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Action of Lecithin–Cholesterol Acyltransferase on Low-Density Lipoproteins in Native Pig Plasma†

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ABSTRACT: The action of lecithin–cholesterol acyltransferase (LCAT, EC 2.3.1.43) on the different pig lipoprotein classes was investigated with emphasis on low-density lipoproteins (LDL). It was demonstrated previously that LDL can serve as substrate for LCAT, probably because they contain sufficient amounts of apoA-I and other non-apoB proteins, known as LCAT activators. Upon a 24-h incubation of pig plasma in vitro in the presence of active LCAT, both pig LDL subclasses, LDL-1 and LDL-2, fused together, forming one fraction, as revealed by analytical ultracentrifugation. This fusion was time dependent, becoming visible after 3 h and complete after 18 h of incubation. Concomitantly, free cholesterol and phospholipids decreased and cholesteryl esters increased. When isolated LDL-1 and LDL-2 were incubated with purified pig LCAT for 24 h, LDL-1 floated toward higher densities and LDL-2 toward lower densities, although this effect was not as pronounced as in incubations of whole serum. In further experiments, pig serum was incubated for various periods of time in the presence and absence of the LCAT inhibitor sodium iodoacetate. The individual lipoproteins then were separated by density gradient ultracentrifugation or by specific immunoprecipitation and chemically analyzed. Both methods revealed that in the absence of active LCAT there was a transfer of free cholesterol from LDL to high-density lipoproteins (HDL) and a small transfer of cholesteryl esters in the opposite direction. In the presence of LCAT the loss of free cholesterol started immediately in all three lipoprotein classes, was most prominent in LDL, and was proportional to the newly synthesized cholesteryl esters incorporated in each fraction. In contrast, the loss of phospholipids was not proportional to the newly formed cholesteryl esters in each lipoprotein class: whereas very low density lipoproteins and LDL lost smaller amounts of phospholipids, HDL lost more phospholipids than necessary for the synthesis of cholesteryl esters incorporated in each class. At any time the greatest amount (60–70%) of newly synthesized cholesteryl ester was found in LDL. From these results it is concluded that in pig serum LCAT acts significantly—if not preferentially—on apoB-containing lipoproteins, even in the presence of physiological concentrations of HDL.

Lecithin–cholesterol acyltransferase (LCAT,[1] EC 2.3.1.43) is responsible for the major portion of cholesteryl esters circulating in plasma (Glomset, 1973, 1979). Recently, the catalytic mechanism of this enzyme from human plasma was elucidated (Jauhiainen & Dolphin, 1986). High-density lipoproteins (HDL) are believed to serve as the main substrate for human LCAT, and apoA-I was found to be a necessary cofactor for the LCAT reaction (Fielding et al., 1972). However, it was shown that besides apoA-I other apolipoproteins, e.g., apoC-I, apoE, and apoA-IV as well as apoA-II, are also able to activate human LCAT to a certain extent (Soutar et al., 1975; Albers et al., 1979; Steinmetz & Utermann, 1984; Zorich et al., 1985; Chen & Albers, 1986). Additionally, it was shown that crude LCAT preparations derived from pig plasma and also purified LCAT derived from either human or pig plasma interact directly with very low density lipoproteins (VLDL) and low-density lipoproteins (LDL) (Barter, 1983; Barter & Hopkins, 1983; Knipping et al., 1986). Finally, it was reported that together with triglyceride-rich particles and cholesteryl ester transfer protein (CETP) LCAT plays a major role in the interconversion of HDL-3 to HDL-2 (Dieplinger et al., 1985; Knipping et al., 1985).

This paper is devoted to the elucidation of the direct action of LCAT on LDL in native serum, which seems to be of particular relevance, as it was recently published that in patients having fisheye disease LCAT acts only on combined VLDL and LDL and not on HDL (Carlson & Holmquist, 1985a,b). In this disease there is a low relative content of cholesteryl esters in HDL, but a normal content of these lipids in VLDL and LDL. Additionally, HDL of fisheye disease plasma are smaller than normal. However, they are good substrates if lipoprotein-deficient plasma of normal subjects is used as the source of LCAT.

