Mechanism of Secretion of Biliary Lipids

I. ROLE OF BILE CANALICULAR AND MICROSOMAL MEMBRANES IN THE SYNTHESIS AND TRANSPORT OF BILIARY LECITHIN AND CHOLESTEROL

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ABSTRACT The role of bile canalicular and microsomal membranes in the synthesis and transport of biliary lipids was investigated by using the isolated perfused rat liver model. Labeled lecithin precursors ([^{3}H]-palmitic acid, [^{14}C]linoleic acid, [^{3}H]choline, and $^{32}PO_4$) and a cholesterol precursor ([^{3}H]mevalonic acid) were administered with and without sodium taurocholate. The incorporation pattern of these labeled precursors into linoleyl and arachidonyl lecithins and cholesterol fractions of microsomes, bile canaliculi, and bile were examined at 30-min intervals up to 90 min. Marker enzymes and electron microscopy indicated that isolated subfractions of plasma membranes were enriched with bile canaliculi (< 10% microsomal contamination). Taurocholate significantly stimulated the incorporation of $^{32}PO_4$, [^{3}H]choline, [^{3}H]palmitic acid, and [^{14}C]linoleic acid into linoleyl and arachidonyl lecithin with parallel incorporation curves for microsomal and bile canalicular membranes throughout the 90-min study period. During the 30–60-min period, however, these same lecithin fractions in bile significantly exceeded the specific activity of the membrane lecithins. The enzyme CDP-choline diglyceride transferase was virtually absent from canaliculi relative to microsomes, indicating that canaliculi lack the capacity for *de novo* lecithin synthesis. Incorporation of [^{3}H]mevalonic acid into membranous and biliary cholesterol followed a pattern similar to that for lecithin. These data provide evidence that (*a*) biliary lecithin and cholesterol are derived from a microsomal subpool regulated by the flux of enterohepatic bile acids, (*b*) the role of the bile canalicular membranes with respect to biliary lipids is primarily transport rather than synthesis, and (*c*) lecithin and cholesterol are transported together from microsomes to bile. The findings are consistent with the existence of a cytoplasmic lipid complex within the hepatocyte which is actively involved in the intermembrane transport of biliary lipid.

INTRODUCTION

It is now recognized that bile salts play an important role in the regulation of biliary phospholipid and cholesterol secretion (1–7). When the hepatic influx of bile salts is low as reported in man and animals with an interrupted enterohepatic circuit (8, 9), there is an associated decrease in the secretion of biliary lipids. However, the reduction in phospholipid secretion exceeds cholesterol so that the resulting bile has a high cholesterol concentration relative to lecithin and bile salts. This combination of events has been associated with cholesterol gallstone formation (10–12). Conversely, when the hepatic influx of bile salts is increased as observed after the oral administration of bile salts (8, 13–15), biliary lipid secretion is enhanced and the resulting bile has reduced concentrations of cholesterol and lecithin relative to bile salts. While these observations support the concept of a homeostatic relationship between the enterohepatic flux of bile salts and the secretion of biliary lipids, the precise hepatocellular site where bile salts exert their effect on biliary lipid metabolism has not been identified and data are not yet available re-

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garding the mechanism by which bile salts increase biliary lipid secretion.

Biliary lecithin is composed mainly of the palmityl-linoleyl species which is synthesized in the liver via the CDP-choline diglyceride pathway (3, 8, 16, 17). However, the predominant hepatic species of lecithin is rich in arachidonic acid and is derived by transacylation or acyl exchange in a particular lecithin via the phosphatidyl ethanolamine pathway (17–21). The studies of Victoria, Van Golde, Hostetler, Scherphof, and Van Deenen (22) and Sarzala, Van Golde, De Kruyff, and Van Deenen (23) indicate that hepatic microsomes may be the only hepatic membrane with CDP-choline transferase activity, which is essential for *de novo* lecithin synthesis. Balint, Beeler, Kyriakides, and Treble (8) have postulated the existence of a microsomal pool of linoleyl lecithin destined for bile. On the other hand, the role of bile canalicular membranes with respect to biliary lecithin metabolism has not been investigated.

The present study critically examines the synthesis of lecithin and cholesterol in microsomal and canalicular membranes during the phase of active biliary lipid secretion. Labeled precursors were administered to the isolated perfused rat liver to label the choline, fatty acid, and phosphate moieties of the lecithin molecule while radioactive mevalonic acid was given as a cholesterol precursor. Bile canalicular membranes, microsomes, and bile were then examined for radioactive labeling of lecithin and cholesterol and for CDP-choline diglyceride transferase activity in an effort to define the role of the membranes with respect to biliary lipid metabolism. Additionally, data are presented which further elucidate the role of taurocholate on the synthesis and transport of the biliary micelle.

