Biochem. J. (1987) 242, 825–834 (Printed in Great Britain)

Solubilization of lipids from hamster bile-canalicular and contiguous membranes and from human erythrocyte membranes by conjugated bile salts

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We have demonstrated *in vitro* the efficacy of the taurine-conjugated dihydroxy bile salts deoxycholate and chenodeoxycholate in solubilizing both cholesterol and phospholipid from hamster liver bile-canalicular and contiguous membranes and from human erythrocyte membrane. On the other hand, the dihydroxy bile salt ursodeoxycholate and the trihydroxy bile salt cholate solubilize much less lipid. The lipid solubilization by the four bile salts correlated well with their hydrophobicity: glycochenodeoxycolate, which is more hydrophobic than the tauro derivative, also solubilized more lipid. All the dihydroxy bile salts have a threshold concentration above which lipid solubilization increases rapidly; this correlates approximately with the critical micellar concentration. The non-micelle-forming bile salt dehydrocholate solubilized no lipid at all up to 32 mM. All the dihydroxy bile acids are much more efficient at solubilizing phospholipid than cholesterol. Cholate does not show such a pronounced discrimination. Lipid solubilization by chenodeoxycholate was essentially complete within 1 min, whereas that by cholate was linear up to 5 min. Maximal lipid solubilization with chenodeoxycholate occurred at 8–12 mM; solubilization by cholate was linear up to 32 mM. Ursodeoxycholate was the only dihydroxy bile salt which was able to solubilize phospholipid (although not cholesterol) below the critical micellar concentration. This similarity between cholate and ursodeoxycholate may reflect their ability to form a more extensive liquid-crystal system. Membrane specificity was demonstrated only inasmuch as the lower the cholesterol/phospholipid ratio in the membrane, the greater the fractional solubilization of cholesterol by bile salts, i.e. the total amount of cholesterol solubilized depended only on the bile-salt concentration. On the other hand, the total amount of phospholipid solubilized decreased with increasing cholesterol/phospholipid ratio in the membrane.

INTRODUCTION

In vivo, bile salts maintain biliary cholesterol in a soluble form by the formation of mixed micelles with phospholipid (Admirand & Small, 1968; Carey & Small, 1970; Hegardt & Dam, 1971; Holzbach *et al.*, 1976; Carey, 1983). When the amount of cholesterol secreted into the bile exceeds the solubilizing capacity of the bile acids, the bile becomes supersaturated with cholesterol: under these conditions cholesterol may crystallize, with the consequent formation of cholesterol gallstones in the gall bladder of humans (Admirand & Small, 1968; Small & Rapo, 1970). The administration of certain bile salts, specifically chenodeoxycholate (CDC) and ursodeoxycholate (UDC), has been found to cause a decrease in cholesterol saturation and dissolution of cholesterol gallstones (Danzinger *et al.*, 1972; Bell *et al.*, 1972; Thistle & Hofmann, 1973; Makino *et al.*, 1975; Maton *et al.*, 1977).

The precise mechanism by which bile salts, cholesterol and phospholipid are secreted into bile remains unclear. In the hepatocyte membrane, three polypeptides which bind bile salts have been identified (Accatino & Simon, 1976; Abberger *et al.*, 1981); moreover, the same polypeptides exist in the sinusoidal and bile-canalicular domains (Accatino & Simon, 1976), in spite of the overall dissimilarity of the protein components of these two membranes. This suggests that the same receptor-mediated process may be involved in transporting bile-salt molecules into the hepatocyte from the blood sinusoid as into the bile canaliculus from the hepatocyte. Bile salts may extract cholesterol and phospholipid from the bile-canalicular membrane as they pass across it (Small, 1970) or they may remove these molecules from the membrane once their concentration in the bile canaliculus exceeds the CMC (critical micellar concentration) (Coleman *et al.*, 1977), in a manner analogous to the solubilization of membrane components *in vitro*. Both these routes would result in the formation of mixed cholesterol/phospholipid bile-salt micelles in the canaliculus; they are two variants of the so-called micellar theory of biliary lipid secretion. O'Maille (1980) has presented evidence that, for translocation of bile-salt molecules across the bile-canalicular membrane, micelle formation is not important, whereas the concentration of bile salts in the hepatic bile may reach 20 mM (Coleman *et al.*, 1980), well above the CMC for many bile salts, although the concentration in the bile canaliculus is not known.

Bile salts are detergents and *in vitro* are capable of solubilizing lipids and proteins from membranes. Their detergent capacity has been used in the dissociation of membrane components and the preparation of functionally active membrane proteins. Certain bile salts are

Abbreviations used: CDC, chenodeoxycholate; UDC, ursodeoxycholate; HBS, Hepes-buffered saline, 10 mM, pH 7.4; CMC, critical micelle concentration; Na^+/K^+-ATPase, $(Na^+ + K^+)$-stimulated, Mg^{2+}-dependent, ATPase; DHC, dehydrocholate; DC, deoxycholate.

known to be highly membrane-active, e.g. deoxycholate, whereas others, such as cholate, are much less active.

A number of workers (e.g. Yousef & Fisher, 1976: Coleman *et al.*, 1979, 1980) have investigated the effect of bile acids or bile salts on the solubilization of phospholipids and proteins from a variety of membrane types. Dihydroxy derivatives were found to be more effective at solubilizing both phospholipids and proteins from human erythrocyte membranes and from rat liver plasma membranes. Our own studies were designed to test the hypotheses that the formation of bile-salt micelles is important in the solubilization of cholesterol and phospholipid from membranes and that different bile salts have different patterns of lipid solubilization, which correlate with their detergent capacity. To investigate whether the type of membrane influences the pattern of lipid solubilization we have tested the effect of bile salts on the bile-canalicular and contiguous membranes from hamster liver and on human erythrocyte membranes. We chose the hamster, because this animal has been described as a good model for human cholesterol-gallstone formation; under certain experimental conditions it can produce gall-bladder bile that is supersaturated with cholesterol, developing cholesterol gallstones that can be prevented by the administration of UDC (Pearlman *et al.*, 1979). Parts of this work have been presented to the Sixth International Bile Acid Meeting, Freiburg, 1980, and to the Annual Meeting of the American Gastroenterology Association, New York, 1981 (Graham *et al.*, 1981).

