Rapid kinetic analysis of the bile-salt-dependent secretion of phospholipid, cholesterol and a plasma-membrane enzyme into bile

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Isolated rat livers were perfused under 'one-pass' conditions and bile was collected at 1 min intervals. After 1 min pulse, taurocholate appeared in the collected bile within 2 min, peak output occurring 2 min later. In contrast, the increased output of phospholipids and cholesterol was slower, peak output occurring 6–11 min after the original pulse of taurocholate. These results suggest that mixed micelles cannot be formed inside the cell or during passage of bile salts through the membrane, since bile salt and lipids should then parallel each other. The bile salts must therefore be pumped into the lumen and the lipids added subsequently, due to the actions of the bile salts in the canalicular lumen. It is suggested that the biliary lipid is obtained from microdomains of biliary-type lipid in the canalculus membrane, which are vesiculated and solubilized by the action of bile salts. It is also suggested that this biliary-type lipid is brought continuously to the membrane via vesicle traffic; this traffic is increased during increased bile-salt output, and is a process that can be inhibited by colchicine.

At physiological rates of output the secretion of phospholipid and cholesterol into bile is dependent on the secretion of bile salts in an approximately linear relationship (Hoffman *et al.*, 1975; Delage *et al.*, 1976; Poupon *et al.*, 1976; Sewell *et al.*, 1979; Casu *et al.*, 1981; Sama *et al.*, 1982); this relationship is not seen, however, at very low or very high secretion rates of bile salts (Hardison & Apter, 1972). The characteristics of the bile-salt-associated lipid secretion are dependent on the physical properties of the bile salt. Thus the more hydrophobic bile salts, such as chenodeoxycholate, deoxycholate and their conjugates, stimulate biliary lipid secretion at lower biliary-bile-salt concentrations than do the more hydrophilic bile salts such as cholate and ursodeoxycholate and their conjugates (Montet & Gerolami, 1978; Schersten & Linblad, 1979; Barnwell *et al.*, 1983a,b). Non-micelle-forming bile salts, such as taurodehydrocholate, do not appear to be able to provoke biliary lipid output (Hardison & Apter, 1972; Barnwell *et al.*, 1984).

Micelle-forming bile salts also stimulate the secretion of several hepatocyte plasma-membrane enzymes into bile (Javitt, 1965; Bode *et al.*, 1973; Layden & Poppe, 1977; Reichen *et al.*, 1979; Fisher *et al.*, 1982; Hatoff & Hardison, 1982;

Barnwell *et al.*, 1983a,b); an exception to this is tauroursodeoxycholate, which, although stimulating biliary lipid output, did not increase plasma-membrane-enzyme secretion (Barnwell *et al.*, 1983b). The non-micelle-forming bile salt taurodehydrocholate is also unable to provoke plasma-membrane-enzyme output (Barnwell *et al.*, 1983a).

In bile, under physiological conditions, the biliary lipids exist as mixed micelles with the bile salts. The actual site of interaction of bile salts with the lipids is, however, not known. Bile salts could form mixed micelles with lipids either: (i) before entry into the canalculus (see Forker, 1977), (ii) during passage through the canalculus membrane (Small, 1970), or (iii) subsequent to entry into the bile canalculus (Coleman *et al.*, 1977). Circumstantial evidence exists for the third hypothesis in that: (a) taurodehydrocholate can be secreted without accompanying phospholipids (Hardison & Apter, 1972; Barnwell *et al.*, 1984), (b) colchicine decreases lipid output without affecting bile-salt or plasma-membrane-enzyme concentration into the bile (Barnwell *et al.*, 1984), and (c) model studies on intact cells show lipid release from plasma membranes with externally added bile salts (Coleman *et al.*, 1977; Billington & Coleman, 1978; Billington *et al.*, 1980). None of these, however, are directly comparable with the normal physiological conditions.