METHODS

Liver perfusion. Male Wistar rats weighing approximately 400 g maintained on Purina rat chow and tap H_2O ad libitum were used as liver donors and as a blood source. The common bile duct was cannulated with no. 10 PE tubing and the liver isolated surgically by a modification of a procedure described earlier (3, 24). A glass cannula was inserted into the portal vein and immediately attached to a reservoir containing an oxygenated solution of 5% red blood cells in saline. A glass outflow cannula was then inserted into the vena cava. This procedure reduced the critical period of liver anoxia to less than 1 min. The liver was then removed, attached to the perfusion system, and perfused with Krebs-Ringer bicarbonate (pH 7.4 medium) containing 3% bovine albumin, 0.1% glucose, 0.3 ml heparin, and 25% washed rat red blood cells. The total perfusion volume was 100 ml and was continuously recirculated. The platform on which the liver rested was adjusted to give a hydrostatic head of pressure of 21 cm water. The flow rate through the liver was 60–75 ml per min. During the entire perfusion period the liver maintained a good appearance and bile production averaged 1–1½ ml/h. An initial 20-min perfusion period was used to establish uniform blood and bile flow rates before the start of the experiments. Two types of liver perfusion experiments were carried out. In one series of experiments a priming dose of 2 mg sodium taurocholate (Calbiochem, San Diego, Calif.) was added to the perfusate and then sodium taurocholate (10 mg/h, 1.6 mg/ml) was infused at a constant rate throughout the duration of the experiment. Previous studies have shown that the isolated perfused rat liver is in a steady state with respect to bile salt secretion of an infusion rate of 10 mg/h (3). In the other series of liver perfusions, saline was substituted for taurocholate. After a 90-min taurocholate or saline infusion period, the labeled precursors were added to the perfusion reservoir in 10 ml of perfusion medium. The precursors were 200 μCi of [9,10-^{3}H]palmitic acid, 50 μCi of [1-^{14}C]linoleic acid, 50 μCi of [*methyl*-^{3}H]choline, 500 μCi of [^{32}P]phosphate, and 100 mCi of [5-^{3}H]mevalonic acid. The labeled compounds were obtained from New England Nuclear, Boston, Mass. After the addition of the labeled precursors the livers were perfused for an additional 30, 60, or 90 min. The saline control livers (without taurocholate) were perfused for 60 min after the precursors were added. Bile samples were collected at 30-min intervals throughout the experiments before and after the addition of the labeled compounds. At the end of the perfusion period, the livers were weighed and immediately placed in chilled 1 mM bicarbonate buffer (pH 7.5) maintained at 2°C.

Membrane isolation. Bile canalicular membranes were isolated according to the procedure of Song, Rubin, Rifkind, and Kappas (25). Livers (weighing 10–12 g) were added to 10 vol of 1 mM bicarbonate and then homogenized with a Dounce homogenizer. The homogenate was centrifuged at 1,500 *g* for 10 min. Residue from this centrifugation contained debris, nuclei, and plasma membranes while the supernatant solution included mitochondria and microsomes. Sucrose was added to the supernatant solution to give a concentration of 0.25 M. This solution was then fractionated by ultracentrifugation into mitochondria (10,000 *g* for 30 min) and microsomes (104,000 *g* for 60 min). Microsomes were washed once with 0.25 M sucrose and centrifuged at 104,000 *g* for 30 min. Bile canalicular membranes were isolated from the residue of the first 1,500-g centrifugation on a discontinuous sucrose density gradient as outlined by Song et al. (25). The canalicular membranes (tight junction complexes) appeared in the upper 1.16 sucrose density layer and the remainder of the liver plasma membranes were localized in the 1.18 sucrose density fraction. Criteria for ascertaining the purity of the membrane fractions included the marker enzymes glucose-6-phosphatase (26), 5′-nucleotidase (27), and Mg^{++}-ATPase (28). The bile canalicular preparations were also examined by electron microscopy. Morphologic criteria for identifying intact bile canaliculi included the presence of adjacent plasma membranes adjoined by tight junctional complexes and associated with microvilli.

Other methods. The protein content of the membrane fractions was determined by the method of Lowry, Rosebrough, Farr, and Randall (29). The bile and liver membrane fractions were extracted with 20 vol of 2:1 chloroform-methanol and partitioned with 0.2 vol of water (30). Free cholesterol and phospholipids were isolated by silicic acid column chromatography (31) and the free cholesterol fraction assayed for mass by gas liquid chromatography (32). The cholesterol ^{3}H activity was determined by adding carrier-free cholesterol and precipitating the mixture as the digitonide. After dissolving the digitonide in methanol

the ^{3}H activity of the digitonide was determined by liquid scintillation counting.