MATERIALS AND METHODS

(*a*) Large-scale harvesting of hamster liver plasma-membrane fractions

Young adult male Syrian hamsters (Shamrock Farms, Henfield, West Sussex, U.K.) were used in this study. They were killed by inhalation of CO_2, and the livers perfused via the hepatic portal vein with 50 ml of phosphate-buffered saline (0.2 g of KCl, 0.2 g of KH_2PO_4, 8 g of NaCl and 0.115 g of Na_2HPO_4, pH 7.4) to remove as much of the blood as possible. The livers from five or six animals were routinely used for a single membrane preparation; they were excised from the animal; the gall bladders were removed and transferred to ice-cold 1 mM-$NaHCO_3$ and washed twice in this medium. All succeeding operations were carried out at 0–4 °C. The contiguous and bile-canalicular membrane fractions were prepared by the method of Evans (1970) and Wisher & Evans (1975). All membranes were stored in 0.25 M-sucrose/5 mM-Tris/HCl, pH 8.0, at −80 °C until required. Enzyme assays were carried out on all sucrose-gradient fractions to determine the efficacy of the separations. In addition, the final preparations of bile-canalicular-membrane vesicles and contiguous-membrane sheets were examined by scanning electron microscopy.

(*b*) Enzyme assays

5′-Nucleotidase (EC 3.1.3.5) and Na^+/K^+-ATPase (3.6.1.3) were assayed radiometrically as described by Avruch & Wallach (1972). Alkaline phosphodiesterase (EC 3.1.4.1) was measured spectrophotometrically by using *p*-nitrophenylthymidine 5′-phosphate (Razzell, 1963). Leucine aminopeptidase (EC 3.4.11.1) was assayed by using 25 mM-leucine *p*-nitroanilide as substrate in the presence of 50 mM-phosphate, pH 7.2 (Wachsmuth *et al.*, 1966). For adenylate cyclase (EC 4.6.1.1) assays, the membrane was incubated in the presence and absence of 10^{-9} M-glucagon in an ATP-regenerating medium as described by Wisher & Evans (1975) and the cyclic AMP formed was estimated in a competitive binding protein assay (Tovey *et al.*, 1974). Succinate:cytochrome *c* reductase (EC 1.3.99.1) was assayed as described by Mackler *et al.* (1962) and protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

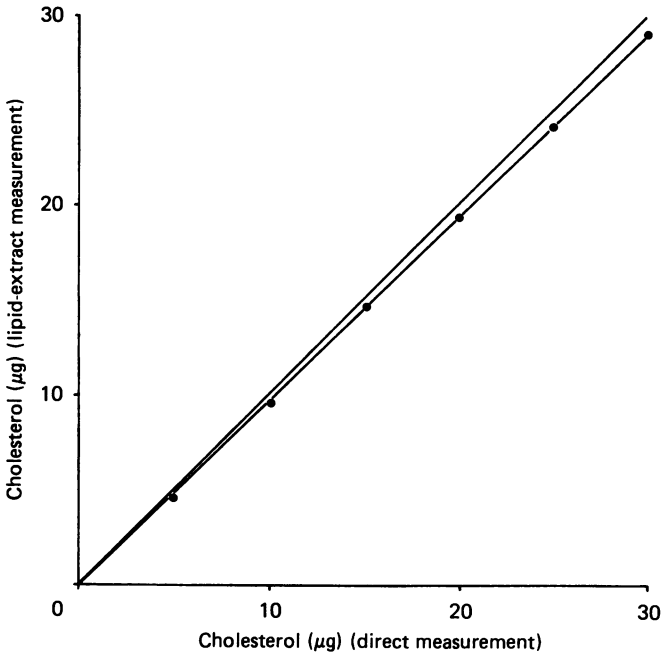

Fig. 1. Determination of erythrocyte membrane cholesterol by the cholesterol oxidase method: comparison of direct measurement with measurement on a lipid extract

●, Measured values; ——, line of identity.

(*c*) Electron microscopy

Membrane samples were centrifuged at 100000 $g_{av.}$ for 40 min and the pellets infiltrated with 25% (v/v) glycerol. The material was rapidly frozen in Freon 22 and then fractured at −110 °C by using a Denton freeze–fracture device. The fracture surfaces were shadowed with platinum and carbon, replicated with carbon, cleared in sodium hypochlorite and mounted on grids. Specimens were examined with a Siemens 101 electron microscope.

(*d*) Human erythrocyte membranes

Fresh human blood (20 ml) was collected in heparinized tubes. Erythrocyte ghosts were prepared by the method of Dodge *et al.* (1963). In the final wash, the hypo-osmotic phosphate buffer was replaced by 0.25 M-sucrose/5 mM-Tris/HCl, pH 8.0. The erythrocyte ghosts were stored in this medium at −80 °C until required.