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The present paper describes the collection and analysis of short (1 min) bile collections from the isolated perfused rat liver under 'one-pass' perfusion conditions. In this way it has been possible to investigate the effect of a short pulse of the micelle-forming bile-salt, taurocholate, on the biliary output of phospholipid, cholesterol and a plasma-membrane enzyme, alkaline phosphodiesterase I. The results indicate that the output of bile salts is the primary event and precedes the output of the lipids, whereas the output of the plasma-membrane enzyme alkaline phosphodiesterase I is more closely coincident with the bile-salt output profile.

Materials and methods

Materials

The following fine-grade chemicals were obtained: taurocholate (Calbiochem-Behring Corp., Bishops Cleeve, Herts., U.K.), [^{14}C]taurocholate (Amersham International, Amersham, Bucks., U.K.), heparin (Weddel Pharmaceuticals, London E.C.1, U.K.), Sagatal (May and Baker, Dagenham, Essex, U.K.). Other fine-grade chemicals were obtained from Sigma (London) Chemical Co., Poole, Dorset, U.K. The assay kit for cholesterol was obtained from Boehringer Corp. (London), Lewes, East Sussex, U.K., and the plastic cannula tubing (PP10 and PP25) was obtained from Portex, Hythe, Kent, U.K.

Methods

Isolated perfused livers. Male Wistar rats, weighing 245–255 g, were used throughout; these had been maintained on a standard laboratory diet and under a constant light cycle. The bile ducts of animals under pentobarbital (Sagatal) anaesthesia were cannulated with 500 mm of PP10 tubing, and their livers were isolated *in situ* (Hems *et al.*, 1966). Liver anoxia was minimized (5–10 s) by commencing perfusion immediately, at a constant flow rate of $15\text{ ml}\cdot\text{min}^{-1}$, 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.*, 1983a) and 40% (v/v) of packed human red blood cells. This solution was recycled, gassed continuously with O_2/CO_2 (19:1) and maintained at $37 \pm 0.5^\circ\text{C}$ within a thermostatically controlled cabinet similar to that recommended by Collins & Skibba (1980).

For the initial 90 min of the perfusion, the isolated liver received bile-salt-free perfusion medium; during this period the output of bile salts, lipids and alkaline phosphodiesterase I declined to the levels indicated by the 0–5 min sample in Fig. 1 (below). The background endogenous bile-salt

secretion was $24 \pm 4\text{ nmol}\cdot\text{min}^{-1}$ ($2.0 \pm 0.2\text{ mM}$) at this time. The effect of perfusing with a 40% (v/v) haematocrit, as opposed to the 10% (v/v) haematocrit used previously, was to increase the basal bile flow after the bile-salt pool had been depleted, from $8.1 \pm 0.5\text{ }\mu\text{l}\cdot\text{min}^{-1}$ (Barnwell *et al.*, 1983a,b, 1984) to $12.2 \pm 0.7\text{ }\mu\text{l}\cdot\text{min}^{-1}$ (0–5 min, Fig. 1 below), similar to the effect observed by Thompsen & Larsen (1983).

After 90 min the liver was converted to a 'one-pass' perfusion of fresh perfusate, and a basal bile sample collected (0–5 min). After 5 min of perfusion with bile-salt-free medium, 5000 nmol of taurocholate, containing $0.1\text{ }\mu\text{Ci}$ of [^{14}C]taurocholate, were infused into the hepatic portal cannula in 60 s. Collections of bile and perfusate were taken into preweighed tubes at 1 min intervals during the 5–25 min period, and then subsequently at 5 min intervals for the remainder of the experiment (25–35 min). Bile and perfusate flows were determined gravimetrically by assuming a density of $1\text{ g}\cdot\text{ml}^{-1}$ (bile) or $1.06\text{ g}\cdot\text{ml}^{-1}$ (perfusion fluid). Samples of bile and perfusate supernatant (erythrocytes were removed by centrifugation for $10000\text{ g}\cdot\text{min}$) were stored at -20°C until required for analysis.

