Studies on the transfer of phosphatidylcholine from unilamellar vesicles into plasma high density lipoproteins in the rat

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Abstract To investigate the metabolic disposition of phosphatidylcholine vesicles, unilamellar vesicles of egg phosphatidylcholine or cholesterol/phosphatidylcholine were injected intravenously into rats. Ten minutes after injection of 0.5 to 3.0 mg phosphatidylcholine vesicles, a major fraction of their radioactivity and mass had been incorporated into high density lipoproteins (HDL). Agarose gel chromatography showed that the phospholipid of vesicles was incorporated into the plasma HDL region, in association with release of a trapped marker, [^{3}H]inulin. Density gradient ultracentrifugtion showed that the highest specific activity of phospholipid was present in HDL of d 1.08–1.12 g/ml, consisting of homogenous spherical particles by negative stain electron microscopy. With increasing cholesterol/phosphatidylcholine ratio of injected vesicles, there was progressively less incorporation of phospholipid into HDL and vesicles retaining [^{3}H]inulin were re-isolated from plasma. Sixty minutes after injection of cholesterol/phosphatidylcholine vesicles, phospholipid appeared to be partly transferred into HDL and partly taken up by the liver. In summary, injection of unilamellar egg phosphatidylcholine vesicles results in a rapid incorporation of vesicle phospholipid into plasma HDL, primarily as a result of insertion of phospholipid into pre-existing HDL. This process is inhibited by a high content of vesicle unesterified cholesterol. These studies may have relevance to the mechanisms of transfer of phosphatidylcholine from chylomicrons into plasma HDL.—**Tall, A. R.** Studies on the transfer of phosphatidylcholine from unilamellar vesicles into plasma high density lipoproteins in the rat. *J. Lipid. Res.* 1980. **21:** 354–363.

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The formation of high density lipoprotein (HDL) recombinants from phospholipid liposomes and isolated HDL apoproteins has been extensively studied as a means of elucidating lipid-protein interactions in the plasma lipoproteins (1–3). Phospholipid liposomes have also been shown to interact with intact plasma HDL in vitro, resulting in incorporation of phospholipid mass into the HDL fraction (4–7). This has been demonstrated for multilamellar liposomes and unilamellar vesicles of dimyristoyl phosphatidylcholine (4, 5, 7) and for unilamellar vesicles of egg yolk phosphatidylcholine (6). The incorporation of phospholipid by the HDL fraction is associated with breakdown of liposome or vesicle structure, resulting in formation of a variety of smaller, phospholipid-rich particles (4–7). Following injection of unilamellar egg yolk phosphatidyl-choline vesicles into the rat, phospholipid radioactivity was incorporated into HDL (8), suggesting that similar interactions between vesicles and HDL may occur in vivo.

The interaction of phospholipid vesicles or liposomes with the isolated apoproteins of plasma HDL is profoundly influenced by the presence of unesterified cholesterol in the phospholipid (9–12). Reports from several laboratories (9–12) indicate that, at least in the presence of higher cholesterol contents, solubilization of phospholipid by apoA-I is markedly inhibited, and the resulting lipoprotein recombinants are relatively cholesterol-poor. These studies raise the possibility that interactions between phospholipid vesicles and intact plasma HDL may be modified by the presence of unesterified cholesterol in the phospholipid vesicles. To study the effects of cholesterol on vesicle-HDL interaction in vivo, small doses of sonicated, fractionated egg yolk phosphatidylcholine vesicles were injected into rats. The aims of these experiments were 1) to determine whether phospholipid *mass* would be incorporated into HDL in vivo; 2) to examine the mechanisms of uptake of phospholipid by HDL, and 3) to investigate the effect of variable vesicle cholesterol/phospholipid ratio on this process.

MATERIALS AND METHODS

Egg yolk phosphatidylcholine (Grade I, Lipid Products) and phosphatidylcholine, (choline-[^{14}C]-methyl), (New England Nuclear) were co-purified by silicic acid chromatography, to >99% purity, as judged by quantitative thin-layer chromatography.

Abbreviations: HDL, high density lipoprotein; PC, phosphatidylcholine.

Cholesterol (Nu-Chek Prep) and [1,2-^{3}H(N)]cholesterol (New England Nuclear) were used without further purification. Aliquots of stock solutions of egg yolk PC and cholesterol in cyclohexane were mixed in appropriate proportions and lyophilized. Five ml of 0.9% saline, pH 8.5, was added to the dried lipids and the lipid suspension was sonicated in a 10 ml water-jacketed sonication cell (Wilbur Scientific) under a stream of N_2. Sonication was continued until the solutions had clarified (about 45 min for PC and 60–180 min for cholesterol/PC). The temperature of the solution was not allowed to rise above 20°C. Following sonication the suspensions were centrifuged at 1,500 *g* for 30 min, then passed over a 40 × 2.5 cm column of cross-linked Sepharose 4B (Pharmacia). The column profiles were similar to those described by Huang (13). For cholesterol-containing vesicles the ratio of ^{3}H/^{14}C was constant across the vesicle peak. Fractions from the top and the descending limb of the vesicle peak were pooled and concentrated to about 1 mg phospholipid/ml by vacuum dialysis or by ultrafiltration (Amicon). These fractions were shown by chemical assay (14, 15) to contain the same ratio of cholesterol/phosphatidylcholine as the material loaded onto the column. Vesicles were stored at 4°C and used within 24 hr of preparation. Only vesicles that had been fractionated on Sepharose 4B were injected into the rats. For preparation of vesicles containing [^{3}H]inulin, about 10 mg of lipid was sonicated in the presence of 100 μCi of [^{3}H]inulin. The liposomes were then fractionated by Sepharose 4B chromatography. The [^{3}H]inulin associated with the vesicle peak was >90% releasable when vesicles were treated with an osmotically active compound, 1.0 M cellobiose, and then reanalyzed on Sepharose 4B. This indicated trapping of [^{3}H]inulin in the internal aqueous compartment of vesicles.