Phospholipid fractions were separated into the major phospholipid classes by thin-layer chromatography on silica gel H impregnated with 1.0 mM sodium carbonate (33) with a solvent system of chloroform-methanol-acetic acid-water (50:25:8:4). Phospholipid standards were banded on either side of the plate. The thin-layer plate was sprayed with 2,7-dichlorofluorescein and the phospholipid zones were visualized under ultraviolet light. The phosphatidylcholine zone was scraped from the plate and eluted three times from the silica gel with 5 ml of 50:39:1:10 chloroform-methanol-acetic acid-water. The eluants were pooled and washed successively with 6 ml of water, 6 ml of NH_4OH, and 6 ml of 50% methanol as described by Arvidson (34). This washing procedure removed the 2,7-dichlorofluorescein dye and the phosphatidylcholine fraction was retained in the chloroform phase. The phosphatidylcholine fraction was then further fractionated into the major lecithin species by thin-layer chromatography on $AgNO_3$-impregnated plates and the system developed with 60:30:5 chloroform-methanol-water (34). Linoleyl- and arachidonyl-rich lecithin fractions were eluted three times from the silica gel with 50:39:1:10 chloroform-methanol-acetic acid-water. The eluants were pooled and washed successively with 6 ml water, 6 ml NH_4OH, and 6 ml 50% methanol (containing 0.5 NaCl). This washing procedure removed the 2,7-dichlorofluorescein dye and small amounts of residual silver ion. The thin-layer argentation procedure resolved the phosphatidylcholine fraction into three major zones based on the degree of fatty acid unsaturation of the phosphatidylcholines. These zones were rich in linoleic, arachidonic, and long-chain polyunsaturated fatty acids. Gas liquid chromatographic analysis (32) of these fractions indicated that the arachidonyl lecithin fraction was devoid of linoleic and other polyunsaturated fatty acids and that arachidonic acid was absent from the linoleyl fraction. The fraction rich in linoleyl lecithin contained 8–10% monounsaturated fatty acids. Phospholipid phosphorus was determined on the fractions by the method of Bartlett (35).

Aliquots of the fractions containing linoleyl lecithin were subjected to mild alkaline hydrolysis with 5% KOH at 75°C for 2 h to remove the fatty acid portion of the molecule. The hydrolysis mixture was then acidified to pH 1 with 7% HCl and extracted three times with diethyl ether. The labeled fatty acids ([^{3}H]palmitic and [^{14}C]linoleic) were extracted into the diethyl ether phase; the aqueous layer contained labeled glycerylphosphoryl choline ([^{32}P]- and [^{3}H]choline). The radioactivity of these fractions was then determined by liquid scintillation counting. The hydrolytic and isotopic separation techniques were validated by several types of experiments. Labeled phosphatidylcholine was prepared biosynthetically by administering labeled precursors ([^{3}H]palmitic and [^{14}C]linoleic or [^{3}H]choline) to isolated perfused rat livers and then isolating the microsomal fraction rich in linoleate. This gave two types of labeled phosphatidylcholines: one with [^{3}H]palmitic and [^{14}C]linoleic and the other with [^{3}H]choline. Gas liquid radiochromatography (32) of the methyl fatty acid esters obtained from the phosphatidylcholine fraction with the labeled fatty acids indicated that virtually all (97%) of the ^{3}H and ^{14}C activities were in palmitic and linoleic acids, respectively. The phosphatidylcholine fraction labeled with fatty acids was also used to check the hydrolytic procedure for quantitative recovery of the labeled fatty acids and for overlap of radioactivity between the diethyl ether and aqueous layers. Complete recovery of the radioactive fatty acids in the diethyl ether fraction was obtained and no fatty acid radioactivity was found in the aqueous layer. Also thin-layer chromatographs of the lipids in the diethyl ether phase showed only free fatty acids and no radioactive phosphatidylcholine. The phosphatidylcholine fraction labeled with [^{3}H]choline was used to check for recovery of glycerylphosphoryl choline radioactivity into the aqueous layer. After hydrolysis all of the ^{3}H activity was found in the aqueous layer and none in the diethyl ether phase. Radioactive recovery experiments were also carried out singly and with mixtures of $^{32}PO_4$ and [^{3}H]choline or with [^{3}H]-palmitic and [^{14}C]linoleic acids. The results also indicated quantitative recovery and separation of the isotopic labels. The radioactivity of the fatty acids (^{3}H and ^{14}C) and glycerylphosphoryl choline (^{32}P and ^{3}H) moieties of the phosphatidylcholine fraction was determined by liquid scintillation counting (Isocap 300, Searle Analytic Inc., Des Plaines, Ill.). External standardization (^{133}Ba) was used to correct for quench. The fatty acid fraction was counted in 10 ml of Liquifluor and the glycerylphosphoryl choline fraction (1 ml) in 15 ml Aquasol (New England Nuclear).

CDP-choline diglyceride transferase activity was determined according to the method of De Kruyff, Van Golde, and Van Deenen (21). The 1,2-diglyceride substrate was prepared from liver lecithin treated with phospholipase C (*Bacillus cereus*). The enzyme digest mixture contained 200 nmol of CDP-choline (^{14}C-methyl, New England Nuclear), 18 μmol Mg^{++}, 8 μmol glutathione, 0.4 mg Tween 20, 2 mg of 1,2-diglyceride, and 0.5–1 mg of membrane protein in tris buffer (pH 7.2) and was brought to a final volume of 1 ml. Incubation time was 20 min at 37°C. 10 vol of 2:1 chloroform-methanol was used to stop the reaction. The phospholipid fraction was isolated by silicic acid column chromatography (30) and assayed for ^{14}C activity.

CALCULATIONS

The specific activities of the phosphatidylcholines were calculated from the radioactivities ([^{32}P], [^{3}H]choline, [^{3}H]-palmitic, [^{14}C]linoleic) and the mass obtained from phospholipid phosphorous. The values are expressed as disintegrations per minute per micromole phosphatidylcholine. The microsomal and bile canalicular membrane fractions rich in linoleyl lecithin were found to have 10% less palmitic and 10% more stearic acids than biliary linoleyl lecithin. The specific activities of the linoleyl lecithin-rich fraction with respect to [^{3}H]palmitic was therefore corrected to a standard 50% palmitic acid to reflect comparability between the linoleyl lecithin fractions of the membranes and bile. This correction was valid since all of the fatty acid ^{3}H activity was associated with palmitic acid.