(*e*) Incubations with bile acids

Before incubation, stored membrane samples were rapidly thawed, centrifuged at 30000 $g_{av.}$ for 20 min to remove any small membrane fragments, and the pellet was resuspended by very gentle homogenization in a loose-

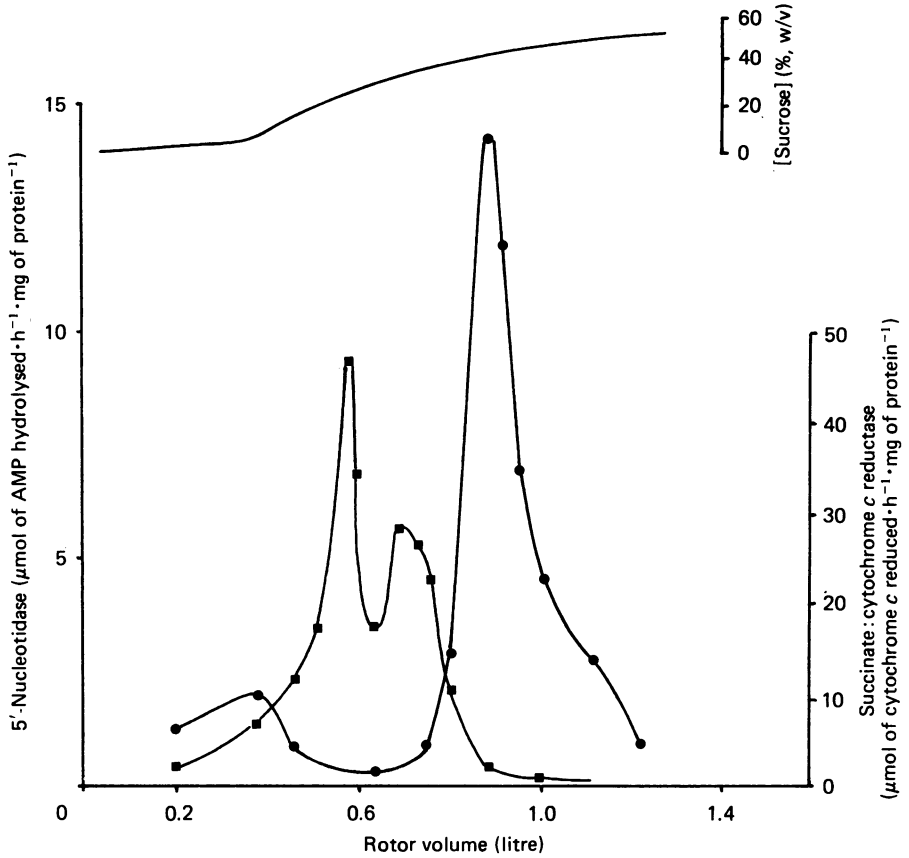

Fig. 2. Isolation of hamster liver plasma membrane in an AXII zonal rotor

Centrifugation was performed as described in the Materials and methods section. ●, 5'-Nucleotidase; ■, succinate:cytochrome *c* reductase. The upper trace shows the sucrose-gradient profile. The data shown are from a single experiment. In 18 separate preparations the precise distribution of 5'-nucleotidase activity was not identical, but the plasma membrane, however, was always well resolved from the mitochondria, and always banded between 0.8 and 1.2 litres.

fitting Dounce homogenizer in 10 mM-Hepes-buffered saline, pH 7.4 (HBS). Taurine or glycine conjugates of cholic acid, CDC, deoxycholic acid, UDC and dehydrocholic acid (obtained from Gipharmex Ltd., Milan, Italy) were dissolved in HBS. The concentration of all the 3α-hydroxy bile salts in solution was checked by the method of Talalay (1960). The bile-salt solutions were incubated with hamster liver bile-canalicular membrane in a total volume of 1 ml under a variety of conditions described in the Results section. After incubation at 37 °C the sample was cooled rapidly to 0 °C and then centrifuged at this temperature for 20 min at 32000 $g_{av.}$. The supernatant was removed, the pellet washed once in HBS and then resuspended in 1 ml of this medium. Preliminary experiments showed that the supernatants from such centrifugations contained no material that sedimented at 100000 $g_{av.}$ for 30 min.

Once the importance of bile-salt CMC to membrane solubilization had been established (see the Results section), it was possible to modify the incubation procedure to take advantage of this so that short periods of incubation could be studied. At the appropriate time, 0.4 ml of the incubation mixture was removed and diluted into 5 ml of ice-cold HBS to decrease the bile-salt concentration below the CMC and thus arrest solubilization. Membrane pellets and supernatants were then prepared as described above.

(*f*) Lipid analysis

Membrane pellets and supernatants from the incubations were lipid-extracted by the method of Folch *et al.* (1957). The lipid extracts were divided and evaporated under nitrogen; one half was assayed for cholesterol by the method of Leffler & McDougald (1963); the other half was digested in 72% $HClO_4$ at 180 °C for 3 h and the released P_i estimated by the method of Bartlett (1959).

In some experiments, in which the amount of cholesterol solubilised was less than 20 μg, an enzymic assay for cholesterol was carried out on the membrane pellet or supernatant without lipid extraction. Cholesterol was determined as cholest-4-en-3-one in a modification of the method of Trinder (1981). The cholesterol oxidase reagent contained 0.93 M-phosphate buffer, pH 6.0, 0.38% lutensol and cholesterol oxidase (0.45 unit/ml; Boehringer Corp., Lewes, Sussex, U.K.). A 2 ml sample (supernatant or pellet) and 0.5 ml of the cholesterol oxidase reagent were incubated at 50 °C for 1 h. The reaction was stopped by addition of 1.5 ml of ethanol, and the ketone extracted into 2 ml of iso-octane. The A_{232} was read against a blank lacking the cholesterol oxidase.

The validity of measuring membrane cholesterol by this method without prior lipid extraction is shown in Fig. 1. In this experiment a range of erythrocyte

Table 1. Isopycnic sucrose-gradient separation of bile-canalicular and contiguous membrane enzymes

Values for specific activities are in the following units: [a]μmol of AMP hydrolysed·h^{-1}·mg of protein^{-1}; [b]μmol of *p*-nitrophenylthymidine 5′-monophosphate hydrolysed·h^{-1}·mg of protein^{-1}; [c]μmol of leucine *p*-nitroanilide hydrolysed·h^{-1}·mg of protein^{-1}; [d]pmol of cyclic AMP produced·h^{-1}·mg of protein^{-1}; [e]μmol of ATP hydrolysed·h^{-1}·mg of protein^{-1}. Values are means (S.D.) for five experiments, except for the adenylate cyclase data, which are means for three experiments. Band 1 contains bile-canalicular membrane; band 3 contains contiguous membrane.

