Effect of diosgenin on biliary cholesterol transport in the rat

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Biliary cholesterol output in rats was stimulated over 3-fold by feeding diosgenin for 5 days, whereas biliary outputs of phospholipid and bile salts were not changed by diosgenin feeding. Isolating and perfusing the liver without bile salts resulted in a rapid and substantial decrease in biliary bile salt output; bile salt depletion abolished the diosgenin-induced increment in biliary cholesterol output, showing that the diosgenin-elevated biliary cholesterol output was bile-salt-dependent. Diosgenin treatment also produced a significant decrease in biliary alkaline phosphodiesterase I. Fresh bile obtained from control and diosgenin-fed rats was subjected to gel-permeation chromatography in order to separate different-sized biliary cholesterol carriers. Two major peaks of cholesterol were eluted, with cholesterol also being eluted between the peaks. The cholesterol peak eluted at the lower molecular mass (20–30 kDa) was observed in all bile samples. The higher-molecular-mass peak, which was eluted at the void volume, was not observed in all biles; control biles contained very little high-molecular-mass form of cholesterol, whereas biles from the diosgenin group contained up to 47% of cholesterol in the high-molecular-mass fraction. Diosgenin treatment produced a range of elevated biliary cholesterol values which positively correlated with the proportion of cholesterol contained in the high-molecular-mass fraction ($r = 0.98$). The results show that diosgenin induced a marked bile-salt-dependent increase in biliary cholesterol output and a shift in biliary cholesterol transport to higher-molecular-mass structures.

INTRODUCTION

The liver plays a prominent role in cholesterol metabolism, displaying functional polarity and compartmentalization. In the hepatocyte, cholesterol is both taken up and secreted across the sinusoidal membrane in numerous lipoprotein forms (Glickman and Sabesin, 1988). In addition, cholesterol is synthesized within the hepatocyte and secreted across the canalicular membrane either directly or after conversion into bile salts (Dietschy and Spady, 1984; Hofmann, 1990). The degree of segregation of these various processes and of targeting towards specific fates is incompletely understood.

The movement of cholesterol into bile is generally coupled to the secretion of bile salts and phospholipid (Lowe et al., 1984; Coleman and Rahman, 1992), although marked species differences have been observed with regard to biliary cholesterol secretion and saturation (Turley and Dietschy, 1988). To what extent these variations in cholesterol secretion represent different mechanisms of control is unknown.

The control and integration of biliary cholesterol secretion is crucial, since an increase in biliary cholesterol relative to bile salts and phospholipid, leading to cholesterol supersaturation, is a prerequisite to cholesterol gallstone formation (Holzbach, 1990). Differential solubilization of secreted biliary cholesterol is also an important factor in the etiology of cholesterol gallstones. Biliary cholesterol has been shown to be solubilized in several forms, including large cholesterol/phospholipid vesicles and lamellae as well as smaller mixed micelles of bile salts and phospholipid (Sömjen et al., 1990a). The distribution of cholesterol between these carriers may be important, since it has been suggested that the bile-salt-poor cholesterol/phospholipid vesicles are involved in gallstone formation (Sömjen and Gilat, 1986; Halpern et al., 1986; Harvey et al., 1987; Holzbach, 1990).

We have used diosgenin to investigate the nature of cholesterol secretion and solubilization in rat bile during normal and elevated cholesterol output. Diosgenin, a plant-derived sapogenin structurally similar to cholesterol, has been shown to decrease cholesterol absorption and to increase biliary cholesterol secretion without altering either serum cholesterol or total biliary bile-salt secretion (Cayen and Dvornik, 1979; Nervi et al., 1988). Although diosgenin administration is associated with increased activity of hepatic 3-hydroxy-3-methylglutaryl-CoA reductase and with inhibition of hepatic cholesterol esterification, the relative contributions of these effects to the increase in biliary cholesterol secretion remain to be established (Cayen and Dvornik, 1979; Nervi et al., 1984). In the present study, variations in hepatic cholesterol handling have been assessed with regard to the coupling of biliary cholesterol output to bile salt secretion and the distribution of cholesterol between biliary carriers during normal and elevated cholesterol output.