Assays. Bile was assayed for phospholipid after lipid extraction by the method of Bligh & Dyer (1959), with phospholipid phosphorus determination by the method of Bartlett (1959). The amount of free cholesterol in bile was determined by using an assay kit based on cholesterol oxidase. Bile-salt concentrations in the 0–5 min samples were determined with hydroxysteroid dehydrogenase (EC 1.1.1.150) as described by Coleman *et al.* (1979). The concentration of taurocholate in the remaining samples was determined by comparing the radioactivity in the bile with the specific radioactivity of the taurocholate injected into the liver.

The plasma-membrane enzyme alkaline phosphodiesterase I (EC 3.1.4.1), was determined as described by Godfrey *et al.* (1981), except that the assay was performed in a total volume of 0.75 ml, and was monitored continuously in a spectrophotometer at 37°C . Enzyme units are μmol of substrate hydrolysed/h at 37°C .

The 1 min bile collections were assayed for taurocholate and phospholipid; the remainders of the samples were then pooled to make 2 min samples before determination of the content of cholesterol and alkaline phosphodiesterase I.

Results

The effect of taurocholate on biliary secretion was determined by the administration of the bile salt into the hepatic portal cannula 5 min after establishing the 'one-pass' liver perfusion;

5000nmol was introduced in 60s, achieving an average concentration of 0.3mM in the perfusate entering the portal vein. Examination of the perfusate leaving the liver showed that $95 \pm 2\%$ (S.E.M., $n = 3$) of the injected taurocholate had been taken up. After 30min, 69% of the injected dose, or 73% of the absorbed dose, had been secreted into the bile.

The effects of the acute administration of taurocholate on bile flow, bile-salt secretion rate, and rates of secretion of phospholipid, cholesterol and alkaline phosphodiesterase I are shown in Fig. 1.

After the injection of taurocholate (at 5–6min), the bile flow rose from the basal level of $12.2 \pm 0.7 \mu\text{l} \cdot \text{min}^{-1}$ to $22.6 \pm 0.8 \mu\text{l} \cdot \text{min}^{-1}$ in the 7–

Fig. 1. Effect of taurocholate administration on the biliary secretion of phospholipid, cholesterol and alkaline phosphodiesterase I

For details of liver perfusion and bile collections, see the Materials and methods section. The values on the graphs are plotted at the midpoint of each collection period, and are means $\pm$ S.E.M., where $n = 12$ for values up to 14 min and $n = 4$ for samples from 14 to 35 min. The hatched area (▨) indicates the time of the taurocholate injection. ●, Amounts of biliary component (left axis); ○, output of taurocholate (right axis).

8 min sample. Since this increase in bile flow was probably caused by the taurocholate entering the bile canaliculus, this indicates that the transport of taurocholate across the hepatocyte occurred within 2 min. The choleresis induced by the taurocholate lasted for 8 min, whereupon the bile flow returned to the basal level. The peak secretion of taurocholate occurred in the 9–10 min bile sample, when 574 ± 14 nmol of taurocholate (at 34 mM) had been secreted; this was 25 times the basal rate of secretion of bile salt, as seen in the 0–5 min sample. The peak secretion of taurocholate occurred 4 min after injection, 2 min after peak bile flow, showing that the time taken for the bile salts to move from the bile canaliculus to the collection tube was 2 min.

The secretion of phospholipid was increased from 23 ± 11 nmol·min⁻¹ to 42 ± 3 nmol·min⁻¹ by taurocholate (Fig. 1), the peak secretion rate occurring in the period 12–16 min. The overall profile of the phospholipid response was very different from that of the taurocholate output curve. The rise from basal phospholipid secretion occurred over a longer period than for taurocholate, with the decline in output of phospholipid occurring about 6 min after the decline in taurocholate output. These phenomena indicated that, although the increased output of phospholipid was caused by the increased output of taurocholate, the phospholipid was not secreted with the bile salt.