	Specific activity			
Enzyme	Homogenate	Band 1	Band 2	Band 3
5′-Nucleotidase[a]	1.5 (0.3)	47.4 (3.0)	10.3 (2.5)	5.2 (0.9)
Alkaline phosphodiesterase[b]	0.8 (0.2)	48.5 (6.3)	3.9 (1.9)	7.5 (1.0)
Leucineaminopeptidase[c]	0.07 (0.02)	6.4 (0.8)	1.0 (0.5)	0.9 (0.1)
Glucagon-stimulated adenylate cyclase[d]	0.4 (0.1)	< 0.05	< 0.05	0.8 (0.4)
Na^+/K^+-ATPase[e]	1.3 (0.3)	< 0.5	3.1 (0.2)	11.3 (2.7)

membrane concentrations were divided and one half was lipid-extracted. Cholesterol was then measured in the lipid extract and the corresponding membrane suspensions directly. The graph relating the values obtained by the new technique is very close to the line of identity.

RESULTS

Isolation of hamster liver bile-canalicular and contiguous membrane

The enzyme profiles of the zonal gradient (Fig. 2) show a clear separation of the plasma membrane and mitochondria (5′-nucleotidase and succinate:cytochrome *c* reductase respectively). The 5′-nucleotidase activity was maximal between 38 and 50% (w/v) sucrose. This material was resolved into three bands and a pellet in the subsequent isopycnic sucrose gradient. Table 1 shows their enzymic composition: the upper band (1) was enriched in enzymes characteristic of the bile-canalicular membrane (alkaline phosphodiesterase, 5′-nucleotidase and leucine aminopeptidase). Neither Na^+/K^+-ATPase nor glucagon-sensitive adenylate cyclase was detectable in the upper band: these two enzymes were found almost exclusively in band 3. All the succinate:cytochrome *c* reductase activity was recovered in the pellet; no enzyme activity was detectable in band 1 or band 3. Freeze–fracture electron micrographs of bands 1 and 3 (Fig. 3) showed that the former contained only small membrane vesicles (Fig. 3A), whereas the latter contained mainly sheets of membrane (Fig. 3B) characteristic of the contiguous membrane. The data on these fractions agree very well with those obtained by Wisher & Evans (1975) for rat liver.

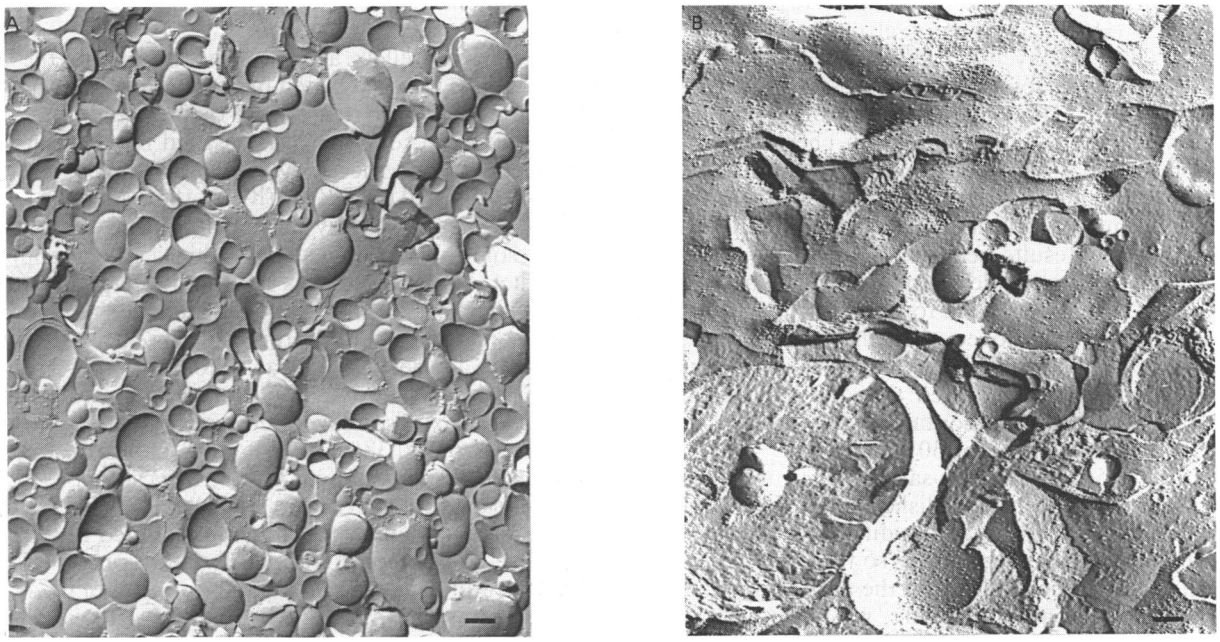

Fig. 3. Freeze–fracture electron micrographs of hepatocyte membrane fractions

A, bile-canalicular membrane vesicles (band 1 from the isopycnic sucrose gradient); B, contiguous membrane sheets (band 3 from the isopycnic sucrose gradient). The bar represents 0.1 μm. For experimental details, see the text.

Table 2. Solubilization of lipid from bile-canalicular membrane by taurine conjugates of CDC, cholate and DHC

Incubations were performed with membranes at 0.18 mg of protein/ml as described in the text. Values are means (S.D.) for three experiments.

Bile salt	Concn. (mM)	Lipid solubilized (%)	
		Cholesterol	Phospholipid
CDC	8	95 (2)	98 (2)
Cholate	8	33 (7)	38 (3)
DHC	8	0	0
DHC	32	0	0

Solubilization of membrane lipids

***(a)* Effect of bile-salt type and bile-salt concentration.** To assess the importance of micelle formation in the ability of bile salts to remove lipid from the bile-canalicular membrane, three compounds were compared: CDC, cholate and DHC, all as the sodium salts of their taurine conjugates. The first two bile salts form micelles, whereas the latter is non-micelle-forming. Table 2 shows that whereas CDC at a concentration of 8 mM solubilized all of the cholesterol and phospholipid from the membranes (0.18 mg of protein/ml) after 10 min, DHC failed to solubilize any lipid whatsoever, even at a concentration of 32 mM. Cholate solubilized approximately a third of both lipids.