EXPERIMENTAL

Materials

Chemicals

All chemicals were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.), except for [^{3}H]cholesterol (43.7 Ci/mmol; Amersham International, Amersham, Bucks., U.K.), Sephacryl 300HR (Pharmacia, Uppsala, Sweden), Ecoscint A (National Diagnostics, Aylesbury, Bucks, U.K.) and pentobarbitone (Sagatal; RMB Animal Health Ltd., Dagenham, U.K.). Plastic cannula tubing was obtained from Portex Ltd. (Hythe, Kent, U.K.).

Animals

Experiments were performed on male Wistar rats weighing approx. 250 g. Animals were allowed free access to water and standard laboratory diet in powdered form. Groups of rats

Abbreviation used: AP I, alkaline phosphodiesterase I.
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received 1% (w/w) diosgenin incorporated into the diet for 5 days (isolated liver perfusion) or 7 days (gel-permeation chromatography). Diosgenin was dissolved in chloroform, mixed with the diet and the solvent was evaporated at room temperature for 24 h under a hood. Corresponding control groups did not receive diosgenin.

Methods

Isolated perfused liver

Rats were anaesthetized with pentobarbitone (6 mg in 0.1 ml/100 g body wt., intraperitoneally) and their bile ducts were cannulated with pp10 tubing. Bile was collected immediately. After 20 min the liver was isolated *in situ* and perfused with a recirculating medium consisting of supplemented Krebs–Ringer bicarbonate buffer (Barnwell et al., 1983b) containing 30% (v/v) sheep erythrocytes. Bile was collected from perfused livers for 2 h in 30 min portions and stored at −20 °C pending analysis of lipids (cholesterol, bile salts and phospholipid).

In an additional series, isolated livers from control and diosgenin-treated rats were perfused with the bile salt sodium taurocholate at a rate of 960 nmol/min. Bile was collected from perfused livers for 1 h, in 15 min portions, and stored at −20 °C pending analysis of lipids.

Gel-permeation chromatography

Rats were anaesthetized with pentobarbitone and their bile ducts were cannulated. A single bile sample was collected for 1 h, a sample of which was taken for the chromatography of fresh bile [the remainder of the bile sample was stored at −20 °C before analysis of alkaline phosphodiesterase I (AP I) and lipids]. For chromatography, samples of fresh bile were added to tubes containing [^{3}H]cholesterol (1.5 μCi/ml of bile) and equilibrated for 1 h at 37 °C with gentle shaking. A 250 μl portion of bile containing [^{3}H]cholesterol was then subjected to chromatographic separation on a Sephacryl S-300 HR column (1.6 cm × 60 cm) with a flow rate of 1 ml/min. Successive 2 min (ml) fractions were collected and analysed for lipid and enzyme either immediately or after storage at −20 °C. The elution buffer contained 50 mM Tris/HCl (pH 8), 1.5 mM EDTA, 150 mM NaCl and 10 mM sodium cholate (Sömjen and Gilat, 1985). The void volume (53 ml) was determined with Blue Dextran (2×10^3 kDa) and the column was calibrated with BSA (66 kDa), ovalbumin (45 kDa) and α-chymotrypsin (25 kDa).

Measurements

Biliary cholesterol was determined by g.l.c. (Rahman and Coleman, 1986). Phospholipid was determined by the method of Bartlett (1959) after lipid extraction (Bligh and Dyer, 1959). Total bile salts were determined with hydroxysteroid dehydrogenase (Coleman et al., 1979). The plasma-membrane enzyme AP I (EC 3.1.4.1) was assayed as described by Godfrey et al. (1981). Units of enzyme activity are μmol of substrate hydrolysed/h at 37 °C. [^{3}H]Cholesterol was measured in Ecoscint A by liquid-scintillation counting.

Statistics

Results are expressed as means ± S.E.M. The significance of differences between groups was assessed by Student's t test.

