Effect of bile salts on hepatic phosphatidylcholine synthesis and transport into rat bile

David L. Young* and Kenneth C. Hanson‡

Department of Medicine, Duke University Medical Center, Durham, North Carolina 27706

Abstract The effect of transport of micelle-forming and nonmicelle-forming conjugated bile salts on phosphatidylcholine synthesis and transport into bile was studied in the ex vivo perfused rat liver. Single additions of sodium taurocholate, a good micelle-forming conjugated bile salt, caused an increase in bile flow associated with increased phosphatidylcholine and taurocholate concentration. The specific activity of phosphatidylcholine with respect to incorporated [1,2-^{14}C]choline and [^{3}H]methyl of L-[*Me*-^{3}H]methionine was not significantly altered by the increased transport of phosphatidylcholine. The data suggested that bile phosphatidylcholine is synthesized to a great extent, although not exclusively, by phosphorylcholine glyceride transferase.

Single additions of the glycine conjugate of dehydrocholate, a poor micelle-forming bile salt, caused an increase in bile flow comparable to that seen with sodium taurocholate administration. However, the concentrations of phosphatidylcholine in bile decreased.

Thin-layer and gas–liquid chromatographic analyses of bile secreted before and after glycodehydrocholate administration revealed no significant increase in bile salt secretion other than the administered glycodehydrocholate.

Investigations utilizing radiochemically pure [^{14}C]glycine dehydrocholate revealed that increased bile flow after [^{14}C]-glycine dehydrocholate administration occurs concomitantly with the secretion of 75–95% of the administered [^{14}C]glycine dehydrocholate as a single peak into bile.

Thus the increased bile flow without increased phosphatidylcholine concentration noted after glycodehydrocholate administration is due to transport of an intact, nonmetabolized, conjugated bile salt with poor micelle-forming properties. The data indicate that the formation of a bile salt–phosphatidylcholine micelle is responsible for phosphatidylcholine transport into bile.

Supplementary key words choline · L-methionine · sodium taurocholate · sodium glycodehydrocholate · micelles · ex vivo perfusion

PHOSPHATIDYLCHOLINE and conjugated bile salts comprise two of the major constituents of bile, and both are important in the solubilization of cholesterol (1). Hepatic enzymes reponsible for the biosynthesis of phosphatidylcholine are located in the endoplasmic reticulum (2). Although some of the enzymes capable of catalyzing steps in the pathway of conversion of cholesterol to bile acids appear in nonmicrosomal fractions of the hepatic cell, much of the de novo biosynthesis and the conjugation of free bile acids are catalyzed by enzymes in the endoplasmic reticulum (3, 4). The transport of conjugated bile salts, as well as of many other organic anions, from the endoplasmic reticulum into the canalicular fluid occurs against a concentration gradient, involves a saturable transport mechanism, and reflects competition by other anions. It is thus considered to represent a carrier-mediated active secretory mechanism (5, 6). However, the mechanism of phosphatidylcholine transport into bile, though associated with bile salts and cholesterol, has remained unclear.

The effect of increased secretion of conjugated bile salts as well as certain other organic anions on increasing the flow of bile is well known and is consistent with the active transport of osmotically active anions and the resultant osmotic choleresis (5, 6). Of the osmotically active anions commonly utilized to study choleresis, only the active transport of bile salts has been noted to be associated with increased lipid secretion. Kay and Entenman (7), while investigating the effect of chronic bile fistulization on bile salt synthesis, noted that the

Presented in part at the meeting of the Southern Society for Clinical Investigation, January 1971.

*Investigator of Howard Hughes Medical Institute.

‡Present address: Department of Medicine, University of Missouri, School of Medicine, Columbia, Mo. 65201.

increased rate of bile salt secretion seen in the chronic fistula rat was associated with increased secretion of phosphatidylcholine and free cholesterol. It was suggested at that time that a bile salt–lipid complex facilitated passage of phospholipid and cholesterol into bile.

The present investigations were undertaken to study the properties of bile salts responsible for the biliary transport of phosphatidylcholine. Investigations of phosphatidylcholine synthesis and release were undertaken in the ex vivo perfused rat liver. This preparation is not influenced by bile salts returning via the portal circulation (8). It thus allows study of the effect of individual bile salts on phospholipid secretion.

The present report describes the effect of the micelle-forming properties of conjugated bile salts on the transport of phosphatidylcholine into canalicular fluid. Also described is the effect of transport of phosphatidylcholine on the specific activity of [1,2-^{14}C]choline and [^{3}H]methyl of L-[*Me*-^{3}H]methionine incorporated into the phosphatidylcholine secreted into bile. [1,2-^{14}C]-Choline and phosphoryl[1,2-^{14}C]choline were both used as choline pathway precursors of phosphatidylcholine and were found to be identical.

METHODS

Materials

[1,2-^{14}C]Choline bromide (1.03 mCi/mmole) and L-[*Me*-^{3}H]methionine (106.5 mCi/mmole) were obtained from Tracerlab, Waltham, Mass. The purity of L-[*Me*-^{3}H]methionine was checked by paper chromatography. Taurocholate was obtained from Calbiochem, Los Angeles, Calif. Glycodehydrocholate was obtained from Mann Research Laboratories, Orangeburg, N.Y. [^{14}C]Glycine dehydrocholate was a generous gift from Leon Lack. The purity of both unlabeled glycodehydrocholate and [^{14}C]glycine dehydrocholate was checked by thin-layer chromatography. Both compounds developed only one spot in iodine vapor, which was superimposable and corresponded to the R_F of authentic glycodehydrocholate. Counting serial sections of the thin-layer plate revealed that all of the radioactivity of [^{14}C]glycine dehydrocholate was within the area identified by iodine vapor. Silica gel G and silica gel H were obtained from E. Merck A.G., Darmstadt, Germany. Reference lipids were obtained from Applied Science Laboratories Inc., State College, Pa. Egg yolk phosphatidylcholine was prepared by the method of Hanahan, Turner, and Jayko (9).