A cannula was placed in the femoral vein of male Sprague-Dawley rats (200–250 g) under ether anesthesia. After recovery from anesthesia animals were injected with 0, 20, 33 or 50 mole% cholesterol/PC vesicles, usually containing 1 mg PC. After a specified time, animals were exsanguinated under ether anesthesia from the abdominal aorta, using a 10 ml syringe containing 1,000 U heparin and 0.1 ml of 20 mM 5,5′-dithionitrobenzoic acid. In experiments designed to study the time course of removal of vesicle radioactivity from plasma (**Fig. 1**), rats were placed in restraining cages and 0.5 ml blood samples drawn at the times shown.

Plasma lipoproteins were isolated by sequential preparative ultracentrifugation. Lipoproteins were separated in a Beckman 40.3 rotor as follows: d 1.006 g/ml, 16hr × 40,000 rpm; d 1.063 g/ml, 24hr × 40,000 rpm; d 1.21 g/ml, 36hr × 40,000 rpm. The HDL (d 1.063 to 1.21 g/ml) was recentrifuged for 36hr × 40,000 rpm to remove albumin. An aliquot of the lipoprotein sample was counted. The rest was diluted, the lipids extracted in chloroform–methanol 2:1 (16) and analyzed for cholesterol, fatty acid, triglyceride, cholesteryl ester, lysolecithin, sphingomyelin, and lecithin by quantitative thin-layer chromatography, using the method of Downing (17). The mass of phospholipid in the HDL fraction was calculated from total HDL phospholipid radioactivity ÷ specific activity of phospholipid in the lipid extract. Masses were corrected for incomplete recovery of plasma, using published values for rat plasma volume as a function of body weight (18).

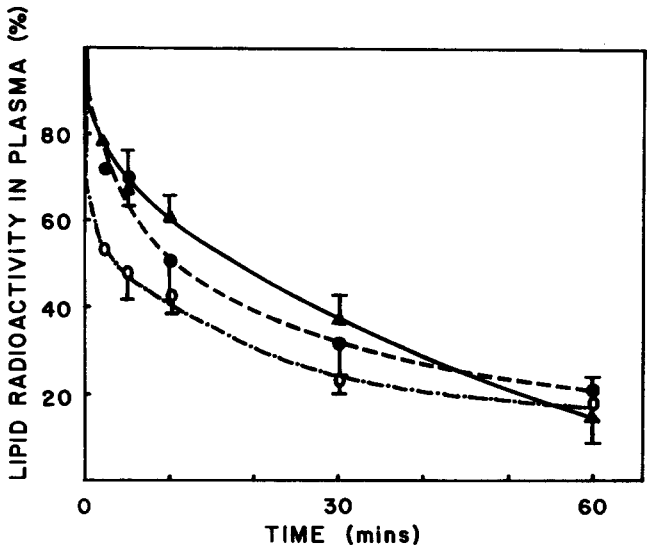

Fig. 1. Time course of disappearance of phospholipid radioactivity from plasma following injection of phosphatidylcholine vesicles (closed circles) or 50 mole% cholesterol/phosphatidylcholine vesicles (triangles) and time course of disappearance of cholesterol radioactivity from plasma following injection of 50 mole% vesicles (open circles). Unilamellar vesicles fractionated by Sepharose 4B column chromatography were injected into rats via a femoral cannula and blood samples withdrawn at the times shown. Results are given as mean ± SEM, with n = four to six rats for each time point, except for 2 min, where n = 3.

Plasma lipoproteins were also analyzed by performing equilibrium density gradient ultracentrifugation of whole plasma. Gradients were formed by layering solutions of different densities, as described by Anderson et al. (19), and were centrifuged for 48 hr in a Beckman SW 50.1. rotor at 50,000 rpm. Densities were determined from refractive index measurements made with an Abbe-3L refractometer at 20°C. Individual fractions from the gradient were extracted by the procedure of Folch, Lees, and Sloane Stanley (16) and their phospholipid (15) and total cholesterol (14) contents were assayed. An aliquot of lipoprotein was dialyzed, lyophilized, and delipidated with

TABLE 1. Distribution of radioactivity following injection of cholesterol/phospholipid vesicles[a]

Injected Vesicles (mole% Cholesterol)	Plasma	Liver	Distribution in Plasma Lipoproteins[a]			
			d < 1.006 g/ml	d 1.006–1.063 g/ml (LDL)	d 1.063–1.21 g/ml (HDL)	d > 1.21 g/ml
			^{14}C-labeled Phospholipid, 10 min			
0	51 ± 8.0	7 ± 1.7	3 ± 1.6	31 ± 3.6	60 ± 3.9	6 ± 1.9
20	45 ± 9.0	9 ± 3.4	2 ± 1.0	52 ± 7.0	42 ± 7.2	4 ± 1.2
33	58 ± 7.0	7 ± 3.9	1 ± 0.6	77 ± 1.1	19 ± 1.3	3 ± 0.6
50	61 ± 5.0	14 ± 2.9	1 ± 0.5	90 ± 1.3	5 ± 1.0	5 ± 1.3
			[^{3}H]Cholesterol, 10 min			
20	33 ± 7.0	12 ± 2.7	2 ± 0.5	52 ± 9.0	46 ± 10	ND[b]
33	43 ± 7.5	13 ± 4.0	3 ± 1.0	74 ± 1.7	23 ± 1.8	ND
50	43 ± 4.2	19 ± 4.0	2 ± 1.7	78 ± 4.7	21 ± 5.0	ND
			^{14}C-labeled Phospholipid, 60 min			
0	22 ± 3.0	14 ± 3.4	3 ± 1.7	45 ± 6.1	42 ± 8.4	11 ± 3.5
20	24 ± 4.8	19 ± 5.1	1 ± 0.5	52 ± 9.0	38 ± 9.2	9 ± 2.8
33	14 ± 1.5	32 ± 4.6	ND	55 ± 6.1	33 ± 5.5	12 ± 4.3
50	14 ± 5.0	38 ± 5.3	ND	65 ± 9.0	22 ± 4.7	13 ± 6.4
			[^{3}H]Cholesterol, 60 min			
20	13 ± 3.0	19 ± 7.0	ND	39 ± 5.2	61 ± 5.2	ND
33	11 ± 2.5	26 ± 5.1	ND	47 ± 8.7	54 ± 8.7	ND
50	17 ± 2.8	39 ± 3.4	ND	58 ± 4.7	42 ± 4.7	ND

[a] Radioactivity recovered in plasma and liver are given as a percentage of the injected dose. The distribution of radioactivity in plasma lipoproteins is given as a percentage of total radioactivity recovered following preparative ultracentrifugation (80–95% of plasma radioactivity was recovered). Results shown are mean ± SEM. For each time point and cholesterol content, four or five rats were used.