RESULTS

Effect of bile salt depletion on biliary lipid outputs

Biliary output values are expressed per g of liver, the liver weights not being significantly different between the groups: control ($n = 6$) 12.00 ± 0.84 g, diosgenin ($n = 5$) 11.58 ± 0.42 g (means ± S.E.M.).

In the initial period before isolated perfusion, when the liver was receiving bile salts from the circulation, bile flow, phospholipid output and total bile salt output were not different between diosgenin and control groups. In contrast, biliary cholesterol output was over 3-fold higher in diosgenin-treated rats (Table 1).

In isolated livers perfused without bile salts, biliary bile salt concentration rapidly declined from around 30 mM to 3 mM during the first 1 h. Subsequently, bile salt output remained constant at a basal (endogenous synthesis) level for the 1–2 h period of perfusion. Values are therefore presented for the isolated perfused liver comprising this 1–2 h period.

Depleting the liver of bile salts resulted in a marked decline in bile flow and biliary lipid output in both control and diosgenin groups. Bile salt depletion also abolished the increased cholesterol output associated with diosgenin treatment (Table 1).

Effect of bile salt infusion on biliary lipid outputs

Biliary output values are expressed per g of liver, the liver weights not being significantly different between the groups: control ($n = 5$) 10.12 ± 0.72 g, diosgenin ($n = 4$) 11.17 ± 0.61 g (means ± S.E.M.).

Table 1 Effect of diosgenin pre-treatment on biliary output from isolated liver perfused without bile salts

Biliary fistula (pre-perfusion) samples were collected before conversion into isolated liver perfusion. Biliary outputs are expressed per g of liver and values are means ± S.E.M. Significant difference between diosgenin group ($n = 5$) and corresponding control group ($n = 6$) is indicated by * ($P < 0.05$).

	Pre-perfusion		Isolated perfused liver	
	Control	Diosgenin	Control	Diosgenin
Bile flow (μl/min per g)	2.03 ± 0.27	2.52 ± 0.23	0.93 ± 0.14	1.32 ± 0.14
Biliary bile salt output (nmol/min per g)	69.37 ± 14.08	73.64 ± 3.53	3.67 ± 0.64	3.72 ± 0.68
Biliary cholesterol output (nmol/min per g)	1.09 ± 0.13	3.67 ± 0.83*	0.142 ± 0.030	0.167 ± 0.033
Biliary phospholipid output (nmol/min per g)	9.81 ± 1.56	8.99 ± 0.78	0.799 ± 0.131	0.564 ± 0.132

Table 2 Effect of diosgenin pre-treatment on biliary output from isolated liver perfused with bile salts

Sodium taurocholate was continuously infused at a rate of 960 nmol/min into isolated perfused livers. Biliary outputs are expressed per g of liver and values are means ± S.E.M. Significant difference between diosgenin group ($n = 4$) and control group ($n = 5$) is indicated by * ($P < 0.05$).

	Control	Diosgenin
Bile flow (μl/min per g)	1.56 ± 0.16	2.23 ± 0.31
Biliary bile salt output (nmol/min per g)	73.6 ± 10.0	86.9 ± 6.7
Biliary cholesterol output (nmol/min per g)	0.57 ± 0.12	2.85 ± 0.92*
Biliary phospholipid output (nmol/min per g)	6.06 ± 0.97	7.90 ± 1.00