Liver perfusions

A modification of the isolated liver perfusion method as described by Miller et al. (10) was used. The method was modified by adding a constant magnetic stirring device to the reservoir portion of the perfusion apparatus. This permitted immediate and constant mixing of the perfusate returning from the liver and insured rapid mixing of substances added to the perfusate reservoir. The oxygenation portion of the perfusion apparatus was modified by the inclusion of silk baffles, thus ensuring adequate oxygenation of the perfusate prior to its passage through the liver. Livers were obtained from Sprague-Dawley rats weighing 250–350 g. These rats were fed Purina Chow up to the time of killing. The time from the ligation of the portal vein to reestablishment of circulation within the perfusion apparatus ranged from 4 to 6 min. The perfusion medium consisted of washed red cells obtained from Sprague-Dawley rats; they were resuspended in Krebs-Ringer bicarbonate buffer to which had been added bovine serum albumin and glucose to give final concentrations of 4 g/100 ml and 100 g/100 ml, respectively. The perfusate volume was 100 ml. The hematocrit of the perfusate ranged from 20 to 25%. The pH of the perfusate was kept constant between 7.3 and 7.4, using 95% oxygen–5% CO_2 oxygenation mixture and the addition of 0.15 N $NaHCO_3$ as needed. Penicillin, streptomycin, and heparin were routinely added to the perfusate. The chamber in which liver perfusion was carried out was maintained at a temperature of 37°C by a thermostatically controlled heating system in the walls of the chamber.

Prior to the removal of the liver from the animal, the bile duct was cannulated with a polyethylene catheter (Intramedic, PE 50; Clay-Adams, Inc., Parsippany, N.J.), thus allowing continuous collection of elaborated bile. The portal vein was cannulated with a PE 200 cannula. The inferior vena cava was cannulated above the liver with a PE 260 cannula. Shortly after being placed in the perfusion apparatus, bile flow rates ranged from 1.2 to 1.8 ml/hr, but within a period of 10–20 min they fell to the range of 600–800 μl/hr. Bile flow rates at the end of the perfusions had a mean of 600 μl/hr. Livers were perfused 3 hr when one bile salt was added to the perfusate and 5 hr when two bile salts were added at different intervals to the same perfusate. Hepatic blood flow rates ranged from 25 to 35 ml/min. Livers not maintaining bile or blood flow within the ranges noted above were discarded.

In some experimental preparations, either [1,2-^{14}C]-choline (25 μCi) or phosphoryl[1,2-^{14}C]choline (25 μCi) or L-[*Me*-^{3}H]methionine (100 μCi) was added to the reservoir after establishment of good bile and blood flow. In other experiments, both labeled precursors were added simultaneously. Labeled methionine was used as the precursor in 12 preparations, labeled choline or phosphorylcholine in 12 preparations, and both labels in 3 preparations. Conjugated bile salts were added to

the reservoir as their sodium salts (18.6 μmoles) in a volume of 1 ml. Bile samples covering a 10-min period were collected in ice-cold glass tubes directly from the bile duct cannula. Bile flow rate and volume were measured both directly and by calculation from drop counting.

Preparation of phosphatidylcholine–cholesterol–bile solutions

Aqueous preparations of phosphatidylcholine and cholesterol used for the comparison of the micelle-forming properties of sodium taurocholate and sodium glycodehydrocholate were prepared from egg yolk phosphatidylcholine and free cholesterol. 300 mg (378 μmoles) of egg phosphatidylcholine and 48.0 mg (122 μmoles) of free cholesterol in 66 ml of 0.15 M NaCl were sonified (Sonifier cell disruptor, Heat Systems-Ultrasonics, Inc., Plainview, N.Y.) for 5 min and adjusted to pH 7.0 with 0.1 N NaOH at 37°C. The resultant semi-opaque preparation was divided into three equal portions of 22 ml each. To one of the portions, 214 mg of sodium taurocholate (398 μmoles) was added. To a second portion, 192.6 mg of sodium glycodehydrocholate (398 μmoles) was added. The three portions were briefly resonicated (1 min) to assure mixing and solubilization of the added bile salts. The resultant phosphatidylcholine–cholesterol–bile salt preparations contained the three constituents in the proportions 22.2%, 7.32%, and 70.4%, respectively.

Analytical procedures

Individual phospholipids from collected bile were isolated by thin-layer chromatography on silica gel G plates as previously described (11). Phospholipid spots on thin-layer plates were located after brief exposure of the plates to an iodine atmosphere. Lipid standards were used in conjunction with all thin-layer chromatographic procedures. After the radioactively labeled lipid spots were free of iodine stain, they were scraped and counted in a Packard Tri-Carb scintillation counter using toluene–Cab-O-Sil solvent with 2,5-diphenyloxazole and 2,2-phenylenebis(5-phenyloxazole) as scintillators. When a single radioactively labeled precursor had been used, quenching was monitored by the channels ratio method. Quenching curves were prepared as previously described (12). When both ^{14}C- and ^{3}H-labeled precursors were used, the counting method described by Okita et al. (13) was used. Phospholipid counts were found exclusively in phosphatidylcholine and lysophosphatidylcholine. Phosphatidylcholine generally contained from 70 to 90% of the biliary phospholipid counts. The remaining counts were found in lysophosphatidylcholine. The radioactivities of the two phospholipids were added together, and the sum is referred to as phosphatidylcholine radioactivity in the Results.

