Behavior of human apolipoprotein A-I: phospholipid and apoHDL:phospholipid complexes in vitro and after injection into rabbits

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Abstract Apolipoprotein A-I was purified from human high density lipoprotein and complexed with polyunsaturated phosphatidylcholine (PC) in deoxycholate (Lipostabil); the bile salt was removed subsequently by dialysis. The behavior of the resultant apoA-I/PC complexes was compared with that of Lipostabil in vitro and after injection into rabbits. In vivo apoA-I/PC complexes had the density of HDL throughout but had both α and preβ electrophoretic mobility, the latter probably reflecting dissociation of apoA-I from PC. Lipostabil initially behaved like LDL but gradually acquired the density of HDL after incubation with plasma and in vivo. Both preparations increased plasma total phospholipids in normolipidemic rabbits to a similar extent, but, increments in HDL phospholipid were greater after apoA-I/PC complexes were injected. ApoHDL/PC complexes, prepared in a similar manner, appeared to promote efflux of cholesterol from perfused rabbit aortas in the presence of lecithin:cholesterol acyltransferase (LCAT) activity, consistent with a stimulatory effect on cholesterol mobilization. Injection of apoHDL/PC complexes into hyperlipidemic rabbits decreased plasma cholesterol but increased HDL cholesterol, whereas Lipostabil decreased both. These findings suggest that human apoA-I/PC complexes resemble HDL in their behavior more closely than does Lipostabil, and show that both types of liposome undergo modification upon interaction with plasma. It remains to be shown whether they possess any therapeutic potential.—**Koizumi, J., M. Kano, K. Okabayashi, A. Jadhav, and G. R. Thompson.** Behavior of human apolipoprotein A-I:phospholipid and apoHDL:phospholipid complexes in vitro and after injection into rabbits. *J. Lipid Res.* 1988. **29:** 1405–1415.

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The inverse association between high density lipoprotein (HDL) and coronary heart disease (CHD), first noted by Barr (1), was subsequently restated by Miller and Miller (2), who suggested that the protective effect of HDL was related to its capacity to act as an acceptor of tissue cholesterol. The major apolipoprotein of HDL is a apoA-I and an inverse association has been shown between plasma apoA-I levels and CHD (3, 4) similar to that between HDL cholesterol and CHD (5–7). Also, both HDL cholesterol (8, 9) and apoA-I (10) levels are inversely correlated with the severity of angiographically demonstrable coronary atherosclerotic lesions. Although it can be argued that high concentrations of HDL in plasma influence CHD by retarding atherogenesis or promoting regression, patients with diseases characterized by reduced levels or absence of HDL do not necessarily develop atherosclerosis, as discussed elsewhere (11). Thus it remains to be resolved whether HDL is causally related to CHD.

The major constituents of HDL are apoA-I and phosphatidylcholine (PC). Complexes of HDL apoproteins, mainly apoA-I, and PC promote the efflux of free cholesterol from cells in vitro, including cultured arterial smooth muscle cells (12, 13). Intravenous infusion of phospholipids into animals with experimentally induced atheroma favourably influences the latter (14, 15) but the effectiveness of this approach may be limited by the rapidity with which phospholipid vesicles are cleared from plasma (16). However, it has been suggested that apoA-I:PC complexes are cleared from the plasma at a slower rate than PC alone, comparable with that of native HDL particles (17), which could be advantageous in the context of therapeutic attempts to mobilize tissue cholesterol.

To investigate this question further, we have examined the properties and behavior of complexes of human apoA-I or apoHDL with phospholipid in vitro and after injection into rabbits and have compared them with those of Lipostabil, a commercial preparation of phospholipid designed for intravenous use. The phospholipid component of Lipostabil is mainly

Abbreviations: HDL, high density lipoprotein; PC, phosphatidylcholine; CHD, coronary heart disease; DOC, deoxycholate; FCR, fractional catabolic rate.

PC (96%), with linoleic acid as its major constituent (18).

MATERIALS AND METHODS

Preparation of human HDL

HDL was obtained by adding 100 ml of 4% Na-phosphotungstate and 25 ml of 2 M $MgCl_2$ to 1 L of hypercholesterolemic plasma obtained during plasma exchange (19). After centrifuging at 6000 *g* for 10 min, 900 ml of 4% Na phosphotungstate was added to the HDL-containing supernatant which was then recentrifuged. HDL was precipitated by adding 175 ml of 2 M $MgCl_2$, allowing the mixture to stand for 2 hr, and centrifuging at 20,000 *g* for 30 min. The precipitate was washed with 500 ml of 1% NaCl containing 0.4% Na-phosphotungstate and 0.1 M $MgCl_2$, centrifuged at 6000 *g* for 10 min, resuspended in 70 ml of 1% NaCl, and then solubilized by dropwise addition of 10% Na_2CO_3 with stirring. After adjusting the density to 1.21 g/ml with solid KBr, HDL was further purified by ultracentrifugation twice in a Beckman 70 Ti rotor at 55,000 rpm for 44 hr. The HDL was dialyzed against 0.15 M NaCl, 0.01% EDTA, pH 7.4, and stored at 4°C after passage through a 0.45-μm Millipore filter (Millex-HA, France).

Preparation of apoA-I and of apoA-I/PC complexes

Human HDL was delipidated by extraction with diethyl ether–ethanol 3:2. ApoA-I was isolated by chromatographing 100 mg of delipidated HDL on a DEAE-Sepharose CL-6B (Pharmacia Fine, Sweden) column (2.5 × 30 cm), which was eluted with an NaCl gradient (0–0.125 M) in a buffer (pH 7.4) that contained 6 M urea, 10 mM Tris-HCL, 1 mM EDTA, 1 mM dithiothreitol (DTT), at a flow rate of 24 ml/hr (20). The apoA-I was dialyzed against 5 mM ammonium bicarbonate and then lyophilized. The apoA-I, which showed a single band on 17% SDS-polyacrylamide gel electrophoresis (21), was dissolved in 0.4% Na deoxycholate (DOC) in 10 mM Tris-HCL, 1 mM EDTA, and 150 mM NaCl at pH 8.0, to give a concentration of less than 2 mg protein/ml. To this was added Lipostabil (5% polyunsaturated PC in 4% DOC) (Natterman, Cologne, West Germany), to give a PC/apoA-I mass ratio of 3.35:1. The mixture was vortexed for 1 min and then stirred under nitrogen at 4°C for 18 hr. The DOC was removed by dialysis (22–24) against 8 L of 10 mM Tris-HCL, 1 mM EDTA, 150 mM NaCl, pH 8.0 (buffer A), and then 2 L of 10 mM Tris-HCL, 1 mM EDTA, 150 mM NaCl, pH 7.4 (buffer B). The sample was concentrated tenfold in an ice bath using a Millipore Immersible CX-10™ concentrating filter; it was then applied to a Sepharose CL-6B column and eluted with buffer B. Fractions comprising the main peak were combined and dialyzed against 150 mM NaCl, pH 7.4. ApoA-I/PC complexes were passed through a Millipore filter (0.45 μm) and stored at 4°C until use.