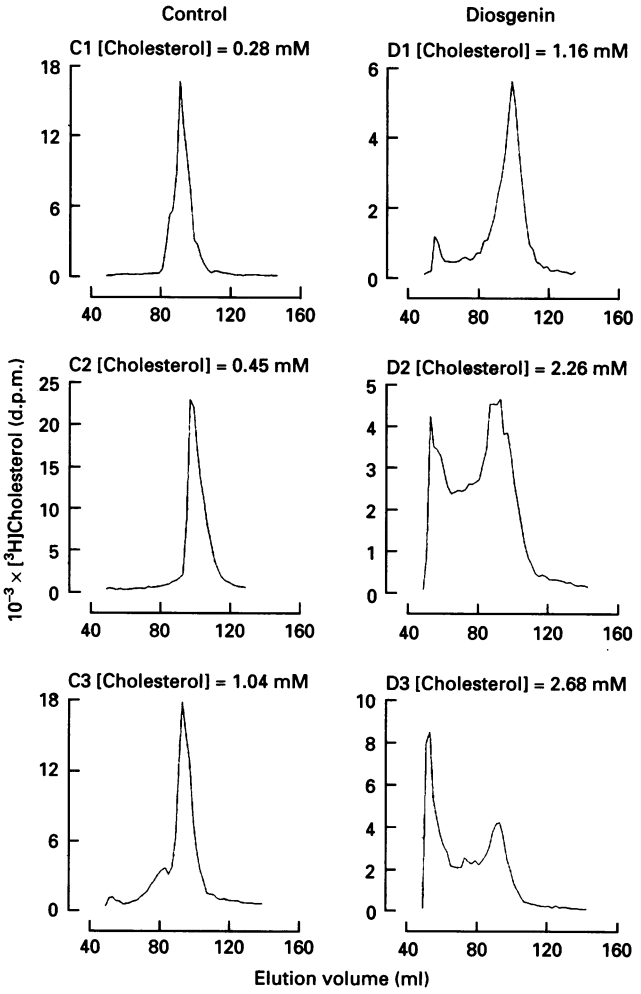

Figure 1 Gel-permeation elution profiles of [³H]cholesterol

Fresh bile, collected from control (C) or diosgenin-fed (D) rats, was equilibrated with [³H]cholesterol before Sephacryl S-300HR separation. Samples were eluted with Tris/saline buffer containing 10 mM sodium cholate. [Cholesterol] values refer to cholesterol concentration of original bile samples.

After an initial equilibration period (15 min), biliary lipid outputs from isolated perfused livers remained constant during the period of perfusion. Values are therefore presented for this 15–60 min period (Table 2). The results show that bile flow,

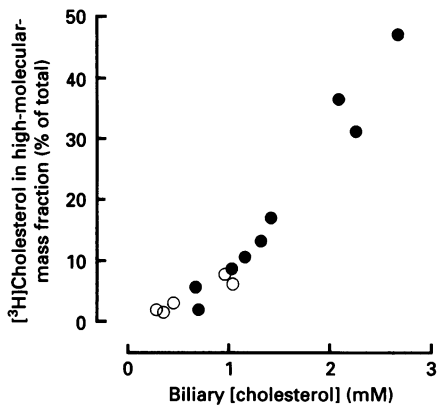

Figure 2 Relationship between biliary cholesterol concentration and cholesterol distribution after gel-permeation chromatography

Fresh bile, collected from control (open circles) or diosgenin-fed (closed circles) rats, was equilibrated with [³H]cholesterol before Sephacryl S-300HR separation. Samples were eluted with Tris/saline buffer containing 10 mM sodium cholate. The high-molecular-mass fraction was derived from the 46–66 ml elution volume and expressed as a percentage of the total (46–126 ml) elution volume.

phospholipid output and total bile salt output were not different between diosgenin and control groups, whereas biliary cholesterol output was over 4-fold higher in the diosgenin group.

The livers remained healthy during the period of perfusion, as assessed by the gross appearance, oxygen uptake and the very low activity of the cytosolic enzyme aspartate aminotransferase in the perfusate and bile (Barnwell et al., 1983b).

