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Effect of Bile Anionic Polypeptidic Fraction on the Fate of Cholesterol Carried by Liposomes in the Rat (42659)

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Abstract. [^{14}C]Cholesterol associated with liposomes with or without anionic polypeptidic fraction was administered intravenously to the rat. The cholesterol originated from liposomes including anionic polypeptidic fraction is secreted in bile much later, is stored in liver in higher quantity, and is metabolized into bile salts in lesser quantity during the 4 hr of experimentation than the cholesterol issued from liposomes exempt of anionic polypeptidic fraction. From these results it can be postulated that the cholesterol associated with liposomes containing anionic polypeptidic fraction might be directed in a particular liver pathway. © 1988 Society for Experimental Biology and Medicine.

Biliary cholesterol originates from a preformed pool of hepatic free cholesterol (1). The relationship between lipoprotein cholesterol and secretion of both biliary cholesterol and bile salts seems to be important since the major part of biliary cholesterol and bile salts is issued from lipoprotein cholesterol. Indeed, only 30% of bile salts and 10 to 20% of biliary cholesterol are derived from newly synthesized hepatic cholesterol (1–3). The remainder is derived from serum lipoprotein cholesterol and from sterol from preformed hepatic storage. The relationship between biliary lipid secretion and lipoprotein cholesterol metabolism has not yet been elucidated. Bile salts (4) and biliary cholesterol (5) are thought to originate mainly from HDL cholesterol. Other authors have observed a direct correlation between LDL cholesterol levels and biliary cholesterol saturation, indicating that biliary cholesterol is derived mainly from LDL (6). Concerning the protein moiety of lipoproteins, Sewell *et al.* have brought evidence that traces of apoproteins A-I, A-II, B, and C were present in bile (7). Results obtained in our laboratory (8–10) indicate a close association between biliary lipids (i.e., bile salts, phosphatidylcholine, cholesterol), an anionic polypeptidic fraction (APF), and fragments of IgA which together form the bile lipoprotein complex (BLC). Recently, APF has been isolated from human bile lipoprotein complex by zonal ultracentrifugation (11). In another study, an immunohistochemical method localized APF on the brush border of enterocytes, in the basolateral intercellar spaces of the absorption epithelium, on the basolateral membranes of the cells, and in the Golgi apparatus region of the cells. These results were interpreted as indicating that APF is taken up by lumen membranes or synthesized in cells, and then secreted into the circulatory system (12). This explains the presence of APF in plasma and in particular the immunological cross-reactivity observed between serum HDL and APF (13). As determined in our laboratory by ELISA using monoclonal anti-APF serum, APF represents 0.6 to 1% of the total proteic component weight of human serum HDL; this percentage is 0.3 to 0.6% for rat serum HDL. APF is also present in bile of rat (unpublished results). However, until now, the physiological function of APF had not been determined. Consequently, the aim of this work was to study, in the rat, a possible effect of APF on the fate of intravenously administered cholesterol associated with liposomes containing APF. These liposomes were obtained by zonal ultracentrifugation of human bile (11), followed by dialysis. They contained APF, and were labeled with [^{14}C]cholesterol. Control rats received [^{14}C]cholesterol–phosphatidylcholine liposomes exempt of APF.

Materials and Methods. *Animals.* Ten male Wistar rats (IFFA-Credo, l'Arbresle, France), aged 11 weeks, were randomly placed in cages of a standard animal house, and were fed a standard diet (UAR, No. A04,

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Villemoisson-sur-Orge, France) for 2 weeks. The rats weighed 350–400 g the day of experimentation.