Preparation of human apoHDL/PC complexes

Human HDL (400 mg) was delipidated as described above and dissolved in 20 ml of 3 M guanidine-HCl. The apoHDL was dialyzed against buffer A (10 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 8.0) and mixed with Lipostabil to give a PC:apoHDL mass ratio of 2:1. The mixture was vortexed for 1 min and stirred under nitrogen at 4°C for 18 hr. The deoxycholate was then removed by dialysis, as described above. After passage through a Millipore filter (0.45 μm), the apoHDL/PC complexes were stored at 4°C until use.

Labeling human apoA-I/PC complexes and HDL with radioiodine

To label human apoA-I/PC complexes and HDL (d 1.063–1.21 g/ml) preparations of apoA-I were labeled with either ^{125}I or ^{131}I, using iodine monochloride (25). A mixture of ^{125}I-labeled apoA-I and apoA-I/PC complexes was incubated in buffer A and a mixture of ^{131}I-labeled apoA-I and HDL was incubated in 0.05 M Na barbital buffer, pH 8.6, at 37°C for 30 min (26). After incubation, each mixture was ultracentrifuged at d 1.225 g/ml at 50,000 rpm for 28 hr in a Beckman 50.3 Ti rotor. The supernatants were then dialyzed against 150 mM NaCl, 1 mM EDTA (pH 7.4). ^{125}I-Labeled apoA-I/PC complexes (4 mg protein) and ^{131}I-labeled HDL (1.9 mg protein) were injected into normal rabbits to compare their half-lives in plasma.

Incubation of ^{14}C-labeled PC/apoA-I complexes or ^{14}C-labeled Lipostabil with rabbit plasma

Lipostabil (2.5 ml containing 125 mg phospholipid) was mixed with 5 μCi phosphatidylcholine labeled with [1-^{14}C]linoleic acid in the *sn*-2 position (Amersham International plc, U.K.). Some of the ^{14}C-labeled Lipostabil was then used to prepare ^{14}C-labeled apoA-I/PC complexes. ^{14}C-Labeled phospholipid (1 mg) as apoA-I/PC complexes or Lipostabil was incubated (at 37°C for periods up to 3 hr) with 3.5 ml of plasma from a heterozygous WHHL rabbit fed a diet containing 0.2% cholesterol. Immediately after incubation, the HDL-containing fraction was isolated by Na phosphotungstate/$MgCl_2$ precipitation of VLDL and LDL. Concomitantly, labeled lipoproteins were separated by single discontinuous density gradient ultracentrifugation (27). This involved adjusting the incubation mixture to d 1.30 g/ml, layering 1.5 ml under 3.5 ml of d 1.006 g/ml NaCl, and then ultracentrifuging at 60,000 rpm for 40 min at 10°C in a Beckman VTi 80

rotor. Successive 0.55-ml fractions were collected, using a Beckman Fraction Recovery System. ^{14}C-Labeled phospholipid (22.5 mg) in the form of apoA-I/PC complexes or Lipostabil was injected into normal rabbits to compare the rate of removal of the labeled phospholipid and its subsequent incorporation into cholesteryl esters.

Injection of human apoHDL/PC complexes and Lipostabil into heterozygous WHHL rabbits fed a diet containing 0.5% cholesterol

Six heterozygous WHHL rabbits, 3.3–4.3 kg in body weight, which were bred locally from homozygous males and heterozygous females kindly provided by Dr. Y. Watanabe, were maintained on a 0.5% cholesterol diet for 5 weeks. Plasma cholesterol levels ranged from 236 to 1366 mg/dl and HDL-cholesterol levels from 4.6 to 17.0 mg/dl.

Human apoHDL/PC complexes or Lipostabil, each containing 200 mg of phospholipid, were made up to 16.5 ml with physiological saline and then infused intravenously into fasted, anesthetized rabbits with an infusion pump to 30 min. Control rabbits received a similar volume of physiological saline. Blood samples were obtained from ear veins at intervals over the next 72 hr and were placed in tubes containing EDTA in an ice bath. The plasma was separated within 3 hr by centrifugation and the HDL-containing fraction was isolated by addition of Na phosphotungstate-$MgCl_2$ (19). Samples of hyperlipidemic plasma were first diluted with an equal volume of saline, in order to obtain a clear supernatant.

Immunoblotting of human apoA-I on agarose electrophoresis

Mixtures of human apoA-I/PC complexes and rabbit plasma were electrophoresed on agarose (28). Immediately afterwards, a piece of cellulose acetate paper (Celagram II, Shandon Southern, U.K.), wetted with 0.01 M phosphate-buffered saline (PBS), pH 7.2, was placed on top of the gel and then rapidly removed. A nitrocellulose membrane (pore size 0.45 μm, Schleicher and Schuell, FRG) was wetted in PBS and placed on the gel. Five pieces of filter paper were then superimposed and compressed with a 2-kg weight for 1 hr at room temperature (29). The nitrocellulose sheet was soaked in 0.05% Tween 20 in PBS overnight at 4°C to block any reactive sites (30) and then incubated for 1 hr at room temperature with a 1:200 dilution in 0.05% Tween 20/PBS rabbit anti-human apoA-I serum, which gave a single precipitation band on an Ouchterlony plate. The sheet was washed with 0.05% Tween 20/PBS (3 times, 5 min each) and then incubated with goat peroxidase-linked anti-rabbit IgG (1:400 dilution in 0.05% Tween 20/PBS) for 1 hr at room temperature. After washing with PBS (3 times, 5 min each) the reaction was developed using 4-chloronaphthol (0.4 mg/ml in 10 mM Tris-HCL, 0.15 M NaCl, pH 7, containing 10 μl of H_2O_2/ml). The sheet was extensively washed in PBS and distilled water.

