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Selective biliary lipid secretion at low bile-salt-output rates in the isolated perfused rat liver

Effects of phalloidin

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At high bile-salt-secretion rates the biliary secretion of phospolipids and cholesterol is dependent on that of the bile salts. However, at low bile-salt outputs some secretion remains. Isolated perfused rat livers were used in these experiments in order to study the bile-salt-independent secretion of biliary lipids. The livers were isolated and saline (0.9% NaCl), or phalloidin dissolved in saline, was added to the perfusion fluid after 1 h of liver isolation. The concentration and output of cholesterol was significantly decreased in phalloidin-treated livers compared with the controls, whereas there was no significant decrease in phospholipids; the secretion of cholesterol and phospholipids can thus be uncoupled from each other by the action of phalloidin. These experiments suggest that a proportion of cholesterol gets into bile independently of bile salts and phospholipids. These findings are discussed in relation to the supersaturation of some biles with cholesterol and its relationship to the bile-salt-independent fraction of cholesterol.

INTRODUCTION

The interrelationships of bile salts, phospholipids and cholesterol during their secretion into bile are not fully understood. One factor that influences both biliary cholesterol and biliary phospholipid secretion is the secretion of bile salts; that is, with increasing bile salt secretion, there is increasing secretion of cholesterol and phospholipid (Hardison & Apter, 1972; Wheeler & King, 1972; Rahman *et al.*, 1986). At high bile-salt-secretion rates the biliary secretion of phospholipids and cholesterol generally appears to be coincident with that of the bile satls (Hardison & Apter, 1972; Redinger & Small, 1972; Wheeler & King, 1972); however, rapid kinetic studies of the sequence of appearance of materials into rat bile have recently shown that the secretion of bile salts is followed by that of phospholipids and cholesterol (Lowe *et al.*, 1984).

When the hepatic influx of bile salts is low, as reported in man and animals with an interrupted enterohepatic circulation (Thureborn, 1962; Balint *et al.*, 1971), there is an associated decrease in the secretion of biliary lipids. However, the decrease in phospholipid secretion exceeds that of cholesterol, so that the resulting bile has a high cholesterol concentration relative to that of phosphatidylcholine and bile salts. This combination of events has been associated in man with cholesterol-gallstone formation (Admirand & Small, 1968; Vlahcevic *et al.*, 1970; Bell *et al.*, 1973).

Hardison & Apter (1972) have demonstrated (in rats) that, at low bile-salt output, relatively more cholesterol and less phospholipid are secreted. An analogous situation has been noted in the monkey (Small, 1968) during interruption of the enterohepatic circulation, when bile-salt excretion is minimal. In both these species and in man (Small, 1968), bile was almost nearly saturated and supersaturated with cholesterol during low bile-salt-excretion rates. Thus, at such low bile-salt-excretion rates, either cholesterol transport and synthesis is increased or small amounts of cholesterol are getting into bile by some other mechanism.

Previous studies have also shown that alterations in the secretion of either cholesterol or phospholipid can be produced independently of one another (Linscheer *et al.*, 1974; Northfield *et al.*, 1975), further suggesting the possibility of a variable relationship in the excretion of cholesterol and phospholipids.

The role of the cytoskeletal and contractile organelles, microtubules and microfilaments, in bile formation and on biliary lipid secretion has been only partially elucidated. Dubin *et al.* (1980) have shown that phalloidin, which causes irreversible polymerization of actin into microfilaments when administered to rats, caused a decrease in the excretion of bile-acid load. Also, phalloidin, when administered to biliary-fistula rats (50 μg/100 g body wt.) for 7 days, induced a decrease in biliary cholesterol, whereas the concentration of bile salt and biliary phospholipid remained unchanged (Dubin & Erlinger, 1980).

The present study was designed to investigate the bile-salt-independent biliary output of phospholipid and cholesterol in the isolated perfused rat liver. Phalloidin was used in order to obtain a better insight into the secretion of biliary lipids.

