Apolipoproteins of high, low, and very low density lipoproteins in human bile[1]

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Abstract We tested the hypothesis that apolipoproteins, the protein constituents of plasma lipoproteins, are secreted into bile. We examined human gallbladder bile obtained at surgery (N = 54) from subjects with (N = 44) and without (N = 10) gallstones and hepatic bile collected by T-tube drainage (N = 9) after cholecystectomy. Using specific radioimmunoassays for human apolipoproteins A-I and A-II, the major apoproteins of high density lipoproteins, for apolipoproteins C-II and C-III, major apoproteins of very low density lipoproteins, and for apolipoprotein B, the major apoprotein of low density lipoproteins, we found immunoreactivity for these five apolipoproteins in every bile sample studied in concentrations up to 10% of their plasma values. Using double immunodiffusion, we observed complete lines of identity between bile samples and purified apolipoproteins A-I, A-II, or C-II. Using molecular sieve chromatography, we found identical elution profiles for biliary apolipoproteins A-I, A-II and B and these same apolipoproteins purified from human plasma. When we added high density lipoproteins purified from human plasma to lipoprotein-free solutions perfusing isolated rat livers, we detected apolipoproteins A-I and A-II in bile. Similarly, when we added low density lipoproteins purified from human plasma to lipoprotein-free solutions perfusing isolated livers of rats treated with ethinyl estradiol in order to enhance hepatic uptake of low-density lipoproteins, we found apolipoprotein B in bile. These data indicate that apolipoproteins can be transported across the hepatocyte and secreted into bile.—**Sewell, R. B., S. J. T. Mao, T. Kawamoto, and N. F. LaRusso.** Apolipoproteins of high, low, and very low density lipoproteins in human bile. *J. Lipid Res.* 1983. **24:** 391–401.

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In bile, bile acid-lecithin complexes solubilize cholesterol, whereas, in plasma, apolipoprotein-phospholipid complexes have a similar function, an analogy first suggested by Small (2). The apolipoproteins, a family of polypeptides, act as detergent molecules and, with phospholipid, solubilize plasma cholesterol, cholesteryl esters, and triglycerides (3). The hepatocyte synthesizes apolipoproteins and secretes them into the plasma (4); whether the liver cell also secrets apolipoproteins into bile is unknown. However, it is known that the hepatocyte can take up high molecular weight compounds, including lipoproteins (5–7). In addition, a number of other endocytosed proteins such as IgA (8, 9), haptoglobin (10), horseradish peroxidase (11), epidermal growth factor (12), and perhaps insulin (11, 13) and asialoglycoproteins (14) may be transported from plasma across the hepatocyte into bile. Such transcellular transport probably involves movement of endocytic vesicles from the sinusoidal pole of the hepatocyte to the biliary canaliculus (11, 15); fusion with lysosomes (11, 16) with direct release of lysosomal contents into bile (17–19) may also occur. Indeed, bile contains a great variety of proteins (17, 20), and up to 4.5% of the total solids of bile are protein (21). For these reasons, we investigated the possibility that the hepatocyte can secrete apolipoproteins into bile, either directly after synthesis or following uptake from plasma and transcellular transport.

In this manuscript, we report that five plasma apolipoproteins, A-I, A-II, C-II, C-III, and B, are detectable by specific radioimmunoassays in human gallbladder and hepatic bile. We also provide preliminary evidence that these apolipoproteins are present in bile as intact polypeptides. Using an isolated perfused rat liver model, we further demonstrate that apolipoproteins A-I and A-II can be recovered in bile following addition of human high density lipoproteins (HDL) to a lipoprotein-free perfusate. Finally, we show that apolipoprotein B is detectable in bile after addition of human low density lipoproteins (LDL) to lipoprotein-free solutions perfusing isolated livers of rats pretreated with ethinyl estradiol, a hormone known to increase receptor-mediated uptake of LDL by hepatocytes (7).

Abbreviations: LDL, low density lipoprotein; HDL, high density lipoprotein.

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METHODS

Bile samples

Human gallbladder bile was obtained from gallbladders removed at surgery or, in some cases, by needle aspiration of these gallbladders prior to cholecystectomy. The presence of gallstones was assessed from the surgical and pathological reports. Human hepatic T-tube bile was collected from patients several days following cholecystectomy. All bile was stored at −70°C prior to analysis.

Preparation of human plasma lipoproteins

LDL (d 1.019–1.063 g/ml) and HDL (d 1.063–1.21 g/ml) were obtained from normal, pooled human plasma (ten females) by sequential ultracentrifugation according to standard procedures (22). The isolated HDL and LDL were refloated at d 1.063 and d 1.21 g/ml, respectively, to remove excess plasma albumin and then dialyzed in buffer containing 0.1 M NaCl, 0.01 M Tris-HCl, 1 mM EDTA, and 0.01% NaN_3, pH 7.4. Lipoproteins prepared for rat liver perfusion studies did not contain NaN_3. The dialyzed LDL was immediately passed through 0.45-μm millipore filters and stored at 4°C. The purified LDL or HDL each showed a single peak following column chromatography on Bio-Gel A-15M (23). Protein content of LDL was determined by a modification of the method of Lowry et al. (24) in the presence of sodium dodecyl sulfate, or by amino acid analysis.

Radioimmunoassay procedures

Apolipoproteins A-I and A-II were obtained by fractionation on Sephadex G-150 in the presence of 5 M guanidine-HCl, pH 8.2 (25, 26). Apolipoproteins C-II and C-III were isolated by DEAE-cellulose chromatography as described previously (27). Homogeneity of the apolipoproteins was established by polyacrylamide gel electrophoresis in the presence of 8 M urea, pH 8.2 (28), and by amino acid analysis.