Preparation of an LCAT-containing fraction

An LCAT-containing fraction was isolated from fresh fasting human plasma as follows (31). Plasma was first adjusted to d 1.21 g/ml with solid KBr and centrifuged at 10°C, at 55,000 rpm, for 40 hr in a Beckman 70 Ti rotor. After tube-slicing, the $d < 1.21$ g/ml supernatant was discarded and the LCAT-containing clear intermediate layer was aspirated, dialyzed against 150 mM NaCl, 0.01% EDTA, pH 7.4, at 4°C and stored at −20°C after passage through a Millipore filter (0.45 μm). This fraction contained 0.93 mg/ml of protein, 0.36 mg/ml of immunoassayable apoA-I (Orion Diagnostica, Helsinki), 0.18 mg/ml of phospholipid, and 0.09 mg/ml of cholesterol. Cholesterol was measured enzymatically (Human, West Germany) or by gas–liquid chromatography (32), phospholipid was measured enzymatically (Wako, West Germany), and protein was assayed by the method of Lowry et al. (33).

Aortic perfusion, in vitro

Aortas from eight heterozygous WHHL rabbits, 3–4 kg in body weight, were perfused in vitro as described by Bowyer and Davies (34). These rabbits had been maintained on a 0.5% cholesterol diet for 44–420 days; their plasma cholesterol levels ranged from 172 to 1190 mg/dl. The rabbits were anesthetized with fentanyl and fluanisone (Janssen Pharmaceuticals, Oxford, U.K.) before removal of the aorta. The aortic perfusate was prepared by the addition of 0.22 ml of 2 M KCl, 0.58 ml of 0.22 M calcium gluconate, 0.46 ml of 25% glucose, and 3.25 ml of 7% Na_2CO_3 to 100 ml of 4.5% human albumin solution (Blood Products Laboratory, Elstree, Herts, U.K.). The mixture was gassed with 5% CO_2, 95% O_2.

After being set up in the perfusion system, the aorta was washed with 50 ml of chilled saline and recirculated with 15 ml of perfusate under 90 mm Hg pressure; this initial perfusate was discarded. Three further perfusions were then carried out sequentially on each aorta; during each perfusion 10 ml of perfusate was recirculated with constant gassing for 40 min at 37°C and 90 mm Hg mean pressure. During periods I and III the effects of adding 0.6 mg/ml of human apoHDL/PC complex alone or plus 50 μl/ml of LCAT fraction or of adding the LCAT fraction alone were compared with the control period II, during which the perfusate contained no additives. At the end of

the perfusion, the aorta was washed with 100 ml of chilled saline, peri-aortic tissue was removed, and the aorta was then opened longitudinally. The inner layer, containing intima and media, was peeled off (35), then homogenized with 2 ml of 0.8 N NH_4OH containing 0.2% Triton X. After measuring the protein content, the homogenate was extracted by the method of Folch, Lees, and Sloane Stanley (36) and total cholesterol was measured by gas–liquid chromatography. To measure the cholesterol content of the perfusate, 4 ml of perfusate was added to 4 ml of ethanolic KOH and 5α-cholestane (Sigma Chemical Co.) as an internal standard. After saponification the lipids were extracted twice with 10 ml of hexane and the pooled extracts were dried under N_2 prior to measurement of cholesterol by gas–liquid chromatography. The increase of cholesterol in the perfusate was calculated by subtracting the amount of cholesterol present before the perfusion.

RESULTS

Properties of human apoA-I/PC complexes

Human apoA-I/PC complexes showed a single peak on Sepharose CL-6B gel chromatography which eluted ahead of HDL, indicating a slightly larger particle size (**Fig. 1**). The phospholipid:protein ratio varied across the peak, indicating some degree of heterogeneity of particle composition. Analysis of the combined fractions comprising the main peak showed a mass ratio of PC–apoA-I–DOC of 2.25 ± 0.51:1:0.26 ± 0.14 (mean ± SD, n = 7).

Human apoA-I/PC complexes and human HDL (d 1.063–1.21 g/ml), labeled with ^{125}I- or ^{131}I-labeled apoA-I, had their fractional catabolic rates (FCR) measured in normal rabbits using a two-compartmental model (37). The FCRs of apoA-I/PC complexes and HDL were similar, 2.05/day and 1.61/day, respectively (**Fig. 2**). Plasma obtained 4 hr after injection of apoA-I/PC complexes and HDL showed sharp peaks of radioactivity with elution profiles similar to those observed prior to injection (**Fig. 3**) except for the presence of a shoulder on the downslope of the pre-injection profile of the apoA-I/PC complex, which disappeared after injection. To compare the rates of removal of their phospholipid component, which was largely PC, apoA-I/PC complexes and Lipostabil were labeled with ^{14}C-labeled PC and injected into normal rabbits. Specific activity time curves showed similar rates of disappearance of labeled phospholipid from plasma (**Fig. 4**). Immediately after injection of apoA-I/^{14}C-labeled PC complexes, the specific activity of phospholipid was higher in HDL than in the remainder of the plasma but this did not occur after the injection of ^{14}C-labeled Lipostabil. Judging from the percentage of radioactivity appearing in the cholesteryl esters of plasma and HDL, the ability of apoA-I/PC complexes and Lipost-