Gel-permeation chromatography

Elution profiles were obtained for biliary lipids and the enzyme AP I. Figure 1 shows examples of elution profiles for [³H]cholesterol, which demonstrate the principal features: either one peak or two peaks were obtained, with the early peak occurring at the void volume and the later peak occurring close to the chymotrypsin elution volume (corresponding to 20–30 kDa). Variable quantities of cholesterol were also eluted between the peaks. The presence and size of the early peak was closely associated with the cholesterol concentration in the original bile sample (Figures 1 and 2). Thus bile samples with cholesterol concentrations less than 0.9 mM did not produce an early peak, whereas biles of greater cholesterol concentration yielded an early as well as a later peak. Additionally, the higher the biliary cholesterol concentration, the greater the size of the early cholesterol peak relative to the second (Figures 1 and 2). Most early peaks and the largest early peaks were observed in the diosgenin group, where biliary cholesterol concentrations were highest [cholesterol concentration for control ($n = 5$) was 0.62 ± 0.16 mM, and for diosgenin ($n = 9$) 1.48 ± 0.24 mM]. Interestingly, a small early peak was obtained from two of five control bile elutions; these biles also showed the highest cholesterol concentrations in their group (approx. 1 mM). In contrast, two of nine biles from the diosgenin group showed no early peak; these two biles contained the lowest cholesterol concentrations in their group (0.7 mM).

All biles were eluted with buffer containing 10 mM cholate, but, in addition, several biles were also eluted with 5 mM cholate from columns equilibrated with 5 mM cholate, in order to assess the effect of cholate concentration. There was a small but consistent difference between biles eluted with 5 mM compared with 10 mM cholate: for any given bile, the first cholesterol peak

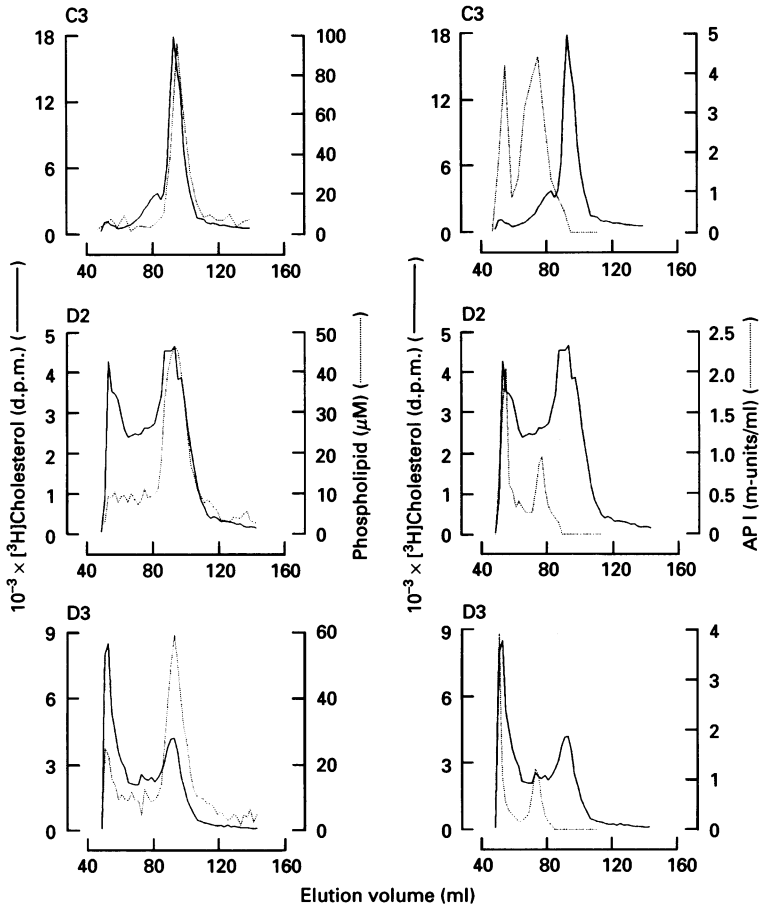

Figure 3 Gel-permeation elution profiles of phospholipid, AP I and [^{3}H]cholesterol

Fresh bile, collected from control (C) or diosgenin-fed (D) rats, was equilibrated with [^{3}H]cholesterol before Sephacryl S-300HR separation. Samples were eluted with Tris/saline buffer containing 10 mM sodium cholate.

was always larger and the second peak was smaller and shifted to the left (higher molecular mass) with 5 mM cholate. The principal features, however, remained the same with either 5 mM or 10 mM cholate elution, namely the absence of an early cholesterol peak at low biliary cholesterol concentration and a positive correlation between the proportion of cholesterol in the early peak and the biliary cholesterol concentration.