Apolipoproteins were iodinated with $Na^{125}I$ using a modification of the chloramine-T method (29). The $Na^{125}I$ (1.0 mCi, Amersham-Searle, Arlington Heights, IL) was added to 25 μg of apolipoprotein in 0.5 M sodium phosphate (pH 7.5). Iodination was initiated by the addition of 10 μl of chloramine-T (1.25 mg/ml). The reaction was allowed to proceed for 30 sec and stopped by the addition of 25 μl of sodium metabisulfide (1.25 mg/ml), at which time the iodinated apolipoprotein was separated from free ^{125}I on a Bio-Gel p-2 column.

LDL was iodinated according to a modification of McFarlane's iodine monochloride technique (26). LDL (250 μg) in 100 μl of 1 M glycine buffer, pH 10.0, was reacted with 1 mCi of $Na^{125}I$ for 30 sec in the presence of 50 μl of ICl (0.05 mg/ml containing 2 M NaCl). Following purification on a Bio-Gel p-2 column, the ^{125}I-LDL was exhaustively dialyzed against 8 l of buffer, described above. The final specific activity of ^{125}I-LDL was approximately 2 μCi/ug. Approximately 90 ± 2% of the ^{125}I-LDL was precipitated by 20% trichloracetic acid, whereas only 6% of the radioactivity remained in the lipid moiety as judged by extraction with ethanol–ether 1:3 (v/v). More than 90% of ^{125}I-LDL was coeluted with unlabeled LDL as a single peak on a Bio-Gel A-15 column (23).

Antibodies to the apolipoproteins or to LDL were raised in New England white rabbits (25–27). Specificity of each antibody was established (25–27, 30) and was determined by radioimmunoassay.

Double antibody radioimmunoassays for apolipoproteins A-I, A-II, C-II, and C-III were established and performed as described previously (25–27, 30). All dilutions of bile, antibodies, and apolipoproteins or LDL were made with a standard radioimmunoassay buffer containing 0.1% bovine serum albumin (Sigma Chemical Co., RIA grade, St. Louis, MO), 0.1 M sodium borate, 1 mM EDTA, and 0.01% NaN_3, pH 8.5. First antibodies, rabbit anti-apolipoproteins, or rabbit anti-LDL, were titered so as to bind 40 to 50% of iodinated tracers (approximately 20,000 cpm), and a carrier of partially purified nonimmune rabbit gamma-globulin was added. The second antibody, goat anti-rabbit gamma-globulin, was titered to produce maximum precipitation. The radioimmunoassay procedure, performed in triplicate, included three steps. *1*) To 100 μl of apolipoprotein or LDL standards or to 100 μl of bile of an appropriate dilution, 100 μl of ^{125}I-labeled apolipoprotein or LDL containing approximately 20,000 cpm was added, along with 100 μl of the appropriate antibody and 100 μl of diluted Tween-20. Tween-20 was used only for radioimmunoassays of apolipoproteins A-I, A-II, C-II, and C-III. Final concentration of Tween-20 was 0.5%, 0.2%, 0.01%, and 0.02% for apolipoprotein A-I, A-II, C-II, and C-III, respectively. Tubes were incubated for 16 hr at room temperature. *2*) One hundred μl of goat anti-rabbit gamma-globulin was added to all tubes followed by a 16-hr incubation. *3*) Two ml of radioimmunoassay buffer was added to all tubes followed by centrifugation at 3,000 rpm (1,000 *g*) for 40 min. The supernatant, containing unbound ^{125}I-labeled apolipoprotein, was aspirated and the precipitates were counted for 1 min. Nonspecific binding was generally less than 3% of the added counts. Intra-assay coefficients of variation were less than 8% for all assays.

For calculations, percentage bound = $B/B_0 \times 100$;

where B is the total radioactivity (cpm) in the presence of standards or unknown samples and B_0 is the total radioactivity bound (cpm) in the absence of standards or unknown samples.

Column chromatography of human bile using Sephadex G-200

Bile samples that contained the most abundant apolipoproteins determined by radioimmunoassays and double gel immunodiffusion were chromatographed on Sephadex G-200. Briefly, 1 ml of gallbladder bile was dialyzed against deionized water (containing 1 mM EDTA) and lyophilized. The dried sample was delipidated by an extraction (ether–ethanol 3:1, v/v) procedure (26). The delipidated bile was dissolved in 0.8 ml of 6 M guanidine, pH 8.0, and applied to a Sephadex G-200 column (1.8 × 85 cm) equilibrated with 5 M guanidine, 0.1 M Tris, 1 mM EDTA, pH 8.0. Human LDL, apoA-I, and apoA-II purified from plasma were used for calibration of the column. Immunoreactivity of each eluted fraction (20–50 μl) of the bile sample was determined by LDL, apoA-I, and apoA-II radioimmunoassays. ApoC-II and C-III were not measured because of interference of guanidine (20–50 μl of 5 M guanidine) with the binding of tracer and antibodies.

Gel immunodiffusion

Gel immunodiffusion was done on commercially prepared plates, with 1.5% agarose in 0.1 M Tris and 0.1% NaN_3 (Helena Laboratories). Undiluted bile samples (10 μl) were tested against anti-apoA-I, anti-apoA-II, anti-apoC-II, and anti-LDL antisera. Bile was not tested against anti-apoC-III antibodies since these antibodies do not precipitate apoC-III-containing lipoproteins from plasma. After 24 hr incubation at room temperature, the immunoprecipitin lines were developed and the plates were photographed.