Preparation of [^{14}C]cholesterol (Ch)–phosphatidycholine (PC)–APF liposomes: APF-[^{14}C]Ch-liposomes. The bile lipoprotein complex (BLC) was obtained from 20 ml of human gallbladder bile by zonal ultracentrifugation according to the method described previously (11). The zonal fraction (4 ml) containing the maximum of PC and APF was incubated with 1 ml of mixed micelles: sodium glycodesoxycholate (GDC, 4.7 mg, A grade, Calbiochem)–PC (2.4 mg, L-α-lecithin from egg yolk, Sigma)–Ch (0.38 mg, Serva)–[^{14}C]cholesterol (free, 5 μCi, CEA France, sp act 50 mCi/mmole). The molar ratio (GDC/PC/Ch = 10/3/1) was similar to that of human gallbladder bile. These mixed micelles were prepared according to the method of Montet and Dervichian (14). The radioactive labeling was performed by molecular exchange according to the method of Nalbone *et al.* (15). The final label represented about 60% of the incubated [^{14}C]cholesterol. The [^{14}C]Ch–BLC was dialyzed for 48 hr with running water and 48 hr with NaCl, 0.9%, so as to transform the discoidal structures into mixed unilamellar vesicles containing APF. The composition of the so-obtained liposomes was 1.87 m*M* PC, 0.6 m*M* Ch, and 74.5 μg APF/ml.

Preparation of [^{14}C]Ch-PC liposomes. [^{14}C]Ch–PC liposomes without APF were prepared according to the protocol described above for preparation of APF–[^{14}C]Ch liposomes. The starting material for preparation of these [^{14}C]Ch liposomes was 20 ml of a micellar solution: GDC (94 mg, A grade, Calbiochem)–PC (48 mg, L-α-lecithin from egg yolk, Sigma)–Ch (7, 6 mg, Serva) instead of 20 ml of human gallbladder bile. This micellar solution was prepared according to the method of Montet and Dervichian (14). The molar ratio GDC/PC/Ch of this micellar solution was similar to that of human gallbladder bile. Lecithin extracted from egg yolk is usually used to prepare liposomes and micellar solution: the nature of lecithin (extracted from bile or egg yolk) has no influence on micellar solubilization of cholesterol or on the behavior (i.e., the size) of the lipidic structures (16). Furthermore, fatty acid composition of lecithin from egg yolk differs only slightly from that of biliary lecithin. So, it can be concluded that the only difference between synthetic liposomes and liposomes obtained from bile lipids was the presence of APF.

The composition of liposomes obtained was 1.93 m*M* PC, 0.6 m*M* Ch. The homogeneity of the prepared liposomes was controlled by A_4 gel filtration. The column was eluted with 0.5 *M* Tris buffer, pH 7.5. Under these conditions, each [^{14}C]Ch liposome and APF–[^{14}C]Ch liposome was eluted in a single peak with the same elution volume. Electrophoresis has demonstrated that liposomes prepared from bile lipoprotein complex show an anodic mobility due to the presence of APF whereas PC liposomes alone have no such mobility (10).

Experimental procedures. Rats were anesthetized with sodium pentobarbital (5 mg/100 g body wt) and a biliary fistula was placed in the bile duct of each animal 10 min before intravenous injection of liposomes. Rats were divided into two groups and received by intravenous injection (jugular vein) respectively 600 μl of APF–[^{14}C]Ch liposomes (six rats, APF group) or 600 μl of [^{14}C]Ch-liposomes (four rats; control group). The quantity of cholesterol injected into rats was about 0.4 μmole, corresponding to 1/30 of total animal plasma cholesterol. Each rat received about 0.4 μCi. The duration of intravenous injection at constant flow was 15 min.

Bile was collected every 15 min during the first hour and every 30 min the next 3 hr. Bile samples were immediately weighed and frozen. Blood samples of 0.15 ml were taken at 30 min and 1, 2, 3, and 4 hr after injection and were immediately placed in heparinized test tubes. Erythrocytes were removed by centrifugation of the blood at 3000 rpm during 10 min at 4°C. The animals were killed by abdominal aortic punctures. Then the liver was excised and rinsed with NaCl, 0.9%. The hepatic tissue (2.5 g) was homogenized with a blender (Polytron PT 10, position 4) in 30 ml of Tris, 1 m*M*, saccharose, 0.25 *M*, buffer.