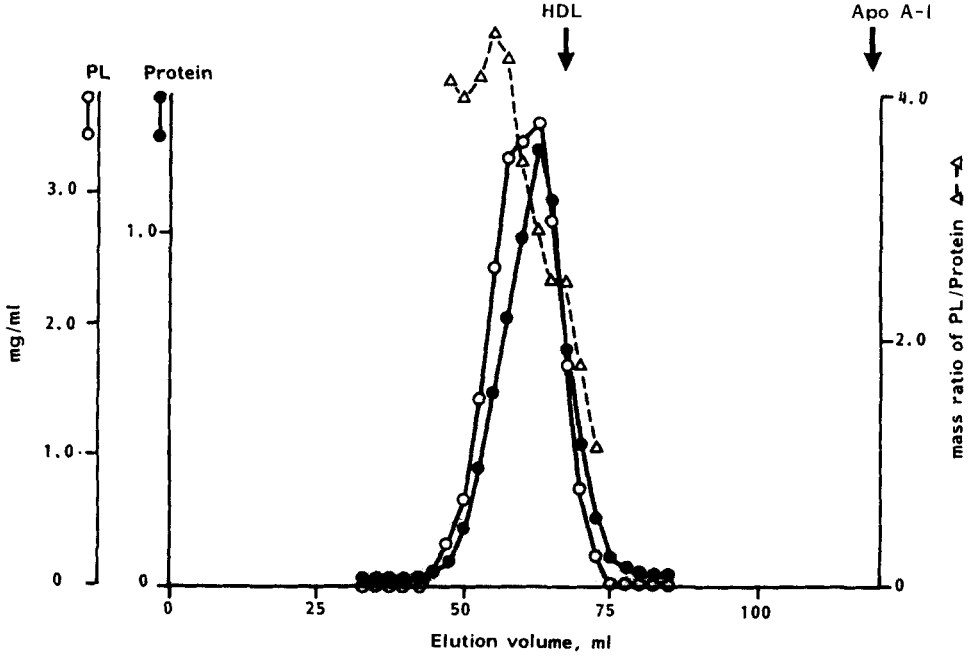

Fig. 1. Elution pattern of human apoA-I/PC complexes chromatographed on a 1.6 × 63 cm Sepharose CL-6B column at room temperature, with a flow rate of 5 ml/hr. The arrows show the elution peaks of human HDL and apoA-I; (●) protein; (○) phospholipid; (△) mass ratio of phospholipid/protein.

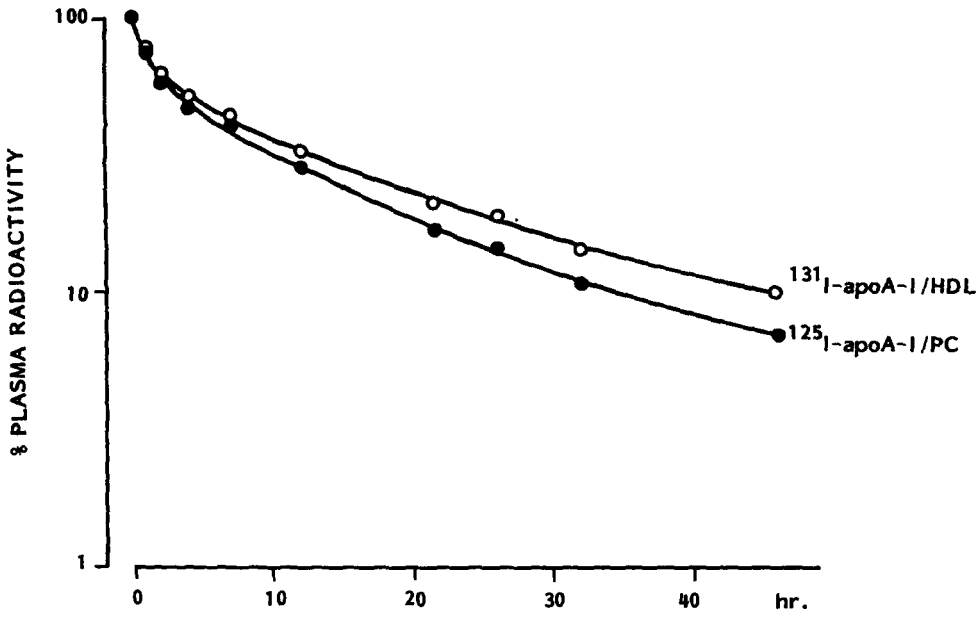

Fig. 2. Disappearance of plasma radioactivity after injection of ^{125}I-labeled human apoA-I/PC complexes (4 mg protein) and ^{131}I-labeled human HDL (1.9 mg protein) into normal rabbits. Data are expressed as the mean of two to four rabbits in each group.

abil to donate PC as substrate for LCAT in vivo was similar (**Table 1**).

When apoA-I^{14} C-labeled PC complexes were incubated with normolipidemic rabbit plasma in vitro, 60% and 75% of the added radioactivity and mass of phospholipid, respectively, were initially recovered in the supernatant after phosphotungstate-$MgCl_2$ precipitation. The percentage of ^{14}C-labeled PC had decreased to 51% by 2 hr but the mass of PL remained constant. With Lipostabil, less PL was initially recovered in the supernatant, despite similar incubation conditions but the proportion of labeled phospholipid associated with HDL gradually increased, as did PL mass (**Table 2**).

The distribution of the apoA-I ^{14}C-labeled PC complexes and Lipostabil after incubation with rabbit plasma was analyzed by single discontinuous density gradient ultracentrifugation (**Fig. 5**). The density of the labeled apoA-I/PC complexes was similar to HDL throughout the incubation period and immunoprecipitation bands against anti-human apoA-I rabbit serum were observed in the fractions (nos. 6–9) corresponding to HDL. In contrast, the pre-incubation density of Lipostabil was initially similar to LDL, but during incubation the distribution of radioactivity progressively shifted from the LDL to the HDL region of the gradient.

Single infusions of human apoHDL/PC complexes and Lipostabil into hypercholesterolemic rabbits

When human apoHDL/PC complexes (100 mg protein:200 mg PL) or Lipostabil (200 mg PL) were infused for 30 min into cholesterol-fed heterozygous WHHL rabits, there were increases in plasma phospholipids (**Fig. 6**). On average, 36% of the increment of plasma phospholipid during the first hour after infusion of apoHDL/PC complexes but only 10% of the increment after infusion of Lipostabil was localized to HDL. Plasma phospholipids remained significantly higher for 2 days after apoHDL/PC complexes as compared with Lipostabil. HDL cholesterol concentration was significantly reduced during the first hour after infusion of Lipostabil, as was total cholesterol at 3 hr (**Fig. 7**). ApoHDL/PC complexes caused a slight rise in HDL cholesterol and a slight decrease in total cholesterol; neither change was significant.

Immunological localization of human apoA-I in rabbit plasma

Immunoblotting showed that human apoA-I exhibited both preβ and α mobility on agarose gel electrophoresis immediately after mixing apoA-I/PC complexes with plasma in vitro, using the original agarose gel stained for lipid as a marker (**Fig. 8**). Immunoreactive material present at the origin both before and after injection of complexes represents rabbit IgG reacting with the goat-derived second antibody.