Other assays of bile samples

Total biliary bile acids were measured by an automated modification of the method of Talalay (31). Biliary cholesterol was measured spectrophotometrically using the enzyme cholesterol analysis kit of Boehringer-Mannheim (32) and biliary phospholipid was measured by the method of Takayama et al. (33) as applied to analysis of bile by the method of Gurantz, Laker, and Hofmann (34) with the use of a commercial kit (PL Kit-K, Nippon Shoji Kaisha, Ltd., Osaka, Japan). Total protein determinations were done by an automated modification of the procedure of Lowry et al. (24); previous experiments in our laboratory had established the accuracy and precision of this method for quantitating total protein in bile.

Saturation of bile samples with cholesterol was calculated from polynomial equations (35) to describe the solubility line proposed by Hegardt and Dam (36) and expressed as percentage saturation, where values above and below 100% represent supersaturation and nonsaturation, respectively.

Isolated perfused rat liver

Isolated rat livers were perfused with a hemoglobin-free, lipoprotein-free medium consisting of a perfluorochemical emulsion as the oxygen carrier in a Krebs-Ringer bicarbonate solution (Fluosol-43, Alpha Therapeutic Corp., Los Angeles, CA) using a modification of the method of Gollan et al. (37). The perfusion solution contained 22 μM sodium taurocholate (Calbiochem, CA) and penicillin (100 U/ml) and was adjusted to pH 7.45 with 0.1 M $NaHCO_3$.

An open recirculating perfusion system was used with a membrane oxygenation system (38). Male Sprague-Dawley rats weighing 300–350 g were the liver donors, and, under pentobarbital anesthesia, the operative technique of Gollan et al. (37) was employed to isolate and perfuse the liver. The portal perfusion pressure was 12 cm H_2O and perfusion flow rates were 20–25 ml/min. Sodium taurocholate was infused into the stirred reservoir at 0.56 μmol/min to stabilize bile flow. After a 30-min equilibration period, human HDL or LDL was injected slowly as a bolus dose into the portal perfusion site. Bile was collected in time aliquots on ice into preweighed vials.

Viability of the perfused livers was satisfactory for the 2.5-hr perfusion as assessed by normal hepatic oxygen extraction (0.56–0.69 ml/min), stable perfusate lactic dehydrogenase activity, and stable bile flow during the control period (1.1 ml/hr) and at the end of the perfusion (0.7 ml/hr).

For some experiments with LDL, ethinyl estradiol (Sigma Chemical Co., St. Louis, MO) dissolved in propylene glycol was administered subcutaneously to rats daily for 5 days at 5 mg/kg per day.

RESULTS

Radioimmunoassays for apolipoproteins A-I, A-II, C-II, and C-III

Typical standard curves obtained in the radioimmunoassay for apolipoproteins A-I, A-II, C-II, and C-III are shown in **Fig. 1.** Specificity of each assay has been established (25–27, 30) as they do not cross-react with other apolipoproteins in the presence of a 1000-fold excess. The sensitivity of each assay is at the range of 2–50 ng of apolipoproteins employed. In order to

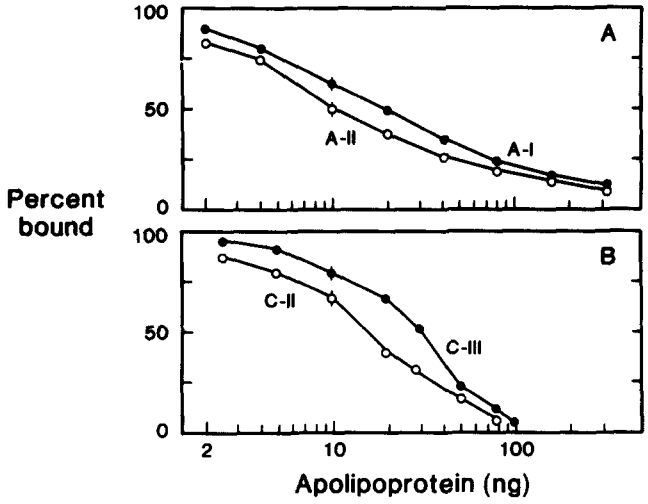

Fig. 1. (A) Standard displacement curves for radioimmunoassays of apoA-I (● ——●) in the presence of 1.5% Tween-20, and apoA-II (○ —— ○). Rabbit antiserum dilutions were 1:1000 and 1:6000, respectively. (B) Standard displacement curves for radioimmunoassays of apoC-II (○ —— ○) and apoC-III (● —— ●) in the presence of Tween-20, 0.2% and 0.1%, respectively. Rabbit antiserum dilutions were 1:800 for apoC-II and 1:1600 for apoC-III.

determine the level of the apolipoproteins in bile, bile samples were diluted to 1:50 with a buffer solution containing 0.1% bovine serum albumin prior to the assay. Undiluted rat bile samples showed no significant immunoreactivity. Quantitative recovery from the addition of known amounts of apolipoprotein standards into human bile, rat bile, and perfusate was investigated. More than 95% of the apolipoproteins on average was recovered in each immunoassay for apoA-I, apoA-II, apoC-II, and apoC-III. A typical example of the apoA-I and apoA-II recovery in human and rat bile is shown in **Table 1.** The level of each apolipoprotein determined was linear with the volume of bile used. Moreover, in the presence of Tween-20 (25, 27), we found that lipids do not affect the immunoreactivity of the apolipoproteins in bile; that is, no difference was found between native and delipidated bile using the various immunoassays.