The response of cholesterol to taurocholate secretion was similar to that of phospholipid (Fig. 1). There was a small peak in cholesterol secretion in the 7–9 min sample, coincident with the peak choleresis; this was similar to the minor peak in the phospholipid-output curve, and was probably caused by the appearance of phospholipid and cholesterol already present in the biliary system before the injection of taurocholate. Peak cholesterol secretion occurred in the 15–17 min bile sample, when 5.3 ± 0.5 nmol of cholesterol·min⁻¹ was secreted. As with phospholipid, the rise in cholesterol output was slower than that of taurocholate, with the decline in cholesterol output occurring about 10–12 min after the decrease in taurocholate output; the difference between the time of peak output of cholesterol compared with that of phospholipid was not statistically significant.

The increased secretion of taurocholate also stimulated the secretion of the hepatocyte plasma-membrane enzyme alkaline phosphodiesterase I. Unlike the response of the lipids, the increase in alkaline phosphodiesterase I was rapid, with peak secretion virtually coincident with peak taurocholate output. The stimulation of the output of this enzyme was about 4-fold over basal (0–5 min sample) levels, unlike the secretion of the lipids,

which were only increased 2-fold. In addition to a rapid rise in output, the decline in the enzyme levels in the bile was also relatively rapid, occurring about 2 min after the decline in taurocholate.

The results indicated that, although the secretion of lipids and a plasma-membrane enzyme into bile was stimulated by the acute injection of taurocholate, the response of each component was different both in relation to each other and in relation to the secretion of the bile salt.

Discussion

The transport of bile salts from perfusate into bile is very rapid. Taurocholate began to appear in collected bile within 2 min and reached peak concentration by 4 min. Taking into account the volume of fluid in the biliary tract which must be pushed ahead of the newly forming bile, the time taken for the bile salt to cross the hepatocyte and enter the canaliculus is 2 min or less.

The biliary lipids, however, appear more slowly, with peak output reached 3–7 min after the peak of bile-salt output. This clearly indicates that the mixed micelles cannot be formed inside the cell or during passage through the membrane, since, if this were so, the peaks of bile salt and biliary lipids should be coincident.

This dissociation of bile-salt secretion from biliary lipid secretion is supported by experiments with the vesicle transport inhibitor colchicine (Gregory *et al.*, 1978; Erlinger *et al.*, 1980; Barnwell *et al.*, 1984), and with taurodehydrocholate (Hardison & Apter, 1972; Barnwell *et al.*, 1984). These demonstrate bile-salt secretion in the absence (or with reduced output) of biliary lipids (see Barnwell *et al.*, 1984).

In the present experiments, the appearance of taurocholate in bile before the biliary lipids must therefore indicate that mixed-micelle formation occurs in the bile canaliculus lumen after the bile salt has been pumped out of the cell. When bile salts are added to intact isolated cells, release of membrane components occurs without the release of intracellular (cytosolic) materials (Coleman *et al.*, 1977, 1979, 1980; Billington & Coleman, 1978; Billington *et al.*, 1980; Lowe & Coleman, 1981). This supports the suggestion that lipids might be removed from the membrane of the bile canaliculus by the action of bile salts within the canaliculus lumen.

The bile-salt-dependent release of components into bile *in vivo* is, however, highly specific: the composition of bile does not reflect the composition of the bile-canalicular membrane (Evans *et al.*, 1976). Bile contains 80–95% phosphatidylcholine (Swell *et al.*, 1968a,b) of a specific fatty acyl

composition (Yousef *et al.*, 1975; Gregory *et al.*, 1975; Kawamoto *et al.*, 1980), whereas the bile-canalicular membrane contains only 33% phosphatidylcholine, together with 25% sphingomyelin and 40% other phospholipids (Kremmer *et al.*, 1976).