After isolation of bile phosphatidylcholine and lysophosphatidylcholine by thin-layer chromatography on washed silica gel H, the lipids were combined and lipid phosphorus was determined by the method of Parker and Peterson (14). The combined phosphatidylcholine–lysophosphatidylcholine lipid phosphorus is referred to as total phosphatidylcholine phosphorus in the Results. Conjugated bile salts were isolated from bile by thin-layer chromatography using two solvent systems: isoamylacetate–propionic acid–*n*-propanol–water 30:30:20:15 (v/v) and isoamylacetate–propionic acid–*n*-propanol–water 40:30:20:10 (v/v) (15). Authentic bile salt standards were used in conjunction with all thin-layer chromatographic procedures. Bile salts separated by thin-layer chromatography were identified by iodine vapor and by charring. After the radioactively labeled bile salts were free of iodine, they were scraped and counted using the solvent noted above for radioactively labeled phospholipids.

Gas–liquid chromatography of bile acids was performed on a Packard gas–liquid chromatograph, with disc integrator and printer, by a modification of the method of Okishio, Nair, and Gordon (16). Prior to gas–liquid chromatography, isolated conjugated bile salts were deconjugated by enzymatic hydrolysis, utilizing the enzyme cholylglycine hydrolase by the method of Okishio et al. (16). Methyl esters were prepared by the method of Schlenk and Gellerman (17).

After isolation by thin-layer chromatography, taurocholate and glycocholate were determined by the modified Pettenkoffer reaction of Irvin, Johnston, and Kopala (18).

RESULTS

Liver perfusions unstimulated by bile salt addition

Perfused liver preparations unstimulated by addition of bile salts maintained a relatively constant bile flow rate, decreasing slightly with time. Bile taurocholate levels fell rapidly after the initial establishment of ex vivo perfusion. The bile salts initially secreted in the bile represented the transportable bile salts present in the liver as a result of the enterohepatic circulation present in the intact animal (8).

By measuring total phosphatidylcholine secreted into bile as well as the radioactivity of the incorporated labeled precursors, it was determined that the specific activity of phosphatidylcholine secreted in bile increased rapidly after the addition of the radioactively labeled precursors to the perfusate reservoir. In experiments in which both precursors were given simultaneously, as

well as in single precursor experiments, the initial specific activity of [^{3}H]methyl in phosphatidylcholine rose more rapidly than that of [1,2-^{14}C]choline. Within a period of 50 min, the specific activity of both [1,2-^{14}C]-choline and [^{3}H]methyl in phosphatidylcholine reached a level at which it then remained relatively constant throughout the period of perfusion. Increased bile flow and phosphatidylcholine transport which occurred after the addition of conjugated micelle-forming bile salts, as noted below, were not associated with a significant change in phosphatidylcholine specific activity with respect to either [1,2-^{14}C]choline or [^{3}H]methyl (Fig. 1).

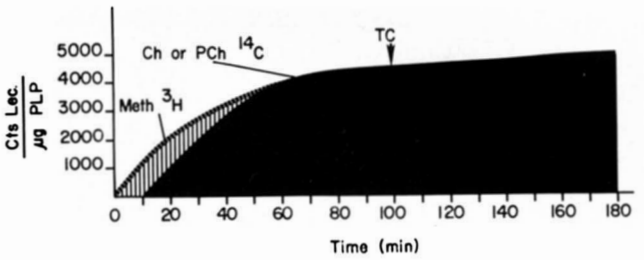

FIG. 1. Effect of single addition of sodium taurocholate (*TC*) on the biliary phosphatidylcholine specific activity. The radioactively labeled precursors were added at zero time. The µg PLP represents the total biliary phospholipid phosphorus.

Effect of sodium taurocholate on phosphatidylcholine transport into bile

As indicated in Fig. 2, single additions of sodium taurocholate (10 mg, 18.6 µmoles) to the perfusate caused a brief increase in bile flow. Associated with this increase was a concomitant increase in bile phosphatidylcholine secretion. Both radioactively labeled and total phosphatidylcholine increased simultaneously and occurred in conjunction with the rapid transport of the administered sodium taurocholate (Fig. 2). The specific activity of phosphatidylcholine did not change significantly. Approximately 70% of the administered taurocholate was recovered as a single peak in bile over a 40-min period after its administration. This period corresponds to the period of increased release of phosphatidylcholine into bile and to the period of increased bile flow.

The effect of sodium taurocholate administration on the concentration of phosphatidylcholine and sodium taurocholate secreted into bile is indicated in the right-hand portion of Fig. 2. After the addition of sodium taurocholate to the perfusate, the concentration of sodium taurocholate in bile abruptly increased. A concomitant increase in the concentration of phosphatidylcholine in bile occurred. These studies demonstrate that there is a proportionately greater increase in both phosphatidylcholine and sodium taurocholate transport than in water secretion after the administration of the micelle-forming bile salt sodium taurocholate.

Comparison of the micelle-forming properties of sodium taurocholate and sodium glycodehydrocholate

The three preparations shown in Fig. 3 permit comparison of the micelle-forming properties of sodium taurocholate with those of sodium glycodehydrocholate. The preparations were prepared as indicated in Methods, so that each of the three tubes contained phosphatidylcholine and cholesterol in millimolar concentrations comparable to those found in rat bile. The percentages of light transmission measured at 520 nm (1-cm light path) were 13.1, 65.5, and 12.9, respectively, in the phosphatidylcholine–cholesterol preparation containing no additions, the preparation to which sodium taurocholate was added, and the preparation to which sodium glycodehydrocholate was added. The molar percentages of phosphatidylcholine, cholesterol, and bile salts present in the latter two solutions place them well in the micelle formation zone when plotted on the triangular coordinates of the quaternary phase system as described by Small, Bourges, and Dervichian (1).