Radioimmunoassay for apolipoprotein B

The standard displacement curve of the immunoassay using LDL as a standard is shown in **Fig. 2.** The sensitive range was from 120 to 1000 ng of added unlabeled LDL. The assay is specific for apoB-containing lipoproteins, since apolipoproteins A-I, A-II, C-II, and C-III, albumin, and HDL up to 2000 ng were ineffective in displacing ^{125}I-LDL. Immunoreactivity of apoB, in bile samples obtained from four subjects, is also shown and each individual bile sample gives a curve parallel

TABLE 1. Analytical recovery of apolipoproteins[a]

		Human Bile		Rat Bile	
	Apolipoprotein Added (ng)	Apolipoprotein Measured	Apolipoprotein Recovery (%)	Apolipoprotein Measured (ng)	Apolipoprotein Recovery (%)
	ng	*ng*	*%*	*ng*	*%*
ApoA-I	16	16.6 (±0.8)	103.6 (±5.4)	14.3 (±0.3)	89.1 (±1.8)
	32	32.4 (±1.5)	101.4 (±4.7)	30.9 (±1.9)	96.4 (±5.7)
	64	69.0 (±4.6)	107.9 (±7.2)	57.1 (±1.2)	89.2 (±1.9)
ApoA-II	20	22.4 (±3.5)	112.2 (±17.7)	19.1 (±1.5)	94.0 (±6.4)
	40	37.1 (±1.6)	92.9 (±4.0)	38.9 (±2.9)	95.8 (±6.6)
	80	87.0 (±4.7)	109.6 (±5.6)	80.9 (±3.8)	102.1 (±4.1)
ApoB	453	501.3 (±31.4)	110.6 (±6.9)	638.0 (±173)	140.8 (±3.8)
	905	911.6 (±191.2)	100.7 (±21.2)	932.7 (±28.9)	103.1 (±3.2)
	1810	2019.4 (±382.4)	111.5 (±20.3)	2009.8 (±57.7)	111.0 (±3.0)

[a] Values are mean ± SD from three experiments.

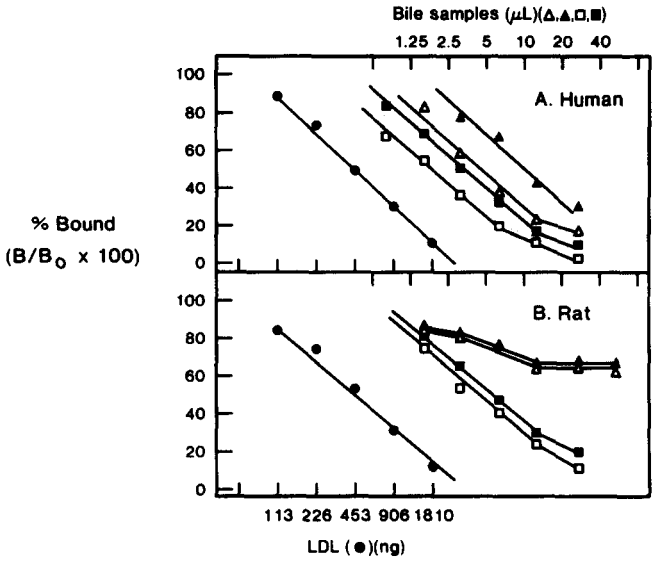

Fig. 2. Displacement curves for radioimmunoassay of human apoB. (A) Human bile samples from four individuals. (B) Bile samples from a rat previously treated with ethinyl estradiol. Immunoreactivity of samples obtained before (Δ, ▲) and after (□, ■) addition of human LDL to the perfusate are shown. Human LDL used for standards (●). Each point represents the mean of three assays.

to the LDL standard (Fig. 2A). In addition, essentially all LDL added to human bile is quantitatively recovered by radioimmunoassay (Table 1). In order to measure immunoreactive apoB in bile, 10 μl of undiluted or diluted (1:10) bile was assayed so that the percentage bound (B/B_0) displaced by bile samples fell into the sensitive range of the assay (Fig. 2A).

Radioimmunoassays of apolipoproteins in human gallbladder and hepatic bile

The levels of immunoreactive apolipoproteins A-I, A-II, C-II, C-III, and B in gallbladder and hepatic bile obtained from patients with and without cholelithiasis and comparisons between the mean apolipoprotein concentrations in bile and in a normal plasma are shown in **Fig. 3** and **Table 2.** Plasma samples, obtained from a pool of ten other normolipemic control subjects, gave apolipoprotein concentrations in close agreement with published values (39). The concentrations of apolipoproteins A-I and A-II in gallbladder bile were 5 to 10 times higher than in hepatic bile ($P < 0.001$), while for apolipoproteins C-II and C-III, the gallbladder and hepatic bile concentrations were not significantly different. The concentration of apolipoprotein B in gallbladder bile was approximately 3 times higher than in hepatic bile ($P < 0.01$). The mean concentrations of apolipoproteins in gallbladder bile, expressed as a percentage of the plasma concentration, were 1.5%, 2.6%, 10%, 2.5%, and 5.0% for apolipoproteins A-I, A-II, C-II, C-III, and B, respectively. When the profiles within each apolipoprotein type were examined, biliary apolipoproteins differed quantitatively from plasma apoli-