[b] ND, not detected.

ethanol–ether 3:2 (vol/vol) at 4°C. The apoproteins were analyzed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and stained with Coomassie Blue, using gels of both 5.6% and 11% polyacrylamide. The HDL fractions were also negatively stained with 2% Na phosphotungstate, pH 7.0, on Formvar-coated Cu grids and examined with an Hitachi 11c electron microscope operated at 75 Kv. Lipoproteins were sized directly from randomly chosen areas of electron micrographs.

RESULTS

Following injection of vesicles, disappearance of phospholipid radioactivity from plasma was initially rapid, then more gradual (Fig. 1). The time course of removal of phospholipid radioactivity was similar for vesicles of phosphatidylcholine or 50 mole% cholesterol/phosphatidylcholine (Fig. 1). Twenty and 33 mole% cholesterol/phospholipid vesicles also showed similar removal of phospholipid radioactivity from plasma at 10 or 60 min (**Table 1**). The removal of cholesterol radioactivity from plasma was initially more rapid than that of phospholipid ractioactivity, but was similar at 60 min (shown for 50 mole% vesicles in Fig. 1). For 20, 33 and 50 mole% cholesterol/PC vesicles there was also less cholesterol than phospholipid radioactivity in plasma at 10 min, but not at 60 min (Table 1).

The distribution of phospholipid and cholesterol radioactivity in the lipoprotein fractions of plasma is shown in Table 1. For control vesicles of PC, more than 95% of radioactivity was isolated at d < 1.063 g/ml. Following injection of vesicles, the distribution of radioactivity was strikingly dependent on the cholesterol content (Table 1). Ten minutes after injection of phosphatidylcholine vesicles, the major plasma fraction containing phospholipid radioactivity was HDL (Table 1). By contrast, for cholesterol-containing vesicles, the major fraction containing radioactivity was the d 1.006–1.063 g/ml fraction, as for control vesicles. Thus, the transfer of vesicle phospholipid radioactivity into HDL was inhibited by the presence of unesterified cholesterol in the vesicles. This effect was more marked with increasing cholesterol content of the vesicles, and was especially pronounced for 33 and 50 mole% vesicles. For cholesterol-containing vesicles the transfer of cholesterol radioactivity into HDL was more marked than that of phospholipid

radioactivity. However, the majority of cholesterol radioactivity remained at d 1.006–1.063 g/ml especially with increasing cholesterol content of the vesicles (Table 1). Sixty minutes after injection of vesicles, the distribution of phospholipid radioactivity in the plasma lipoproteins was similar to the 10 min time points, i.e., with increasing cholesterol content of the injected vesicles, a greater amount of radioactivity remained in the d 1.006–1.063 g/ml fraction (Table 1). However, the differences between phosphatidylcholine and cholesterol/phosphatidylcholine vesicles were less marked.

Analysis of phospholipid radioactivity in liver showed a greater hepatic uptake with increasing cholesterol content of the vesicles (Table 1). A similar percentage of cholesterol and phospholipid radioactivity were taken up by the liver, consistent with uptake of intact vesicles. However, less than half of the injected radioactivity was recovered in the liver at 10 min or 60 min, and phospholipid radioactivity recovered in the spleen was less than 1%. Less than 2% of phospholipid radioactivity was present in the wall and luminal contents of the small intestine. For all vesicles, the sum of plasma plus hepatic radioactivity recovered at 60 min was 36–52% of that injected, indicating uptake or exchange of radioactivity involving other organs or tissues.

The relative lipid composition of the plasma lipoproteins after injection of vesicles is shown in **Table 2.** For PC and 20 mole% cholesterol/PC vesicles there was a marked increase in relative content of PC in HDL 10 min after injection of vesicles. This was reflected by increased ratios of phospholipid/cholesteryl ester, which were significantly greater ($p < 0.01$) than for control HDL. By contrast, for 33 and 50 mole% vesicles there were smaller increments in PC in HDL at 10 min and changes in the ratio of phospholipid to cholesteryl ester were not statistically significant. For the d 1.006–1.063 g/ml fraction, changes in lipid composition were the converse of those in HDL, i.e. the highest phospholipid/cholesteryl ester ratios were observed for 33 and 50 mole% vesicles, consistent with persistence of vesicles at d 1.006 to 1.063 g/ml in these samples. This was also reflected in a small increase in cholesterol content with increasing cholesterol content of injected vesicles.

Sixty minutes after injection of the vesicles, the phospholipid content of HDL was still increased for the PC and the 20 mole% cholesterol/PC vesicles. In contrast to the 10 min time point, for the 33 and 50 mole% vesicles, there was also an increased content of phospholipid, resulting in significant ($p < 0.05$) increases in the ratio of phospholipid/cholesteryl ester.