After injection of the complex into rabbits, the mobility of the preβ component appeared slower and that of the α component appeared faster than was evident immediately after admixture of complexes with plasma in vitro (Fig. 8). Both components had virtually

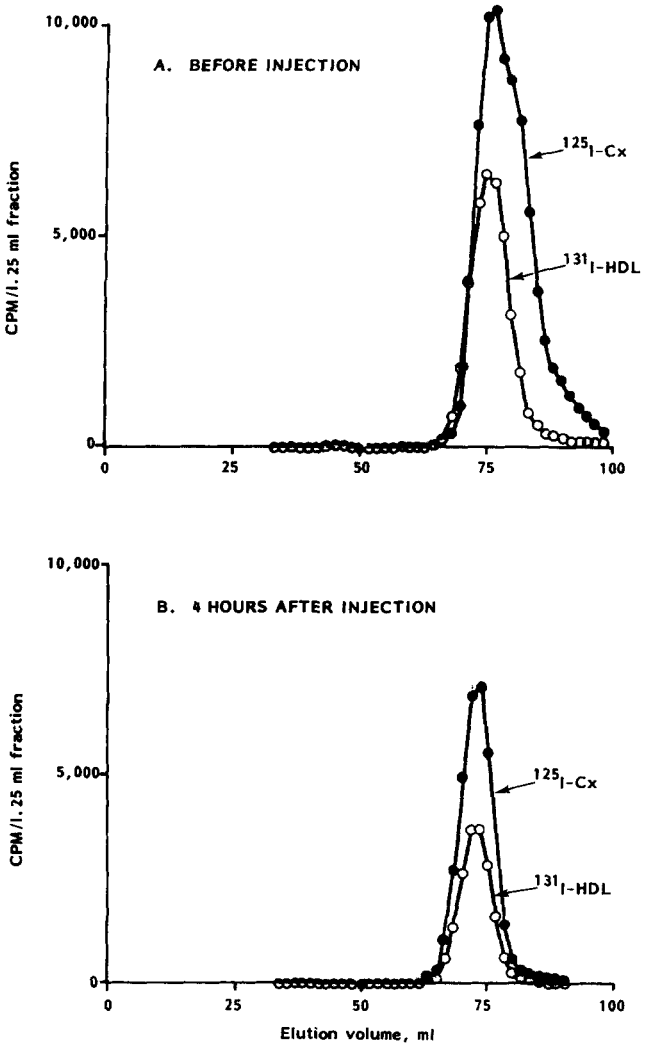

Fig. 3. Elution pattern of apoA-I/PC complexes and HDL before and after injection into normal rabbits. Lipoproteins (d < 1.225 g/ml) were obtained by ultracentrifugation, performed immediately after mixing ^{125}I-labeled apoA-I/PC complexes (●) and ^{131}I-labeled HDL (○) with normal rabbit plasma (upper panel), and also from plasma obtained 4 hr after injection (lower panel). The d < 1.225 g/ml fractions were chromatographed on a 1.6 × 70 cm Sepharose CL-6B column.

disappeared by 3 hr after injection. Despite the apparent predominance of the preβ component during the first 30 min after injection, 90% of the labeled apoA-I on density gradient ultracentrifugation of apoA-I/PC complexes was recovered from the bottom 2 ml of the gradient over this time period (see Fig. 5). None of the label was adsorbed when the 3-hr incubation mixture was passed through a dextran sulfate affinity column, as might have been expected if the preβ mobility component mainly represented apoE-containing complexes. Similarly, only 12.6% of the radioactivity was precipitated by Na phosphotungstate-$MgCl_2$ immediately after mixing the labeled complexes with plasma, which decreased to 6.8% when precipitation was carried out after 3 hr of incubation.

Cholesterol efflux during aortic perfusions

The cholesterol content of the perfusate increased more during the first perfusion period than during the subsequent ones (**Fig. 9**) and also varied with the wet weight and cholesterol content of each individual aorta perfused. To correct for these variables and to assess the effect of the various additives, cholesterol efflux has been expressed as the ratio of the increment in the cholesterol content of the perfusate during periods I or III, when human apoHDL/PC complexes and/or an LCAT-containing fraction of plasma were added to the perfusate, to the increment during period II, the control period, when the perfusate contained no additives. The results, given in **Table 3,** suggest that the highest ratios occurred when apoHDL/PC complexes and an active LCAT fraction were both added to the perfusate, whereas either of these alone or apoHDL/PC complex plus heat-inactivated LCAT fraction did not promote efflux to a greater extent than perfusate without additives. However, none of these differences quite achieved statistical significance, owing to the limited number of WHHL rabbit aortas available for these experiments.

DISCUSSION

The main purpose of these studies was to compare the in vitro properties and in vivo behavior of apoA-I/PC complexes with those of HDL and Lipostabil. Sequential precipitation of plasma provided a convenient source of human HDL and thus of apoA-I. The latter was solubilized in deoxycholate and complexed with polyunsaturated phospholipids dissolved in deoxycholate, i.e., Lipostabil, most of the bile salt being removed subsequently by dialysis. In some experiments, the protein moiety was labeled by mixing apoA-I/PC complexes with ^{125}I-labeled apoA-I and in others the phospholipid was labeled by the addition of ^{14}C-labeled PC. It was also possible to follow the fate of the human apoA-I in rabbit plasma by immunoblotting.

Human apoA-I/PC complexes were slightly larger than HDL on gel chromatography, had a similar density on ultracentrifugation, and a mass ratio of protein to phospholipid comparable to that reported by others (22). After injection into normal rabbits, the FCR of labeled human apoA-I was slightly faster when injected as an apoA-I/PC complex than when injected as HDL but the difference, which was slight, may have reflected