EXPERIMENTAL

Materials

Phalloidin and hydroxysteroid dehydrogenase (grade II, from *Pseudomonas testosteroni*) were obtained from Sigma (London) Chemical Co., Poole, Dorset, U.K. Sagatal was obtained from May and Baker, Dagenham, Essex, U.K. Heparin was obtained from C.P. Pharmaceuticals, London E.C.1, U.K. All other reagents were from Fisons, Loughborough, Leics, U.K., and were of the highest grade available. Plastic cannula tubing (PP10) was obtained from Portex, Hythe, Kent, U.K.

Methods

Male Wistar rats, weighing 240–280 g, were used throughout. These had been maintained on a standard laboratory diet and under a constant light/dark cycle. The bile ducts of animals under pentobarbitone (Sagatal) anesthesia were cannulated with PP10 tubing and their livers were then isolated *in situ* (Hems *et al.*, 1966). Liver anoxia was minimized (5–10 s) by commencing perfusion immediately, at a constant flow rate of 16 ml·min^{-1}, with 150 ml of Krebs–Ringer bicarbonate buffer, pH 7.4 (Krebs & Henseleit, 1932); this buffer also contained 2 mM-$CaCl_2$, 5 mM-glucose, 1% (w/v) bovine serum albumin, a physiological amino acid mixture (see Barnwell *et al.*, 1983) and 20% (v/v) of packed human red blood cells. This solution was recycled, gassed continuously with O_2/CO_2 (19:1, v/v) and maintained at 37 ± 0.5 °C within a thermostatically controlled cabinet similar to that recommended by Collins & Skibba (1980).

Bile was collected in preweighed tubes on ice at 60 min intervals for 3 h. After the first 60 min of isolation, the perfusion fluid was changed to fresh medium to replace substrates used by the liver. At the same time phalloidin, when used (dissolved at 0.5 mg/ml in 0.15 M-saline) at a dose of 0.20 mg/250 g of rat (this dose was arrived at from a preliminary concentration curve) was added with complete mixing to the perfusion fluid and bile collected for another 2×60 min. Samples of perfusion medium were obtained at 60 min and 180 min and were centrifuged at 400 ***g*** for 5 min to remove red blood cells. After completion of the perfusion the livers were weighed, a sample of liver taken for electron microscopy and liver homogenates made. The volume of bile was determined gravimetrically, a density of 1 g/ml being assumed. All bile samples, homogenates and samples of centrifuged perfusion fluid were stored at −20 °C until required for analysis.

Chemical determinaitons

Phospholipid present in bile was determined by the method of Bartlett (1959) after lipid extraction by the method of Bligh & Dyer (1959).

Cholesterol was analysed as trimethylsilyl ether derivatives essentially as described by Vanlerenberghe & Cassaigne (1968). This method was used because of the small quantity of cholesterol present in bile and in order to prevent loss of parent compound on the column; also, low cholesterol cannot be detected satisfactorily by other methods. Bile (up to 0.15 ml) was placed in a glass-stoppered centrifuge tube and diluted with 0.8 ml of 80% (v/v) ethanol. This solution was extracted twice with 3.0 ml of light petroleum (b.p. 40–60 °C) and the combined light-petroleum extracts were evaporated to dryness in vials containing 1 nmol of 5α-cholestane as an internal standard. Trimethylsilyl ethers were made by adding 0.05 ml of pyridine/hexamethyldisilazane/trichlorosilane (9:3:1, by vol.). A 2 μl portion of this solution was used for g.l.c. The g.l.c. system used was a Pye–Unicam series-304 chromatograph equipped with a flame-ionization detector. The column [152 cm (5 feet) × 3 mm] was 1.5% SE30 on diatonite CQ (80–100 mesh). Operating conditions were as follows: injection temperature, 250 °C; column temperature, 235 °C; detector temperature, 270 °C; carrier gas (N_2) flow rate, 50 ml/min. The cholesterol concentration was calculated by peak-area ratios of cholesterol to the internal standard, cholestane.

Bile-salt concentrations were determined with hydroxysteroid dehydrogenase (EC 1.1.1.1.50) as described by Coleman *et al.* (1979).

