# Solubilisation of cholesterol in human bile

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Two non-disruptive separation techniques (gel filtration, and CsCl density gradient ultracentrifugation) were used to characterise the mode of cholesterol transport in human bile. Both methods showed that biliary cholesterol is solubilised as a high $-M_r$ non-micelle (lipoprotein) complex as well as the mixed micelle. The lipoprotein complex was separated between the densities 1.01 and 1.08, had a cholesterol to phospholipid ratio of $0.95 \pm 0.41$ and contained protein (7–20% by wt). The mixed micelle on the other hand sedimented at $d \geqslant 1.18$, and had a cholesterol to phospholipid ratio of $0.41 \pm 0.13$. Eighteen bile samples, obtained from either T-tube, gallbladder or endoscopy, were analysed, and without exception they all contained a fraction of biliary cholesterol in the non-micelle form. The results indicate that cholesterol is secreted, at least in part, as a lipoprotein complex independently of bile acids (mixed micelles).

*Bile* *Cholesterol* *Colloid micelle* *Lipoprotein*

## 1. INTRODUCTION

An important function of bile is the excretion of water-insoluble cholesterol, which is thought to be kept in solution by forming mixed micelles composed of bile acids and phospholipids. Much of the evidence for this model comes from artificial bile systems [1], however little information is available on whether the same phenomenon occurs in native bile. Previous attempts to fractionate these aggregates from native bile using gel filtration and electrophoresis have resulted in disruption of the micelle due to dilution by buffer [2,3]. Thus, the presence of micelles has not been directly demonstrated in native bile. Further, a recent study using quasi-elastic light scattering to measure particle sizes in bile found lipid particles considerably larger than the mixed micelle particles of model systems [4].

How then is cholesterol solubilised in native bile?

To answer this question two separation techniques designed to minimise mixed micelle disruption were used. The results indicate that cholesterol in native bile is transported as both a mixed micelle and a larger, less dense, lipoprotein complex.

## 2. MATERIALS AND METHODS

Gallbladder bile was obtained from patients at cholecystectomy and hepatic bile from patients with indwelling T-tubes or from endoscopic retrograde cholangiopancreatography (ERCP). Fresh bile was kept at room temperature (22°C) and either immediately spun for 1 h in a Sorvall SS 34 rotor at 18000 rpm or filtered using a 0.22 $\mu$m microdisc. The supernatant was taken for gel filtration or CsCl ultracentrifugation.

### 2.1. *Gel filtration*

This method was based on that of Mansbach et al. [5] designed to minimise the disruption of the mixed micelle on gel filtration. Bile was concentrated (2×) in an ultrafiltration cell (UM 10, Amicon Corp.; $M_r$ cut-off, 10000) and the ultrafiltrate, containing the monomeric components of bile at their equilibrium concentration, used to preequilibrate the column (AcA 34,

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Ultrogel-LKB; 0.8 × 10.0 cm). Bile retentate (400 μl) was applied to the column and the biliary components eluted with more ultrafiltrate. Flow rate was 3 ml/h and the fraction volume, 500 μl. Sample fractions were either immediately extracted or stored frozen at −20°C prior to analysis. ERCP bile samples were eluted on microcolumns (8 × 65 mm), preequilibrated with 2.5 mM glycocholate in phosphate-buffered saline, pH 7.4.

### 2.2. *CsCl density gradient ultracentrifugation*

Bile was subjected to CsCl density ultracentrifugation at 22°C in a Beckman SW 50.1 rotor at 37000 rpm for 48–65 h. To 0.688 g CsCl was added either whole bile (5.32 ml) or an aliquot of bile (gel filtration fraction) layered on top of bile ultrafiltrate in which the CsCl had been previously dissolved. After centrifugation the tubes were sliced into 4 fractions, designated 1–4 in ascending density (fraction volume 1.5 ml) using a gel slicer.

### 2.3. *Chemical analysis*

Lipid analysis was performed after total lipid extraction by chloroform and methanol (2:1, v/v) (phospholipid analysis) or ethanolic KOH and hexane (cholesterol analysis). Cholesterol was determined chemically [6] and phospholipids by total phosphorus analysis [7]. Bile acids were determined enzymatically using 3α-hydroxysteroid dehydrogenase [8] and protein by the method of Lowry et al. [9] after sample dialysis to remove CsCl.

