Cohen, Leonard, and Carey (14) demonstrated that the PC transfer activity of this protein is stimulated markedly in vitro by submicellar concentrations of bile salts. They concluded that this could provide an intracellular selection mechanism, under bile salt control, for the delivery of highly specific PCs to the canalicular plasma membrane for secretion into bile.

Concerning the original source of biliary cholesterol, Stone et al. (15) demonstrated that infusion of cholesterol-rich lipoproteins into rats does not actually alter biliary cholesterol secretion. The majority of cholesterol delivered into the liver in these lipoproteins is esterified and stored, or secreted in VLDL. Schwartz et al. (16) reported that about one third of biliary cholesterol is newly synthesized. In 1989, Bilhartz, Spady, and Dietschy (17) reported that after chronic administration of mevinolin, an inhibitor of HMG-CoA reductase, the key regulatory enzyme in cholesterol synthesis, withdrawal of the drug results in a marked increase in the enzyme activity in the microsomal membrane cholesterol and a threefold increase in biliary cholesterol secretion. Robins et al. (18) reported that hepatic free cholesterol is in metabolic equilibrium in one single kinetic pool, and that all hepatic free cholesterol is potentially available for secretion into bile.

The asymmetric distribution of BCM phospholipid is another interesting phenomenon that must have its implications for biliary lipid secretion. The luminal leaflet is rich in PC while the cytoplasmic leaflet is rich in phosphatidylethanolamine and phosphatidylserine (19). The presence of a flippase on the BCM has been demonstrated by Berr, Meier, and Stieger (19) in the rat, using the water-soluble dibutyroyl-PC. They assumed that it was also competent in translocating long chain PCs, by analogy to the previous studies in the microsomes. They concluded that the flippase would be predominantly occupied in the vectorial in-to-out translocation of biliary PCs. The selective depletion of PCs from the luminal leaflet of BCM by bile salts would provide the driving force for the overall vectorial phospholipid secretion, while the flip-flop movement would follow passively by facilitated diffusion. The discovery of the importance of mdr2 P-glycoprotein in biliary phospholipid secretion by Smit et al. (20) was an important milestone in understanding canalicular biliary lipid transport. The bile of the mdr2 (-/-) mice did not contain detectable phospholipid and the cholesterol was markedly reduced. The homozygous animals suffered from liver disease. The authors hypothesized that the mdr2 P-glycoprotein may work as a phospholipid flippase, facilitating the transport of phosphatidylcholine from the cytoplasmic to the luminal leaflet of BCM. They also speculated that the protein might facilitate the entry of phospholipid into BCM by promoting fusion of the lipid transport vesicles with the membrane, or by acting as a

Table 1. Enzymic charcaterization of isolated fractioans

| | Homogenate; | Relative Enrichment[b] | | |
|---|---|---|---|---|
| | Sp Act[a]; | BCM | BLM | Vesicles V3 |
| Ikaline phosphatase; | 4.7 | 138.2 | 5.1 | 123.1 |
| 5'-Nucleotidase | 8.1 | 161.3 | 7.3 | 147.2 |
| Na-K ATPase | 8.8 | 8.3 | 42.3 | 10.8 |
| NADPH cyt. C reductase | 15.1 | 1.8 | 2.3 | 158.2 |
| Succinate cyt. C reductase | 11.4 | 1.3 | 12.3 | 5.8 |
| Acid phosphatase | 7.8 | 8.3 | 8.8 | 10.7 |

Values given as mean; n = 6.
[a]Specific activity: nmol/mg protein per min.
[b]Relative enrichment: ratio of specific activity in subfraction to specific activity in homogenate.

TABLE 2. Protein recovery and marker enzyme distribution per cent in isolated fractions

| | Homogenate | BCM | BLM | Vesicles V3 | Others | Recovery |
|---|---|---|---|---|---|---|
| | | | *mg protein/g liver tissue;* | | | % |
| Protein | 229.2 | 0.325 | 1.437 | 0.225 | 198.5 | 87.5 |
| Alkaline phosphatase | 100 | 19.6 | 3.2 | 12.1 | 47.4 | 82.3 |
| 5′-Nucleotidase | 100 | 22.8 | 4.6 | 14.4 | 43.9 | 85.7 |
| Na-K ATPase; | 100 | 1.2 | 26.5 | 1.1 | 53.1 | 81.9 |
| NADPH cyt. C reductase | 100 | 0.2 | 1.5 | 15.5 | 58.8 | 76.0 |
| Succinate cyt. C reductase | 100 | 0.2 | 7.7 | 0.6 | 71.6 | 80.1 |
| Acid phosphatase | 100 | 1.2 | 5.5 | 1.1 | 68.1 | 75.9 |

Values given as mean; n=6

direct acceptor for phosphatidylcholine bound to phosphatidylcholine transport protein. Recently, the flippase function of the mouse mdr2-P glycoprotein has been demonstrated using secretory vesicles from mutant yeast (21) and a fluorescent phosphatidylcholine analogue. The activity is time- and temperature-dependent and specific for phosphatidylcholine.

Little is known, however, about cholesterol and phospholipid transport from the endoplasmic reticulum to bile across BCM, or about the lipid composition of BCM and other hepatocyte membranes in man. It is also not known whether the transport and structures are affected in cholesterol gallstone disease and obesity, which are known to be associated with increased cholesterol synthesis and secretion. The aim of this study was to test the hypothesis that cholesterol and phospholipid are co-transported across the hepatocyte in vesicular form from the endoplasmic reticulum to the site of biliary secretion in BCM. If so, cholesterol transport should be increased in obesity and in cholesterol cholelithiasis, in which both synthesis and secretion are increased.

## MATERIAL AND METHODS

### Subjects

We studied 15 cholesterol gallstone patients with radiolucent stones and functioning gallbladders, scheduled for elective cholecystectomy (age: 56 ± 3.6, F/M: 8/7, IBW%: 116 ± 5.0) and 10 control subjects scheduled for laparotomy for other reasons (age 58 ± 2.9, F/M: 2/8, IBW%: 94 ± 2.9). The patients gave written consent