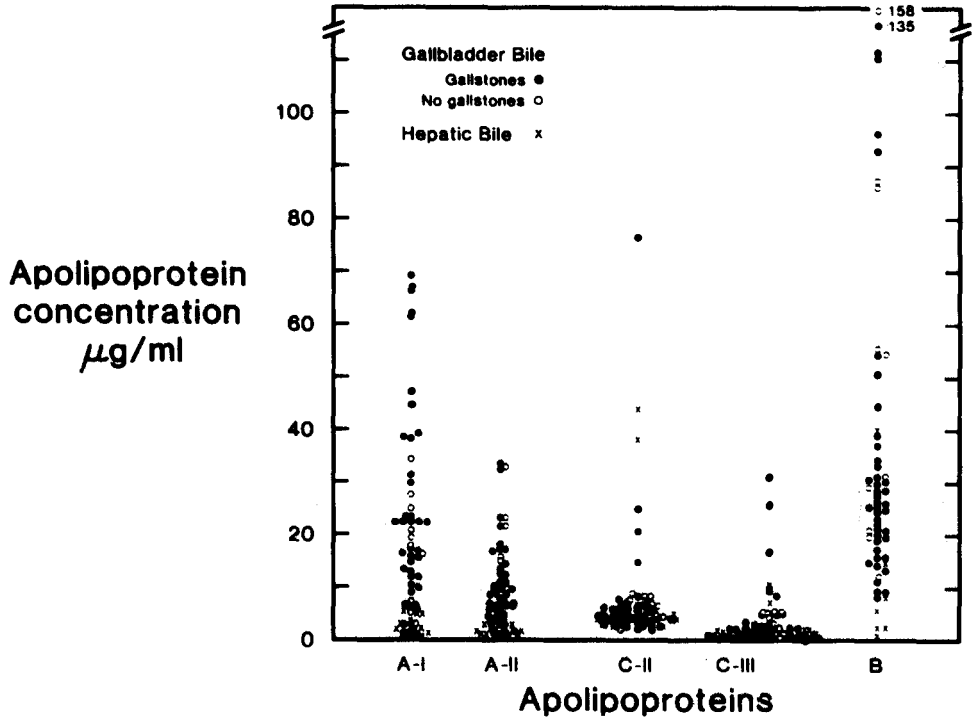

Fig. 3. The concentrations of apolipoproteins (μg/ml) in human gallbladder bile in patients with (●) and without (○) gallstones and in hepatic bile (×) collected from T-tubes after cholecystectomy.

TABLE 2. Apolipoprotein concentrations in bile and plasma by radioimmunoassay

	ApoA-I	ApoA-II	ApoC-II	ApoC-III	ApoB
			μg/ml		
Pooled plasma	1315	400	79	158	766
Gallbladder bile (N = 54)	19.1[a] (±2.2)	10.4 (±1.1)	7.8 (±1.4)	3.9 (±0.8)	38.5 (±4.7)
Hepatic T-tube bile (N = 9)	2.9 (±0.5)	1.5 (±0.4)	12.4 (±5.5)	3.4 (±1.1)	10.6 (±5.2)
P value for gallbladder versus hepatic bile	<0.001	<0.001	NS	NS	<0.01

[a] Values are means (±S.E.).

poproteins. The ratio of apoC-III to apoC-II in plasma was 2:1 (40), whereas in gallbladder bile, it was 1:2; the plasma apoA-I to apoA-II ratio was 3.2:1 (40), whereas in gallbladder bile it was 1.9:1. Among the apolipoproteins in gallbladder bile, significant correlations were found between apoA-I and apoA-II ($r = 0.73$, $P < 0.001$), between apoC-II and apoC-III ($r = 0.82$, $P < 0.001$) and between apoB and apoA-I ($r = 0.43$, $P < 0.001$), apoA-II ($r = 0.47$, $P < 0.001$), apoC-II ($r = 0.54$, $P < 0.001$), and apoC-III ($r = 0.65$, $P < 0.001$). No significant differences were found between A and C apoproteins or in the mean apolipoprotein concentrations in gallbladder bile between patients with and without gallstones.

Double immunodiffusion

Bile was studied by double immunodiffusion for detection of the presence of apolipoproteins A-I, A-II, C-II, and B. Apolipoproteins A-I, A-II, and C-II purified from plasma showed complete lines of identity with the bile samples against each of the specific antibodies. Examples of gel immunodiffusion plates, using bile and purified apolipoproteins A-I and A-II from plasma are shown in **Fig. 4.** However, bile did not give a precipitin line against rabbit or goat anti-human LDL antibodies (data not shown).

Chromatography of human bile on Sephadex G-200

In order to estimate the molecular weights and assess the integrity of the immunoreactive apolipoproteins found in bile, a delipidated bile sample was chromatographed on a Sephadex G-200 column containing 5 M guanidine that had been calibrated with purified plasma LDL, apoA-I, and apoA-II. Immunoreactive apoB, apoA-I, and apoA-II from bile were measured by radioimmunoassays on each fraction eluted from the column. In **Fig. 5,** the elution profiles of immunoreactive biliary apoA-I, apoA-II, and apoB were found to be nearly identical to those of purified plasma apoA-I, apoA-II, or LDL.