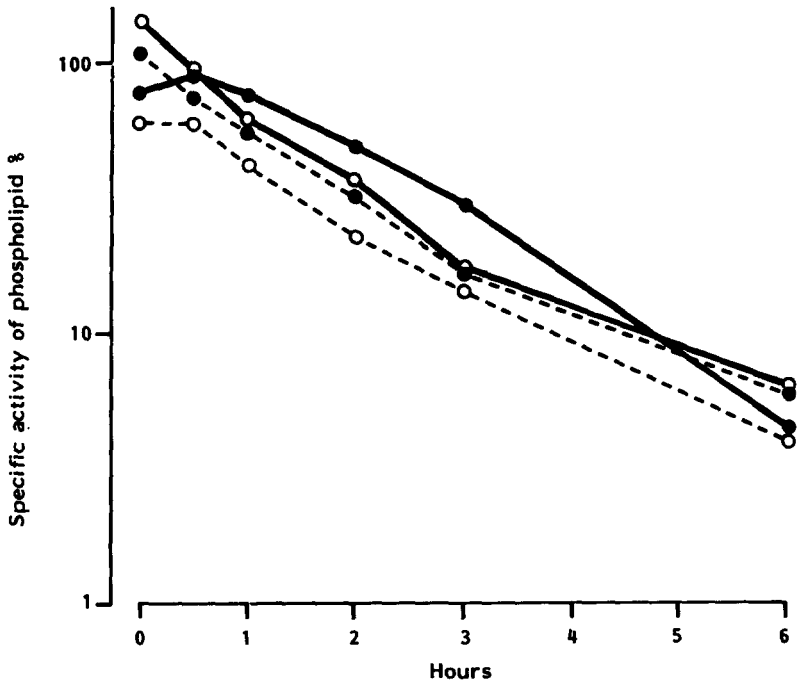

Fig. 4. Changes in specific activities of phospholipid in HDL and non-HDL fraction after injecting apoA-I/PC complexes or Lipostabil, labeled with phosphatidylcholine with [1-^{14}C]linoleic acid in the *sn*-2 position into normal rabbits. Radioctivity in plasma and HDL phospholipid was assayed after lipid extraction and TLC separation. Plasma and HDL phospholipids were measured enzymatically and specific activity of phospholipid in the HDL and non-HDL fractions was calculated. Values shown are relative to the initial specific activity of plasma phospholipid; (●) apoA-I/PC complexes; (○), Lipostabil; (————) non-HDL fraction; (- - - -) HDL fraction.

a degree of heterogeneity of apoA-I/PC complexes or dissociation of free apoA-I, which is known to be rapidly metabolized by the kidney (38, 39).

In vitro, both before and after incubation with rabbit plasma, most of the phospholipid mass and radioactivity of apoA-I/^{14}C-labeled PC complexes was resistant to precipitation with Na phosphotungstate and $MgCl_2$. In contrast, most of the Lipostabil was precipitable before incubation, behaving in this respect like LDL, although the proportion resistant to precipitation increased gradually with time. Analogous differences between apoA-I/PC complexes and Lipostabil

TABLE 1. Cholesterol esterification using ^{14}C-labeled PC/apoA-I complexes and ^{14}C-labeled PC Lipostabil as substrates of LCAT *in vivo*

Time	Plasma		HDL	
	PC/ApoA-I	Lipostabil	PC/ApoA-I	Lipostabil
	% of total counts in cholesteryl ester			
3 min	2.2	2.2	2.4	3.1
30 min	5.8	5.5	5.5	5.7
60 min	10.0	10.4	8.3	9.9
120 min	19.3	19.5	13.6	17.1
180 min	27.4	30.6	19.7	21.7
360 min	46.6	50.0	21.1	25.8

PC/apoA-I complex or Lipostabil (22.5 mg PL), labeled with phosphatidylcholine with [1 – ^{14}C] linoleic acid in the *sn*-2 position, was injected into normal rabbits intravenously. Radioactivity in cholesteryl ester in plasma and HDL (isolated by phosphotungstate-Mg Cl_2 precipitation) was separated by thin-layer chromatography after chloroform-methanol extraction.

TABLE 2. Percent recovery of ^{14}C-labeled phospholipid added as human apoA-I/PC complex or Lipostabil to rabbit plasma *in vitro*

Incubation time	DPM		Mass	
	ApoA-I/PC	Lipostabil	ApoA-I/PC	Lipostabil
	% recovery in HDL			
0 min	60.1	14.6	75.9	14.7
30 min	64.6	37.5	(96.8)	28.3
60 min	57.7	39.2	75.3	37.4
120 min	51.2	41.1	72.3	53.2
180 min	52.3	42.6	74.9	57.4

One mg of phospholipid of apoA-I/PC or Lipostabil, each labeled with phosphatidylcholine with [1 – ^{14}C] linoleic acid in the *sn*-2 position, was incubated at 37°C in 3.5 ml of normolipidemic heterozygous WHHL rabbit plasma in which 64.7% of total phospholipid was in HDL before addition of exogenous PL (plasma PL, 51 mg/dl), HDL-PL, 33 ml/dl). After incubation, HDL was isolated by phosphotungstate-$MgCl_2$ precipitation. Radioactivity was assayed by adding 0.2 ml of solution to 10 ml of scintillator and phospholipid mass was measured enzymatically.

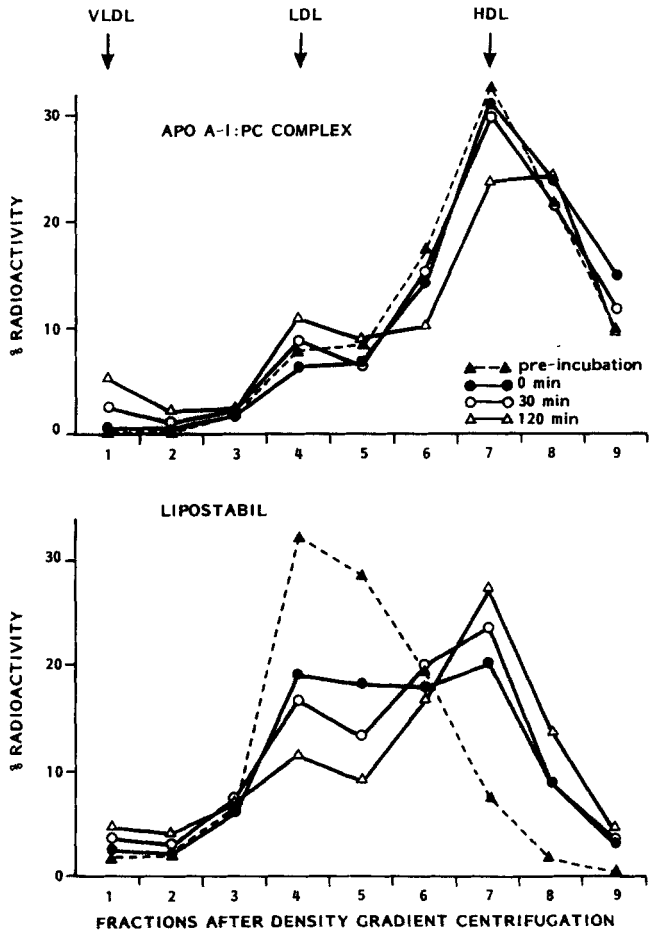

Fig. 5. Fractionation of [^{14}C] phosphatidylcholine-labeled apoA-I/PC complexes and Lipostabil by single discontinuous density gradient ultracentrifugation. Prior to centrifugation, mixtures of ^{14}C-labeled PC/apoA-I complexes or ^{14}C-labeled PC/Lipostabil and normolipidemic rabbit plasma were incubated in a similar manner to that described in Table 2. The radioactivity in each fraction was counted after dissolving 0.2 ml of sample in 10 ml Beckman Ready Solv MP; (▲- - - -▲) apoA-I/PC complexes or Lipostabil in salt solution; (●———●) apoA-I/PC complexes or Lipostabil immediately after the mixture with rabbit plasma; (○———○); after 30 min incubation at 37°C; (△———△) after 120 min incubation at 37°C.