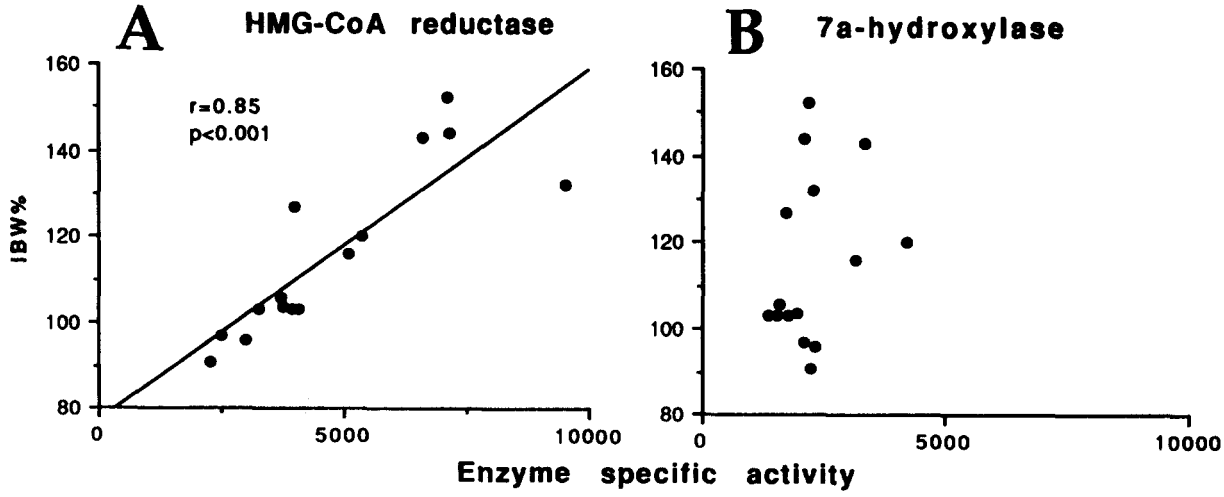


**Fig. 2.** The relationship between IBW% and the activity of the microsomal enzymes HMG-CoA reductase (A) and cholesterol 7α-hydroxylase (B) in gallstone patients. The increased activity of HMG-CoA reductase in gallstone patients relates directly to IBW% whereas the decreased activity of cholesterol 7α-hydroxylase is independent of IBW%.

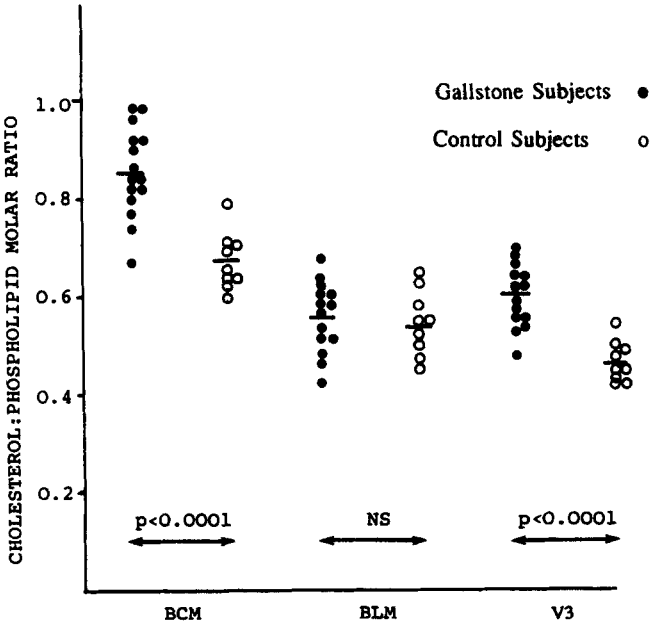


**Fig. 3.** The cholesterol/phospholipid molar ratio is higher in the bile canalicular membrane and the microsomal vesicular fraction (V3) from cholesterol gallstone patients compared with controls. The ratio is not different in the basolateral membrane from both groups.

and the protocol was approved by the local hospital ethical committee.

### Clinical procedures

One day before operation, a sample of gallbladder bile was obtained by nasoduodenal intubation and cholecystokinin infusion from control subjects (22). Surgical bile was obtained from all gallstone patients. Bile samples were stored at -20°C for subsequent analysis.

### Human liver fractionation (23, 24)

*Homogenization.* A wedge liver biopsy (0.8–1.2 g) was taken as the first procedure at laparotomy in chilled homogenization buffer (0.25 M sucrose in 5 mM Tris-HCl, pH8, 4°C) and washed. The liver tissue was finely chopped by scissors and homogenized in Potter-Elvehjem homogenizer, in 15 ml buffer, by 5–6 up and down strokes while the pestle was revolving at 500 rpm and with clearance of 0.3–0.5 mm, keeping the homogenate in ice during the procedure. The homogenate was checked under light microscopy and filtered through gauze.

*Differential centrifugation.* Using an SS-34 fixed-angle rotor in a Sorval RC-5B refrigerated superspeed centrifuge, the homogenate was pelleted at 1000 *g*, 5000 *g*, and 20,000 *g*, each for 10 min. The 20,000 *g* supernatant was spun at 100,000g for 40 min using 10 × 10 fixed-angle rotor in MSE Superspeed 65 ultracentrifuge. The pellets P1 through P4 were resuspended in the homogenization buffer to make 1 ml each. The microsomal pellet P4 was partly kept at -70°C, suspended in the buffer suitable for the microsomal enzyme assays (HMG-CoA reductase and cholesterol 7α-hydroxylase), which were performed within 48 h.

*Gradient centrifugation.* The densities of the suspended pellets P1 through 4 were increased by adding 55% w/w sucrose in a gradual manner while checking the refractive index using a refractometer until they were equivalent to the density of 35% w/w sucrose solution. The gradients were prepared of 55,45,35,25, and 15% w/w sucrose in 5 mM Tris-HCl, pH 8 (4°C), substituting the adjusted pellet for the 35% sucrose solution, i.e., applying the suspended pellet in the middle of the gradient. The gradients were spun at 100,000 *g* in 6 × 14 swing-out rotor in an MSE- Superspeed Ultracentrifuge for 18 h. The bands were visualized against light, taken by a syringe into polycarbonated tubes, washed, and finally suspended in homogenization buffer and kept at -20°C. The subfractions were characterized by marker enzymes and checked by transmission electron microscopy (25).

### Biochemical techniques

*Enzyme assays.* Cholesterol 7α-hydroxylase (EC 1.14) was assayed as described by Goodwin, Cooper, and Margolis (26). The microsomal pellet was washed and resuspended in a buffer containing 100 mM sucrose, 40 mM potassium phosphate, pH 7.2, 30 mM EDTA, 20 mM dithiothreitol, and 50 mM sodium fluoride. The microsomes were then preincubated at 37°C for 10 min with β-mercaptoethylamine (final concentration of 10 mM in the assay mixture), 2 μg of cholesterol, and 10 μl of [$^{14}$C]cholesterol, 0.5 μCi/ml in a suspension with 600 μg of lutensol. The assay mixture contained 400–600 μg microsomal protein in buffer, 1 mM $NADP^+$, 10 mM glucose-6-phosphate, 0.15 unit of glucose-6-phosphate dehydrogenase, 10 mM β-mercaptoethylamine in a final assay volume of 0.5 ml. The mixture was incubated at 37°C for 30 min with mild regular shaking and the reaction was stopped by the addition of 10 ml of chloroform–methanol 2:1 (v/v). The tubes were mixed vigorously for at least 5 min. After adding 2 ml of 0.9% sodium chloride, the tubes were mixed and centrifuged at 1000 *g* for 10 min. The chloroform (lower) phase was collected, evaporated under nitrogen, and the solid residue was carefully redissolved in 100 μl of chloroform and spotted on activated silica gel G plates, 20 × 20 cm (250 microns) (Anachem, Luton, Beds., UK) and developed using toluene–ethylacetate 2:3 (v/v), using 7α- and 7β-hydroxycholesterol and 7-ketocholesterol as standards. Sterols were stained with Rhodamine B and visualized under UV light. Zones corresponding to 7α-hydroxycholesterol were scraped into scintillation vials