In a comparison of taurine conjugates of DC, CDC, UDC and cholate (Fig. 4), a higher concentration of membrane protein (0.4 mg/ml) was used to prevent total lipid solubilization at the highest bile-salt concentration. No solubilization of either phospholipid or cholesterol was obtained with DC below 1.6 mM, whereas with CDC, phospholipid (but not cholesterol) solubilization always commenced at a lower concentration (0.8 mM) of bile salt. Both of these bile salts were more effective in solubilizing phospholipid than cholesterol. Lipid solubilization by CDC and DC showed a pronounced concentration threshold, around the CMC, which for CDC is 0.8–2.5 mM and for DC is 1.0–3.1 mM (Small, 1971; Carey, 1983). On the other hand, cholate showed no such feature; this bile salt showed little discrimination between the two lipids over the range of concentrations tested. UDC solubilized significant amounts of cholesterol only above 3.2 mM; although small amounts of phospholipid were removed below this concentration, the solubilization of this lipid also increased markedly above 3.2 mM-bile salt.

The data shown in Fig. 4 were obtained by the modified incubation procedure (see subsection *e* of the Materials and methods section) in which the incubation mixture was diluted with buffer before centrifugation. Previous experiments with CDC and cholate in which the mixture was centrifuged without prior dilution showed the same patterns of lipid solubilization. Indeed, the sudden increase in lipid solubilization by CDC at concentrations of bile salt above the CMC was exaggerated by the exposure of the membrane to such concentrations during both the incubation and the subsequent centrifugation. Dilution before centrifugation prevented this additional solubilization and did not affect the characteristic lipid solubilization by each bile salt.

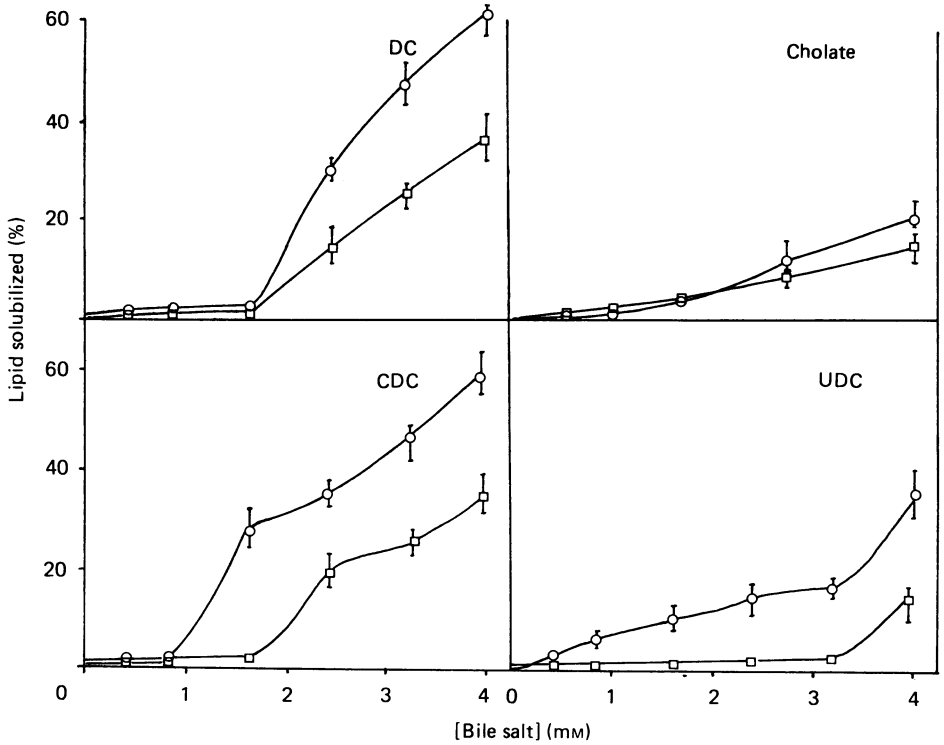

Fig. 4. Solubilization of lipids from bile-canalicular membrane by taurine-conjugated bile salts (0–4 mM)

○, Phospholipid; □, cholesterol. Values are means (±S.E.M.) for three experiments. For experimental details, see the text.

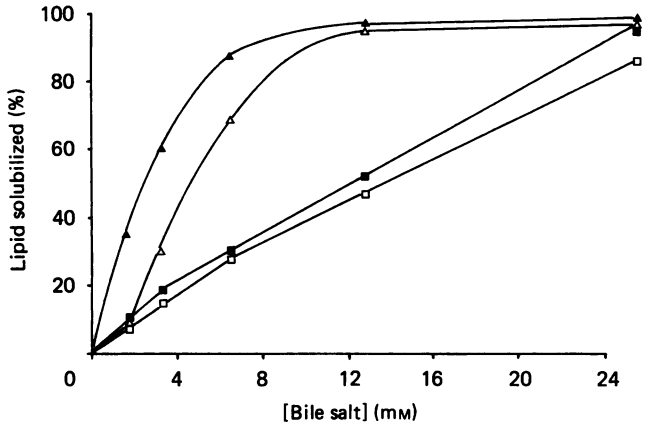

Fig. 5. Solubilization of cholesterol and phospholipid from bile-canalicular membrane by taurine-conjugated bile salts

△,▲, Solubilization by CDC of cholesterol (△) and of phospholipid (▲). □,■, Solubilization by cholate of cholesterol (□) and of phospholipid (■). Values are means for three experiments. For experimental details, see the text.

In a comparison of CDC and cholate over a wider range of bile-salt concentrations (Fig. 5), using a membrane protein concentration of 0.4 mg/ml, further differences between the two bile salts were apparent. The solubilization of phospholipid by CDC was virtually complete at 8 mM, and that of cholesterol by 12 mM, and the solubilization of both lipids by cholate was linear up to 24 mM.