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[1] Abbreviations: HDL, high-density lipoproteins; LDL, low-density lipoproteins; pig LDL-1, buoyant density fraction 1.020–1.063 g/mL; pig LDL-2, buoyant density fraction 1.063–1.080 g/mL; VLDL, very low density lipoproteins; LCAT, lecithin–cholesterol acyltransferase; CETP, cholesteryl ester transfer/exchange protein.

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To reevaluate the direct action of LCAT on the individual lipoprotein classes, we performed in vitro incubation experiments using pig plasma, because it is known that this animal species lacks CETP activity to a major extent (Barter et al., 1981). Additionally, it was shown that purified pig LCAT behaves similar to purified human LCAT (Knipping, 1986). Therefore, using pig plasma for our studies, we were able to follow solely the action of LCAT without interference of the exchange or transfer of the newly formed cholesteryl esters. Because of the similarity of the enzymes from both pig and human plasma, we may draw conclusions from our results on how LCAT acts in human plasma.

In contrast to human LDL, pig LDL comprises two subclasses designated LDL-1 and LDL-2 (Janado & Martin, 1973), which differ in size, hydrated density, and chemical composition. Both subclasses are physicochemically well characterized (Jackson et al., 1976; Jürgens et al., 1981; Nöthig-Laslo & Knipping, 1984; Herak et al., 1985). However, little is known about their origin or physiological function. Recently, we demonstrated that when isolated LDL-1 and LDL-2 or human LDL was a substrate for LCAT purified from either pig or human plasma, the esterification of free cholesterol in both pig LDL subfractions and in human LDL was about 38–44% that of HDL. Pig LDL-1 and human LDL became even better substrates if the molar phosphatidylcholine/free cholesterol ratio was raised to >3:1 (Knipping et al., 1986). In the present paper we study the direct action of pig LCAT on different lipoproteins by incubating freshly isolated pig serum or individual pig LDL subfractions.

Materials and Methods

Blood from individual fasting pigs (*Sus domesticus*) was obtained from the local slaughterhouse. Serum or plasma was prepared by low-speed centrifugation at 4 °C. In some cases, 10 mM sodium iodoacetate was immediately added to whole blood for inactivation of LCAT.

Incubations for Studying the Action of LCAT. All incubations were performed in a shaking water bath in sealed Erlenmeyer flasks under nitrogen for 24 h, unless stated otherwise, in darkness and in the presence of 50 mg/L streptomycin and penicillin to avoid microbial growth. If necessary, fatty acid poor albumin (Serva, Heidelberg, FRG) was used in the incubation mixtures, which were found to be essentially free from LCAT or CETP activity.

Fresh pig plasma was divided into LCAT-active and -inactive samples by addition of 5 mM sodium iodoacetate to one part of plasma. Aliquots of LCAT-active and of LCAT-inactive plasma were incubated at 37 °C; another aliquot of the LCAT-inactive sample was stored at 4 °C as control. After incubation at different time intervals, LCAT was inhibited in the LCAT-active samples by the addition of 5 mM sodium iodoacetate, and the lipoproteins were isolated as described below (methods 1 and 2) and chemically analyzed.

Lipoprotein Separation. Method 1a: Pig VLDL, LDL, and HDL were isolated from 2 mL of pig plasma in an SW 41 rotor under the conditions described by Terpstra et al. (1981) with the minor modification that sucrose was replaced by NaBr. To make lipoprotein banding visible, plasma was prestained with 30 μL of an aqueous 0.1% Serva blue solution. Method 1b: LDL-1 and LDL-2 were separated by adjusting 2 mL of plasma or total LDL to d = 1.080 g/mL with solid NaBr. Then 5 mL of a NaBr density solution of 1.050 g/mL was overlaid, and the tubes were filled with a tris(hydroxymethyl)aminomethane (Tris)-buffered 0.15 M NaCl solution and spun for 24 h in the SW 41 rotor. The lipoproteins were removed by aspiration.

All lipoprotein fractions used in this study were exhaustively dialyzed against 0.15 M NaCl containing 10 mM Tris-HCl (pH 7.4) and 1 mM ethylenediaminetetraacetic acid (EDTA).