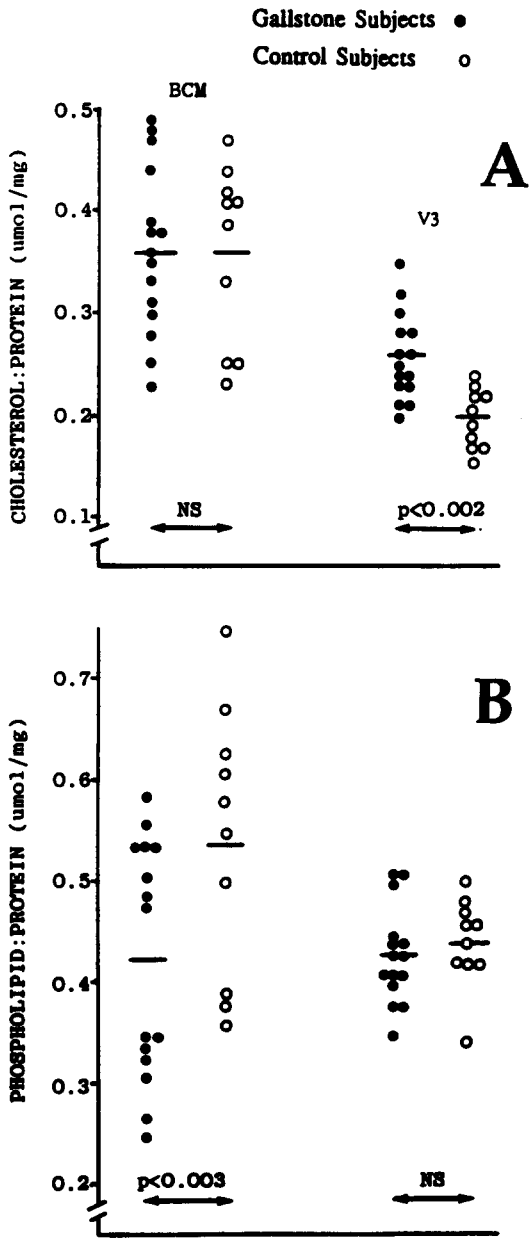


**Fig. 4.** The absolute cholesterol (A) and phospholipid (B) content (per mg protein) in membrane preparations. The BCM cholesterol content is not different, while the phospholipid content is decreased, in gallstone patients compared with control subjects. By contrast, the microsomal vesicular fraction V3 has a raised cholesterol content and a phospholipid content that is not different from that in controls.

and counted. Blank values were determined by assaying all enzyme samples in absence of $NADP^+$ and subtracted before calculating enzyme activity. Results are expressed as dpm incorporated into 7α-hydroxycholesterol per mg protein at 37°C. $R_f$ value for 7α-hydroxycholesterol was 0.24–0.26. HMG-CoA reductase (EC 3.6.1.3) was assayed as described before (27, 28). The microsomal pellet was washed and suspended in a buffer containing 0.25 M sucrose, 30 mM EDTA, 1 mM dithiothreitol, and 20 mM imidazole HCl, pH 7. The assay mixture contained 30 μmol of glucose-6-phosphate, 2 units of glucose-6-phosphate dehydrogenase, 3 μmol of $NADP^+$, 300 nmol of DL-HMG-CoA (3-$^{14}$C, sp act 400 cpm/nmol), and 400–600 μg of microsomal protein in a total volume of 0.5 ml. Samples were incubated at 37°C for 15 min and the reaction was stopped by the addition of 0.2 ml of concentrated HCl. The tubes were then left for another 15 min with occasional mild mixing to allow complete lactamization of mevalonic acid produced. The mixture in the tubes was then saturated with solid sodium sulfite to facilitate the extraction of mevalonolactone with ether, which was then evaporated under a stream of nitrogen. The solid residue was then dissolved in 100 μl acetone–water 9:1 (v/v), spotted on activated silica gel G plates, 20 × 20 cm, 250 microns, (Anachem, Luton, Beds., UK) and developed using toluene–acetone 1:1 (v/v) using standards of mevalonolactone, cholesterol, and HMG-CoA. Plates were sprayed with Rhodamine B and visualized under UV light. Mevalonolactone bands were scraped into scintillation vials and counted. Blank values were determined by assaying all enzyme samples in absence of $NADP^+$ and subtracted before calculating enzyme activity. Results are expressed as dpm incorporated into mevalonolactone per mg microsomal protein at 37°C. $R_f$ value for mevalonolactone was 0.45–0.48. Sodium-potassium-ATPase (EC 3.6.1.3), 5′-nucleotidase (EC 3.1.3.5), (29), acid (EC 3.1.3.2), and alkaline (EC 3.1.3.1) phosphatase (30), succinate cytochrome C reductase (EC 1.3.99.1), (31) and NADPH cytochrome C reductase (EC 1.6.2.4), (32) were assayed by radioisotope and colorimetric methods.

*Chemical measurements.* Protein (33), cholesterol (34), and phospholipid (35, 36) were measured by conventional colorimetric and enzymic methods.

*Fatty acid pattern.* Phospholipids were extracted from BCM, BLM, vesicular fraction, and bile (35), and separated by thin-layer chromatography, using silica gel G plates, 20 × 20 cm, 250 microns (Anachem, Luton, Beds., UK) and a mixture of chloroform–hexane–methanol–acetic acid–water 12:7:4:3:0.3 as a developing solvent. The spots were visualized with iodine and phosphatidylcholine (PC) spots were scraped and extracted with chloroform–methanol 2:1 (v/v) and dried under nitrogen. Using phospholipase $A_2$ and thin-layer chromatography (37), fatty acids from positions 1 and 2 were obtained separately, boiled in 14% boron trifluoride in methanol to produce fatty acid methyl esters (38), which were extracted in hexane and dried under nitrogen. They were analyzed by gas–liquid chromatography using a DB-23 Megabore 30 meter column, with 0.5 μm film thickness, (J&W Scientific, CA) in GC-6000 (Vega series 2, ICU 600, Carlo Erba Instruments) gas chromatography machine.