Calculation of the total mass of phospholipid in

TABLE 2. Lipid composition of plasma lipoproteins following injection of cholesterol/phosphatidylcholine vesicles[a]

Vesicle (mole% Cholesterol)	Cholesterol	Triglyceride	Cholesteryl Ester	Sphingomyelin	Phosphatidylcholine	Phospholipid/ Cholesteryl Ester
			% of total lipids			
LDL, 10 min						
Control (n = 6)	10 ± 2.5	5 ± 1.2	39 ± 4.0	10 ± 1.9	34 ± 1.8	1.1 ± .10
0 (n = 5)	11 ± 0.9	8 ± 1.1	35 ± 1.2	9 ± 1.1	35 ± 1.1	1.2 ± .10
20 (n = 4)	11 ± 0.8	10 ± 3.0	34 ± 2.6	7 ± 2.3	35 ± 1.1	1.2 ± .10
33 (n = 4)	13 ± 1.8	8 ± 1.2	34 ± 1.8	8 ± 0.8	36 ± 0.3	1.3 ± .08
50 (n = 4)	14 ± 0.8	5 ± 1.1	32 ± 2.4	9 ± 1.1	41 ± 2.6	1.6 ± .19
HDL, 10 min						
Control (n = 12)	7 ± 1.1	2 ± 0.6	38 ± 1.8	8 ± 0.4	43 ± 2.0	1.4 ± .09
0 (n = 5)	8 ± 1.4	1 ± 0.3	27 ± 2.3	10 ± 1.5	55 ± 2.4	2.5 ± .36
20 (n = 4)	8 ± 1.3	ND[b]	26 ± 2.3	10 ± 2.2	55 ± 1.7	2.6 ± .25
33 (n = 4)	5 ± 1.3	2 ± 0.6	35 ± 3.2	10 ± 2.2	47 ± 1.1	1.6 ± .17
50 (n = 4)	7 ± 2.5	1 ± 0.3	33 ± 3.3	7 ± 2.0	51 ± 2.6	1.8 ± .12
LDL, 60 min						
0 (n = 4)	10 ± 0.4	9 ± 3.0	34 ± 1.8	10 ± 0.6	37 ± 1.5	1.4 ± .07
20 (n = 4)	11 ± 0.5	11 ± 2.9	32 ± 1.6	10 ± 0.8	36 ± 3.4	1.5 ± .19
33 (n = 4)	11 ± 0.7	10 ± 4.7	33 ± 0.3	10 ± 1.0	35 ± 3.2	1.4 ± .15
50 (n = 4)	12 ± 0.2	8 ± 2.5	34 ± 2.1	10 ± 0.5	35 ± 2.3	1.3 ± .13
HDL, 60 min						
0 (n = 4)	7 ± 0.5	ND	31 ± 1.6	9 ± 0.3	54 ± 1.7	2.1 ± .18
20 (n = 4)	8 ± 1.4	1 ± 0.3	26 ± 2.3	12 ± 0.5	52 ± 2.2	2.6 ± .30
33 (n = 4)	9 ± 1.5	ND	30 ± 1.7	10 ± 0.9	50 ± 1.3	2.0 ± .12
50 (n = 4)	8 ± 0.4	1 ± 0.3	28 ± 1.7	11 ± 1.1	52 ± 2.1	2.2 ± .18

[a] Relative lipid composition of plasma lipoproteins determined by quantitative TLC.
[b] ND, not detected.

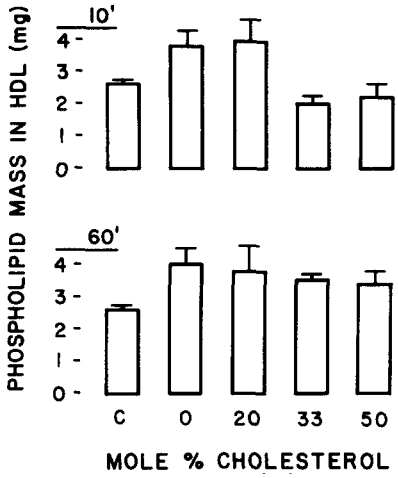

Fig. 2. Mass of phospholipid in HDL 10 min or 60 min after injection of 1 mg of phosphatidylcholine vesicles containing 0, 20, 33, or 50 mole% cholesterol. C indicates control rats (n = 12), not injected with vesicles. For each cholesterol dose and time point four to five rats were used. Values shown are mean ± SEM.

HDL showed an increase of approximately 1.0 mg 10 min after injection of phosphatidylcholine or 20 mole% cholesterol/phosphatidylcholine vesicles (**Fig. 2**). By contrast, there were no significant changes in mass of phospholipid in HDL 10 min after injection of 33 or 50 mole% vesicles. At 60 min there was still an increased mass of phospholipid in HDL for 0 and 20 mole% vesicles. In contrast to the 10-min values, the mass of phospholipid in HDL was increased by 0.5 to 0.6 mg 60 min after injection of 33 and 50 mole% cholesterol/PC vesicles (Fig. 2), indicating a partial transfer of phospholipid into HDL.

To investigate further the disposition of vesicles following injection, vesicles containing a trapped, water-soluble marker ([^{3}H]inulin) were injected. These vesicles were prepared by sonication of PC or cholesterol/PC in the presence of [^{3}H]inulin. Free [^{3}H]inulin was removed by Sepharose 4B chromatography, then the vesicles were concentrated and injected. After 10 min of circulation the recipient rat's plasma was analyzed by chromatography on 6% agarose. In order to control for the loss of [^{3}H]inulin due to spontaneous leakage, an identical aliquot of vesicles was also injected into saline and analyzed simultaneously on an identical 6% agarose column.

In **Fig. 3** is shown the distribution of ^{14}C-labeled PC and [^{3}H]inulin radioactivity following injection of PC vesicles into saline or a rat, and following injection of 25 to 50 mole% cholesterol/PC into a rat. Following injection of PC vesicles into saline, most of the PC radioactivity was re-isolated in the small vesicle region; a smaller amount eluted just after the column void volume. [^{3}H]inulin was present in both regions but