Effect of sodium glycodehydrocholate on phosphatidylcholine transport into bile

The effect of sequential, single additions of equimolar amounts of sodium taurocholate and sodium glyco-

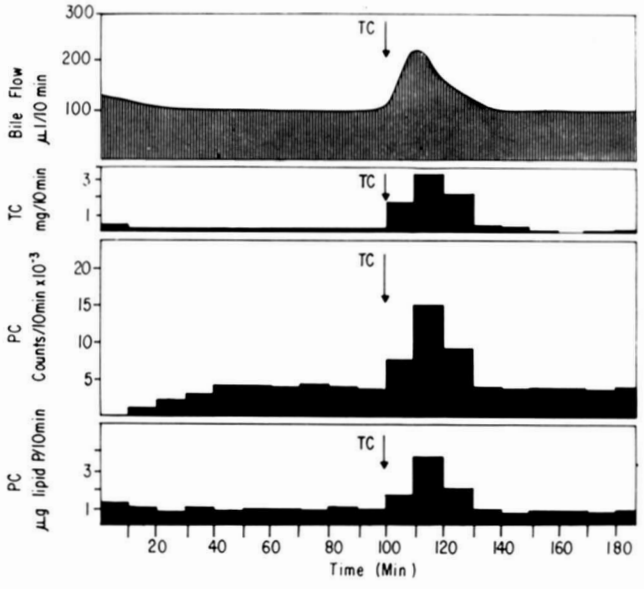

FIG. 2. Effect of single additions of sodium taurocholate on bile flow and on phosphatidylcholine (*PC*) and taurocholate (*TC*) transport into bile. The ex vivo perfused liver preparations were as described under Methods. Phosphatidylcholine counts shown are from incorporated [1,2-^{14}C]choline or phosphoryl[1,2-^{14}C]choline, but they are representative of [^{3}H]methyl of L-[*Me*-^{3}H]methionine as well. The radioactively labeled precursors were added at zero time. Sodium taurocholate was added at the time indicated.

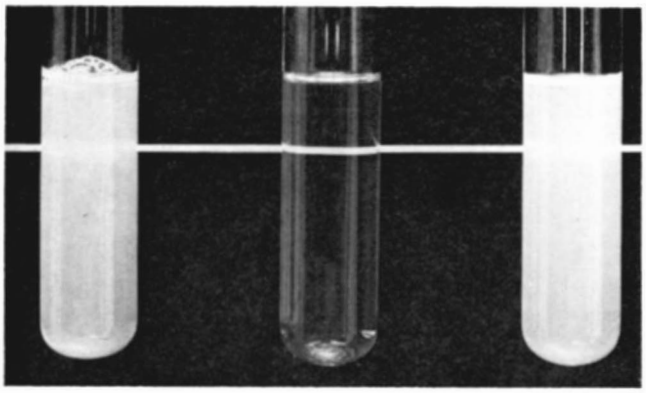

FIG. 3. Comparison of micelle-forming properties of sodium taurocholate and sodium glycodehydrocholate on preparations of phosphatidylcholine and cholesterol. Preparation was as described in Methods. All three tubes contain 100 mg (126 μmoles) of phosphatidylcholine and 16 mg (40.66 μmoles) of cholesterol in 22 ml of 0.15 M NaCl. The center tube contains in addition 214 mg (398 μmoles) of sodium taurocholate. The tube on the right contains in addition 192.6 mg (398 μmoles) of glycodehydrocholate.

dehydrocholate on phosphatidylcholine transport is indicated in Fig. 3. As previously noted (Fig. 2), and as indicated on the left and right portions of the four panels in Fig. 4, the administration of sodium taurocholate (10 mg, 18.6 μmoles) before and after the administration of sodium glycodehydrocholate resulted in an increase in bile flow. Total and radioactively labeled phosphatidylcholine secretion, however, was only briefly and minimally increased. Sodium taurocholate secretion increased very slightly as well. The small increments in secretion of phosphatidylcholine and sodium taurocholate were consistent with the canalicular washout associated with the increase in bile flow. In association with the increase in flow after sodium glycodehydrocholate administration, the concentrations of both phosphatidylcholine and sodium taurocholate decreased. As noted previously, sodium taurocholate resulted in an increase in phosphatidylcholine and sodium taurocholate concentrations.

Effect of added glycodehydrocholate on the release of other bile salts

After perfused livers had been secreting bile for 40 min, bile samples were obtained during the 60-min periods immediately before and after single additions of glycodehydrocholate to the perfusate. These samples were subjected to thin-layer chromatography, and areas corresponding in R_F to taurocholate and glycocholate were assayed for the quantity of trihydroxy bile salts by the method referred to under Analytical Procedures. No free cholic acid was found either prior to or after the administration of glycodehydrocholate. During the 60-min period after administration of sodium glycodehydrocholate, an increment of 20–100 μg of taurocholate was noted when compared with a comparable period prior to glycodehydrocholate administration. The increase generally occurred during the first 20 min. Only trace quantities of glycocholate could be detected