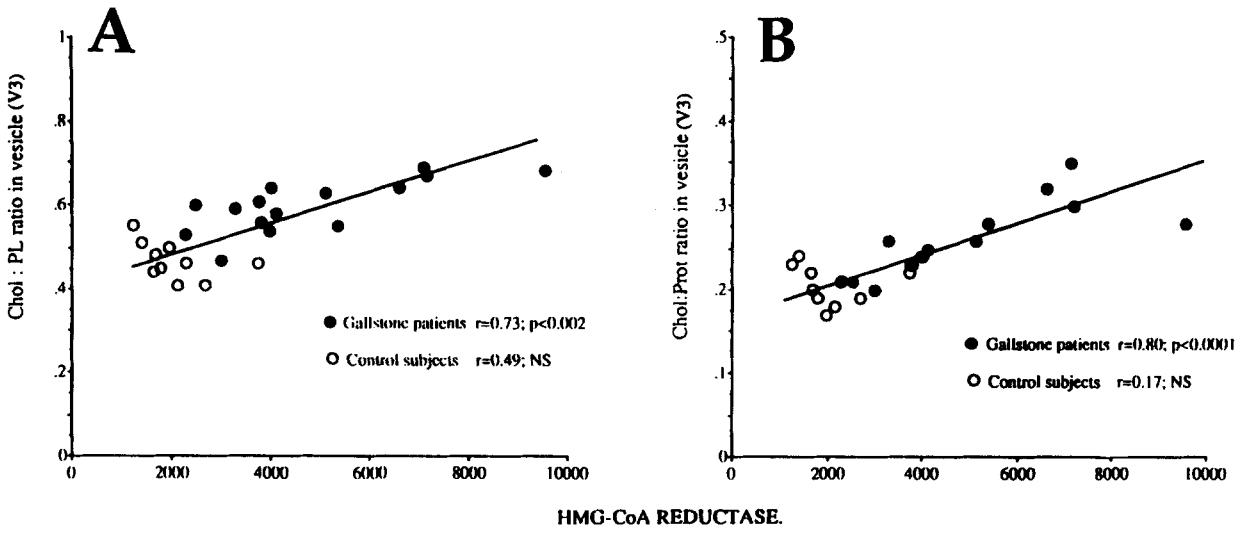


**Fig. 5.** Correlation between cholesterol/phospholipid ratio in BCM and both the vesicular fraction V3 (A) and gallbladder bile (B). A direct relationship can be seen in both cases.

### Statistical analysis

Results are presented as mean values and their standard errors (SEM). Differences between groups were tested for statistical significance with the Student's *t*-test and correlation coefficients were calculated by the Spearman method.

## RESULTS

A BCM-rich fraction (**Fig. 1, A**) was separated at a buoyant density of 1.16 g/ml and showed relative enrichment in alkaline phosphatase and 5′-nucleotidase, the marker enzymes for the bile canalicular membrane, 138 and 161 times, respectively, over homogenate (**Table 1** and **Table 2**). This fraction was minimally contaminated by the ouabain-sensitive, sodium potassium ATPase, the marker enzyme for the BLM, which was obtained in another band at a buoyant density of 1.18. This fraction showed enrichment in the above-mentioned ATPase 42 times over homogenate (Tables 1 and 2). Both membrane preparations were minimally contaminated by marker enzymes from mitochondria (succinate cytochrome C reductase), lysosomes (acid phosphatase), and smooth endoplasmic reticulum (NADPH cytochrome C reductase), (Tables 1 and 2). One of the

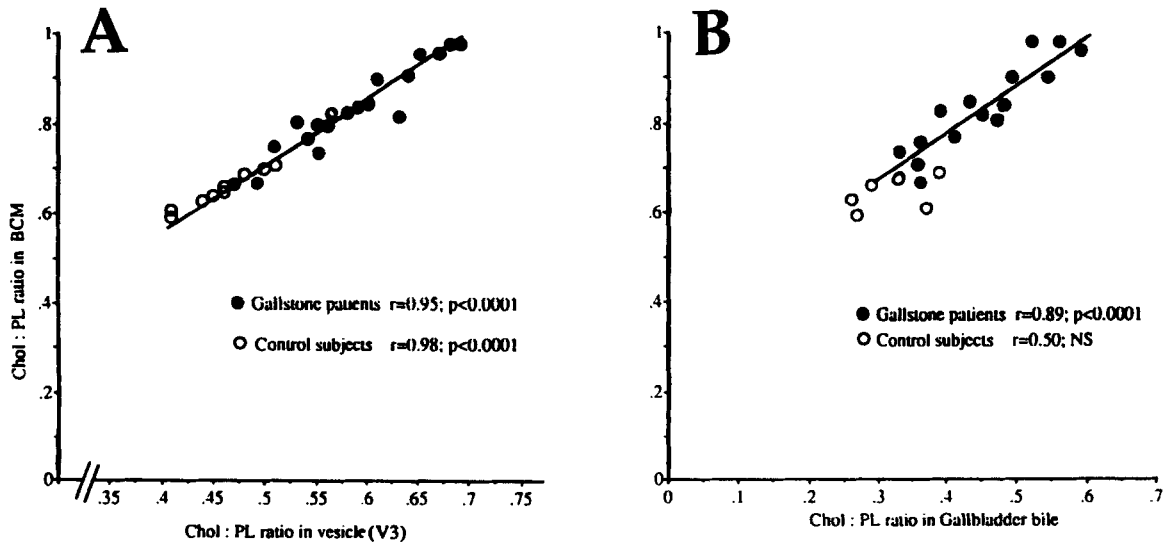


**Fig. 6.** Relationship between the IBW% and both the cholesterol/phospholipid (A) and the cholesterol/protein (B) ratio in the transport vesicles (V3). The rise in the cholesterol/phospholipid ratio with the increase in IBW% can be explained by a concomitant rise in the cholesterol content in the vesicle V3.

microsomal vesicular fractions (Fig. 1, B) showed enrichment in the marker enzymes for BCM (alkaline phosphatase and 5′-nucleotidase) 123 and 147 times over homogenate, (Tables 1 and 2) and was also rich in the microsomal marker enzyme NADPH-cytochrome C reductase 158 times over homogenate. This microsomal fraction was separated at a buoyant density of 1.16 g/ml similar to BCM, but morphologically it looked very different on electron micrography (Fig. 1, B). This vesicular fraction was also unique among other vesicular fractions in other characteristics consistent with it having a role in transhepatic biliary cholesterol and phospholipid transport. These characteristics will be discussed in the paper and summarized at the end.