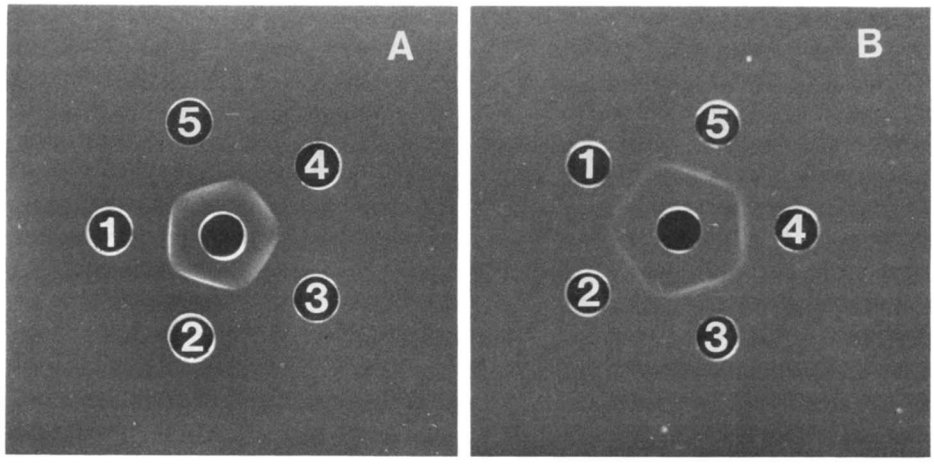

Fig. 4. Presence of apolipoprotein A-I and A-II in human bile as evidenced by immunodiffusion technique. (A) The central well contained rabbit anti-apoA-I antibodies. Wells 1 and 2 contained undiluted human bile. Wells 3–5 contained apolipoprotein A-I isolated from human plasma. (B) Similar to (A), except rabbit anti-apoA-II (central well) and apoA-II (wells 3–5) isolated from human plasma were employed. One μg of purified apoA-I or apoA-II was filled in wells 3–5. Complete lines of identity were found between bile samples and apoA-I and apoA-II, respectively.

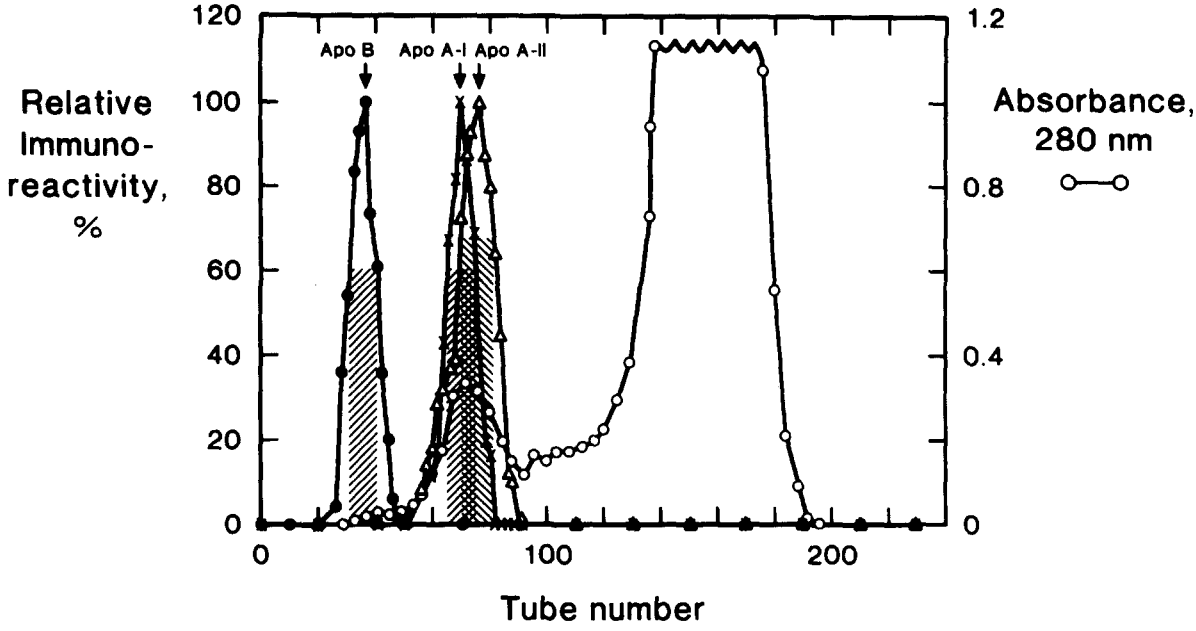

Fig. 5. Gel filtration chromatography of delipidated human bile on a Sephadex G-200 column. The shaded areas indicated the fractions from the column that contained LDL, ^{125}I-apoA-I and ^{125}I-apoA-II purified from human plasma and used to calibrate the column. Elution profiles of apoB (● —— ●), apoA-I (× —— ×), and apoA-II (△ —— △), as well as total protein (○ —— ○) are shown.

Biliary lipid, total protein, and apolipoproteins

The mean values for the bile acid, phospholipid, cholesterol, and total protein concentrations and the calculated lithogenic indices in gallbladder bile for patients with and without gallstones are shown in **Table 3.** Bile from patients with gallstones had a lower total protein concentration ($P = 0.05$) than bile from patients without gallstones. In neither group of patients, however, did biliary lipid concentrations correlate significantly with any of the apolipoprotein concentrations. Total protein concentrations in bile did show significant correlations ($P < 0.005$) with concentrations of apoA-II ($r = 0.55$) and apoC-III ($r = 0.44$), but not with other lipoproteins. Also, no significant correlations were found between the cholesterol saturation index of bile and any of the apolipoproteins in bile. As expected, biliary lipids correlated strongly ($P < 0.001$) among themselves, but not with biliary total protein levels.

Isolated rat liver perfused with human high density and low density lipoproteins

Human HDL, at one of two concentrations, or saline was added to the perfusate in three isolated liver perfusions. The higher perfusate concentration (0.8 mg/ml) was two-thirds that of the A-I concentration in human plasma (40), the lower (0.2 mg/ml) was one-fifth that concentration. The results for the three experiments are shown in **Fig. 6.** In all cases, perfusate concentrations of apoA-I and apoA-II by radioimmunoassay remained stable from 5 min after injection. No apoA-I or A-II was detected in bile by radioimmunoassay prior to injection of HDL, and in the control experiment, none was detected in bile following injection

TABLE 3. Lipid and total protein concentrations and lithogenic index in gallbladder bile

	Bile Acid	Phospholipid	Cholesterol	Total Protein	Lithogenic Index
		μmol/ml		*mg/ml*	*%*
Gallstone patients (N = 44)	112.7[a] (±10.6)	29.3 (±2.6)	10.7 (±1.1)	17.5 (±1.9)	119.5 (±8.7)
Non-gallstone patients (N = 10)	148.2 (±25.1)	38.3 (±6.5)	13.2 (±3.1)	31.0 (±8.7)	97.0 (±12.9)
P value	NS	NS	NS	$P = 0.05$	NS

[a] Values are means (±S.E.).