Data were analyzed by the paired *t* test to determine the significance of isotope incorporation into the various lipid and membrane fractions by the same liver. The group *t* test was used for comparing data from different group of livers (i.e., taurocholate-perfused livers vs. saline controls) (36).

RESULTS

Enzyme activities and electron microscopy of the liver cell membranes. The relative purity of each membrane fraction was determined by marker enyzme activity and electron microscopy. As shown in Table I, 5′-nucleotidase and Mg^{++}-dependent ATPase were found

TABLE I

Enzyme Activity Units of Liver Cell Membranes*

Enzymes	Activity	
	Microsomes	Biliary canaliculi
Glucose-6-phosphatase	83±16	9±1
5′-Nucleotidase	50±8	516±125
Mg^{++}-ATPase	37±5	444±96
CDP-CDT		
Preparation (1)	1.46	0.008
(2)	2.52	0.007

* Represents the average±SD of cell preparation. Each preparation was a pool of three livers with all assays performed in duplicate. Enzymatic activity for glucose-6-phosphatase, 5′-nucleotidase, and Mg^{++}-ATPase is expressed as nanomoles P per milligram protein per minute. Enzymatic activity for CDP-choline 1,2-diglyceride transferase (CDT) expressed as nanomoles CDP-choline incorporated into lecithin per milligram protein per minute.

almost exclusively within the canalicular membrane fraction. As previously reported (25) these enzymes were concentrated primarily in the plasma membranes of the hepatocyte. The specific activity of glucose-6-phosphatase in microsomes was more than nine times higher than in bile canaliculi indicating that there was very little contamination of the canalicular preparation with microsomal membranes. Also shown in Table I is the fractional distribution of CDP-choline diglyceride transferase activity. Relative to microsomes, this enzyme was virtually nonexistent in bile canalicular membrane preparations. Comparison of the relative distribution patterns for CDP-choline diglyceride transferase with glucose-6-phosphatase (Table I) shows a higher relative activity of the latter in bile canaliculi. It would thus appear from these data that CDP-choline diglyceride transferase may be a more sensitive indicator for ascertaining the purity of canalicular membrane preparations. While these marker enzymes are helpful in distinguishing plasma from microsomal membranes, they are not specific for distinguishing the portion of plasma membranes which form bile canaliculi. Fig. 1*a* is a representative low-power electron photomicrograph of a typical bile canalicular preparation which contains a virtually homogeneous collection of intact bile canalicular membranes resembling their original *in situ* morphology in hepatocytes. Fig. 1*b* is a higher-power magnification of a typical intact bile canaliculus and clearly demonstrates membranous layers surrounded by characteristic fibrillar cytoplasm joined together to form a classical tight junction which then separates and extends into a microvillous pattern.

Lipid synthesis and transport. To establish the optimal conditions for studying the synthesis and secretion of biliary lecithin and cholesterol, sodium taurocholate was infused into the liver system for 90 min before the addition of the labeled precursors; the taurocholate infusion was then continued throughout the study periods. Without taurocholate, biliary lipid secretion was extremely low (phospholipid 0.2 μmol/h and cholesterol 0.05 μmol/h). Addition of taurocholate to the system resulted in the immediate appearance of phospholipid and cholesterol into bile. During a 90-min perfusion period the average secretion rate was 2 μmol/h for phospholipid and 0.2 μmol/h for cholesterol. These data confirm our earlier findings (3) and emphasize the necessity of bile salts for biliary lipid transport.

Fig. 2 shows the incorporation of [^{3}H]palmitic and [^{14}C]linoleic acids into linoleyl lecithin. Incorporation of [^{14}C]linoleic and [^{3}H]palmitic acids into linoleyl lecithin of the membranes was very rapid. The highest recorded specific activity was obtained in approximately 30 min. At that time the specific activity relationships with respect to [^{3}H]palmitic indicated that the linoleyl lecithin of microsomes had a significantly ($P < 0.01$) higher specific activity than linoleyl lecithin isolated from bile canalicular membranes. There were no significant differences in the [^{14}C]linoleic specific activities of linoleyl lecithin at 30 min. During the second study period (30–60 min) specific activities of [^{14}C]- and [^{3}H]-fatty acids from biliary linoleyl lecithin exceeded that of both microsomal and biliary canalicular membranes. These specific activity differences between bile and the membrane fractions were most pronounced by the last study period (60–90 min) for [^{3}H]palmitic ($P < 0.01$) and during the 30–60-min biliary collection period for [^{14}C]-linoleic ($P < 0.01$) at which time there was a significantly higher specific activity in bile than in the membranes.