In the crude microsomal preparation, the activity of HMG-CoA reductase was higher in gallstone patients (4763 ± 525 dpm/mg protein) as compared to control subjects (2067 ± 231 dpm/mg protein, $P < 0.001$). The activity showed a direct correlation in gallstone patients, with the IBW% ($r = 0.85$, $P < 0.001$, **Fig. 2, A**). There was no similar correlation in control subjects who, unlike gallstone patients, did not have a wide scatter in their IBW%. The activity of cholesterol 7α-hydroxylase was lower in gallstone patients (2263 ± 195 dpm/mg protein) as compared to control subjects (5125 ± 405 dpm/mg protein, $P < 0.001$). The activity did not show any correlation with the IBW% in gallstone patients (Fig. 2, B) or in control subjects.

The cholesterol/phospholipid ratio was higher in gallstone patients compared to control subjects in the putative transport vesicle (V3) (0.60 ± 0.03 vs. 0.47 ± 0.06, $P < 0.001$), BCM (0.85 ± 0.10 vs. 0.67 ± 0.13, $P < 0.001$), but not in BLM (0.55 ± 0.02 vs. 0.53 ± 0.03, NS, **Fig. 3**). The absolute cholesterol content per mg protein was higher in the vesicular fraction (V3) from gallstone patients (0.26 ± 0.03) compared to control subjects (0.21 ± 0.05, $P < 0.002$). However, the BCM cholesterol content was not different in gallstone patients (0.36 ± 0.03) compared to control subjects (0.36 ± 0.04, NS), (**Fig. 4, A**). The absolute phospholipid content in the vesicles was not different in gallstone patients (0.43 ± 0.14) compared to control subjects (0.44 ± 0.09, NS), while it was lower in BCM from gallstone patients (0.43 ± 0.08) compared to control subjects (0.54 ± 0.14, $P < 0.01$), (Fig. 4, B). None of the other microsomal fractions showed a difference in the cholesterol/phospholipid or cholesterol/protein ratios in gallstone patients compared to control subjects.

A direct correlation between cholesterol/phospholipid molar ratio in the transport vesicles (V3) and BCM was shown in gallstone patients ($r = 0.95$, $P < 0.001$) and controls ($r = 0.98$, $P < 0.001$) (**Fig. 5, A**). Both groups lie on the same regression line, with the control subjects occupying the left hand side of the scale, being less obese. Similarly, a direct correlation was shown between the cholesterol/phospholipid molar ratio in BCM and gallbladder bile from gallstone patients ($r = 0.89$, $P < 0.001$), but not from control subjects ($r = 0.50$, NS), (Fig. 5, B). Also, a direct correlation was shown between the IBW% and the cholesterol/phospholipid ratio ($r = 0.81$, $P < 0.001$) (**Fig. 6, A**) and cholesterol/protein ratio ($r = 0.91$, $P < 0.001$) (Fig. 6, B) in the transport vesicle (V3) in gallstone patients. In control subjects, a direct correlation was only seen with cholesterol/protein ratio ($r = 0.65$, $P < 0.05$) (Fig. 6, B). The activity of HMG-CoA reductase also showed a direct correlation with the vesicular (V3) cholesterol/phospholipid ratio ($r = 0.73$, $P < 0.002$) (**Fig. 7, A**) and cholesterol/protein ratio

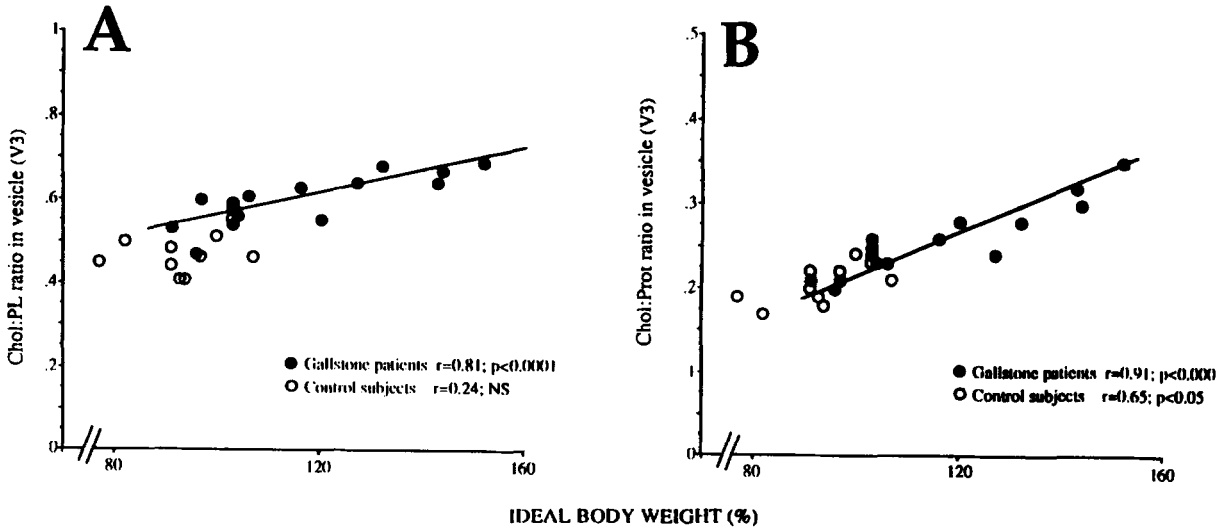


**Fig. 7.** Relationship between the activity of the HMG-CoA reductase and that of the cholesterol/phospholipid (A) and the cholesterol/protein (B) ratio in the transport vesicles (V3). The rise in the cholesterol/phospholipid ratio with the increase in the activity of HMG-CoA reductase can be explained by a concomitant rise in the cholesterol content in the vesicle V3.

TABLE 3. Fatty acids in position 1 (n = 6)

| Fatty Acid | Bile | Vesicle V3 | BCM | BLM |
|---|---|---|---|---|
| | | *% of total* | | |
| Myristic | 2.1 | 2.2 | 3.1 | 4.1 |
| Palmitic | 74.3 | 64.1 | 33.6 | 50.0 |
| Stearic | 7.1 | 12.0 | 23.2 | 30.4 |
| Oleic | 12.6 | 17.0 | 30.2 | 6.3 |
| Linoleic | nil | nil | nil | 4.3 |
| Linolenic | nil | nil | 5.3 | nil |
| Unidentified | 3.9 | 4.5 | 4.3 | 4.9 |

($r = 0.80$, $P < 0.001$) (Fig. 7, B) in gallstone patients but not in control subjects. Absence of correlation in controls is probably due to a narrow range of values of IBW% and HMG-CoA reductase activity.

The positional fatty acid patterns in phosphatidylcholine from gallbladder bile, vesicles (V3), BCM, and BLM are shown in **Table 3** and **Table 4.** A marked similarity is noted between bile and vesicles (V3) especially for palmitic and myristic acids (Table 3) and palmitoleic, linoleic, arachidonic acids (Table 4). Differences were, however, noted especially for linolenic, oleic, and eicosatrienoic acids (Table 4), and stearic and oleic acids (Table 3). BCM showed less similarity to the pattern in bile. BLM showed a high stearic, low oleic acids in position 1 and much lower linoleic acid in position 2 compared to the pattern in bile.