Assays. Biliary phospholipids were assayed by the colorimetric method (17) and enzymatic methods were used for bile salts (18)

TABLE I. PLASMA DISAPPEARANCE AND LIVER UPTAKE OF [^{14}C]CHOLESTEROL ASSOCIATED WITH LIPOSOMES WITH OR WITHOUT APF, AFTER INTRAVENOUS INJECTION

Groups	Times after injection (hr)	[^{14}C]Cholesterol (percentage injected dose)	
		Plasma	Liver
Control	$\frac{1}{2}$	54.7 ± 1.0	25.9 ± 1.2
	1	45.6 ± 0.9	27.2 ± 1.5
	2	29.5 ± 0.6	30.1 ± 2.2
	3	21.5 ± 0.9	31.2 ± 1.4
	4	13.4 ± 0.4	34.7 ± 3.2
APF	$\frac{1}{2}$	40.3 ± 0.9	51.5 ± 2.4
	1	30.6 ± 0.6	54.5 ± 2.0
	2	14.6 ± 0.8	55.8 ± 1.6
	3	7.5 ± 1.0	56.8 ± 2.2
	4	5.6 ± 0.6	60.4 ± 1.9

Note. Liposomes without APF (control group) or with APF (APF group) were labeled with [^{14}C]cholesterol and injected intravenously. At indicated times, radioactivity was determined in liver and plasma. Results are expressed as means ± SEM (n = 6 for the APF group; n = 4 for the control group).

and cholesterol (19). The bile samples (20 μl), serum samples (100 μl), and liver homogenates (500 μl) were dissolved in scintillation liquid (Beckman Ready Solv MP) to determine radioactivity.

Bile salts and cholesterol in the bile were separated by thin-layer silica gel chromatography (Schleicher-Schull, F 1500) using a migration medium consisting of 40% isoamyl acetate, 30% propionic acid, 20% isopropylic alcohol, and 10% water (v/v). Identification was performed by using ultraviolet light (at 350 nm). The spots were scraped and counted in 10 ml of scintillation liquid.

After Folch extraction (20) from liver homogenates, free and esterified cholesterols were separated by thin-layer silica gel chromatography using a migration medium containing 90% heptane, 30% sulphuric ether, 1% acetic acid (v/v). Identification was carried out by iodine vapors and the spots were scraped and counted in 10 ml scintillation liquid.

Results. *Bile.* Bile flow (1.06 ml ± 0.05/hr) was not affected by either APF–[^{14}C]Ch liposome or [^{14}C]Ch liposome injection. No significant variations in rate of biliary cholesterol or bile salt mass secretion were observed between the two groups of rats. These values remained constant throughout the experiment, 30 ± 1.40 and 0.39 ± 0.03 μmole/ml/hr for bile salts and cholesterol secretion, respectively, for the two groups of rats.

Total radioactivity. The disappearance in plasma of [^{14}C]cholesterol associated with liposomes including APF was faster than that of liposomes without APF (Table I).

A short time after injection ($\frac{1}{2}$ hr) the percentage of total radioactivity recovered in the liver was twofold higher for the APF group (51.5%) than for the control group (25.9%) (Table I). For the two groups of rats, liver uptake of [^{14}C]cholesterol progressively increased over time.

Figure 1 shows the biliary excretion rate. Radioactivity appeared rapidly in the bile for the control group and was at its peak from 30 min to 1 hr after the injection. The peak occurred later (4 hr) for the APF group.

The total amounts of radioactivity recovered at the end of experimentation in bile, serum, and liver are indicated in Table II.