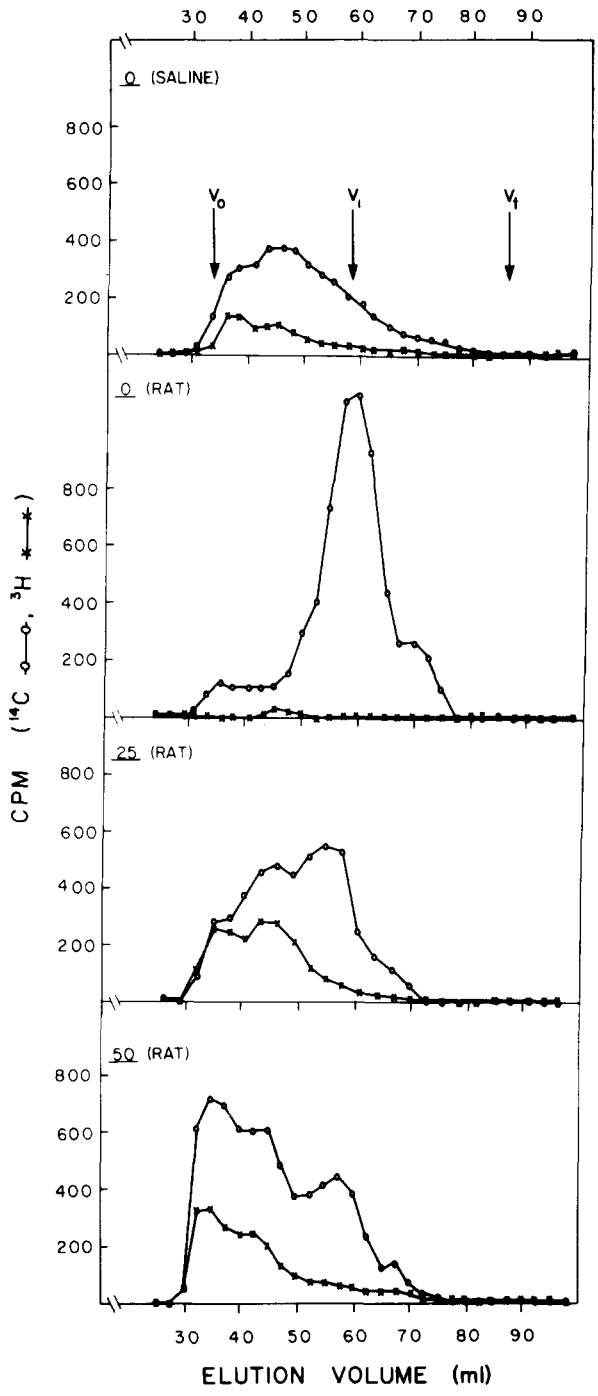

Fig. 3. Gel chromatography of rat plasma or saline after injection of cholesterol/[^{14}C]phosphatidylcholine vesicles containing trapped [^{3}H]inulin. The distribution of [^{14}C]phosphatidylcholine and [^{3}H]inulin radioactivity is shown after injection of phosphatidylcholine vesicles into saline (0 (SALINE)) or into a rat (0 (RAT)) or after injection of 25 or 50 mole% cholesterol/phosphatidylcholine vesicles into a rat. The preparation of vesicles containing trapped [^{3}H]inulin is described in the legend to Table 3. Plasma or saline were fractionated by chromatography on a 120 cm column of Sepharose CL-6B. Vo and Vt indicate, respectively, the void and total column volumes and V_1 the elution volume of a control preparation of rat HDL.

TABLE 3. Fate of [^{3}H]inulin radioactivity after injection of cholesterol/[^{14}C]phosphatidylcholine vesicles[a]

Mole% Cholesterol	Into Rat		Into Saline		Ratio 1/ Ratio 2[d]
	^{3}H in Vesicles[b]	^{3}H/^{14}C[c]	^{3}H in Vesicles	^{3}H/^{14}C	
	cpm	*ratio 1*	*cpm*	*ratio 2*	
0	75	.01	928	.24	.08
25	2161	.46	1,720	.87	.53
33	2394	.88	1,655	1.07	.82
50	2451	.35	1,684	.43	.81

[a] Vesicles were prepared by sonication of about 10 mg of lipid in the presence of 100 μCi [^{3}H]inulin, followed by chromatography on Sepharose CL-4B. Fractions from the vesicle peak were pooled and concentrated by ultrafiltration (Amicon). Aliquots containing 1 mg PC were injected into rats or into a volume of saline equivalent to the plasma volume of the rat (~9 ml). Following 10 min of circulation in the rat or incubation in saline, 1.5 ml of plasma or 1.5 ml saline were simultaneously passed over identical 120 cm columns of Sepharose CL-6B.

[b] ^{3}H in vesicles is the total [^{3}H]inulin radioactivity, corrected for spillover from the ^{14}C channel, associated with the vesicle region of the column elution profile. Results are the means of duplicate experiments. About 70% of the total [^{3}H]inulin radioactivity injected into saline was recovered from the columns.

[c] ^{3}H/^{14}C is the ratio of total [^{3}H]inulin to total [^{14}C]phosphatidylcholine radioactivity recovered from the vesicle and HDL regions of the elution profile.

[d] Ratio 1/ratio 2 is the ^{3}H/^{14}C ratio following injection of vesicles into the rat divided by the ^{3}H/^{14}C ratio following injection into saline. This fraction expresses the retention of [^{3}H]inulin in vesicles injected into the rat, normalized for recovery of ^{14}C radioactivity and spontaneous leakage of [^{3}H]inulin from vesicles. A ratio 1/ratio 2 of 1.0 would indicate that injection into the rat resulted in no leakage of [^{3}H]inulin.

more was associated with the larger liposomes reflecting their bigger internal volumes. Following injection of PC vesicles into the rat, almost all of the phospholipid radioactivity was transferred into the HDL region, with release of [^{3}H]inulin (Fig. 3). The failure to recover free [^{3}H]inulin from plasma (Fig. 3) probably reflects rapid dilution of released inulin in the relatively large extracellular fluid volume. With increasing cholesterol content of the injected vesicles, there was progressively less transfer of PC radioactivity into the HDL region and persistent association of [^{3}H]inulin with the vesicles. Analysis of phospholipid mass in these experiments showed that transfer of radioactivity into the HDL peak was paralleled by mass transfer of phospholipid (not shown). A small peak of phospholipid radioactivity (Fig. 3) and mass eluted after the HDL peak; possibly representing lysophosphatidylcholine bound to albumin.

The recoveries of [^{3}H]inulin following injection of PC or cholesterol/PC vesicles into rats or into saline are summarized in **Table 3.** Following injection into the rat, increasing cholesterol content of the vesicles was associated with an increase in the absolute amount of [^{3}H]inulin recovered in the vesicle region (^{3}H in vesicles, Table 3). However, an increased amount of [^{3}H]inulin was also recovered for cholesterol/PC vesicles injected into saline, reflecting their decreased permeability to inulin.