Method 2: In two experiments the VLDL + LDL fraction was removed by precipitation with anti-pig apoB (Knipping et al., 1975) after incubation. The amount of antibody necessary for total precipitation of VLDL + LDL was determined in previous experiments. The quantity of removal of VLDL + LDL was followed by lipid electrophoresis and by immunochemical techniques and was found to be complete by addition of 5 mg of the particular anti-apoB batch to 100 μL of serum. Thus, 100 μL of serum was incubated with 100 μL of antibody solution, containing 5 mg of anti-apoB, for 1 h at 37 °C. After incubation, the samples were centrifuged, and the amount of free and esterified cholesterol was determined from total serum and the supernatant (=HDL). The difference of these values was taken as VLDL + LDL free and esterified cholesterol.

For investigation in the analytical ultracentrifuge, lipoproteins were isolated by ultracentrifugation at 4 °C, 115000g for 48 h in a Sorvall OTD 75 B ultracentrifuge. VLDL were obtained at d < 1.006 g/mL, LDL at d = 1.020–1.080 g/mL, and HDL at d = 1.080–1.21 g/mL.

Isolation of Pig LCAT. Pig LCAT was isolated and purified essentially as described by Knipping (1986). Briefly, pig serum was ultracentrifuged for 48 h at a density of 1.21 g/mL. The clear middle zone was immediately subjected to phenyl-Sepharose chromatography, and LCAT activity was eluted with doubly distilled water. The phenyl-Sepharose step was repeated twice. The LCAT-active fraction was exhaustively dialyzed against 10 mM potassium phosphate buffer, pH 6.8, in the presence of 1 mM sodium deoxycholate and then subjected to hydroxylapatite chromatography. LCAT was eluted with 10 mM potassium phosphate buffer, pH 6.8. The purity of LCAT was checked by 10% sodium dodecyl sulfate (SDS)–gel electrophoresis (Weber & Osborn, 1969) and isoelectrofocusing using a pH gradient of 4–6.

Chemical Analyses. Free cholesterol and esterified cholesterol were determined with enzymatic test kits from E. Merck, Darmstadt, FRG. Triacylglycerols and phospholipids were assayed enzymatically (Biomerieux, Carbonniere les Bains, France). Protein was measured according to the method of Lowry et al. (1956) using bovine serum albumin as a standard. Analyses of apolipoproteins on individual LDL fractions were performed by immunoelectrophoresis using HDL, apoE, and apoC-II as reference and antisera against pig apoA-I, apoC-II, apoE, and LDL (Knipping et al., 1975, 1984). Lipoprotein electrophoresis was performed using the lipidophor electrophoresis kit of Immuno AG (Vienna, Austria). Cholesteryl ester transfer/exchange (CETP) activity was measured according to the method of Barter et al. (1981). Lysolecithin was determined by thin-layer chromatography (TLC) according to the method of Kostner and Holasek (1972).

Physicochemical Analyses. The LDL analyses were performed in a Beckman Model E analytical ultracentrifuge equipped with Schlieren optics and using a four-hole rotor An-F. Runs were carried out at 48 000 rpm and 20 °C for 3 h. Photographs were taken every 16 min as described (Zechner et al., 1982). The lipoprotein fractions were analyzed at a concentration of 10–15 mg/mL in a NaBr density solution of 1.090 g/mL, pH 7.4. Under these conditions the concentration dependence of the flotation constants is negligible.

Results

Action of LCAT on LDL. Pig plasma was incubated in the presence of active LCAT at 37 °C and in the absence of active

Table I: Chemical Composition of Total LDL (LDL-1 and LDL-2) in the Absence and Presence of Active LCAT[a]