## DISCUSSION

A relatively simple technique has been applied for the first time to the fractionation of human liver homogenate that allows the separation of BCM and BLM with minimal cross contamination, based on methods used in animals (23, 24). The membrane preparations were minimally contaminated by other subcellular fractions, as judged by the marker enzymes and electron microscopy. One of the microsomal vesicular fractions (V3) was not only enriched in the microsomal marker enzyme NADPH cytochrome C reductase but also in the marker enzymes for the BCM, alkaline phosphatase, and 5′-nucleotidase. The bile canalicular membrane preparation had a much smaller relative enrichment in NADPH cytochrome C reductase enzyme (1.8 times) compared to the vesicular fraction V3 (158 times) over homogenate. However, the orientation of the microsomal fraction is expected to be reversed when it joins the BCM (inside out) which might affect the enzyme activity. Also, inhibitory effects of bile acids cannot be excluded as the BCM is normally exposed to high levels of bile acids in the canaliculus. The enzyme is also known to be weakly bound to membranes and can be lost during transport. Because of these special enzymic characteristics, different morphology and gravitational force used in pelleting, and other characteristics summarized at the end of the discussion, we suspected that this vesicular fraction may be involved in cholesterol and phospholipid transport from the endoplasmic reticulum, the site of pooling of newly synthesized and lipoprotein-delivered cholesterol and phospholipid, to bile across the BCM. The biochemical techniques used would obviously allow us to comment on the composition of the subcellular organelles studied in a "snap shot" analogous way as dynamic characterization was not performed.

In order to test this hypothesis further, we investigated whether a clinical condition known to be associated with increased biliary cholesterol, namely cholesterol cholelithiasis with obesity, also caused increased cholesterol content of these putative cholesterol transport vesicles. In this condition, the microsomal enzymes involved in cholesterol synthesis (HMG-CoA reductase) and conversion to bile acids (cholesterol 7α-hydroxylase) were both deranged in such a way as to increase the amount of cholesterol available for incorporation into the microsomal membranes. Some laboratories have previously reported increased HMG-CoA reductase activity in cholesterol gallstone patients (39), while others (40) did not report a statistically significant difference. The activity of HMG-CoA reductase showed a direct correlation with obesity as indicated by the IBW% and we concluded that obese gallstone patients synthesize more cholesterol than control subjects. The activity of the enzyme also correlated positively with the absolute cholesterol content and with the cholesterol/phospholipid ratio in the vesicular fraction (V3) from gallstone patients, which, in general, showed higher cholesterol/phospholipid and cholesterol/protein ra-

TABLE 4. Fatty acids in position 2 (n = 6)

| Fatty Acid | Bile | Vesicle V3; | BCM | BLM |
|---|---|---|---|---|
| | | *% of total* | | |
| Palmitic | 2.0 | 2.3 | 2.5 | 18.1 |
| Palmitoleic | 3.5 | 3.2 | 1.5 | 3.5 |
| Stearic | 2.1 | 1.6 | 3.3 | 11.6 |
| Oleic | 26.5 | 15.0 | 18.0 | 23.3 |
| Linoleic | 43.5 | 44.4 | 25.7 | 18.0 |
| Linolenic | 3.5 | 13.0 | 30.1 | 10.4 |
| Eicosatrienoic | 3.1 | 6.1 | 7.1 | 2.5 |
| Arachidonic | 11.5 | 10.3 | 7.5 | 7.5 |
| Unidentified | 4.3 | 4.1 | 4.3 | 5.1 |

tios over controls. No correlation was found between the enzyme activity and any of the other microsomal vesicular fractions isolated. Thus, the increased cholesterol synthesis in obese gallstone patients manifests itself in an increased cholesterol content in the vesicular fraction (V3), and this is directly related to obesity. These findings provide additional support for the concept that the vesicular fraction that was enriched in both microsomal and BCM marker enzymes is indeed involved in the transport of cholesterol from the endoplasmic reticulum to the site of biliary secretion.

In its turn, the BCM, but not the BLM, cholesterol/phospholipid molar ratio correlated with the same ratio in the vesicular fraction (V3) and in gallbladder bile. A correlation between cholesterol/phospholipid ratio in BCM and vesicles isolated from bile would have been more supportive. However, the correlation is still holding for the whole gallbladder bile (Fig. 5 B). It is known that biliary micelles are derived from biliary vesicles (during bile concentration), and are likely to be affected by their components. The BCM and the vesicular fraction V3, unlike the BLM, showed a higher cholesterol/phospholipid ratio in gallstone patients. The increased cholesterol/phospholipid ratio in the BCM but not in the BLM might be interesting, as the BCM is the pole of the hepatocyte that is more intimately and directly linked to biliary secretion. However, while the increased ratio in the vesicles (V3) reflected an increase in the cholesterol content, the increased ratio in the BCM reflected a decrease in the phospholipid content. This paradox may be discussed in the context that while the transport vesicles respond solely to what is delivered to them by the endoplasmic reticulum, the bile-canalicular membrane responds to the balance between what is delivered to it by the transport vesicles and what is removed from it by bile acids (41). In our in vitro experiments (42), we found that bile acids were more effective in solubilizing phospholipid than cholesterol from hamster BCM. Moreover, in a perfusion study in the rat, Yousef et al. (43) suggested that the increase in cholesterol/phospholipid ratio in BCM was due to membrane phospholipid depletion by cholic acid.

The positional analysis of fatty acids in phosphatidylcholine from gallbladder bile, which constitutes more than 95% of total phospholipid, and from the vesicles (V3), which, unlike the other microsomal vesicular fractions, constitutes more than 70% of total phospholipid, showed a remarkable similarity. Some differences between the two preparations could be explained by a counter difference in the structure of the BCM. In that sense, the higher proportions of stearic and oleic acids in position 1 in the transport vesicles (V3) over bile is reflected in a higher proportion of these fatty acids in the BCM. Similarly, in position 2, the higher proportion of linolenic acid in the transport vesicles (V3) over bile is reflected in the high content of linolenic acid in the BCM. The BCM phospholipid is reported to be composed of more than one pool (7,41) and it seems unlikely that every species in the BCM phospholipid can reach bile. A structural component and another functional or secretory component that can supply biliary type lipids are likely to co-exist. Moreover, it is difficult to imagine that the vesicle V3 is the only vesicular traffic going to the BCM, as we know that bile acids can, in certain conditions, be transported by vesicles separate from cholesterol and phospholipid. Other vesicular and carrier protein traffic pathways can also supply structural and secretory lipid and proteins to the BCM. The interesting asymmetric distribution of phospholipid between the cytoplasmic and luminal leaflets of BCM should also be taken carefully into account. An intra-membrane phospholipid selective transport process, through a flip-flop movement of phosphatidylcholine, is becoming accepted thanks to the elegant demonstration of the phospholipid flippase activity in the rat BCM by Berr et al. (19), the discovery of the role of mdr2 P-glycoprotein in biliary phospholipid secretion in the mice by Smit et al. (20), and the recent direct demonstration of its phospholipid flippase activity in yeast vesicles by Ruetz and Gros (21). This creates a specific phospholipid pattern in the luminal leaflet of BCM, ready to be solubilized by bile salts.