Fig. 3 shows the incorporation of [^{3}H]choline into the linoleyl and arachidonyl lecithins of bile and the membrane fractions. The [^{3}H]choline was incorporated into linoleyl lecithin of microsomes, canaliculi, and bile from the same source more rapidly than it was into arachidonyl lecithin. There was a close parallel between the specific activity relationships of the lecithins from bile canaliculi and microsomal membranes throughout the 90-min period, with microsomal lecithin activity slightly exceeding the specific activity of the biliary canalicular lecithin. The very rapid attainment of similar lecithin specific activities in each of these membranes indicates a very rapid transfer of biliary lecithin from microsomal to canalicular membranes. The incorporation of [^{3}H]-choline into the linoleyl and arachidonyl lecithins of bile was markedly greater than the incorporation into the membranous fractions. By the 30–60-min period the specific activity of the biliary lecithins significantly ($P <$ 0.01) exceeded the specific activity of the membrane

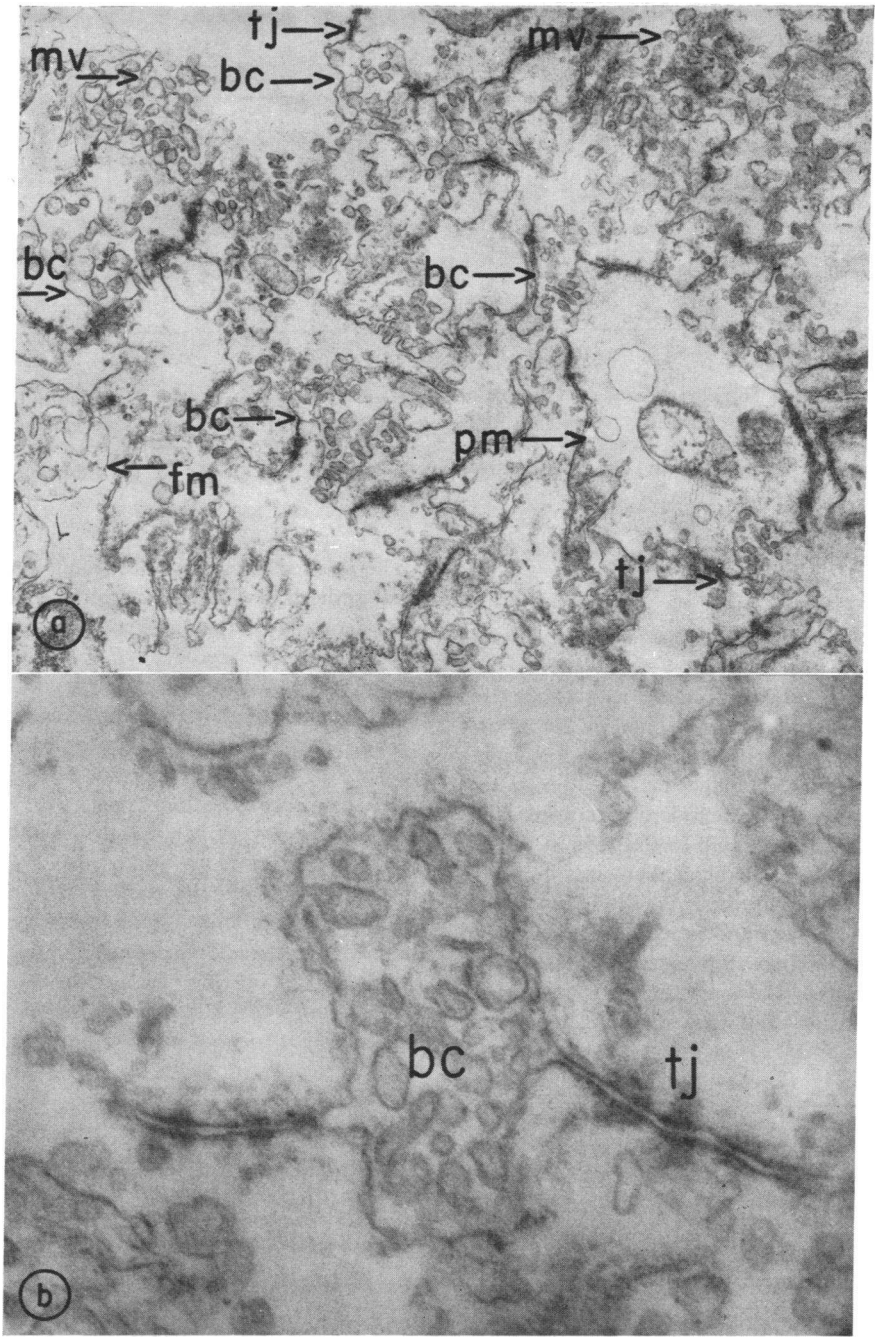

FIGURE 1 Representative electron micrographs of bile canaliculi-enriched liver fraction obtained by discontinuous gradients of sucrose. (*a*) × 10,000. (*b*) × 48,000. Intact bile canaliculi (bc) with microvilli (mv) and tight junctional complexes (tj) from adjoining plasma membranes (pm). Fragmented plasma and bile canalicular membranes (fm).

lecithins. This difference was even more significant during the final study period (60–90 min) ($P < 0.001$).