Electron microcopy

Liver tissue was fixed for 1 h in 2.5% (v/v) glutaraldehyde in 0.1 M-cacodylate buffer, pH 7.2. After being washed in cacodylate buffer, the tissue was fixed in 1% osmic acid in 0.1 M-cacodylate buffer for 1 h and then dehydrated in successive ethanol concentrations, transferred to propylene oxide and then embedded in epoxy resin. Thin sections were then counterstained for 1 h in aq. uranyl acetate and for 1–2 min in Reynolds lead citrate. The tissues were examined on a Philips 301 transmission electron microscope at 80 kV.

RESULTS

After isolation of the liver, bile was collected at three 60 min intervals, phalloidin or saline being added to the perfusion medium 60 min after liver isolation; the effects of phalloidin would therefore only be expected in the appropriate 60–120 and 120–180 min fractions (see Table 1).

Bile-salt output decreased in both sets of livers with time, owing to interruption of the enterohepatic circulation, and in the 120–180 min fractions probably represented bile salts mainly from synthesis *de novo*. The bile-salt output in the phalloidin-treated livers was appreciably, but not significantly, less than that in the controls.

Bile flow decreased with respect to time in the control livers largely owing to a decrease in bile-salt-dependent flow occasioned by loss of bile salts resulting from interruption of the enterohepatic circulation; the 120–180 min fraction is mainly a bile-salt-independent flow. In the phalloidin-treated livers the decline in flow between 60 and 120 min was similar to the control value, but the flow between 120 and 180 min was significantly less.

Bile phospholipid output and concentration decreased with decreasing bile-salt outputs in both groups of livers to approximately the same extent at all time points. Cholesterol output and concentration decreased similarly in control and phalloidin-treated groups up to 120 min, but both concentration and ouput were significantly lower in the 120–180 min fraction from phalloidin-treated livers than from corresponding controls.

The cholesterol/phospholipid ratio was therefore significantly decreased in the 120–180 min fraction from 0.142 to 0.042, suggesting that phalloidin has a more specific effect upon cholesterol output than phospholipid output.

Phalloidin had no effect on liver weight in these experiments (Table 1), and electron micrographs of phalloidin-treated livers showed that bile cannaliculi appeared relatively normal, with intact microvilli and only a slight deposition of microfilaments around the bile canaliculus at 180 min. The cytoplasm also appeared to be similar to the control in respect of the morphology of mitochondria and other organelles. One striking difference between phalloidin-treated and control livers in the 120–180 min time interval was the larger number of lysosomes present in the phalloidin-treated livers (Fig. 1).

Table 1. Effect of phalloidin on biliary output in the isolated perfused rat liver

Bile was collected after liver isolation at 60 min intervals up to 180 min. Phalloidin (0.20 mg/250 g body wt. of rat) was added to the perfusion fluid after 60 min of liver isolation. Values are means ± S.E.M. for the number of observations given; significant differences from controls are indicated by *($P < 0.05$).

Parameter	Time of bile collection (min)...	Control ($n = 3$)			Phalloidin ($n = 4$)		
		0–60	60–120	120–180	0–60	60–120	120–180
Bile (μl/min)		13.29 ± 0.14	9.58 ± 1.64	7.99 ± 0.88	12.46 ± 0.89	9.58 ± 0.49	*3.64 ± 0.54
Bile salt							
Concn. (mM)		4.01 ± 0.75	2.72 ± 0.67	3.44 ± 0.69	3.49 ± 0.61	1.83 ± 0.21	2.40 ± 0.21
Output (nmol/min)		53.36 ± 10.46	27.46 ± 10.03	28.30 ± 7.61	44.76 ± 9.82	17.43 ± 1.68	8.69 ± 1.42
Phospholipid							
Concn. (mM)		1.01 ± 0.22	0.34 ± 0.08	0.26 ± 0.09	1.25 ± 0.03	0.37 ± 0.05	0.29 ± 0.07
Output (nmol/min)		13.48 ± 3.01	3.09 ± 0.60	2.22 ± 1.01	15.61 ± 1.45	3.51 ± 0.47	1.13 ± 0.43
Cholesterol							
Concn. (mM)		0.101 ± 0.010	0.032 ± 0.010	0.037 ± 0.010	0.082 ± 0.040	0.03 ± 0.01	*0.013 ± 0.002
Output (nmol/min)		1.34 ± 0.10	0.32 ± 0.11	0.31 ± 0.10	1.05 ± 0.46	0.30 ± 0.10	*0.05 ± 0.01
Cholesterol/phospholipid ratio (mol/mol)		0.100 ± 0.014	0.095 ± 0.031	0.142 ± 0.030	0.065 ± 0.028	0.082 ± 0.023	*0.045 ± 0.016
Liver weight (g)			11.06 ± 0.30			11.09 ± 0.18	