***(b)* Effect of conjugation.** Table 3 shows that the amounts of cholesterol and phospholipid solubilized from two different bile-canalicular membrane preparations (0.35 mg of protein/ml) gave no consistent difference between the glycine and taurine conjugates of cholate during a 5 min incubation; however, the glycine conjugate of CDC was able to solubilize more phospholipid and cholesterol than was the taurine conjugate.

***(c)* Effect of membrane type**

To test whether the amount of lipid solubilized was in any way membrane-specific, the amount of cholesterol and phospholipid solubilized by 4 mM-tauro-CDC from the bile-canalicular and contiguous membranes of the hamster hepatocyte and the human erythrocyte membrane was studied. Although each incubation contained the same amount of membrane (in terms of protein mass), the total amount of cholesterol in each incubation depended on the cholesterol/phospholipid molar ratio of the membrane. Table 4 shows that the percentage of total cholesterol in the membrane that was solubilized in any given incubation by the bile salt was inversely related to the cholesterol/phospholipid molar ratio of that membrane. Thus the bile salt extracted approximately the same amount of cholesterol in each incubation; the product of the molar fraction of cholesterol in the membrane and the percentage of cholesterol solubilized was the same for each membrane: 21.7, 23.2 and 22.1% for the bile-canalicular, contiguous and erythrocyte membranes respectively.

The greater availability of human erythrocyte membranes permitted us to study the lipid solubilization patterns at considerably higher membrane protein concentrations. The pattern of cholesterol and phospholipid solubilization by tauro-CDC using erythrocyte membranes at 1 mg of protein/ml was identical with that observed with the same membranes at 0.5 mg of protein/ml and with the bile-canalicular membrane, i.e. phospholipid was solubilized at a lower concentration of tauro-CDC than was cholesterol and the amount of phospholipid extracted at 4 mM-tauro-CDC was approximately twice that of the cholesterol. This pattern was therefore not influenced by the amount or type of membrane in the incubation (see Figs. 4 and 6).

Table 3. Effect of conjugation (taurine or glycine) on the lipid solubilization from bile-canalicular membrane by cholate and CDC at 5 min

Values are the means for two experiments, each with two different preparations of hamster bile-canalicular membrane (A and B).

			Lipid solubilized (%)			
			Cholesterol		Phospholipid	
Bile salt	Conjugate	Bile acid concn. (mM)	A	B	A	B
Cholate	Taurine	1	2	2	3	3
		2	5	4	8	8
		4	18	17	20	18
	Glycine	1	2	2	3	3
		2	5	4	10	8
		4	16	18	22	24
CDC	Taurine	1	2	1	8	10
		2	15	13	45	49
		4	35	31	65	75
	Glycine	1	2	4	7	6
		2	21	26	60	52
		4	40	43	84	81

Table 4. Comparison of lipid solubilization from mammalian plasma membranes by tauro-CDC (4 mM)

The solubilization values are means (S.D.) for three experiments.

Membrane...	Erythrocyte	Contiguous	Bile-canalicular
Membrane cholesterol/phospholipid molar ratio	0.92	0.53	0.64
Cholesterol solubilized (%)	24 (2)	44 (2)	34 (3)
Phospholipid solubilized (%)	68 (5)	71 (4)	71 (7)

***(d)* Effect of time.** The use of the enzymic assay for cholesterol permitted us to measure amounts of cholesterol below 20 μg; we were therefore able to study the lipid solubilization at short time intervals. The pattern of solubilization (i.e. greater percentage of phospholipid than of cholesterol) was maintained at all times studied, and the bulk of the lipid removal was complete within 1 min (Fig 7). There did appear, however, to be a small, but significant, increase in cholesterol solubilization over the subsequent 4 min, although no further increase in phospholipid solubilization could be detected. Interestingly, the solubilization of lipid from the erythrocyte membrane by 4 mM-taurocholate was, over the time range studied, almost linear.

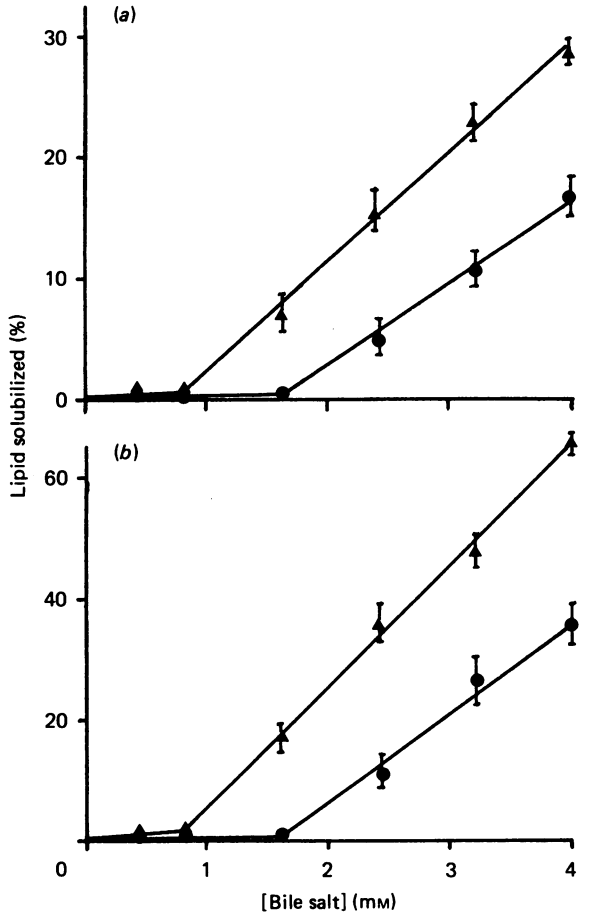

Fig. 6. Solubilization of cholesterol and phospholipid from human erythrocyte membrane by tauro-CDC

(*a*) 1.0 mg of membrane protein·ml^{-1}; (*b*) 0.5 mg of membrane protein·ml^{-1}. ●, Cholesterol; ▲, phospholipid. Values are means (±S.E.M.) for three experiments. For experimental details, see the text.