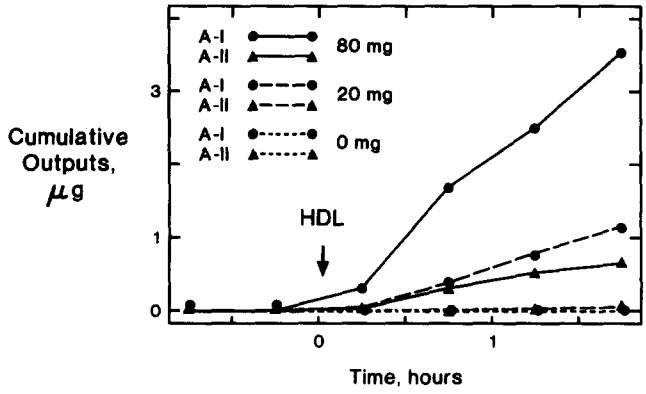

Fig. 6. Cumulative outputs of apolipoproteins A-I and A-II into rat bile after injection of human HDL into three isolated perfused rat livers. Eighty, 20, or 0 mg of HDL protein were added to the 100 ml perfusate.

of saline. After injection of HDL, apoA-I and A-II were present in all bile samples at progressively increasing concentrations over the next 2 hr; the 2-hr biliary excretion of apoA-I represented 0.2% of the injected dose. The ratio of A-I to A-II in rat bile (3.9/1) was relatively constant at each time point after injection of HDL and was similar to the ratio of A-I to A-II in native HDL (39).

Human LDL, at a concentration (0.16 mg/ml) of apoB approximating one-fifth the plasma concentration, or saline was added to the perfusate of three isolated liver perfusions. In one study, the rat had been pretreated with ethinyl estradiol for 5 days prior to the experiments. No significant amounts of apoB were detected in bile by radioimmunoassay prior to injection of LDL (**Fig. 7**). Also, no apoB was detected in bile after injection of saline or after injection of LDL into the perfusate of the liver from the untreated rat. In contrast, after injection of LDL into the perfusate of the liver from the rat previously treated with ethinyl estradiol, apoB was present in all bile samples at progressively increasing concentrations over the next 2 hr; the 2-hr biliary excretion of apoB in this experiment represented 0.24% of the injected dose.

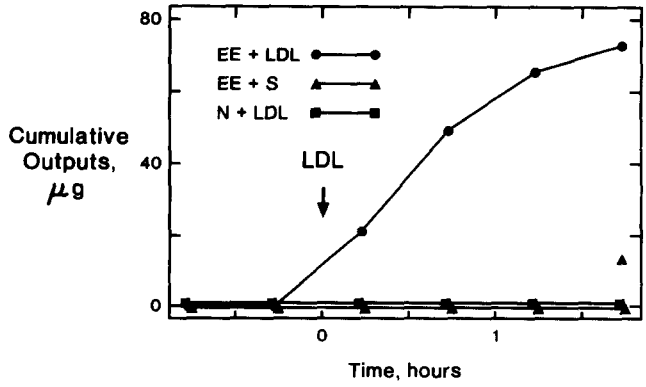

Fig. 7. Cumulative output of apolipoprotein B into rat bile after injection of human LDL into three isolated perfused rat livers. EE, ethinyl estradiol; S, saline; N, normal.

As discussed previously, we had extensively validated the radioimmunoassay for apoB in human and rat bile (Table 1 and Fig. 2). In Fig. 2B, we show that bile collected from a liver isolated from an ethinyl estradiol-treated rat before addition of human LDL to the perfusate only slightly displaces ^{125}I-labeled human LDL from the antibodies. This displacement probably represents cross-reactivity of endogenous rat apoB in bile with the antibodies raised against human LDL. Considerably greater displacement of ^{125}I-labeled human LDL occurs if bile collected from the liver of an ethinyl estradiol-treated rat after the addition of human LDL to the perfusate is used (Fig. 2B). Moreover, the displacement curves using these bile samples are parallel to the displacement curve using human LDL purified from plasma (Fig. 2B). For calculating baseline levels (time 0) of apoB in rat bile (Fig. 7), we subtracted the very small amounts of presumably endogenous rat apoB in bile that cross-react with the antibodies to human apoB.

DISCUSSION

The main finding of this study is that apoproteins of high, low, and very low density lipoproteins are present in human bile. We have shown that apolipoproteins A-I, A-II, C-II, C-III, and B are detectable by sensitive and specific radioimmunoassays and by gel immunodiffusion in both gallbladder and hepatic bile. The identical elution profiles by molecular sieve chromatography of biliary apolipoproteins A-I and A-II with those of apolipoproteins purified from human plasma suggest that these apolipoproteins are present in bile as intact polypeptides.

We have also demonstrated that apolipoproteins A-I and A-II are detectable in bile by radioimmunoassay after addition of HDL to lipoprotein-free solutions perfusing isolated rat livers. Finally, we have shown that apoB is present in bile after addition of LDL to a lipoprotein-free solution perfusing the liver of a rat previously treated with ethinyl estradiol, a hormone that increases receptor-mediated uptake of LDL by hepatocytes.