incubation (h)		T (°C)	P	FC	CE	PL	TG	molar PC/FC ratio
0	+LCAT	37	22.5	9.6	43.5	19.7	4.7	1.28
0.5	+LCAT	37	22.6	9.1	44.7	19.5	4.1	1.08
1	+LCAT	37	22.5	8.5	46.1	18.9	4.0	1.11
2	+LCAT	37	22.4	7.8	47.1	18.5	4.2	1.19
3	+LCAT	37	22.6	7.4	48.0	18.1	3.9	1.22
6	+LCAT	37	23.5	6.2	49.1	17.2	4.0	1.39
9	+LCAT	37	24.2	5.2	49.9	16.3	4.4	1.61
12	+LCAT	37	24.6	4.5	50.9	15.9	4.1	1.76
18	+LCAT	37	25.6	3.6	51.5	14.8	4.5	2.10
24	+LCAT	37	25.6	2.6	53.6	14.0	4.2	2.75
24	-LCAT	37	21.4	8.3	42.9	22.9	4.5	1.39
24	-LCAT	4	22.0	9.9	43.4	21.0	3.7	1.06

[a] Pig serum was incubated in the absence and presence of 5 mM sodium iodoacetate at the indicated time intervals at 37 °C. For control, one part of the LCAT-inactive sample was incubated at 4 °C. The values are represented as mean weight percent of two experiments performed in triplicate. P = protein; FC = free cholesterol; CE = cholesteryl esters; PL = phospholipids; TG = triglycerides.

LCAT at 4 and at 37 °C for 24 h. Total LDL were isolated at d = 1.020–1.080 g/mL by preparative ultracentrifugation, dialyzed, and chemically analyzed.

We found that upon a 24-h in vitro incubation at 37 °C in the presence of LCAT the cholesteryl esters not only in HDL (Knipping et al., 1985) but also in the LDL fraction increased markedly with a concomitant decrease of free cholesterol and phospholipids. In contrast, nearly no change was found in the LCAT-inactive samples incubated at either 4 or 37 °C. Since we could not detect any remarkable activity of CETP, we interpreted this finding to represent the direct action of LCAT on LDL. By analyzing the LDL fraction in the analytical ultracentrifuge, we found that LDL-1 and LDL-2, originally well separated into two peaks, had fused together in the LCAT-active samples. This newly formed LDL fraction floated at a density of 1.052 g/mL, which is in between that of LDL-1 and LDL-2. Electron microscopy revealed that these LDL particles had diameters of 23.7 ± 1.44 nm, whereas those of control LDL-1 and control LDL-2 were 25.9 ± 1.89 and 22.4 ± 1.55 nm, respectively.

To study this phenomenon precisely, incubation experiments were performed in the presence and absence of active LCAT at different time intervals. The isolated LDL fractions were analyzed in the analytical ultracentrifuge. In comparison to control LDL, virtually no change in the flotation behavior of the LDL subclasses was obtained with samples incubated at 37 °C in the presence of the LCAT inhibitor sodium iodoacetate. The fusion of LDL-1 and LDL-2 was found to be time dependent, starting to become visible after 3 h of incubation in the presence of active LCAT. After 12 h, the fusion was nearly complete. No change in the flotation profile was observed after further incubation up to 24 h (Figure 1). In contrast, the change in the content of cholesteryl esters, free cholesterol, and phospholipids started immediately and proceeded for 24 h of incubation (Table I).

In order to get a deeper insight into the action of LCAT on the pig LDL subfractions, we isolated prior to incubation LDL-1 and LDL-2 from LCAT-inactive serum and incubated the fractions in the presence of 4 fatty acid poor albumin and in the absence or presence of purified pig LCAT (50 units/mL) at 37 °C for 24 h. When these fractions were reisolated after incubation in the SW 41 rotor, the flotation behavior of LDL-1 changed toward higher densities and that of LDL-2 toward lower densities upon incubation with LCAT (Figure 2). However, these changes in the flotation behavior were not pronounced to the same degree as in incubations of total plasma. Although physiological concentrations of lipoprotein and LCAT were used and the incubation lasted for 24 h, the alterations occurring in the chemical composition of LDL were