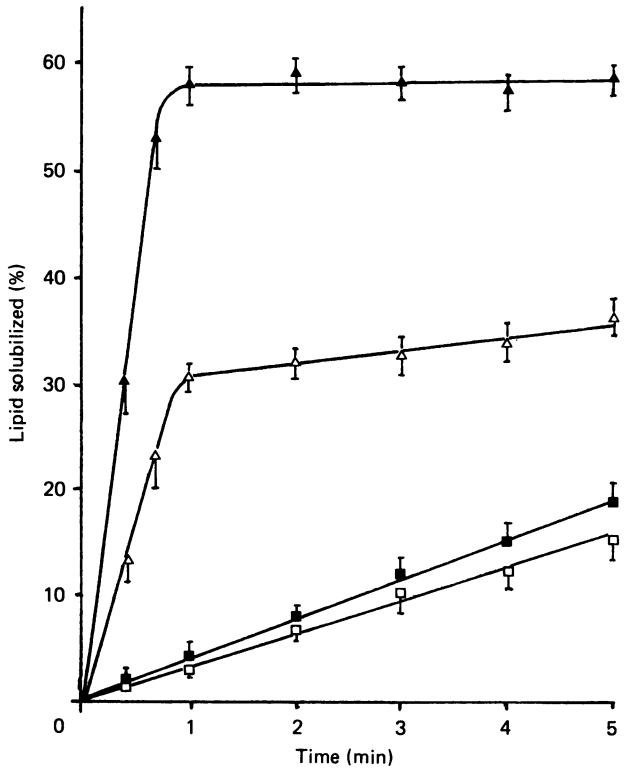

Fig. 7. Time-dependence of lipid solubilization from human erythrocyte membrane by tauro-CDC and taurocholate

△,▲, Solubilization by 4 mM-CDC of cholesterol (△) and of phospholipid (▲). □,■, Solubilization by 4 mM-cholate of cholesterol (□) and of phospholipid (■). Values are means (±S.E.M.) for three experiments. The incubations contained 1 mg of protein/ml. For other experimental details, see the text.

DISCUSSION

Enzyme analysis and electron microscopy of the membrane fractions from the isopycnic sucrose gradient showed that band 1 contained membrane vesicles derived from the bile-canalicular membrane: the high activities of 5′-nucleotidase, alkaline phosphodiesterase and leucine aminopeptidase, and absence of Na^+/K^+-ATPase and glucagon-sensitive adenylate cyclase in the membrane are in accord with the results found in rat liver by Wisher & Evans (1975) and Poupon & Evans (1979). Although other workers (Yousef *et al.*, 1977) have reported not only high levels of 5′-nucleotidase and leucine aminopeptidase in their bile-canalicular membrane preparation from rat liver but also the highest activity for the Na^+/K^+-ATPase, Blitzer & Boyer (1982) and Latham & Kashgarian (1979) proposed a non-canalicular location for the ATPase. The reason for this divergence in results is not clear.

Whereas other groups, e.g. Yousef & Fisher (1976) and Vyvoda *et al.* (1977), have investigated the solubilization of phospholipids from rat liver membranes by bile salts (conjugated and unconjugated) *in vitro* at 0–4 °C, we chose to study conjugated salts only at 37 °C (this being a more 'physiological' temperature), and we have studied their effect on solubilization of cholesterol as well as of phospholipid from three types of membrane: hamster liver bile-canalicular and contiguous membranes and human erythroycte membrane.

The total inability of the non-micelle-forming DHC to solubilize any lipid, even at a concentration as high as 32 mM, suggests the importance of micelle formation, since CDC, at a much lower concentration (8 mM), totally solubilized all the membrane lipid. This is consistent with the observation that DHC fails to release any lipids in the perfused liver (Barnwell *et al.*, 1984). The use of concentrations of bile salt in the range 0–4 mM has shown that, at least for DC and CDC, there is a clear threshold concentration (0.8–1.6 mM) below which neither cholesterol nor phospholipid is solubilized. Although estimates of the CMC of these compounds vary considerably, most fall in the range 1–3 mM. For the taurine conjugates of CDC, values of 0.8–2.5 mM and for taurine conjugates of DC values of 1.0–3.1 have been reported [for reviews of these data, see Small (1971) and Carey (1983)]. Data on the CMCs of taurocholic acid are less clear: values between 1.5 mM and 14.0 mM have been reported, although most values are in the range 3.4–6.0 mM (Small, 1971; Carey, 1983). Our data on the solubilization of both cholesterol and phospholipid by cholate is clearly quite distinct from that by DC and CDC in that no threshold concentration is apparent, the solubilization of both lipids being virtually linear over the bile-salt concentration range tested (0–32 mM). The phospholipid- and cholesterol-solubilizing capacity of cholate compared with that of DC and CDC shown here is in keeping with the observations on phospholipid solubilization by Yousef & Fisher (1976) and Vyvoda *et al.* (1977). Actual values for the amount of phospholipid solubilized vary among the studies because of different membrane/bile-salt ratios and different periods and temperatures of incubation.

In the case of UDC the almost linear solubilization of phospholipid over the range 0–3.2 mM resembles that observed with cholic acid. However, like the other dihydroxy bile salts (CDC and DC), UDC demonstrated a threshold concentration above which there occurred a marked increase in solubilization of both phospholipid and cholesterol. The CMC of UDC has been reported to be between 1.3 and 3.8 mM (Carey *et al.*, 1981). UDC also resembled CDC and DC in that phospholipid was solubilized considerably more efficiently than was cholesterol; indeed UDC solubilized no cholesterol whatsoever below 4 mM.