To normalize for spontaneous leakage of inulin, the recoveries in plasma and saline were compared. Recoveries were also normalized for the total amount of ^{14}C-labeled PC across the column profile (^{3}H/^{14}C ratio). The ratio of ^{3}H/^{14}C ratios in plasma and saline expresses the relative retention of [^{3}H]inulin in vesicles injected into the rat compared to saline, normalized for recoveries of ^{14}C radioactivity. A value of 1.0 would indicate that there was similar spontaneous leakage of inulin after injection into the rat or into saline and therefore no leakage of inulin *as a result of injection into the rat*. The results (ratio 1/ratio 2 in Table 3) indicate release of [^{3}H]inulin as a result of injection into the rat, especially from 0 and 25 mole% vesicles compared to 33 and 50 mole% vesicles. These experiments demonstrate that following injection of 0 and 25 mole% vesicles, there is disruption of the vesicle structure, with incorporation of PC into HDL-sized particles. With increasing cholesterol content, the injected vesicles tend to persist in plasma as vesicles and there is decreased incorporation of PC into HDL.

To assess possible changes in the structure or composition of HDL resulting from acquisition of PC, the HDL region of plasma was analyzed by density gradient ultracentrifugation. Ten or 60 min after

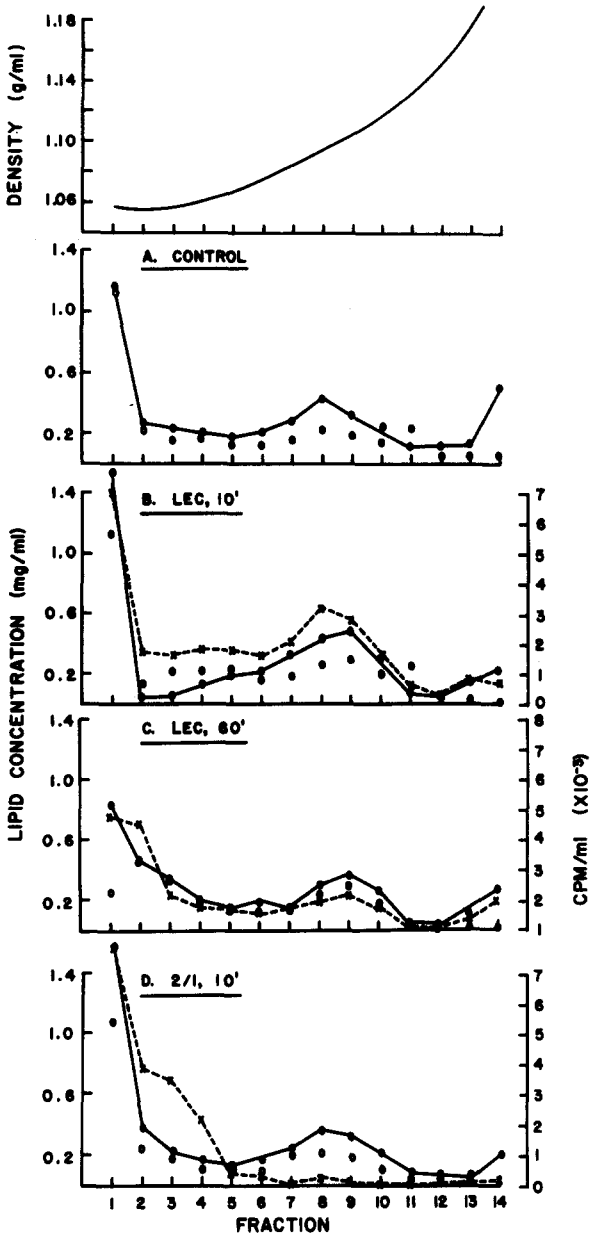

Fig. 4. Density gradient ultracentrifugation of rat plasma from control animals or from rats 10 min after injection of phosphatidylcholine vesicles (LEC, 10′), or 50 mole% cholesterol/phosphatidylcholine vesicles (2/1, 10′), or 60 min after injection of phosphatidylcholine vesicles (LEC, 60′). The concentration of phospholipid (closed circles) and total cholesterol (open circles) was determined in individual fractions. The phospholipid radioactivity is shown with crosses. The density of individual fractions was determined in each experiment by refractometry. The line joining the mean values for each fraction is shown in the top panel.

injection of PC or 50 mole% cholesterol/PC vesicles, plasma was analyzed between densities 1.06 to 1.18 g/ml. The profiles of phospholipid radioactivity and phospholipid, cholesterol and protein concentrations were determined across the gradient. For control rats, the major HDL peak was present between densities 1.08 to 1.12 g/ml as shown in **Fig. 4.** In some animals a smaller peak was present at density 1.06 to 1.08 g/ml (not shown). Ten minutes after injection of PC vesicles, most of the PC radioactivity and mass had been incorporated into the major HDL peak (Fig. 4). The radioactivity of control PC vesicles was confined to the top two fractions of the gradient (not shown). In the phospholipid-enriched HDL, the concentrations of phospholipid, total cholesterol and total protein (not shown) described a smoothly contoured peak (Fig. 4) suggesting that phospholipid combined with pre-existing HDL.

Sixty minutes after injection of PC vesicles, the HDL peak still had a smooth contour (Fig. 4). Ten minutes after injection of 50 mole% cholesterol/PC vesicles, there was no transfer of PC mass or radioactivity into the major HDL peak (Fig. 4D). The extra phospholipid and cholesterol present in fractions 1–4 of the gradient (Fig. 4D) was also present following density gradient ultracentrifugation of control vesicles of 50 mole% cholesterol/PC (not shown), suggesting that fractions 1–4 contained unreacted vesicles. These findings reflect the greater hydrated density of cholesterol-containing vesicles. Sixty minutes after injection of 50 mole% vesicles, there was a peak containing primarily phospholipid and protein in fractions 3 and 4 (not shown). This was not defined further.