were evident in vivo. Both before and up to 2 hr after injection, most of the phospholipid of apoA-I/^{14}C-labeled PC complexes remained in the HDL region on density gradient ultracentrifugation. In contrast, Lipostabil with ^{14}C-labeled PC was mainly located in the LDL region before injection, although it became increasingly associated with the HDL region after injection, achieving a distribution comparable to that of apoA-I/PC complexes by 2 hr. The half-life in plasma of labeled PC was similar regardless of whether it was injected as apoA-I/PC complexes or Lipostabil, as was the extent to which it was incorporated into cholesteryl esters. These findings suggest that the phospholipid moiety of Lipostabil gradually acquires the physicochemical characteristics of apoA-I/PC complexes after injection into rabbits, presumably as a result of the removal of the deoxycholate component by hepatic uptake (40) and by adsorption to the residual PC of some of the apoA-I present in HDL (41).

Adsorption of apoE to liposomes is well documented (42) but we found no evidence that ^{125}I-labeled apoA-I/PC complexes were susceptible to precipitation with Na phosphotungstate-$MgCl_2$ or to adsorption by dextran sulfate, as would have been expected if the preβ electrophoretic mobility component of human apoA-I observed after admixture with rabbit plasma was mainly attributable to uptake of apoE. Instead, it seems likely that the particles with preβ

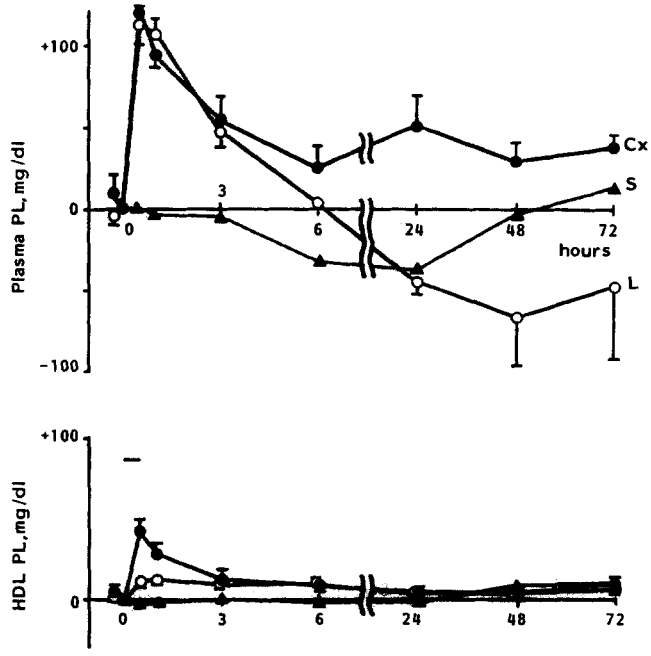

Fig. 6. Changes in plasma and HDL phospholipid after infusion of apoHDL/PC complexes, Lipostabil, and saline into heterozygous WHHL rabbits fed a 0.5% cholesterol diet. One hundred mg protein of apoHDL/PC complexes (Cx, ●), 200 mg of phospholipid as Lipostabil (L, ○), or 16.5 ml of 0.15 M saline (S, ▲) was infused over the period indicated by the horizontal bar. Plasma and HDL phospholipid levels before the infusions were 281 ± 76 mg/dl (mean ± SE), 28.0 ± 1.5 mg/dl (mean ± SE) in the Cx group (n = 3), 345 ± 77, 24.9 ± 3.5 in the L group (n = 3), and 264, 26.9 (mean) in the S group (n = 2). Data show changes in concentration relative to preinfusion values (mean ± SE). Statistically significant changes were: plasma PL at 30 min: Cx versus S, $P<0.001$; L versus S, $P<0.01$; at 1 hr: Cx versus S, $P< 0.01$; L versus S, $P <0.01$; at 3 hr: L versus, S, $P< 0.05$; at 24 hr: Cx versus L, $P< 0.01$; at 48 hr: Cx versus L, $P<0.05$; HDL PL at 30 min: Cx versus S, $P<0.01$; Cx versus L, $P<0.02$; L versus S, $P<0.02$; at 1 hr: Cx versus S, $P<0.05$; L versus S, $P<0.02$.

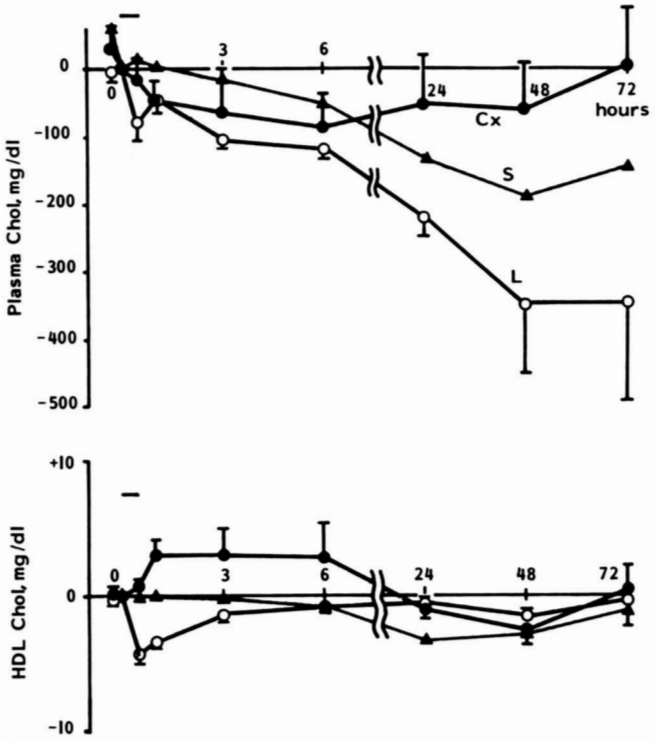

Fig. 7. Changes in plasma and HDL cholesterol after infusion of apoHDL/PC complexes, Lipostabil, and saline into heterozygous WHHL rabbits fed a diet containing 0.5% cholesterol. Symbols as in Fig. 6. Initial plasma and HDL cholesterol levels were 791 ± 370 mg/dl (mean ± SE), 9.8 ± 6.4 mg/dl (mean ± SE) in the Cx group (n = 3), 1037 ± 400, 8.7 ± 2.0 in the L group (n = 3) and 761, 9.8 in the S group (n = 2). Statistically significant changes were: plasma chol. at 3 hr: L versus S, $P<0.02$; HDL chol. at 30 min: L versus S, $P<0.01$; Cx versus L, $P<0.01$; at 1 hr: L versus S, $P<0.02$.