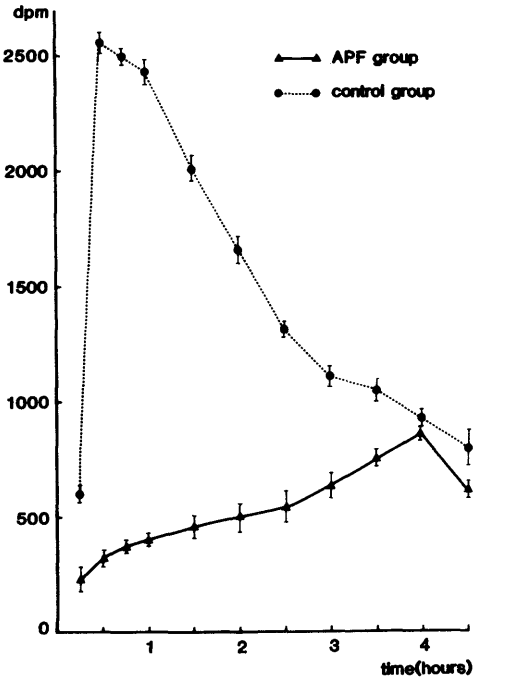

FIG. 1. Biliary excretion rate of [^{14}C]cholesterol-derived radioactivity. The rate of excretion is expressed as dpm/100 μl of bile samples at various times.

TABLE II. TOTAL RADIOACTIVITY (EXPRESSED IN PERCENTAGE INJECTED DOSE) RECOVERED IN BILE, SERUM, AND LIVER AT THE END OF THE EXPERIMENT

	APF group		Control group	
Bile	7.1 ± 0.3	BS: 5.9 Ch: 1.2	16.3 ± 1.4[a]	BS: 14.9 Ch: 1.4
Serum	5.6 ± 0.6		13.4 ± 0.4[a]	
Liver	60.4 ± 1.9	FC: 57.0 EC: 3.4	34.7 ± 3.2[a]	FC: 29.4 EC: 5.3
Total	73.1		64.4	

Note. Liposomes with or without APF were labeled with [^{14}C]cholesterol and injected intravenously. At the end of experiment, radioactivity was determined in bile, serum, and liver. Thin-layer silica gel chromatography was used to separate bile salts (BS) and cholesterol (Ch) in bile and to separate free cholesterol (FC) and esterified cholesterol (EC) in liver. Results are expressed as means ± SEM (n = 6 for the APF group; n = 4 for the control group). Differences were analyzed with Student's t test and were significant at 2 $P \leq 0.001$.

[a] APF group vs control group.

The percentages of total radioactivity recovered for the APF group in bile (7.1%) and in serum (5.6%) were significantly lower than those of the control group (respectively 16.3 and 13.4%). With regard to radioactivity excreted in bile, 82.7 and 91.4% were found in bile salts for APF group and control group, respectively; the remainder was free cholesterol. The percentage of total radioactivity recovered in the liver was higher for the APF group (60.4%) than for the control group (34.7%). This variation between the two groups was significant (2 $P \leq 0.001$). For the APF group, 94.4% of the radioactivity recovered in the liver was in free cholesterol and 5.6% was in esterified cholesterol. For the control group, these percentages were 84.8 and 15.2%, respectively.

Specific radioactivity in the bile. Figure 2 shows the maximum values of specific radioactivity for bile salts synthesized from [^{14}C]cholesterol and for biliary cholesterol. These specific radioactivities reached their highest values 30 min after injection for the control group and 4 hr after injection for the APF group. For the two groups, labeled bile salts and cholesterol appeared simultaneously in the bile, and the specific radioactivity values were greater for cholesterol than for bile salts. The ratios of specific radioactivity of bile salts to biliary cholesterol at 30 min and 4 hr after injection for the two groups of rats are indicated in Fig. 2. They were constant over time for the two groups and were lower for the APF group than for the control group.

Discussion. The distribution of radioactivity in plasma, bile, and liver was determined

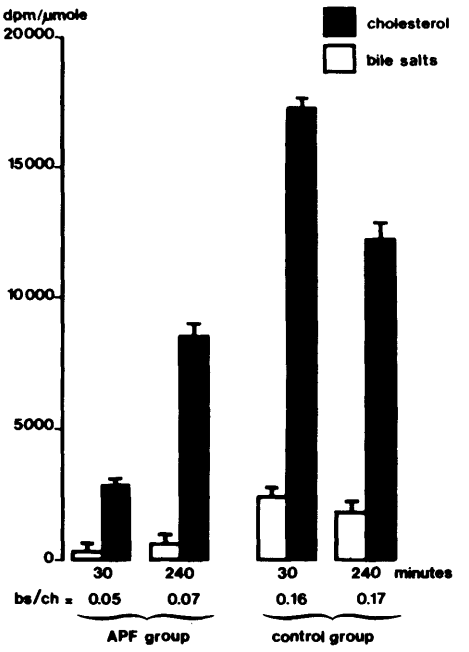

FIG. 2. Specific radioactivity of labeled bile salts and cholesterol in bile. Specific radioactivity is expressed as dpm/1 μmole of bile salts or dpm/1 μmole of cholesterol. bs/ch; ratio of specific radioactivity of bile salts to cholesterol at 30 min and 4 hr after injection for the two groups.