The total mass of PC in the major HDL peak was determined by summing the individual fractions of the density gradients. The total masses of PC were 2.5 mg from control animals; 3.4 mg, from rats 10 min after injection of PC vesicles; 2.6 mg, from rats 60 min after injection of PC vesicles; 2.5 mg from rats 10 min after injected of 50 mole% vesicles; and 3.1 mg from rats 60 min after injection of 50 mole% vesicles. Except for the PC mass in HDL at 60 min, these results are in reasonable agreement with Fig. 1, suggesting that the major mechanism of transfer of PC into the HDL density range was by incorporation into particles of density 1.08 to 1.12 g/ml. The total mass of protein recovered under the major HDL peak (1.7 to 2.1 mg) did not appear to be altered by aquisition of PC, but these measurements could not be made precisely due to contaminating plasma proteins in fractions 11–14.

Fractions of the HDL obtained by density gradient ultracentrifugation were examined by negative stain electron microscopy and analyzed by SDS polyacrylamide gel electrophoresis. The major peak of HDL of control rats or of HDL from rats 10 or 60 min after injection of PC vesicles, consisted of a uniform population of apparently spherical particles, with no

disks or vesicles. In **Fig. 5** is shown the size distribution of particles in the major HDL peak from control rats or 10 min after injection of 1 mg of PC vesicles. There was no major size change in the peak fraction of HDL following acquisition of 1 mg of PC. The HDL apoproteins of rats injected with PC and 50 mole% cholesterol/PC vesicles were analyzed in both 5.6% and 11% SDS polyacrylamide gels. The apoprotein patterns were identical to those reported previously (20, 21) with major bands of apoA-I and apo-E and lesser bands of apoA-IV and apoC. There was no appreciable alteration in the percentage distribution of these apoproteins after injections of 1mg of vesicles, as determined by gel scanning.

To explore further the mechanisms of incorporation of PC into HDL, rats were injected with different doses of unilamellar egg PC vesicles and the plasma HDL region was analyzed by density gradient ultracentrifugation. The specific radioactivity (cpm/mg phospholipid) of individual fractions in the HDL region was determined 10 min after injection of 0.5, 1.0, 2.0, or 3.0 mg of PC vesicles (**Fig. 6**). The specific activity of the injected vesicles was 100,000 cpm/mg phospholipid. For all doses there was an increase in phospholipid specific activity in HDL, primarily between densities 1.08 to 1.12 g/ml. With the smaller doses of phospholipid, the specific activity was broadly distributed within this density range; with the larger doses the specific activity formed a sharper peak. The center of distribution of specific activity moved from about density 1.110 g/ml (0.5 mg dose) to about density

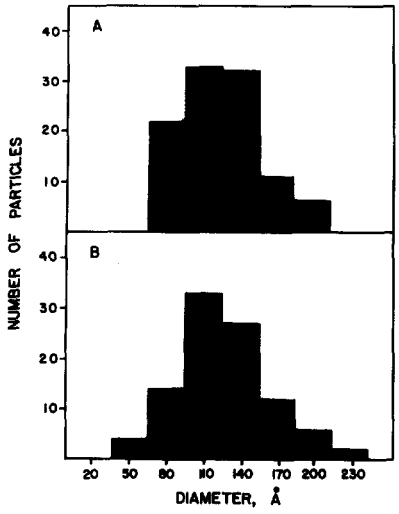

Fig. 5. Size distribution of HDL particles from uninjected control rats (A) or 10 min after injection of 1 mg phosphatidylcholine vesicles (B). The HDL fraction from the density gradients (Fig. 4) containing the highest concentration of phospholipid was examined on Formvar coated grids, negatively stained with phosphotungstic acid.

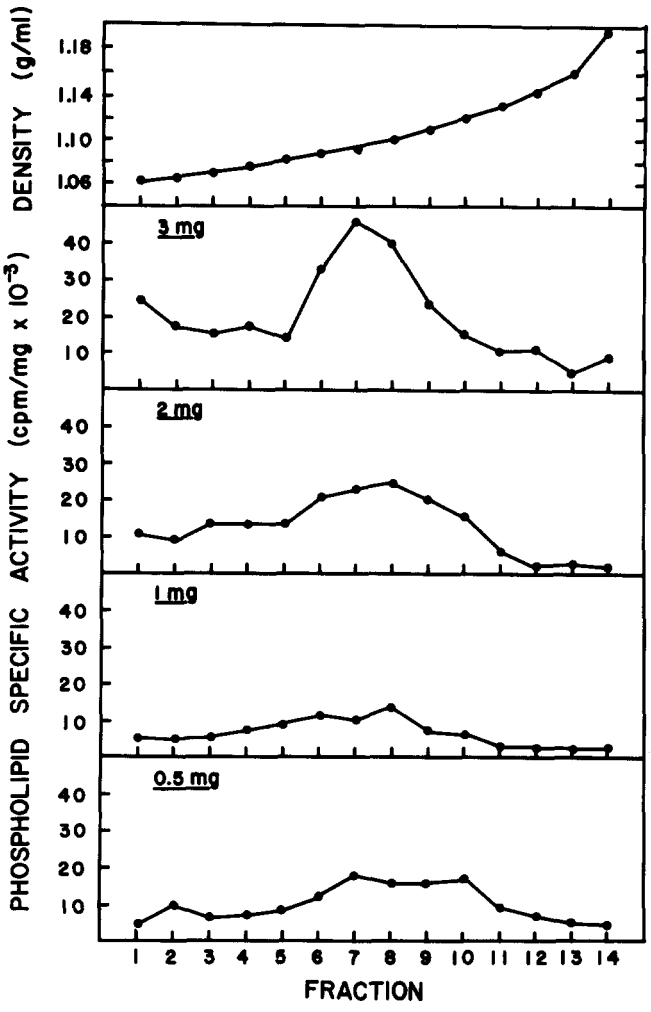

Fig. 6. Density gradient ultracentrifugation of plasma from rats 10 min after injection of different doses of vesicles. The specific radioactivity of phospholipid in individual fractions of the gradient is shown. The injected vesicles had a specific activity of 100,000 cpm/mg phospholipid. The dose of vesicles is shown in the upper left hand corner of each panel. Densities were determined on individual fractions by refractometry, and did not vary more than 0.002 g/ml between gradients.

of 1.095 g/ml (3 mg dose), suggesting incorporation of phospholipid into particles of progressively lower density with increasing uptake of phospholipid. In this group of experiments the total mass of phospholipid represented by the major HDL peak was calculated to be 2.2 mg (control), 2.1 mg (0.5 mg dose), 3.6 mg (1.0 mg dose), 3.9 mg (2.0 mg dose) and 5 mg (3.0 mg dose). Thus, most or all of the mass of PC vesicles is incorporated into HDL even up to a 3.0 mg dose.