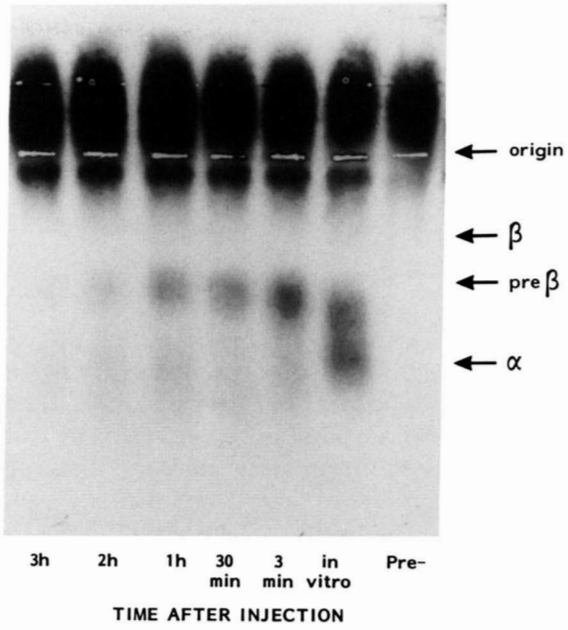

Fig. 8. Immunoblotting of human apoA-I on agarose gel electrophoresis after injection in vivo. Human apoA-I/PC complexes (containing 22.5 mg of PL) were intravenously injected into normal rabbits and blood samples were obtained at various times during and thereafter. Agarose gel electrophoresis and immunoblotting were carried out as described in Material and Methods. Immunoblots of rabbit plasma before (pre) and immediately after addition of human apoA-I/PC complexes (in vitro) are also shown.

mobility consisted of partially delipidated (43) or free apoA-I (44), whereas those with α mobility consisted of intact complexes.

Infusion of 200 mg of phospholipid as apoHDL/PC complexes or as Lipostabil into hyperlipidemic rabbits resulted in comparable increases in plasma phospholipid. However, there was less of an increase in HDL phospholipid after Lipostabil than after apoHDL/PC complexes had been infused. Both preparations caused a slight and transient increase in plasma total cholesterol compared with saline and this was accompanied by a small fall in HDL cholesterol after Lipostabil. Infusion of apoHDL/PC complexes into hyperlipidemic rabbits resulted in an insignificant decrease in plasma total cholesterol and an increase in HDL cholesterol, the fall in total cholesterol possibly reflecting interaction between complexes and LDL (45). Lipostabil caused significant decreases in both total and HDL cholesterol.

The ability of phospholipids to act as an acceptor of cholesterol from cells in tissue culture was demonstrated by Burns and Rothblat (46). This was con-

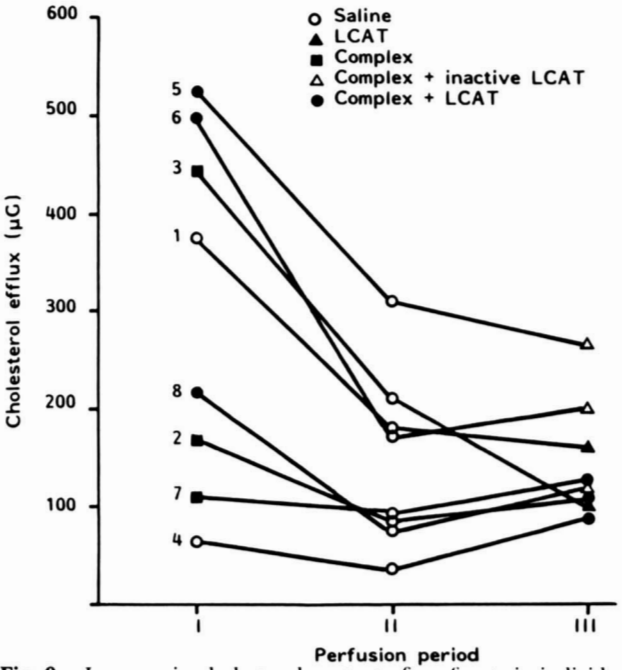

Fig. 9. Increase in cholesterol content of perfusate in individual aortas (Arabic numerals) during each perfusion period (Roman numerals). There were no additives during period II. Additives during periods I and II, indicated by the symbols, were as follows: saline, 1.5 ml of 0.15 M NaCl; Complex, 6 mg (1 ml) of human apoHDL/PC complexes; LCAT, 0.5 ml of an LCAT-rich fraction of plasma; inactive LCAT, 0.5 ml of heat-inactivated LCAT-rich fraction. The total volume of perfusate was 10 ml.

TABLE 3. Cholesterol efflux from perfused rabbit aortas

Additive to Perfusate	Mass ratio of cholesterol in perfusate		
	Periods I : II	Periods III : II	Mean
Nil or LCAT	1.94 (n = 2)	0.71 (n = 2)	1.32 ± 0.74
Complex or Complex + inactive LCAT	1.79 (n = 3)	1.13 (n = 3)	1.46 ± 0.50
Complex + LCAT	2.37 (n = 3)	1.69 (n = 3)	2.03 ± 0.68

firmed subsequently by Stein and Stein (12) who also showed that this effect could be enhanced by first complexing the phospholipid with apoHDL. These workers later showed removal by apoHDL/PC complexes of cholesterol from aortic smooth muscle cells (13). During the present studies there was a suggestion that addition of apoA-I/PC complexes and LCAT to the perfusion medium promoted efflux of cholesterol from the aorta of hypercholesterolemic rabbits. The effect of Lipostabil was not tested because it was considered that its high concentration of deoxycholate could damage the endothelium and thus spuriously increase the cholesterol content of the perfusate. However, this would be prevented from occurring in vivo by rapid removal of deoxycholate from the circulation by the liver (40).

Although these studies provide some support for the concept that Lipostabil or HDL analogues can act as cholesterol acceptors in vivo, it remains to be shown whether their administration is capable of promoting sufficient mobilization of cholesterol in the arterial wall to be therapeutically useful in the context of human atherosclerosis.

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