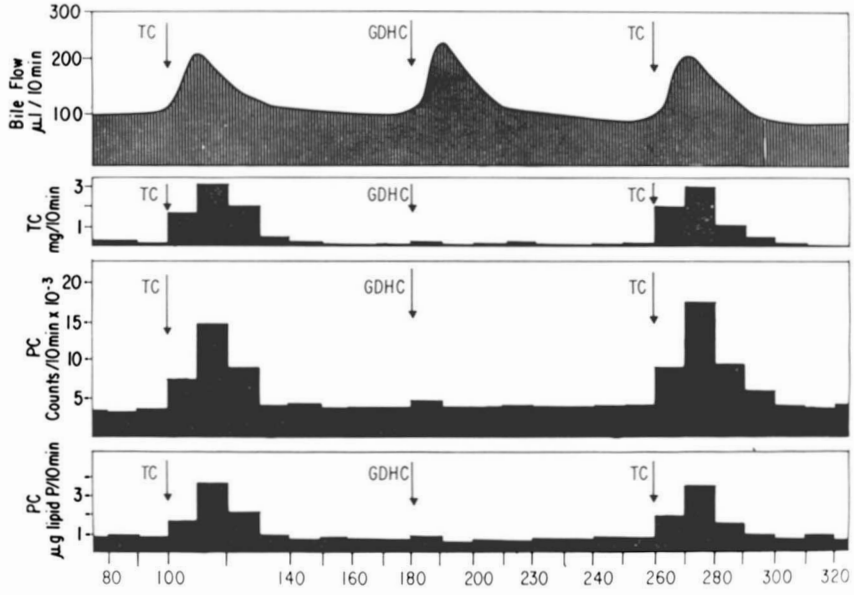

FIG. 4. Effect of single sequential additions of sodium taurocholate and glycodehydrocholate on bile flow and on phosphatidylcholine (*PC*) and taurocholate (*TC*) transport into bile. The ex vivo perfused liver preparations were as described under Methods. Phosphatidylcholine counts shown are from incorporated [1,2-^{14}C]choline or phosphoryl[1,2-^{14}C]choline, but they are representative of [^{3}H]methyl of L-[*Me*-^{3}H]-methionine as well. The radioactively labeled precursors were added at zero time (not shown). Sodium taurocholate and sodium glycodehydrocholate (*GDHC*) were added at the times indicated.

in the bile prior to the administration of glycodehydrocholate, and no significant difference in the quantity of this conjugated bile salt was found after the administration of the glycodehydrocholate. Glycodehydrocholate spots were regularly and easily visible on iodine-stained and charred thin-layer chromatographs of bile obtained 10–30 min after addition of glycodehydrocholate to the perfusate reservoir.

Bile obtained during the periods 30 min prior to and 60 min after the addition of glycodehydrocholate was subjected to gas–liquid chromatographic analysis as described under Methods. Only small peaks of cholic acid were detected both prior to and after the administration of sodium glycodehydrocholate. The small increment in cholic acid after glycodehydrocholate administration noted by thin-layer chromatography was also detected by this method. Because the enzyme cholylglycine hydrolase does not utilize glycodehydrocholate as substrate (16), the methyl ester of dehydrocholate would not be expected to be formed during preparation of the bile samples for gas–liquid chromatography. However, a small peak estimated to represent approximately 50 μg of bile salt with a retention time 1.55 times that of cholic acid appeared on gas–liquid chromatographs of the samples obtained during the 30–60-min period after glycodehydrocholate administration. This compound was not further characterized; however, its appearance in bile at a time later than that of the majority of the administered glycodehydrocholate and after the peak increase in flow rate suggests that its presence in bile is unrelated to the osmotic effect noted. Because of the lability of 3-keto bile acids to strong reagents and heat (19), an alkaline hydrolysis method for the deconjugation of bile salts prior to methylation was not used in these investigations.

Transport of [^{14}C]glycine dehydrocholate

The recovery of [^{14}C]glycine dehydrocholate in bile after the addition of 9 mg (18.6 μmoles, 56.9 $\times$ 10^4 cpm) of the radiochemically pure conjugated bile salt to the perfusate is shown in Fig. 5. Concomitant with the increase in bile flow, the administered labeled glycodehydrocholate is transported as a single peak into bile. 75–95% of the counts added to the perfusate were recovered in the bile. The [^{14}C]glycine dehydrocholate recovered in bile chromatographed as a single radioactive band on thin-layer chromatography with an R_F identical to the administered sodium [^{14}C]glycine dehydrocholate and superimposed nonlabeled sodium glycodehydrocholate. Serial areas of gel from the thin-layer plates from the origin to the solvent front revealed that the radioactivity was in the glycodehydrocholate identified by iodine vapor. No other areas contained significant radioactivity.

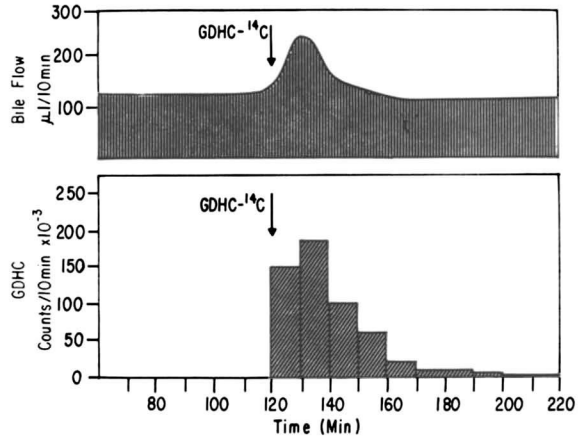

FIG. 5. Recovery of [^{14}C]glycine dehydrocholate in bile. At the time indicated, 9.0 mg (18.6 μmoles, 56.9 $\times$ 10^4 cpm) of [^{14}C]-glycine dehydrocholate (*GDHC-^{14}C*) was added to the perfusate. The ex vivo perfused liver preparations were as described under Methods.