at various times after intravenous injection of [^{14}C]Ch liposomes including APF. Rats from control group received [^{14}C]Ch liposomes exempt of APF. The Ch–PC liposomes are more stable in the plasma than the PC liposomes, and they are taken up by the hepatocytes independently of the presence of specific receptors (21).

Differences in biliary excretion were observed during the 4 hr following cholesterol injection. Cholesterol from liposomes containing APF is secreted in the bile much later than cholesterol from liposomes exempt of APF. Recent studies have shown that LDL cholesterol appears later in the biliary secretion than HDL cholesterol or liposome cholesterol (22). These kinetic differences might depend on specific receptors for apolipoproteins and APF on the hepatocytes. Specific receptors for LDL have been detected on liver membranes (23, 24). To our knowledge, however, HDL receptors have not been evidenced on these membranes even if high-affinity saturable HDL-binding sites have been found on rat liver membranes (25). Complementary research on the possible existence of APF receptors on liver membranes would be necessary to elucidate our results.

Whether or not APF is included in liposomes, the radioactivity excreted in bile is recovered mainly in bile salts. These results are in good agreement with those of Kuipers *et al.* (26); for them, 94–96% of radioactivity excreted in bile was found in bile salts after intravenous injection of unilamellar or multilamellar cholesterol-labeled liposomes. Our results, expressed as total radioactivity and specific radioactivity, demonstrate that cholesterol from liposomes including APF is metabolized into bile salts in lesser quantity than cholesterol from APF-free liposomes. It cannot be excluded that Kupffer cells are able to uptake the liposomes as hepatocytes do. Nevertheless, it is well known that there are more hepatocytes than Kupffer cells in the liver. Furthermore, small unilamellar vesicles are known to be preferentially taken up by the hepatocytes, whereas multilamellar vesicles end up primarily in the Kupffer cells (21). In this case, the biliary excretion of multilamellar vesicle-associated cholesterol is delayed and the cholesterol label appears in bile from 7 to 12 hr after injection (26). In the present study, the biliary secretion of liposome-associated cholesterol is rapid; the cholesterol label and bile salts appear in bile from 10 to 240 min after injection. This rapid biliary excretion of liposome-associated cholesterol strongly suggests the preferential uptake of these liposomes by hepatocytes. Furthermore, results indicated that cholesterol carried by liposomes including APF is taken up by the liver in higher quantity than cholesterol from APF-free liposomes. The liver uptake of [^{14}C]cholesterol associated with liposomes including APF progressively increases over time. APF does not seem to block [^{14}C]cholesterol uptake by the liver. These results suggest that the two lipidic structures, one with APF and the other without, might use their own specific hepatic intracellular pathway or might be excreted from the hepatocyte at different rates.

In conclusion, the presence or absence of APF leads to differences in the hepatic uptake, the transformation into bile salts, and the rates of biliary elimination of cholesterol originating from the two lipidic structures. APF seems to have a function in the control of bile-destined cholesterol. This APF might act at different levels in the hepatic cell: in the cholesterol uptake by the sinusoidal pole exchanging plasmatic constituents, in the intracellular pathway, or in cholesterol excretion by the biliary pole. Further experiments are being carried out on the potential role of APF in the uptake of cholesterol by the sinusoidal membranes of hepatocytes. Recent results obtained in our laboratory (unpublished data) suggest that rat hepatocytes take up and degrade APF through a process mediated by saturable binding sites. As an immunological cross-reaction occurs between serum HDL and APF (13), it is possible that the apoprotein-like character of APF is the cause of its specific liver uptake.

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