Baseline phospholipid values were sufficiently low as to be difficult to determine accurately; however, for all biles, a phospholipid peak was co-eluted with the later cholesterol peak (Figure 3). Similarly, an early peak of phospholipid was not detected in the absence of an early cholesterol peak. Thus phospholipid and cholesterol tended to be co-eluted, although where clearly detectable early peaks occurred, the cholesterol/phospholipid ratio was always higher in the earlier peak compared with the later peak (Figure 3).

The enzyme AP I was eluted in two peaks (Figure 3), the early one of which corresponded to the void volume and the later peak occurred before the elution volume of BSA (equivalent to approx. 80 kDa).

AP I was also measured in samples of bile that had not been chromatographed. The secretion rate (m-units/min) was significantly lower ($P < 0.05$) in the diosgenin group (24.7 ± 3.3, $n = 9$) than the control group (74.6 ± 18.7, $n = 5$). To test further the nature of the decreased AP I activity in diosgenin-treated rats, bile samples were incubated in cholesterol-coated tubes to produce biles identical except for cholesterol content. It was found that cholesterol concentrations over the range 0.2–1.2 mM did not alter AP I activity in bile.

DISCUSSION

The isolated perfused liver provides a means for rapidly depleting the liver of bile salts by eliminating the primary source of these bile salts, i.e. the enterohepatic circulation. This technique enabled us to study the coupling of cholesterol to bile salt secretion during both normal (control) and elevated (diosgenin) cholesterol output. The present finding that bile salt depletion led to an abolition of the elevated cholesterol output observed in the diosgenin group when compared with the control group suggests that bile salts are necessary for the elevated cholesterol secretion. It is known that absence of bile salts from the perfusion fluid causes a decrease in biliary cholesterol and phospholipids (Barnwell et al., 1983a; Rahman and Coleman, 1986). Addition of bile salts increases the output of biliary lipids resulting from either pulses (Rahman et al., 1986; Rahman and Coleman, 1987) or an extended perfusion (Rahman et al., 1986; Monte et al., 1990). The results of our bile-salt-infused liver perfusions are in agree-

ment with these findings and further show that biliary cholesterol output from diosgenin-treated rats is significantly higher than that of control rats. These results show that in the rat, where bile-salt-independent bile flow is relatively high, a bile-salt-independent mechanism is not involved in the secretion of elevated biliary cholesterol in diosgenin-treated animals. Therefore, although biliary cholesterol output is markedly increased with diosgenin, despite no change in total bile salt output, bile salts nevertheless play an essential permissive role in the secretion of this elevated cholesterol.

The precise nature of cholesterol secretion into bile has not been fully determined, but may involve the formation or exocytosis of phospholipid/cholesterol vesicles from the canalicular membrane (Coleman, 1987). Biliary lipid is not, however, derived indiscriminately from the canalicular membrane, since the cholesterol/phospholipid ratio and phospholipid profiles are different between the bile and canalicular membrane (Coleman and Rahman, 1992). It is likely, however, that an enriched biliary cholesterol output reflects an increased cholesterol density in at least part of the canalicular membrane. The present finding that AP I activity is decreased in diosgenin-treated rats might suggest a cholesterol-related change in the normal distribution or function of some membrane constituents destined for biliary excretion.

The inhibitory effect on AP I is unlikely to be due to an inhibition of membrane-bound enzyme activity, since the elution profile of AP I activity revealed separation from the cholesterol and phospholipid profiles. Taken together with the finding that a range of cholesterol concentrations did not alter AP I activity in bile, these data suggest that the decrease in AP I activity seen in the diosgenin group resulted from a decreased content of AP I rather than an inhibition of activity, although the cause of this effect is not apparent.