Fig. 4 illustrates the incorporation of [^{32}P]phosphate into linoleyl and arachidonyl lecithins of the membranes and bile. There was a greater incorporation of [^{32}P]-phosphate into microsomal linoleyl lecithin than arachidonyl lecithin. The pattern of incorporation of ^{32}P into the lecithin of the membranes and bile was markedly

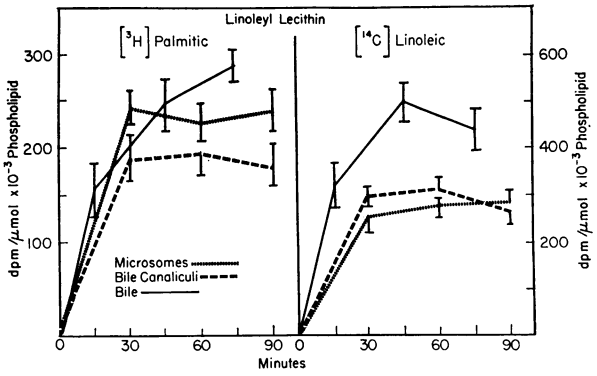

FIGURE 2 Incorporation of [^{3}H]palmitic and [^{14}C]linoleic acid into linoleyl lecithin of microsomes, bile canaliculi, and bile. Each point represents 7–9 perfused livers (±SE) per time period. Bile samples are plotted at the midpoint of each collection period.

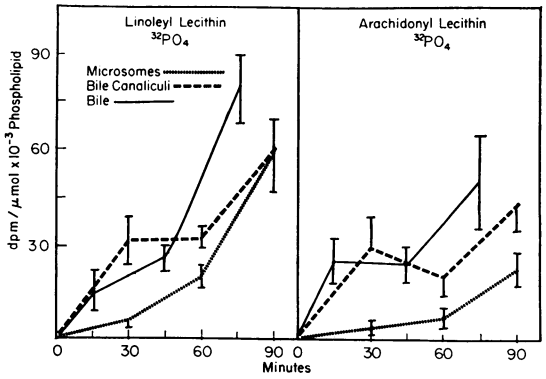

FIGURE 4 Incorporation of $^{32}PO_4$ into linoleyl and arachidonyl lecithin of microsomes, bile canaliculi, and bile. Each point represents 7–9 perfused livers (±SE) per time period. Bile samples are plotted at the midpoint of each collection period.

different than for the incorporation of the labeled fatty acids (Fig. 2) and choline (Fig. 3). Within 30 min of administering the ^{32}P precursors, the specific activities of the lecithin from the bile canalicular membranes significantly ($P < 0.02$) exceeded those from the microsomal fractions. At this time the linoleyl lecithin specific activity in bile was also higher than that in the microsomal fraction, but was less than that in the bile canalicular membrane fraction. The specific activities of microsomal ^{32}P in linoleyl and arachidonyl lecithins were similar at 30 min. After the rapid initial rise in the incorporation of ^{32}P into bile canaliculi there was a 30-min plateau period followed by a second rise in the incorporation of ^{32}P into both linoleyl and arachidonyl lecithin of the membranes. By the final study period (60–90 min) the ^{32}P specific activities of biliary linoleyl lecithin exceeded the ^{32}P specific activities of the membrane fractions.

The marked effect of taurocholate on microsomal lecithin synthesis is shown in Fig. 5. Taurocholate significantly stimulated the incorporation of $^{32}PO_4$ and [^{3}H]-choline into the linoleyl ($P < 0.05$) and arachidonyl ($P < 0.025$, 0.05) lecithin species at 60 min as compared to nontaurocholate-perfused controls (saline).

The incorporation of [^{3}H]mevalonic acid into cholesterol (Fig. 6) shows that the microsomal and biliary canalicular incorporation curves were virtually identical and indicate a very rapid transfer of the newly synthesized cholesterol from microsomes to biliary canaliculi in the presence of taurocholate. The cholesterol specific activity of the biliary fraction was the same as that for the membrane fractions during the first two secretion periods (0–60 min), but during the last secretory period (60–90 min) the bile activity significantly ($P < 0.02$) exceeded that in both microsomes and biliary canaliculi.

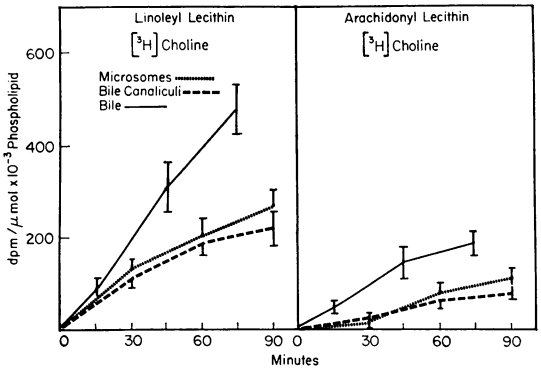

FIGURE 3 Incorporation of [^{3}H]choline into linoleyl and arachidonyl lecithin of microsomes, bile canaliculi, and bile. Each point represents 7–9 perfused livers (±SE) per time period. Bile samples are plotted at the midpoint of each collection period.