## 3. RESULTS

Gel filtration of hepatic T-tube bile on a column of AcA 34, preequilibrated and eluted with bile ultrafiltrate, resolved two cholesterol-containing fractions, designated FI and FII (fig.1). Fraction I (non-micelle) emerged at the void volume ($M_r \geqslant 500000$) and fraction II (mixed micelle) close to the total volume ($M_r$ 40000–60000) together with a peak of bile acid.

Similarly the same bile subjected to CsCl density gradient ultracentrifugation resolved two cholesterol fractions, one of $d \leqslant 1.08$ and the other of $d \geqslant 1.18$ associated with bile acid (fig.2). To confirm the identity of each fraction and to validate further the separation procedures, fractions I and II resolved on gel filtration were ultracentrifuged

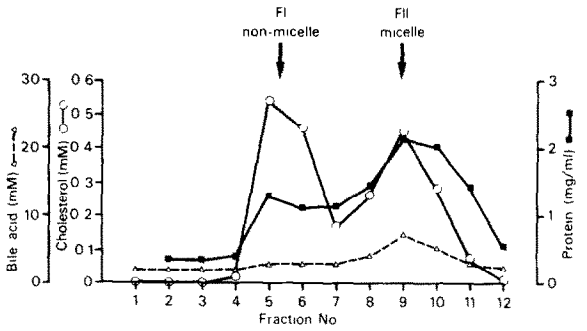


Fig.1. Gel filtration of T-tube bile on AcA 34 preequilibrated and eluted with biliary ultrafiltrate.

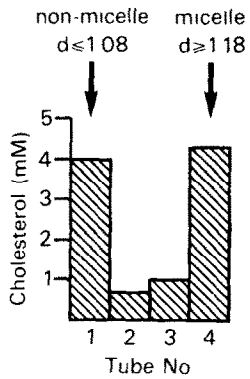


Fig.2. CsCl density gradient ultracentrifugation of whole bile. Samples were centrifuged at 22°C in a Beckman SW 50.1 rotor at 37000 rpm for 48–65 h.

(fig.3). Fraction II (mixed micelle) predictably sedimented at $d \geqslant 1.18$ with a coincident peak of bile acid (fig.3B) and fraction I (non-micelle) separated principally at $d \leqslant 1.08$ (fig.3A) although a minor second peak of cholesterol coincided with a peak of bile acid ($d \geqslant 1.18$) (fig.3A). This latter finding suggests that the concentration of bile acid in the ultrafiltrate was slightly greater than the critical micelle concentration, thereby allowing some micellar solubilisation [5]. Thus, both techniques show good agreement and confirm the presence of a quantitatively important non-micelle form of cholesterol transport.

The micelle/non-micelle ratio, saturation index and lipid compositions of a number of different bile samples are given in table 1. All samples contained the non-micelle complex. In those biles in which the bile acid concentration was below its

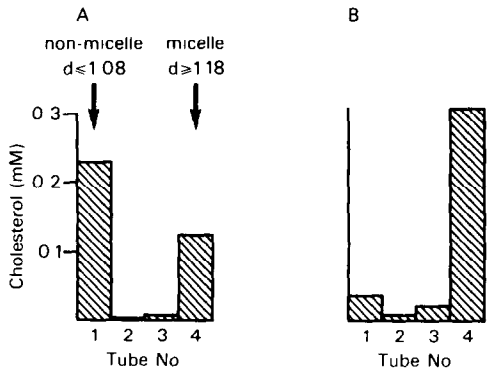


Fig.3. CsCl density gradient ultracentrifugation of cholesterol-containing fractions eluted from the gel filtration column. Each fraction was layered on top of bile ultrafiltrate in which CsCl had been previously dissolved. (A) Gel filtration fraction I; (B) gel filtration fraction II.