The efficiencies of cholesterol and phospholipid solubilization by CDC and DC were similar and much greater than those by UDC and cholate in our studies. Similar trends have been observed in the micellar solubilization of aqueous dispersions of cholesterol by bile acid/mono-oleoylglycerol mixtures (Montet *et al.*, 1983) and the solubilization of cholesterol and phospholipid from model membranes perfused by bile salts (Salvioli & Carey, 1982). Broadly, the magnitude of solubilization of these bile salts correlates well with their detergent capacity (Carey *et al.*, 1981) and their degree of hydrophobicity (Carey, 1983), CDC and DC being considerably better detergents and much more hydrophobic than UDC or cholate.

The hydrophobicity of the glycine-conjugated bile salts has also been established to be greater than that of the taurine analogues (Carey, 1983). This is in accord with our own observations that the glycine-conjugated CDC is more effective than the taurine conjugate in solubilizing both phospholipid and cholesterol from the bile-canalicular membrane, although this difference was only noticeable with CDC and not with cholate.

Carey (1983) also showed that as the hydrophobicity of the bile salt decreased, so the three-phase region (saturated micelles + liquid crystal + solid crystal) in quaternary-phase diagrams, expanded and the micellar phase became reduced. It is possible that the lack of a clear threshold concentration for the solubilization of both phospholipid and cholesterol by cholic acid and for the solubilization of phospholipid by UDC at concentrations below 3.2 mM reported here may reflect the formation of a liquid-crystal phase in addition to micellar solubilization.

The other significant differences between lipid solubilization by cholate and CDC, i.e. the rapid (less than 1 min) and complete solubilization of both lipids by 12 mM-CDC and its pronounced concentration-dependence only above the CMC, in contrast with the slow solubilization (about 5 min) and linear concentration-dependence (0–32 mM) of cholate, may also reflect the formation of a more extensive liquid-crystal phase by cholate compared with CDC.

No obvious specificity was apparent between the two membranes of the hepatocyte or the erythrocyte membranes. Although the percentage of the total cholesterol solubilized by tauro-CDC from the three membranes increased in the order erythrocyte membrane, bile-canalicular membrane, contiguous membrane, when the concentration of cholesterol in the membrane (which decreased in the same order) was taken into account, the actual amount of cholesterol removed from all three membranes was very similar. It therefore appeared that the major controlling influence on lipid solubilization from membranes was the bile salt itself, which extracted similar amounts of cholesterol from the membrane irrespective of the ratio of cholesterol to phospholipid in the membrane. The almost identical value for the percentage of phospholipid solubilized from all three membranes suggests that, as the phospholipid/cholesterol ratio in the membrane falls (contiguous membrane > bile canalicular membrane > erythrocyte membrane), the absolute amount of phospholipid extracted per mg of protein also falls. Some degree of membrane specificity in phospholipid solubilization has also been observed by other workers. Yousef & Fisher (1976) reported that, at bile-salt concentrations producing more than 60% solubilization, the phospholipid from the contiguous membrane was more easily extracted than that of the bile-canalicular membrane from rat liver, and Coleman *et al.* (1980) have shown that the patterns of phospholipid solubilization from erythrocyte membranes of different species are quite distinct. The relative amounts of individual phospholipids in bile are very different from those of the bile-canalicular membrane (Yousef *et al.*, 1977); the type of phospholipid which is solubilized in our system *in vitro* requires further investigation.

The results of incubation studies *in vitro*, such as we

have described in the present paper, in which single bile salts are permitted to solubilize lipid from an isolated membrane, are difficult to extrapolate to the situation *in vivo*, since bile contains not only a mixture of bile salts but also bile pigments and specific proteins such as immunoglobulins (Orlans *et al.*, 1983). Solubilization *in vitro* removes lipid from the membrane which is not replenished, in contrast with the situation *in vivo*, where a steady state probably exists in which lipid removal from the membranes is balanced by a flow of newly synthesized lipid from within the cells (Barnwell *et al.*, 1984). Moreover, although the bile-salt concentration in hepatic bile is well documented, its concentration in the canaliculus is unknown. Nevertheless, under conditions that eliminated the conversion of cholate into DC by intestinal bacteria, some studies *in vivo* (Ponz de Leon *et al.*, 1981; Loria *et al.*, 1982) have demonstrated that biliary cholesterol output decreased in the order DC, CDC, cholate, UDC (i.e. in the order of decreasing hydrophobicity), confirming our own observations *in vitro*. Unfortunately the possible contribution of phospholipid secretion was not examined in these studies *in vivo*. At low bile-salt output, Einarsson & Grundy (1980) have shown an increased biliary cholesterol/phospholipid ratio in patients on cholate therapy but not on CDC therapy, a finding in keeping with those of our studies *in vitro*. Furthermore, von Bergmann (1983) has reported raised phospholipid secretion during CDC and UDC therapy, although other workers (LaRusso *et al.*, 1975) have been unable to show such an increase.

Our own studies have demonstrated the ability of both UDC and CDC (*a*) to solubilize phospholipid at bile-acid concentrations lower than those required to solubilize cholesterol, and (*b*) to solubilize cholesterol less efficiently than phospholipid at all concentrations, and are thus consistent with the data of Hardison & Apter (1972), who suggested that, in the intact animal, the availability of phospholipid limits biliary cholesterol secretion.

The results support a micellar mechanism for biliary lipid secretion, whereby bile salts extract phospholipid and cholesterol from the bile-canalicular membrane once they have reached the canaliculus (Coleman *et al.*, 1977). In this context it is very relevant to note that, in liver-perfusion studies (Barnwell *et al.*, 1984), bile-salt molecules are secreted from the hepatocyte unaccompanied by cholesterol and phospholipid.

We thank the Cell Surface Research Fund and Weddel Pharmaceuticals for financial support, Mr. R. Bird for expert technical assistance and Dr. S. Knutton for his kind help with the electron microscopy. We also acknowledge Gipharmex for generously providing conjugated bile acids.

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Received 12 June 1986/28 October 1986; accepted 24 November 1986