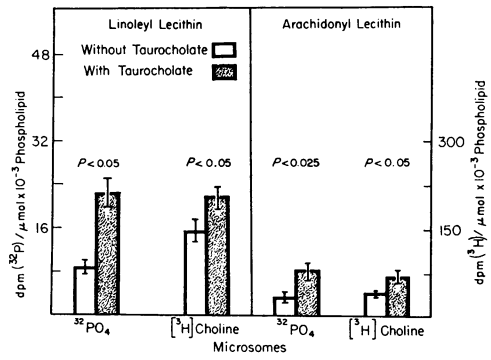

FIGURE 5 Effect of sodium taurocholate on the incorporation of $^{32}PO_4$ and [^{3}H]choline into microsomal arachidonyl and linoleyl lecithin at 60 min compared to controls (saline). Each bar represents eight taurocholate perfusions (±SE) or five saline controls.

DISCUSSION

Data have been presented which elucidate the possible role of the bile canalicular and microsomal membranes in the formation of biliary lecithin and cholesterol. An essential prerequisite for this study was the availability of a method for isolating a liver cell fraction rich in these bile canalicular membranes. The procedure of Song et al. (25) yielded a preparation enriched with biliary canaliculi (tight junction complexes) as evidenced by marker enzymes and electron microscopy. The absence of CDP-choline diglyceride transferase activity in the canalicular membranes and the high concentration of this enzyme in microsomes further attest to the very low contamination of the canalicular preparation with endoplasmic reticulum. Another critical factor for studying hepatic lipid synthesis and biliary transport was the availability of a model (the isolated perfused rat liver) which was relatively inactive with respect to biliary lipid secretion until the system was perfused with sodium taurocholate (1–4).

Several lines of evidence from the present report suggest that bile canalicular membranes do not synthesize biliary lecithin. First, CDP-choline diglyceride transferase activity was not found in bile canaliculi. This enzyme is essential for the *de novo* synthesis of lecithin. The isotopic data can also be interpreted to indicate a lack of participation by the canalicular membrane in the synthesis of biliary lecithin. If the canalicular membranes were the source of biliary lecithin, one would expect that the linoleyl lecithin fraction of this membrane should have a very rapid turnover during the active phase of biliary lecithin secretion. Additionally, since linoleyl lecithin from the canalicular membranes is rapidly flowing down the canaliculus during this active phase of biliary lipid secretion, the specific activity of the linoleyl lecithin fraction in this membrane should be approximately equivalent to bile. Therefore, the significantly higher specific activity of linoleyl lecithin in bile compared to this same lecithin species in the bile canalicular membrane indicates that linoleyl lecithin synthesis probably did not occur in this membrane. Alternatively, the bile canalicular membrane could be the site of biliary lecithin synthesis if the lecithin destined for bile was rapidly secreted without prior mixing in the canalicular linoleyl lecithin pool. In this manner a fraction of the newly synthesized biliary lecithin could be rapidly released into bile while another portion could be incorporated into a more slowly turning over structural pool of membranous canalicular linoleyl lecithin. However, the inability to demonstrate CDP-choline diglyceride transferase in the bile canalicular membrane provides strong evidence that this is an unlikely possibility. Although linoleyl lecithin is the major biliary lecithin, arachidonyl lecithin constitutes 10% of the biliary lecithin fraction. The incorporation of [^{3}H]choline into arachidonyl lecithin of bile canalicular and microsomal membranes paralleled linoleyl lecithin and suggests that both of these biliary lecithins were derived from microsomes. While these data show that bile canalicular membranes do not actively participate in biliary lecithin synthesis, it is clear that newly synthesized lecithin is rapidly transferred from microsomes to the canalicular membranes before being released into bile. Thus, it seems that the major role of the bile canalicular membrane with respect to biliary lipid metabolism is transport rather than synthesis.

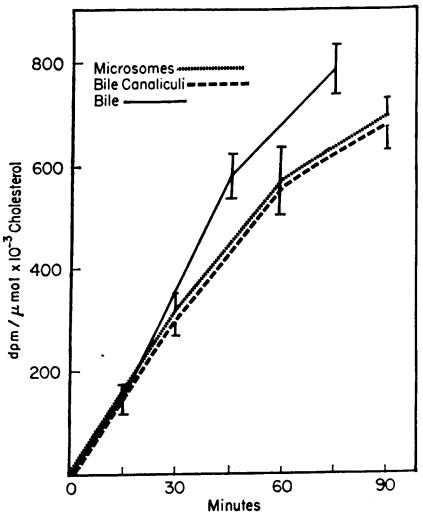

FIGURE 6 Incorporation of [^{3}H]mevalonic acid into cholesterol of microsomes, bile canaliculi, and bile. Each point represents 7–9 perfused livers (±SE) per time period. Bile samples are plotted at the midpoint of each collection period.