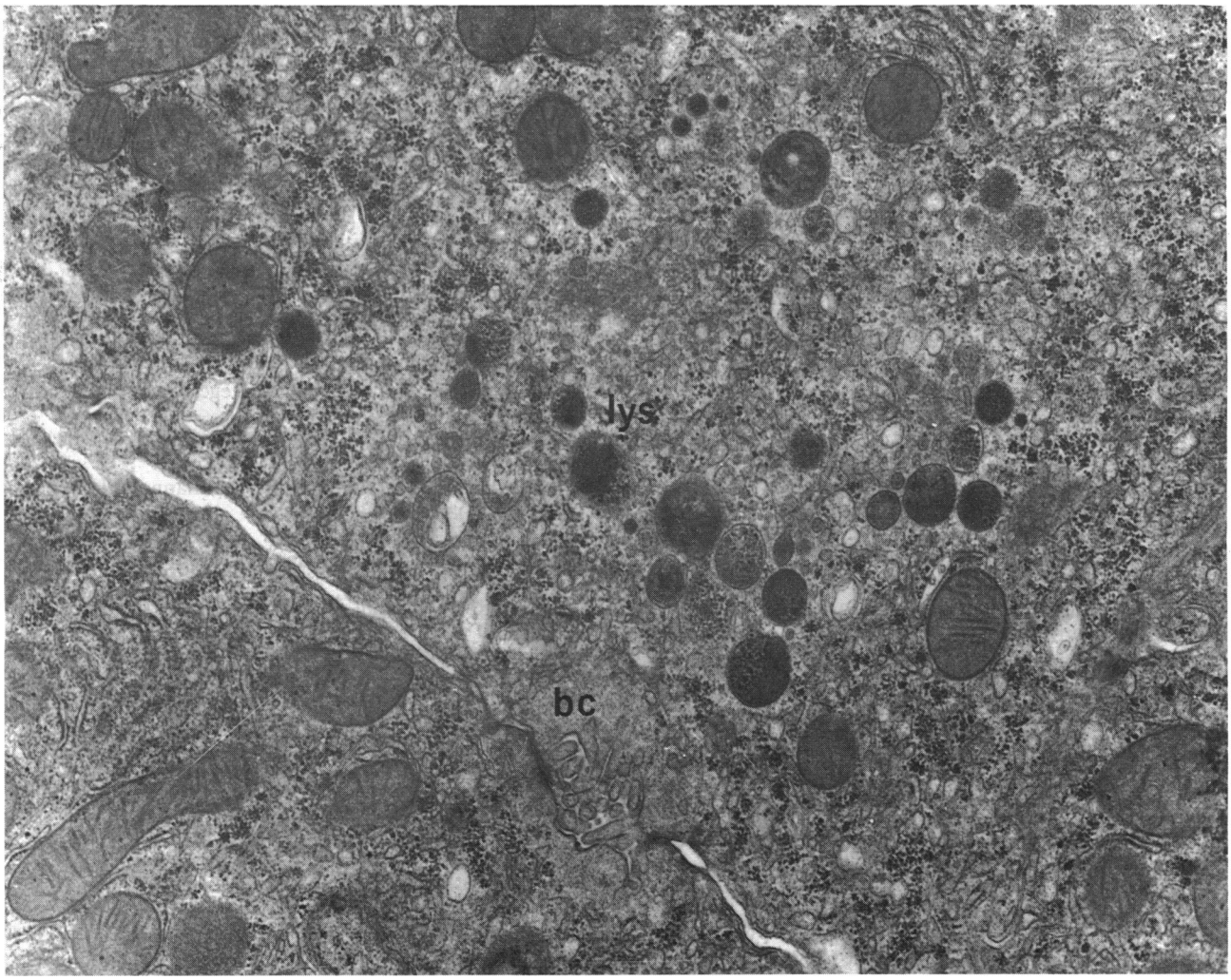

Fig. 1. Transmission electron micrograph of an example of hepatocytes from phalloidin-treated livers

The sample was taken from a liver that had been exposed to phalloidin for 2 h (i.e. after 120–180 min bile sample). There is some deposition of microfilaments around the bile canaliculus (bc), but there is still a good complement of microvilli. A number of lysosomes (lys) are present close to the bile canaliculus. Magnification × 20700.

DISCUSSION

Compared with controls, there appears to be little observable effect of phalloidin until the 120–180 min fraction. At this time, bile-salt output was at its lowest, and therefore most of the phenomena observed are probably bile-salt-independent.

Decrease in bile flow in phalloidin-treated biliary-fistula rats (a combination of bile-salt-independent and -dependent flows) has been noted by Dubin *et al.* (1978) and by Dubin & Erlinger (1980). The effect noted here is largely on bile-salt-independent flow and is of a more rapid onset (60 min) and at a lower phalloidin exposure than in these biliary-fistula rats, demonstrating the usefulness of the perfused liver system.

Hardison & Apter (1972) and Wagner *et al.* (1976) have described a bile-salt-independent output of cholesterol in which cholesterol appears to be secreted into bile without accompanying equivalent amounts of phospholipids. The effects of phalloidin in selectively depressing cholesterol output at low bile-salt outputs in the present experiments confirm that some of the cholesterol is entering the bile by a mechanism separate from that by which phospholipids and bile salts enter. A selective depression of cholesterol secretion relative to phospholipid, caused by chronic phalloidin administration, can be seen in the experiments of Dubin & Erlinger (1980), but these experiments relate to higher bile-salt-secretion rates and are therefore less able to separate effects on bile-salt-dependent from -independent rates.