For all doses of injected PC, the peaks of highest phospholipid specific activity (fractions 5–9) were examined by negative stain electron microscopy. All fractions consisted of apparently uniform spherical particles. No discs or vesicles were seen.

DISCUSSION

The present experiments show that, following injection of egg yolk PC vesicles into the rat, there was a rapid transfer of both radioactivity and mass of phospholipid into HDL. Krupp, Chobanian, and Brecher (8) reported a similar transfer of vesicle phospholipid radioactivity into HDL, but did not determine HDL composition. Therefore, their results did not differentiate between phospholipid exchange and mass transfer. The changes in HDL lipid composition (Table 2) and the increase in HDL phospholipid mass (Fig. 2) show that the transfer of phospholipid radioactivity into HDL was accompanied by phospholipid mass transfer. Ten minutes after injection of 1 mg of PC vesicles there was an increment in HDL phospholipid of about 1 mg (Fig. 2), showing transfer of a major fraction of the injected phospholipid into HDL. Even with injection of 3 mg of PC, most or all of the phospholipid was transferred into HDL (Fig. 6), showing an extensive capacity of rat plasma to incorporate phospholipid into HDL.

A number of potential mechanisms of transfer of PC into HDL were considered: 1) a change in density of vesicles due to adsorption of protein; 2) dissociation of apoA-I from HDL and the formation of discoidal complexes of PC/apoA-I, as described for the interaction of dimyristoyl PC and HDL (4, 5); and 3) direct insertion of PC into pre-existing HDL. Vesicle PC was transferred into particles of similar size to HDL (Figs. 3 and 5), associated with release of [^{3}H]-inulin from vesicles. Therefore, PC transferred into HDL was no longer in vesicle form. Analysis of plasma by density gradient ultracentrifugation showed that most of the PC was transferred into HDL particles of density 1.08 to 1.12 g/ml (Figs. 4 and 6) forming lipoproteins of homogeneous spherical morphology. Thus, there is probably insertion of PC into pre-existing HDL.

With increasing phospholipid uptake the HDL showed a progressive decrease in density (Fig. 6). Incubation of egg PC vesicles in human plasma shows that PC can combine with pre-existing HDL, converting HDL_3 into less dense particles resembling HDL_{2a} (22). The reservoir for uptake of PC in human plasma exists largely in the HDL_3.[1] In the present study, vesicle phospholipid presumably combined with particles of $d > 1.12$ g/ml, forming less dense HDL particles.

The in vitro studies of Chobanian, Tall, and Brecher (6) and Nichols et al. (7) have demonstrated the importance of phospholipid to apoHDL ratio in determining the products of interaction of vesicles and HDL. At higher ratios of vesicles/HDL phospholipid/apoprotein complexes, which may be vesicular or discoidal, can be readily identified (6, 7). In the present experiments, where a small dose of phospholipid was injected relative to the total mass of plasma HDL_3, separate phospholipid/apoprotein complexes were not observed in the HDL density range. However, our experiments do not rule out the possible presence in the top fractions of the density gradients (Figs. 4 and 6) of vesicular or discoidal particles containing small amounts of HDL proteins.

The presence of cholesterol in the injected vesicles had a marked effect on their subsequent metabolic disposition. For vesicles of 33 and 50 mole% cholesterol/PC there was very little transfer of phospholipid into HDL at 10 min. Injection of vesicles containing [^{3}H]inulin verified that 33 and 50% mole% cholesterol/PC were still largely present in plasma as intact vesicles (Fig. 3). The less stable cholesterol/PC vesicles tended to form larger liposomes eluting in the column void volume when reisolated following injection into saline (Fig. 3). However, almost all of the radioactivity of PC vesicles was incorporated into HDL whether from the small or larger vesicle regions. Much less of the PC of cholesterol/PC vesicles was incorporated into HDL, even for material reisolated in the small vesicle region (Fig. 3). Thus, the observed differences between PC and cholesterol/PC vesicles were due to differences in cholesterol content rather than size.

Sixty minutes after injection of cholesterol-containing vesicles, there was probably a partial transfer of PC mass into HDL (Fig. 2), suggesting that cholesterol delayed the uptake of vesicle PC. Uptake of some of PC from cholesterol/PC vesicles may have occurred secondary to removal of cholesterol from vesicles by transfer to cell membranes. However, there was also substantially greater uptake of vesicle radioactivity by the liver with increasing cholesterol/PC ratio (Table 1). Thus, the delayed clearance of cholesterol/PC vesicles by HDL was probably associated with increased hepatic uptake. The uptake of vesicle radioactivity by liver (and not spleen or small intestine), with a similar ratio of isotopes to the injected vesicles (Table 1), raises the possibility of uptake of intact cholesterol-rich vesicles by hepatocytes.

Studies of liposome-HDL interactions are relevant to the mechanisms of disposition of endogenous or exogenous PC in both humans and rats. For example, vesicles may be introduced into human plasma as a result of cholestasis (23) and, potentially, following injection of vesicles containing enzymes or drugs (24). Following ingestion of fat, humans show a rise in HDL phospholipid, suggesting transfer of chylomicron PC into HDL (25). Studies in the rat have verified the

[1] Tall, A. R. Unpublished results.

transfer of a major fraction of chylomicron PC into HDL during lipolysis (26–29). Following lipolysis of injected chylomicrons, PC forms lamellar structures, including vesicles, which are subsequently incoporated into HDL (27, 29). The results of the present study suggest that the physiological transfer of chylomicron PC into HDL may occur primarily by insertion of PC into pre-existing HDL, producing particles of decreased density. The normal disposition of chylomicron PC probably depends on a low cholesterol/PC ratio in the chylomicron surface and on the presence of adequate levels of receptor HDL.

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