Table 1

Micelle/non-micelle cholesterol ratio, saturation index and lipid composition of biles

| Bile (T-tube) | Bile acid (mM) | Lecithin (mM) | Cholesterol (mM) | Saturation index[c] | Micelle/non-micelle |
|---|---|---|---|---|---|
| 1 | 26 | 3.1 | 1.1 | 1.31 | 0.14 |
| 2 | 50 | 6.9 | 1.8 | 0.83 | 3.40 |
| 3 | 8 | 0.8 | 0.6 | 3.89 | 0.06 |
| 4 | 17 | 2.0 | 1.3 | 2.60 | 0.16 |
| 5 | 3 | 0.5 | 0.2 | 4.28 | 0.00 |
| 6[a] | 103 | 28.2 | 10.3 | 1.13 | 14.94 |
| 7 | 4 | 0.5 | 0.3 | 5.78 | 0.00 |
| 8 | 23 | 12.0 | 2.9 | 1.21 | 1.00 |
| 9 | 33 | 4.3 | 0.9 | 0.74 | 1.00 |
| 10 | 42 | 5.8 | 1.7 | 0.98 | 0.80 |
| 11[a] | 147 | 56.1 | 17.5 | 0.99 | 50.00 |
| 12 | 13 | 3.9 | 2.4 | 2.82 | 0.02 |
| 13[b] | 43 | 12.0 | 4.8 | 1.44 | 1.95 |
| 14[a] | 106 | 18.8 | 9.1 | 1.38 | 1.99 |
| 15[b] | 33 | 16.3 | 8.2 | 2.08 | 0.79 |
| 16[a] | 123 | 40.4 | 15.3 | 1.17 | 4.65 |
| 17 | 67 | 17.9 | 4.5 | 0.87 | 13.92 |
| 18 | 40 | 14.0 | 6.6 | 1.76 | 0.83 |

[a] Gallbladder bile
[b] ERCP (endoscopic retrograde cholangiopancreatography) – common bile duct
[c] Derived from critical tables [14]

critical micelle concentration (nos 5,7) only the non-micelle complex was present.

To test the kinetic stability of the non-micelle complex, biliary samples obtained at ERCP (nos 13,15) and T-tube sample (no.18) (table 1) were subjected to gel filtration immediately after aspiration (within 15–20 min) and again 24 h later. No change in the micelle/non-micelle ratio occurred over this time period.

The composition of the mixed micelle and non-micelle components were quite distinct. The cholesterol/phospholipid ratio was markedly higher and more variable in the non-micelle form ($0.95 \pm 0.45$ cf. $0.41 \pm 0.13$), and protein made up 7–20% of its total weight (purified using CsCl density gradient ultracentrifugation).

The $M_r$ of the non-micelle complex was $\geqslant 500000$ as determined by gel filtration. However, the complex is not homogeneous in size since recovery of biliary lipoprotein after filtration through a millipore filter of pore size 220 nm varied from 60 to 100% between different samples.

## 4. DISCUSSION

Two non-disruptive separation techniques were used to demonstrate that cholesterol in native bile is solubilised as a non-micelle (lipoprotein) complex in addition to the mixed micelle. This confirms the earlier findings of Somjen and Gilat [4] who used quasi-elastic light scattering to measure lipid particles in bile. These authors found particles of $d$ 700 Å (cf. mixed micelles, 60 Å) indicating the presence of a bile acid-independent mode of cholesterol transport. However, using their technique they were unable to examine the relative contribution of the non-micelle form to the mixed micelle in total cholesterol solubilisation.

All the 18 biles examined contained the non-micelle complex. In 11 of these 18 samples $\geqslant 50\%$ of total biliary cholesterol was solubilised in the non-micelle form and in those biles in which the bile acid concentration was below its critical micelle concentration (nos 5,7) only the non-micelle complex was present. Cholesterol is secreted into bile, therefore, at least in part, independently to bile acids (mixed micelles) in the form of a kinetically stable non-micelle complex.

Between individuals, both the composition and size of the non-micelle complex varied con-

siderably. The factors determining this are unknown, however, as bile acid hydrophobicity can greatly effect the cholesterol/phospholipid ratio in bile [10,11] it might also have important implications in the form cholesterol is solubilised. In addition, it is interesting to note that lipoprotein-X, which refluxes from the hepatic bile canaliculus during cholestasis [12], exhibits a very similar lipid composition to the non-micelle complex separated here.

The other important component of the non-micelle complex is the protein moiety which makes up 7–20% of its total weight. The identity of the protein or proteins is presently under investigation. They could have an analogous role to the apolipoproteins of serum and it may be speculated that it is the levels of these proteins, in conjunction with bile acid concentration and species, that determines the ultimate solubility limits of cholesterol in bile. Apolipoproteins have recently been identified in bile [13].

In conclusion the mixed micelle theory used to predict the lithogenicity of bile needs to be re-evaluated in that it does not take into account the important role of the biliary lipoprotein complex.

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