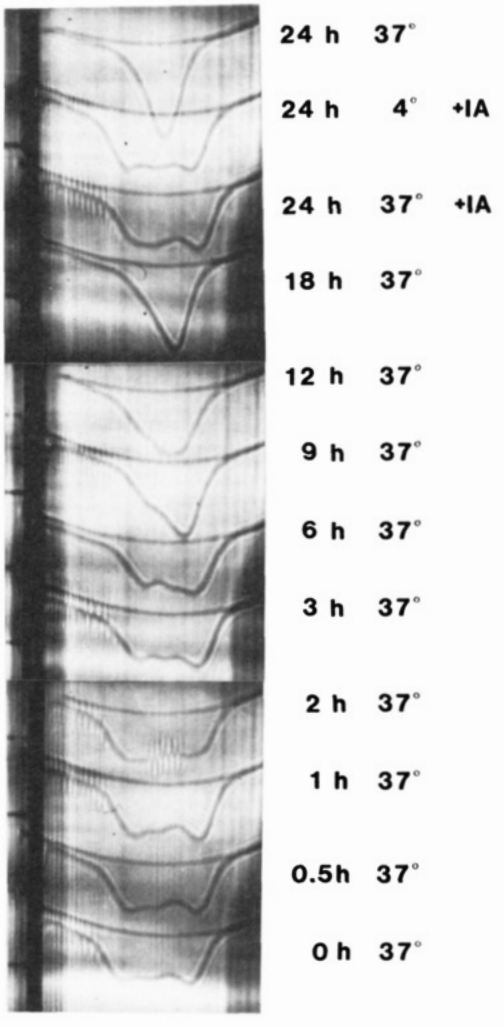

FIGURE 1: Influence of LCAT on LDL. Schlieren pattern of pig LDL obtained from serum that was incubated in the presence or absence of the LCAT inhibitor sodium iodoacetate at 37 °C at different time intervals. One aliquot of the LCAT inactive serum was stored at 4 °C for 24 h as control. Samples 2 and 3 from the top were incubated in the presence of 5 mM sodium iodoacetate.

comparable to those obtained by incubation of total serum for about 3 h. The absolute changes in the chemical composition are given in Table II.

Effect of LCAT on Total Serum. After establishing that LCAT has a marked effect on LDL, we wanted to investigate in detail the direct action of LCAT in whole plasma. For these experiments we had to find methods allowing us to determine exactly the total amount of protein and lipids of the different

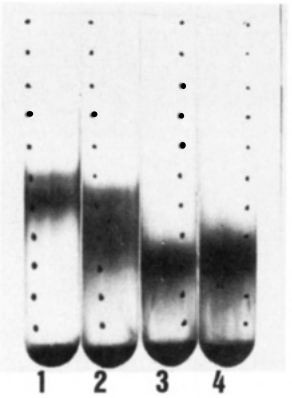

FIGURE 2: Influence of purified pig LCAT on isolated LDL subfractions. Equal amounts of either pig LDL-1 or pig LDL-2 were incubated in the presence or absence of purified pig LCAT for 24 h at 37 °C. (1) LDL-1 in the absence of LCAT; (2) LDL-1 in the presence of LCAT; (3) LDL-2 in the absence of LCAT; (4) LDL-2 in the presence of LCAT.

Table II: Incubation of Isolated Pig LDL-1 and LDL-2 with Purified LCAT[a]

		FC	CE	PL
LDL-1	-LCAT	166 ± 11	626 ± 13	197 ± 12
	+LCAT	134 ± 18	658 ± 16	166 ± 15
		-32	+32	-31
LDL-2	-LCAT	145 ± 12	649 ± 14	196 ± 16
	+LCAT	98 ± 9	697 ± 12	151 ± 14
		-47	+48	-45

[a] Either LDL-1 or LDL-2 was incubated with purified pig LCAT for 24 h at 37 °C in the presence or absence of 5 mM sodium iodoacetate and in the presence of 4% albumin. The values are presented in mean micromolar per liter ± SD from three experiments performed in triplicate. FC = free cholesterol; CE = cholesteryl esters; PL = phospholipids.

lipoprotein classes in the plasma. The following two methods were used.

First, we tried method 1a originally applied by Terpstra et al. (1981). Chemical analyses of the lipoprotein fractions obtained by this method revealed that 80–90% of the total amount of free and esterified cholesterol, phospholipids, and triglycerides could be recovered.