DISCUSSION

The present report indicates that the micelle-forming properties of conjugated bile salts transported into bile are important in the transport of phosphatidylcholine from its site of biosynthesis in the hepatic cell into the canalicular fluid.

The ex vivo perfused liver preparations utilized in the present investigations were free of the enterohepatic circulation and thus were not influenced by the variety of conjugated and unconjugated bile salts returning from the intestinal tract via the portal circulation (8). Additionally, the rate of hepatic de novo bile salt biosynthesis in acute fistula animals is relatively low (7). Thus, the ex vivo perfused liver permitted study of the effect on phosphatidylcholine synthesis and transport of single additions of individual bile salts with different physicochemical properties. Because the single additions of the conjugated bile salts were rapidly transported, the effect of increasing and decreasing quantities of these substances transported into bile could be observed. The initial period of ex vivo perfusion, as noted in Fig. 2, was characterized by the transport into the bile of a decreasing quantity of taurocholate, the major primary conjugated bile salt in rat bile. This secretion of the remaining intrahepatic portion of the bile salt pool was associated with a lesser decrease in the rate of bile flow. Total phosphatidylcholine transport decreased concomitantly and, as with taurocholate and bile flow, reached a relatively constant secretion rate.

After the addition of the labeled precursors of phosphatidylcholine to the perfusate, the specific activity of biliary phosphatidylcholine increased progressively over a period of 50 min (Fig. 1). Previous studies in this laboratory (11) and others (20, 21) have indicated that

hepatic microsomal phosphatidylcholine attains a maximum specific activity within 60–90 min after the in vivo administration of both [1,2-^{14}C]choline and L-[*Me*-^{3}H]-methionine to rats. In the ex vivo perfused liver, the added labeled precursors are utilized only by the liver and reach the liver rapidly and in high concentration so that a more rapid attainment of maximum specific activity of hepatic phosphatidylcholine might be expected. In the present studies, phosphatidylcholine transported into bile maintained a relatively constant specific radioactivity when either [1,2-^{14}C]choline or L-[*Me*-^{3}H]methionine was used as the labeled precursor (Fig. 1). The increase in phosphatidylcholine transport which occurred in association with transport of added micelle-forming conjugated bile salts was not associated with any significant change in the specific activity of bile phosphatidylcholine. Both phosphatidylcholine radioactivity and total phosphatidylcholine transport returned to prestimulated levels following the transport of the micelle-forming conjugated bile salt. These data suggest that a bolus of micelle-forming bile salt does not stimulate the synthesis of phosphatidylcholine destined for bile but just increases the transport of this phosphatidylcholine into bile. These results differ from those of the studies of Balint et al. (22). The difference between our biliary lecithin specific activities and those of Balint et al. (22) are probably related to the short term infusions of bile salts in our experiments versus the prolonged bile salt infusions in their experiments. In our experiments we find essentially the same biliary specific activity of phosphatidylcholine when [1,2-^{14}C]-choline, phosphoryl[1,2-^{14}C]choline, or L-[*Me*-^{3}H]methionine is used as the labeled precursor.

Swell, Bell, and Entenman (23) and Entenman et al. (24), while studying phosphatidylcholine secretion during continuous bile salt infusion in the ex vivo perfused dog and rat liver, respectively, utilized ^{32}P as the labeled precursor of phosphatidylcholine. Their studies indicated that very little ^{32}P-labeled phospholipid was transported into bile up to 200 min after ^{32}P was added to the perfusate when no bile salt was infused. 30–45 min after the beginning of a bile salt infusion, a progressive increase was noted in biliary phosphatidylcholine specific activity with respect to ^{32}P. However, ^{32}P is known to be rapidly incorporated into hepatic phosphatidylethanolamine but rather slowly into hepatic phosphatidylcholine. The investigations of Balint et al. (25) indicate that 15 min after intravenous administration of ^{32}P to rats, the specific activity of hepatic phosphatidylethanolamine is approximately 40 times greater than that of hepatic phosphatidylcholine and at 1 hr is still 12 times that of phosphatidylcholine. Contributions to the phosphatidylcholine of endoplasmic reticulum by the pathway involving the stepwise methylation of phosphatidylethanolamine (11) would result in a progressive rise in specific activity of [^{32}P]phosphatidylcholine. Continuous administration of micelle-forming bile salts, resulting in a demand for continuous replenishment of exportable phosphatidylcholine, would thus result in a sustained progressive increase in the specific activity of bile phosphatidylcholine with respect to ^{32}P. Data from the present investigations, in conjunction with the findings of Swell et al. (23) and Entenman et al. (24), show a marked increase of biliary transport of phosphatidylcholine synthesized via the methylation of phosphatidylethanolamine, as a result of administration of a micelle-forming bile salt. In contrast, there is a difference in the rate of attainment of maximum specific activity of biliary phosphatidylcholine with respect to ^{32}P as noted by Swell et al. (23) and Entenman et al. (24). Our more rapid rate of attainment of maximum specific activity suggests that the [^{3}H]methyl of L-[*Me*-^{3}H]methionine exchanges to some extent directly with the methyl groups of choline in CDP-choline as well as acting as substrate in the SAME:PE methyltransferase pathway (11), whereas ^{32}P is incorporated only through this latter pathway.