One can summarize the possible biliary lipid selection processes as follows. A specific vesicular traffic from the endoplasmic reticulum joins the BCM, with relative enrichment in biliary type phospholipid and in cholesterol. The phospholipid is mainly phosphatidylcholine with fatty acid patterns very similar to biliary phospholipid fatty acid patterns. The cholesterol content is increased in a condition characterized by increased biliary cholesterol secretion. A similar role for specific phospholipid transport protein(s) is also supported by experimental evidence. The relative contribution and the importance of the two pathways under physiological and pathological conditions are open for further investigations.

Intra-canalicular membrane selective transport process will further select and purify biliary type lipid from other structural elements. The canalicular membrane flippase occupies a central role in this mechanism. However, other mechanisms can be speculated, e.g., a phospholipid methyltransferase, capable of methylating phosphatidylethanolamine into phosphatidylcholine.

Selective solubilization of phosphatidylcholine species by bile acids can then take place. Chenodeoxycholate has been shown to preferentially solubilize typical biliary *sn*-1-palmitoyl-phosphatidylcholine species from

uni- and multilamellar vesicles (44). Moreover, in isolated rat liver, intrabiliary injection of taurocholate has been shown to stimulate phosphatidylcholine output into bile (45).

One can summarize the points in favor of a role for the vesicular fraction (V3) in intrahepatic biliary cholesterol and phospholipid transport as follows. The putative transport vesicles (V3), though originally separated in the microsomal fraction at 100,000 g unlike the BCM which separated at 1000 g, and showing its microsomal marker enzyme pattern, were also rich in BCM marker enzymes. Morphologically, however, the vesicles (V3) were quite distinct from BCM which strongly argues against cross contamination.

The cholesterol content in the vesicles (V3) expressed per mg protein was increased while the phospholipid content was not different in obese gallstone patients who had increased cholesterol synthesis, and was related to IBW% and to the activity of HMG-CoA reductase. The cholesterol content of the BCM was not increased in these patients, while its phospholipid content was decreased. The different alteration in cholesterol and phospholipid content in the vesicles (V3) and BCM preparation provide another evidence against cross contamination.

A large proportion of the vesicular (V3) phospholipid was in the form of phosphatidylcholine, the main biliary phospholipid. This was not the case for the BCM preparation. The positional fatty acid patterns were similar in phosphatidylcholine from vesicles and gallbladder bile.

*Manuscript received 17 May 1995 and in revised form 22 August 1995.*

## REFERENCES


1. Gregory, D. H., Z. R. Vlahcevic, P. Schatzki, and L. Swell. 1975. Mechanism of secretion of biliary lipids. I. Role of bile canalicular and microsomal membranes in the synthesis and transport of biliary lecithin and cholesterol. *J. Clin. Invest.* **55:** 105–114.
2. Kuipers, F., J. P. T. Derksen, A. Gerding, G. L. Scherphof, and R. J. Vonk. 1987. Biliary lipid secretion in the rat. The uncoupling of biliary cholesterol and phospholipid secretion from bile acid secretion by sulphated glycolithocholic acid. *Biochim. Biophys. Acta.* **922:** 136–144.
3. Rutgeerts, P., Y. Ghoos, and G. Vantrappen. 1987. Effect of partial ileocolectomy and Crohn's disease on biliary lipid secretion. *Dig. Dis. Sci.* **32:** 1231–1238.
4. Pattinson, N. R., K. E. Willis, and B. A. Chapman. 1987. Inhibition of biliary phospholipid and cholesterol secretion by cefoperazone. *Dig. Dis. Sci.* **32:** 615–619.
5. Salen, G., G. Nicolau, S. Sheffer, and E. H. Mosbach. 1975. Hepatic cholesterol metabolism in patients with gallstones. *Gastroenterology.* **69:** 676–684.
6. Maton, P. N., H. J. Ellis, M. J. Higgins, and R. H. Dowling. 1980. Hepatic HMG-CoA reductase in human cholelithiasis: effect of chenodeoxycholic and ursodeoxycholic acids. *Eur. J. Clin. Invest.* **10:** 325–332.
7. Yousef, I. M., D. L. Bloxam, and M. J. Phillips. 1975. Liver cell plasma membrane lipids and the origin of biliary phospholipid. *Can. J. Biochem.* **53:** 989–997.
8. Coleman, R., K. Rahman, M. E. Bellringer, K. Kan, and S. Hamlin. 1989. Trends in Bile Acid Research. G. Paumgartner, A. Stiehl, and W. Gerok, editors. Kluwer, London. 161–176.
9. Lowe, P. J., S. G. Barnwell, and R. Coleman. 1984. Rapid kinetic analysis of the bile salt-dependent secretion of phospholipid, cholesterol and a plasma membrane enzyme into bile. *Biochem. J.* **222:** 631–637.
10. Kaplan, M. R., and R. D. Simoni. 1985. Transport of cholesterol from the endoplasmic reticulum to the plasma membrane. *J. Cell Biol.* **101:** 446–453.
11. Wirtz, K. W. A., and D. B. Zilversmit. 1968. Exchange of phospholipids between liver mitochondria and microsomes in vitro. *J. Biol. Chem.* **243:** 3596–3602.
12. Teerlink, T., T. P. van der Krift, M. Post, and K. W. A. Wirtz. 1982. Tissue distribution and subcellular localisation of phosphatidylcholine transfer protein in rats as determined by radioimmunoassay. *Biochim. Biophys. Acta.* **713:** 61–67.
13. Wirtz, K. W. A. 1991. Phospholipid transfer proteins. *Annu. Rev. Biochem.* **60:** 73–99.
14. Cohen, D. E., M. R. Leonard, and M. C. Carey. 1994. In vitro evidence that phospholipid secretion into bile may be coordinated intracellularly by the combined actions of bile salts and the specific phosphatidylcholine transfer protein of liver. *Biochemistry.* **33:** 9975–9980.
15. Stone, B. G., D. Schreiber, L. D. Alleman, and C. Y. Ho. 1987. Hepatic metabolism and secretion of a cholesterol-enriched lipoprotein fraction. *J. Lipid Res.* **28:** 162–172.
16. Schwartz, C. C., M. Berman, Z. R. Vlahcevic, G. Halloran, D. H. Gregory, and L. Swell. 1978. Multicompartmental analysis of cholesterol metabolism in man. *J. Clin. Invest.* **61:** 408–423.
17. Bilhartz, L. E., D. K. Spady, and J. M. Dietschy. 1989. Inappropriate hepatic cholesterol synthesis expands the cellular pool of sterol available for recruitment by bile acids in rat. *J. Clin. Invest.* **84:** 1181–1187.
18. Robins, S. J., J. M. Fasulo, R. Leduc, and G. M. Patton. 1989. The transport of lipoprotein cholesterol into bile: a reassessment of kinetic studies in the experimental animal. *Biochim. Biophys. Acta.* **1004:** 327–331.
19. Berr, F., P. M. Meier, and B. Stieger. 1993. Evidence for the presence of a phosphatidylcholine translocator in isolated rat liver canalicular plasma membrane vesicles. *J. Biol. Chem.* **268:** 3976–3979.
20. Smit, J. J. M., A. H. Schinkel, R. P. J. Oude Elferink, A. K. Groen, E. Wagenaar, L. Van Deemter, C. A. A. M. Mol, R. Ottenhoff, N. M. T. van der Lugt, M. A. van Roon, M. A. van der Valk, G. J. A. Offerhaus, A. J. M. Berns, and P. Borst. 1993. Homozygous disruption of the murine mdr2 P-glycoprotein gene leads to a complete absence of phospholipid from bile and to liver disease. *Cell.* **75:** 451–462.
21. Ruetz, S., and P. Gros. 1994. Phosphatidylcholine translocase: a physiological role for the mdr2 gene. *Cell.* **77:** 1071–1081.
22. Jazrawi, R. P., R. M. Kupfer, C. Bridges, A. Joseph, and T. C. Northfield. 1983. Assessment of gallbladder storage function in man. *Clin. Sci.* **65:** 185–191.
23. Berman, H. M., W. Gram, and M. A. Spirtes. 1969. An improved, reproducible method of preparing rat liver plasma cell membranes in buffered isotonic sucrose. *Biochim. Biophys. Acta.* **183:** 10–18.