Although these studies indicate that microsomes are the site of biliary lecithin synthesis, the findings also show that the hepatic metabolism of linoleyl lecithin is a complex and heterogeneous process. The more rapid incorporation of [^{3}H]choline and [^{14}C]linoleic into biliary linoleyl and arachidonyl lecithins rather than microsomal and canalicular membrane lecithins provide evidence for the existence of a very rapidly turning over subpool of these lecithins that are secreted in bile. Further evidence of a microsomal subpool of biliary lecithin is the incorporation of ^{32}P into bile and canalicular linoleyl and arachidonyl lecithin. At 30 min the specific activity of $^{32}PO_4$ in the canalicular membrane exceeded microsomes and bile for both linoleyl and arachidonyl lecithin and was disassociated from the incorporation of [^{3}H]choline. The much higher specific activity of $^{32}PO_4$ in bile canalicular membranes compared to microsomes could result from incomplete equilibration of $^{32}PO_4$ with a large ATP pool if the newly synthesized linoleyl lecithin destined for bile was selectively transferred to the bile canalicular membrane. Alternatively,

transformation of linoleyl lecithin involving high-energy phosphate could occur within the bile canalicular membrane. By 90 min the $^{32}PO_4$ had equilibrated within the ATP pool and the specific activity relationship indicated a homogeneous incorporation pattern of $^{32}PO_4$ into both lecithin species of bile. Balint et al. (8, 17) compared the specific activities of hepatic and biliary lecithin in bile fistula rats after administration of labeled lecithin precursors. They observed that biliary linoleyl lecithin had a higher specific activity than hepatic linoleyl lecithin and also postulated the existence of hepatic subpool of biliary lecithin.

The findings of the present study indicate that taurocholate has an essential role in the transport of lipids into bile, and at the same time probably regulates the rate of synthesis of biliary lecithin. The incorporation of [^{3}H]choline and $^{32}PO_4$ into microsomal lecithin was markedly greater in taurocholate-perfused livers as compared to the saline-infused control livers. The effect of taurocholate on increasing lecithin synthesis was very rapid, and was demonstrable within 90 min of taurocholate infusion. Infusion of taurocholate for 90 min before the addition of the labeled precursors was essential for stimulating lecithin synthesis and transport. The stimulatory effect of taurocholate was common to both arachidonyl and linoleyl lecithin but was more pronounced on the linoleyl species. Balint et al. (8, 17) have previously reported a similar effect of taurocholate on linoleyl lecithin synthesis and attributed their findings to a specific effect on the CDP-choline pathway. From the available evidence (8, 17, 18, 37) it appears that palmityl linoleyl lecithin is the principal lecithin synthesized via this pathway. Arachidonyl lecithin is probably derived from a combination of several pathways, including deacylation and acylation reactions involving the palmityl linoleyl species and the transmethylation of phosphatidyl ethanolamine (37–39). Since arachidonyl lecithin is probably not synthesized de novo, the observed increase in labeling of the phosphoryl choline moiety of that species in the presence of taurocholate most likely resulted from transacylation with the more highly labeled palmityl linoleyl species.

In addition to stimulating microsomal lecithin synthesis, taurocholate also appeared to facilitate the intracellular transport of biliary lipids as evidence by the rapid transfer of labeled microsomal linoleyl lecithin to bile via the bile canalicular membrane. Thus bile salts probably have a dual role in the metabolism of biliary lipids which involves both synthesis and transport. Although intracellular cytoplasmic proteins have been identified (40, 41) which appear to participate in the transfer of lecithins from microsomes to mitochondrial and plasma membranes, no attempt was made in the present study to isolate such a carrier protein. However, the rapid exchange of lecithin observed between microsomes, bile canaliculi, and bile make such a transfer mechanism a distinct possibility. Whether the primary effect of bile salts is on synthesis or transport is not presently known. It is possible that microsomal lecithin could be depleted as a result of increased transport. If this were the case, microsomal linoleyl lecithin synthesis could be initiated as a consequence of a negative feedback effect on a rate-limiting step in the linoleyl lecithin biosynthetic pathway such as the CDP-choline diglyceride transferase enzyme.

As noted by others (3, 6, 7), taurocholate was also shown to have a marked effect on biliary cholesterol secretion. This effect was similar to that observed for lecithin as the [^{3}H]cholesterol rapidly equilibrated between microsomal and canalicular membranes. After the initial washout (low specific activity) of [^{3}H]cholesterol into bile there was a progressive increase in biliary [^{3}H]-cholesterol specific activity which by 90 min exceeded both microsomal and canalicular membranes. This phenomenon suggests the presence of a microsomal subpool of cholesterol destined for bile. The apparent dependence of both cholesterol and lecithin transport on the availability of bile salts provides further support for the existence of an intracellular lipoprotein complex that is involved in the transport of lipids from microsomes to bile. Soluble proteins have been isolated from the supernate of rat liver homogenates with binding affinity for bile acids (42) and cholesterol precursors (43). Such a complex could originate in the microsomes and be dependent on the concentration of taurocholate entering the liver cell for its specific composition and rate of formation. After coupling with carrier protein this complex could be transported to other intracellular membranes, including the bile canalicular membrane. Alternatively, the biliary lipids could be transported independently to the canalicular membrane or to an assembly site such as the Golgi body and then subsequently to the bile canaliculi. Whether the biliary lipids are independently and directly transported to canalicular membranes or arrive together as a lipoprotein macromolecular complex is not yet known. It also remains to be determined how the biliary lipids are transported across the canalicular membrane. Since it has been shown that liver plasma membranes have the enzymatic capacity for the transacylation of lecithins (22), it is possible that microsomal lecithin destined for bile could be altered in the bile canalicular membranes before its appearance in bile.

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