As noted in Fig. 2, the single addition of a bolus (18.6 μmoles) of sodium taurocholate, a micelle-forming conjugated bile salt, to the perfusate resulted in a brief increase in bile flow. There was a concomitant but proportionately greater increase in bile phosphatidylcholine transport which occurred simultaneously with the transport into bile of the administered sodium taurocholate. The canalicular transport of both of these components occurred over the 40-min period immediately following the administration of sodium taurocholate. The concentration of phosphatidylcholine and sodium taurocholate transported into bile abruptly increased, demonstrating a proportionately greater increase in both phosphatidylcholine and taurocholate transport than in water formation. Substances, such as mannitol, which equilibrate rapidly between plasma and liver cell water maintain a relatively constant concentration in bile in the face of increasing water movement into bile canaliculi (26). The present studies indicate that in addition to the osmotic effect noted with increased micelle-forming bile salt transport there is an associated greater increase in phosphatidylcholine transport. Sodium taurocholate is an excellent micelle-forming bile salt, and the concomitant secretion of both taurocholate and phosphatidylcholine in increased concentrations is consistent with the formation of a micelle-forming bile salt–phosphatidylcholine micelle at or near the canalicular membrane with resultant simultaneous transport of both components. To further investigate this possibility, the studies were done utilizing the glycine conjugate of dehydrocholate, a bile salt

which shows no, or only a slight, tendency to micelle formation in the concentration range occurring in bile (6, 27, 28). The absence of any significant effect of sodium glycodehydrocholate on the phosphatidylcholine–cholesterol preparation, as noted in Fig. 3, suggests this bile salt has poor micelle-forming properties. Although the phosphatidylcholine–cholesterol–glycodehydrocholate preparation was artificially prepared, the molar ratios of the constituents are ideal for a micellar solution (1), as in the case of the preparation containing sodium taurocholate (Fig. 3).

The increase in bile flow which occurs following the addition of sodium glycodehydrocholate to the perfusion preparation clearly indicates the osmotic properties of this bile salt. The slight increase in taurocholate and phosphatidylcholine secretion noted immediately after the administration of glycodehydrocholate (Fig. 4) does not necessarily indicate increased canalicular transport of these bile constituents. Sperber (6) has indicated that when transfer of a substance into the canaliculi occurred at a constant rate but the flow increased, the amount of the substance excreted per unit time increased during the period when the change in bile flow occurred. The increased quantities of both taurocholate and phosphatidylcholine secreted after glycodehydrocholate administration are consistent with this canalicular washout. The absence of a significant increase in phosphatidylcholine transport after glycodehydrocholate administration is emphasized by the decrease in the concentration of phosphatidylcholine after glycodehydrocholate administration. The counts of phosphatidylcholine/μl of bile decreased 100% from base line after glycodehydrocholate administration. In contrast, when taurocholate was administered the concentration increased 60% above base line. These data indicate that phosphatidylcholine transport into bile is not causally related to the osmotic properties of bile salts but is clearly related to their micelle-forming properties. The fact that thin-layer and gas–liquid chromatography of bile obtained during the period following the administration of sodium glycodehydrocholate revealed no significant increase in the transport of bile salts other than glycodehydrocholate indicates that the increased flow was not due to the stimulated transport of other bile salts.

As indicated in Fig. 4, administration of sodium taurocholate prior to and after the administration of sodium glycodehydrocholate results in an abrupt increase in bile flow and taurocholate and phosphatidylcholine transport. Such experiments indicate that the perfused livers were capable of transporting phosphatidylcholine in association with the transport of micelle-forming bile salts rather than an osmotically active, non-bile-salt metabolite of glycodehydrocholate. The rapid secretion of 75–95% of the added radiolabeled non-micelle-forming bile salt concomitant with the increase in bile flow thus indicates that most of the administered bile salt was not metabolized during passage through the liver. Investigations by Hardison (29) and by Soloway et al. (30) indicate that the in vivo intravenous administration of unconjugated ^{14}C-labeled dehydrocholate results in metabolism of all of this bile salt to substances other than dehydrocholate.

Entenman et al. (31), in using a liver preparation similar to ours, noted increased phospholipids secreted into bile with unconjugated dehydrocholate infusions. A probable explanation for the difference between their results and ours is that their dehydrocholate was converted to various metabolites, one of which was taurocholate (29, 30). In our preparation, the liver was unable to break the peptide bond of glycodehydrocholate, and it was secreted in the same form as administered, as shown by the [^{14}C]glycodehydrocholate secretion peak.

The present data indicate that the formation of a bile salt–phosphatidylcholine micelle is responsible for phosphatidylcholine transport into bile.

This work was supported by U.S. Public Health Service grants HE11443 and T0105093.

Manuscript received 29 March 1971 and in revised form 15 September 1971; accepted 21 October 1971.

REFERENCES

1. Small, D. M., M. Bourges, and D. G. Dervichian. 1966. Ternary and quaternary aqueous systems containing bile salts, lecithin and cholesterol. *Nature* (London). **211:** 816–818.
2. Wilgram, G. F., and E. P. Kennedy. 1963. Intracellular distribution of some enzymes catalyzing reactions in the biosynthesis of complex lipids. *J. Biol. Chem.* **238:** 2615–2619.
3. Bergström, S., H. Danielsson, and B. Samuelsson. 1960. Formation and metabolism of bile acids. *In* Lipid Metabolism. K. Bloch, editor. Wiley, New York. 291–336.
4. Danielsson, H. 1963. Present status of research on catabolism and excretion of cholesterol. *Advan. Lipid Res.* **1:** 335–385.
5. Sperber, I. 1959. Secretion of organic anions in the formation of urine and bile. *Pharmacol. Rev.* **11:** 109–134.
6. Sperber, I. 1965. Biliary secretion of organic anions and its influence on bile flow. *In* The Biliary System. W. Taylor, editor. Blackwell, Oxford. 457–467.
7. Kay, R. E., and C. Entenman. 1961. Stimulation of taurocholic acid synthesis and biliary excretion of lipids. *Amer. J. Physiol.* **200:** 855–859.
8. Cronholm, T., and J. Sjövall. 1967. Bile acids in portal blood of rats fed different diets and cholestyramine. *Eur. J. Biochem.* **2:** 375–383.
9. Hanahan, D. J., M. B. Turner, and M. E. Jayko. 1951. The isolation of egg phosphatidylcholine by an absorption column technique. *J. Biol. Chem.* **192:** 623–628.
10. Miller, L. L., C. G. Bly, M. L. Watson, and W. F. Bale. 1951. The dominant role of the liver in plasma protein