24. Wisher, M. H., and W. H. Evans. 1975. Functional polarity of the rat hepatocyte surface membrane. Isolation and characterisation of plasma-membrane subfractions from the blood-sinusoidal, bile - canalicular and contiguous surfaces of the hepatocyte. *Biochem. J.* **146:** 375–388.
25. Song, C. S., W. Rubin, and A. B. Rifkind. 1969. Plasma membranes of the rat liver. Isolation and enzymatic characterisation of a fraction rich in bile canaliculi. *J. Cell Biol.* **41:** 124–132.
26. Goodwin, C. D., B. W. Cooper, and S. Margolis. 1982. Rat liver cholesterol 7α-hydroxylase, modulation of enzyme activity by changes in phosphorylation state. *J. Biol. Chem.* **257:** 4469–4472.
27. Linn, T. C. 1967. The demonstration and solubilization of β-hydroxy-β-methylglutaryl coenzyme A reductase from rat liver microsomes. *J. Biol. Chem.* **242:** 984–989.
28. Shapiro, D. J., R. L. Imblum, and V. W. Rodwell. 1969. Thin-layer chromatographic assay for HMG-CoA reductase and mevalonic acid. *Anal. Biochem.* **31:** 383–390.
29. Avruch, J., and D. F. H. Wallach. 1971. Preparation and properties of plasma membrane and endoplasmic reticulum fragments from isolated rat fat cells. *Biochim. Biophys. Acta.* **233:** 334–347.
30. Engstrom, L. 1961. Studies on calf intestinal alkaline phosphatase. I. Chromatographic purification, microheterogeneity and some other properties of the purified enzyme. *Biochim. Biophys. Acta.* **52:** 36–48.
31. Mackler, B., P. J. Collip, H. M. Duncan, N. Appaji Rao, and F. M. Heunnekens. 1962. An electron transport particle from yeast, purification and properties. *J. Biol. Chem.* **237:** 2968–2974.
32. Williams, C. H., and H. Kamin. 1962. Microsomal triphosphopyridine nucleotide-cytochrome C reductase of liver. *J. Biol. Chem.* **237:** 587–595.
33. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
34. Trinder, P. 1981. Oxidase determination of plasma cholesterol as cholest-4-en-3-one using iso-octane extraction. *Ann. Clin. Biochem.* **18:** 64–70.
35. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226:** 497–509.
36. Bartlett, G. R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* **234:** 466–468.
37. Haverkate, F., and L. L. M. Van Deenen. 1965. Isolation and chemical characterisation of phosphatidyl glycerol from spinach leaves. *Biochim. Biophys. Acta.* **106:** 78–92.
38. Morrison, W. R., and L. M. Smith. 1964. Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride–methanol. *J. Lipid Res.* **5:** 600–608.
39. Coyne, M. J., G. G. Bonorris, L. I. Goldstein, and L. J. Schoenfield. 1976. Effect of chenodeoxycholic acid and phenobarbital on the rate limiting enzymes of hepatic cholesterol and bile acid synthesis in patients with gallstones. *J. Lab. Clin. Med.* **87:** 281–291.
40. Carulli, N., P. Loria, M. Bertolotti, M. Ponz de Leon, D. Menozzi, G. Medici, and I. Piccagli. 1984. Effects of acute changes of bile acid pool composition on biliary lipid secretion. *J. Clin. Invest.* **74:** 614–624.
41. Graham, J. M., H. A. Ahmed, and T. C. Northfield. 1989. Unidirectional pathway for vesicular cholesterol transport. *In* Trends in Bile Acid Research. G. Paumgartner, A. Stiehl and W. Gerok, editors. Kluwer Academic Publishers, Dordrecht, The Netherlands. 177–187.
42. Graham, J. M., and T. C. Northfield. 1987. Solubilisation of lipids from hamster bile-canalicular and contiguous membranes and from human erythrocyte membranes by conjugated bile salts. *Biochem. J.* **242:** 825–834.
43. Yousef, I. M., S. Barnwell, F. Gratton, B. Tuchweber, A. Weber, and C. C. Roy. 1987. Liver cell membrane solubilization may control maximum secretory rate of cholic acid in the rat. *Am. J. Physiol.* **252:** G84–91.
44. Cohen, D. E., M. Angelico, and M. C. Carey. 1990. Structural alterations in lecithin-cholesterol vesicles following interactions with monomeric and micellar bile salts: physical-chemical basis for selection of biliary lecithin species and aggregative states of biliary lipids during bile formation. *J. Lipid Res.* **31:** 55–70.
45. Coleman, R., K. Rahman, K. S. Kan, and R. A. Parslow. 1989. Retrograde intrabiliary injection of amphipathic materials causes phospholipid secretion into bile. *Biochem. J.* **258:** 17–22.