The way in which phalloidin suppresses biliary cholesterol output is unknown. Four mechanisms are possible: (i) decreased hepatic cholesterol uptake from plasma (unlikely in the present experiments, since isolated perfused rat livers use used), (ii) decreased heptocellular cholesterol synthesis, (iii) decreased delivery of cholesterol to the canalicular membrane and (iv) decreased extraction from the canalicular membrane. It is unlikely (although possible) that phalloidin could substantially affect cholesterol synthesis in 1 h. A more plausible explanation is that phalloidin decreases delivery of cholesterol to, and across, the canalicular membrane, or extraction from it. Cholesterol may be transported in vesicles similar to those employed in the transport of phospholipids (Rahman *et al.*, 1986). Another possible mode of delivery of cholesterol is via lysosomes. This is suggested by the electron micrograph of phalloidin-treated livers (Fig. 1). A large number of vesicles/lysosomes are visible around the bile canaliculus, and these could be enriched in cholesterol derived from the catabolism of low-density lipoprotein. Movement of these could have been blocked as a result of microfilament dysfunction around the bile canaliculus caused by phalloidin; phalloidin causes irreversible polymerization of actin into microfilaments. A third possibility is that phalloidin induces changes in the ultrastructure of the bile canalicular membrane that inhibit the extraction of cholesterol by bile salts in the canalicular lumen.

It is very likely that the bile-salt-independent cholesterol (which appears to enter bile by a pathway separate from that by which bile salt and phospholipid enter) plays an important part in cholesterol saturation of bile; a better understanding and control of this bile-salt-independent biliary cholesterol transport might lead a better understanding and management of cholelithiasis.

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