synthesis. A direct study of the isolated perfused rat liver with the aid of lysine-ϵ-C^{14}. *J. Exp. Med.* **94:** 431–453.
11. Young, D. L., G. Powell, and W. O. McMillan. 1971. Phenobarbital-induced alterations in phosphatidylcholine and triglyceride synthesis in hepatic endoplasmic reticulum. *J. Lipid Res.* **12:** 1–8.
12. Young, D. L., and F. Lynen. 1969. Enzymatic regulation of 3-*sn*-phosphatidylcholine and triacylglycerol synthesis in states of altered lipid metabolism. *J. Biol. Chem.* **244:** 377–383.
13. Okita, G. T., J. T. Kabara, F. Richardson, and G. V. LeRoy. 1957. Assaying compounds containing H^3 and C^{14}. *Nucleonics.* **15:** 111–114.
14. Parker, F., and N. F. Peterson. 1965. Quantitative analysis of phospholipids and phospholipid fatty acids from silica gel thin-layer chromatograms. *J. Lipid Res.* **6:** 455–460.
15. Hofmann, A. F. 1962. Thin-layer adsorption chromatography of free and conjugated bile acids on silicic acid. *J. Lipid Res.* **3:** 127–128.
16. Okishio, T., P. P. Nair, and M. Gordon. 1967. The microquantitative separation of cellular bile acids by gas–liquid chromatography. *Biochem J.* **102:** 654–659.
17. Schlenk, H., and J. L. Gellerman. 1960. Esterfication of fatty acids with diazomethane on a small scale. *Anal. Chem.* **32:** 1412–1414.
18. Irvin, J. L., C. G. Johnston, and J. Kopala. 1944. A photometric method for the determination of cholates in bile and blood. *J. Biol. Chem.* **153:** 439–457.
19. Sobotka, H. 1938. Chemistry of the Steroids. Williams and Wilkins Co. Baltimore, Md. 7–46.
20. Hallinan, T., T. Duffy, S. Waddington, and H. N. Munroe. 1966. Incorporation of ^{14}C-choline into phospholipids of rat liver microsome subfractions: evidence against formation of endoplasmic reticulum from the nuclear membrane. *Quart. J. Exp. Physiol. Cog. Med. Sci.* **51:** 142–150.
21. Bjørnstad, P., and J. Bremer. 1966. In vivo studies on pathways for the biosynthesis of lecithin in the rat. *J Lipid Res.* **7:** 38–45.
22. Balint, J. A., D. A. Beeler, E. C. Kyriakides, and D. H. Treble. 1971. The effect of bile salts upon lecithin synthesis. *J. Lab. Clin. Med.* **77:** 122–133.
23. Swell, L., C. C. Bell, Jr., and C. Entenman. 1968. Bile acids and lipid metabolism. III. Influence of bile acids on phospholipids in liver and bile of the isolated perfused dog liver. *Biochim. Biophys. Acta.* **164:** 278–284.
24. Entenman, C., R. J. Holloway, M. L. Albright, and G. F. Leong. 1969. Bile acids and lipid metabolism. II. Essential role of bile acids in bile phospholipid excretion. *Arch. Biochem. Biophys.* **130:** 253–256.
25. Balint, J. A., D. A. Beeler, D. H. Treble, and H. L. Spitzer. 1967. Studies in the biosynthesis of hepatic and biliary lecithins. *J. Lipid Res.* **8:** 486–493.
26. Forker, E. L. 1967. Two sites of bile formation as determined by mannitol and erythritol clearance in the guinea pig. *J. Clin. Invest.* **46:** 1189–1195.
27. Reinhold, J. G., and D. W. Wilson. 1934. The acid–base composition of hepatic bile: I. *Amer. J. Physiol.* **107:** 378–387.
28. Reinhold, J. G., and D. W. Wilson. 1934. The acid–base composition of hepatic bile. III. The effect of the administration of sodium taurocholate, sodium cholate and sodium dehydrocholate (Decholin). *Amer. J. Physiol.* **107:** 400–405.
29. Hardison, W. G. 1970. Metabolism of dehydrocholate in the rat liver: its effect on micelle formation in bile. *Proc. Cent. Soc. Clin. Res.* **43:** 41. (Abstr.)
30. Soloway, R. D., A. F. Hofmann, P. J. Thomas, and L. J. Schoenfield. 1971. Dehydrocholic acid: hepatic metabolism and effect on biliary secretion in man. *Gastroenterology.* **60:** 754. (Abstr.)
31. Entenman, C., R. J. Holloway, M. L. Albright, and G. F. Leong. 1968. Bile acids and lipid metabolism. I. Stimulation of bile lipid excretion by various bile acids. *Proc. Soc. Exp. Biol. Med.* **127:** 1003–1006.