Separation of biliary lipid carriers by Sephacryl elution cannot be considered to be entirely non-perturbing (Sömjen and Gilat, 1985). Ideally each bile sample should be eluted from a column pre-equilibrated with bile salts identical in composition and concentration with those in the aqueous phase of that bile (Stone et al., 1992). However, such analysis would take many hours, which might allow changes to occur in cholesterol distribution in biles not at equilibrium at the time of collection. In practice, cholate at a concentration of 5–10 mM is commonly used (Kibe et al., 1984; Sömjen and Gilat, 1985; Stone et al., 1992). We found that the same principal findings were obtained with either 5 mM or 10 mM cholate, as described in the Results section.

The early cholesterol and phospholipid peaks obtained by Sephacryl separation are likely to be vesicular lipid carriers, whereas the later peaks are likely to be mixed micelles of bile salts, cholesterol and phospholipid (Sömjen and Gilat, 1985). The higher cholesterol/phospholipid ratio observed in the early lipid peak compared with the later peak would be in agreement with this interpretation (Sömjen and Gilat, 1985). Lipid also is eluted between the early and later peaks, which is of intermediate cholesterol/phospholipid ratio, and this could correspond to cholesterol-carrying phospholipid lamellae recently identified by Sömjen et al. (1990b) in human gall-bladder bile.

Cholesterol/phospholipid vesicles have been identified in human bile, where cholesterol saturation is usually higher than that occurring in rat bile (Sömjen and Gilat, 1985), and vesicular carriers of biliary cholesterol have also been identified in cholesterol-unsaturated rat bile (Cohen et al., 1989). Results from a study by Ulloa et al. (1987) implied that cholesterol/phospholipid vesicles occurred in cholesterol-unsaturated rat bile and identified such vesicles in cholesterol-saturated rat bile produced by bile salt depletion. In addition, Stone et al. (1992) demonstrated a cholesterol shift from micellar to vesicular peak when the cholesterol saturation was increased via bile salt depletion. In the present study we have demonstrated a similar shift of cholesterol from lower- to higher-molecular-mass forms when cholesterol saturation was increased by elevating biliary cholesterol concentration without bile salt depletion.

Cholesterol saturation has been shown to have a similar effect in model bile systems (Sömjen et al., 1991), where increasing cholesterol saturation in the presence of controlled bile salt and phospholipid contents results in the formation of high-molecular-mass lamellar stacks. In the more complex situation of human bile containing a wide range of bile salt and phospholipid concentrations, some studies have found no correlation between the proportion of cholesterol in the vesicular fraction and the cholesterol saturation index of the bile (Sömjen and Gilat, 1985; Schrier and Jüngst, 1989), although a positive correlation was noted by Peled et al. (1988). These findings suggest that the ability of cholesterol saturation to influence the prevalence of specific lipid structures in bile is modulated by various factors, including biliary lipid concentrations and proportions (Sömjen and Gilat, 1986). Compared with the wide range of bile salt and phospholipid concentrations observed in human hepatic and gallbladder bile, the values obtained in the current study of rat hepatic bile were relatively uniform. Under these conditions (bile salt concn. 37.3 ± 1.7 mM; phospholipid concn. 4.24 ± 0.17 mM) we have used diosgenin feeding to show that the proportion of cholesterol contained in the high-molecular-mass fraction correlates positively with both biliary cholesterol concentration ($r = 0.98$, $P < 0.001$) and biliary cholesterol molar percentage ($r = 0.93$, $P < 0.001$).

In summary, in agreement with other studies, diosgenin markedly increased biliary cholesterol output, but was without effect on the total biliary output of bile salts and phospholipids. Although no concomitant increase in biliary bile salt output occurred, we have nevertheless demonstrated that the increase in cholesterol secretion is entirely bile-salt-dependent and does not occur at very low bile salt secretion rates.

Diosgenin treatment also decreased the biliary activity of the enzyme AP I, which does not appear to have resulted from a cholesterol-induced inhibition of activity.

Finally, we have shown that the solubility and transport of biliary cholesterol are substantially influenced by diosgenin-induced increases in biliary cholesterol output. Thus, at low cholesterol concentrations, biliary cholesterol was carried entirely in low-molecular-mass aggregates, whereas with increased cholesterol concentration proportionally more cholesterol was carried in higher-molecular-mass cholesterol/phospholipid aggregates.

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