Previous reports have indicated that protein–lipid complexes may be present in human bile (41, 42), and, by using immunodiffusion techniques, an immunogenic apolipoprotein has been proposed within these complexes; however, the molecular species of this apolipoprotein in bile was not identified (42). Because our radioimmunoassays are specific and sensitive, we have

been able, for the first time, to identify and quantitate the amounts of apolipoproteins in bile. Biliary apolipoprotein concentrations were present in amounts up to 10% of plasma values, whereas most plasma proteins found in bile are present at <0.2% of their serum concentrations (10).

The origin of these biliary apolipoproteins is unknown. It seems unlikely that contamination of bile samples with blood can explain our results for two reasons. First, the ratios of the apolipoproteins in bile differed from their ratios in plasma. Second, immunoreactive apolipoproteins were present in every sample of gallbladder bile tested, as well as in samples of bile passively collected from biliary T-tubes several days after cholecystectomy. Indeed, the relatively higher biliary concentrations of C apolipoproteins, particularly in hepatic T-tube bile, in comparison to A apolipoproteins, indicate that the origin or fate of these apolipoprotein classes may be different. We propose that either preformed apolipoproteins are specifically transported from plasma into bile or that newly synthesized apolipoproteins are directly released from the hepatocyte into biliary canaliculi; direct secretion from cells lining the biliary tree seems less likely. At present, we cannot distinguish between these possibilities, which are not necessarily mutually exclusive.

There is a growing body of data indicating that some plasma proteins which are also detectable in bile, such as secretory IgA (9), haptoglobin (10), glycoproteins (14), and perhaps insulin (11), are taken up by the hepatocyte via receptor-mediated endocytosis (15), transported across the hepatocyte in vesicles, and released into bile by exocytosis either directly or after fusion with and partial degradation by hepatocyte lysosomes. Moreover, considerable evidence indicates that plasma lipoproteins are also taken up by the hepatocyte via a receptor-mediated pathway for processing in the lysosomal system (5–7, 43–46). The subsequent fate of the components of these intralysosomal lipoproteins is, however, not entirely clear. Whether they are completely disassembled into their lipid and apolipoprotein components, and whether at least some of these components are destined for biliary excretion, either by exocytosis from lysosomes into biliary canaliculi (as has recently been proposed (17–19)) or after permeating the lysosomal membrane and entering the cell cytoplasm, is unknown. In our isolated perfused rat liver experiments, apolipoproteins A-I and A-II were detectable in bile by radioimmunoassay after the addition to a lipoprotein-free perfusate of human HDL at concentrations comparable to that of plasma. Moreover, we also detected apoB in bile by radioimmunoassay after addition of LDL to a lipoprotein-free solution perfusing the isolated liver of a rat pretreated with ethinyl estradiol. Thus, we believe that biliary apolipoproteins may be derived, at least in part, from preformed plasma lipoproteins. Whether passage into bile is via a specific vesicular transport mechanism across the hepatocyte or via a paracellular pathway (47) had not been determined. However, our observation that, after injection of LDL, apoB was detected only in the bile of rats pretreated with ethinyl estradiol, a hormone known to increase receptor-mediated uptake of LDL by hepatocytes (7), strongly suggests that a specific, transhepatocyte, receptor-mediated pathway is involved in the biliary excretion of apoB.

The possible function(s) of biliary apolipoproteins is unknown. Obviously, one possibility is that they have no particular function but that their presence in bile merely represents lysosomal discharge of material previously sequestered in hepatocyte lysosomes. We doubt this explanation because our gel filtration data suggest that apolipoproteins A-I and A-II in bile are probably present as intact polypeptides, a finding which would be unlikely if their biliary output were simply a disposal mechanism by which the hepatocyte excretes hydrolyzed lysosomal residue. A statement about the integrity of apoB in bile cannot be made with similar confidence because both plasma LDL and biliary immunoreactive apoB eluted in the void volume of the column. However, it was of interest that, while human bile contained the apoB immunoreactivity, biliary apoB was not able to precipitate LDL antibodies.

A second, more attractive and testable hypothesis, is that apolipoproteins are somehow involved in the transport, secretion, and solubilization of biliary cholesterol and phospholipid. Plasma HDL and LDL have been proposed as major sources of cholesterol and phospholipid destined for biliary secretion (48, 49). If this is true, then the apolipoprotein components of these lipoproteins might serve to direct the lipid components into bile. Such a sequence of events might explain the presence of apolipoproteins in bile. In our human experiments, no correlations between biliary apolipoprotein and biliary lipid concentrations were seen. However, we have not as yet examined whether fluctuations in biliary lipid secretion are accompanied by parallel changes in apolipoprotein secretion; such a finding would be consistent with a role for apolipoproteins in the transport and biliary secretion of cholesterol and phospholipid. Whether apolipoprotein patterns in bile relate to the formation of gallstones cannot be determined from this study. It was of interest, however, that biliary total protein concentrations were lower in gallstone patients, as has been previously reported (21).

Apolipoproteins may also function to solubilize cholesterol and phospholipid in bile in a fashion analogous to their role in plasma (2), possibly as a "back-up" or

supplementary system to bile acids and lecithin for solubilizing biliary cholesterol. Indeed, it has already been proposed that additional factors, which contribute to the stabilization of cholesterol in micelles, are probably present in bile (50), since, in more than 50% of subjects without gallstones, bile is supersaturated with cholesterol (50, 51).

Clearly, however, our data are only an initial step in examining the possible relationships between biliary lipids and apolipoproteins. Our results encourage further studies of the physical characteristics of biliary apolipoproteins and support an examination of the physiological significance of apolipoproteins in human bile.

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