Thus mechanisms must exist whereby specific biliary lipid components are transported to the bile-canalicular membrane and are secreted into bile in a bile-salt-dependent manner. The output of biliary lipid is inhibited by colchicine (Gregory *et al.*, 1978; Erlinger *et al.*, 1980; Barnwell *et al.*, 1984), indicating that the transport of bile-directed lipid is likely to be mediated by vesicles. The fusion of such vesicles with the bile-canalicular membrane would result in the presentation to the bile of microdomains of biliary-type lipid (Fig. 2).

The results presented here indicate a delay between the appearance of the bile salt in the canalicular lumen and the extraction of lipid into the bile. There are two points at which this delay could occur, either (i) before fusion of the biliary lipid vesicles with the canalicular membrane (Fig. 2, process A), or (ii) in the processing of the membrane by the bile salt (Fig. 2, process B or C). Which of these alternatives is correct, however, cannot be resolved from the present data.

There are two possible mechanisms whereby the biliary lipid could be released into the bile from the canalicular membrane. One possibility is the direct extraction by bile salts of outer-leaflet lipid (Fig. 2, process B) (although this presents problems with

an increase in inner-leaflet lipids, unless these are subsequently removed, or flip to the outer leaflet). The other possibility is the removal of lipid by outward vesiculation and subsequent reorganization into mixed micelles (Fig. 2, process C), although both processes may act at the same time.

Bile-salt-induced vesiculation of plasma membranes can be observed with erythrocytes (Billington & Coleman, 1978; Coleman *et al.*, 1980), hepatocytes (Billington *et al.*, 1980) and thymocytes (Ferber *et al.*, 1980). In these model systems, vesiculation occurs from very fluid membranes, but not from ordered membranes (Coleman *et al.*, 1980); thus, if regions of biliary-type lipid, which would be very fluid owing to being mainly shorter-chain-length unsaturated phosphatidylcholines, were present in the otherwise-ordered bile-canalicular membrane (Lowe & Coleman, 1982), these regions should vesiculate preferentially. It has previously been shown that, when exocytic vesicles fuse with plasma membranes, the lipids and proteins of the two membranes do not always intermix (Meldolesi & Ceccarelli, 1981; Orci *et al.*, 1981; Steinman *et al.*, 1983). Coated pits are also thought to possess highly fluid membranes, different from the bulk plasma membrane (Bretscher, 1976; Montesano *et al.*, 1979).

The situation with the hepatocyte plasma-membrane enzyme alkaline phosphodiesterase I, is different from that of the lipids. The output profile parallels that of the taurocholate much more

Fig. 2. Possible mechanisms for the secretion of lipid into bile

Bile salt (BS) is secreted via a transport protein. Vesicles, composed of biliary-type lipid, are moved to and fuse with the bile-canalicular membrane by a process that involves the cytoskeleton. The microdomain of lipid thus produced persists until the action of the bile salts, already present in the lumen of the canalculus, causes the release of the membrane components. Processes A, B and C are discussed in the text.

closely, although there is a significant delay in the decline in output. Thus it follows that this plasma-membrane enzyme is probably already present in the membrane of the bile canaliculus before the secretion of taurocholate, and is then removed by the bile salt entering the canaliculus, possibly by a vesiculation process (see Bouchier, 1971; Billington & Coleman, 1978); only low concentrations of bile salt are required to initiate this vesiculation process involving membrane proteins (Coleman *et al.*, 1980).

Experiments tracing the transcellular vesicle transport of horseradish peroxidase (Renston *et al.*, 1983) and polymeric immunoglobulin A (Birbeck *et al.*, 1979; Mullock & Hinton, 1981) have shown that maximum output of these components occurs 40 min after injection into the portal vein. Thus the increased output of lipid and plasma-membrane enzyme is unlikely to originate, in the short term, from the sinusoidal plasma membrane. The source of the biliary lipid in the present experiments is thus most likely to be from within the hepatocyte, perhaps originating from the endoplasmic reticulum (Yousef *et al.*, 1975; Gregory *et al.*, 1975; Kawamoto *et al.*, 1980), possibly from a class of vesicles destined specifically for bile.

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