Similar results were obtained by use of another gradient (method 1b), where LDL-1 and LDL-2 were separated from each other and HDL sedimented to the bottom. However, with this method the change in phospholipids in HDL could not be quantitatively estimated because the amount of phospholipids in HDL and the arising lysolecithin were determined together. The reason we used this method was to investigate the changes in the composition of LDL-1 and LDL-2, expecting to get an explanation for the fusion of these subfractions.

Second, immunoprecipitation of VLDL and LDL after incubation of serum in the presence and absence of LCAT at differing time intervals was performed (method 2). Here none of the free or esterified cholesterol was lost since ultracentrifugation was omitted.

The results obtained with methods 1a and 1b are displayed in Figures 3–8. The control sample was the plasma incubated in the absence of active LCAT at 4 °C for 24 h. As already known, the chemical composition of HDL changed markedly upon incubation with LCAT: free cholesterol and phospholipids decreased, whereas cholesteryl esters increased. No significant changes were found in the triglyceride content. Only after a 24-h in vitro incubation was the triglyceride content in HDL slightly increased.

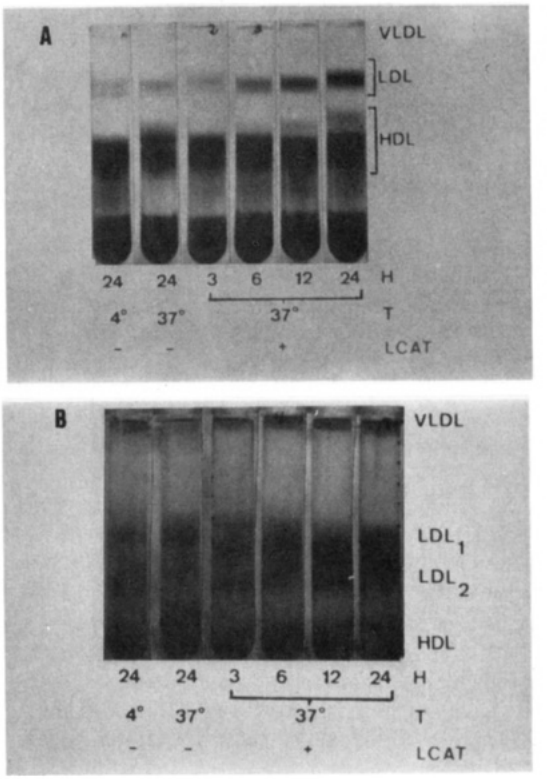

FIGURE 3: Flotation pattern of pig lipoproteins by gradient ultracentrifugation. Pig serum was incubated in the presence and absence of the LCAT inhibitor sodium iodoacetate at 37 °C at different time intervals. As control, one aliquot of the LCAT-inactive serum was stored at 4 °C. (A) Pig lipoproteins isolated according method 1a. (B) Pig lipoproteins isolated according method 1b.

Similar alterations were detected in LDL and were comparable to those in Table I: a pronounced decrease of free cholesterol, a minor decrease of phospholipids, and an increase in cholesteryl esters. Concomitantly, with all these changes in the chemical composition, the flotation behavior of the LDL and HDL classes was altered (Figure 3). As shown above, at between 3 and 6 h of incubation both LDL subclasses started to fuse and floated after 24 h as a single band. In contrast, the previous more or less homogeneous HDL started after 6 h to become inhomogeneous. The colored HDL separated into three subclasses: one resembling human HDL-2 with regard to flotation behavior and chemical composition, the next resembling HDL-3, and the third resembling very high density lipoproteins (VHDL). From the chemical composition it seemed that "HDL-2" and VHDL contained most of the newly formed cholesteryl esters. After 24 h of incubation in the presence of LCAT, HDL-2 represented about 5–10%, HDL-3 70–80%, and VHDL 14–28% of the total mass of HDL. A rearrangement of HDL after a 24-h incubation in the absence of active LCAT was also noticed, and the previously homogeneous HDL broadened. This part, which floated in the HDL-2 region, although not clearly separated from HDL-3, represented about 25–30% of the total mass of HDL, whereas no change or only a slight loss of the mass of VHDL was obtained. In the control plasma, which was incubated for 24 h at 4 °C in the absence of active LCAT, no or only 1% of HDL-2-like particles were found; HDL-3 represented 88–93% of the total HDL mass, and the remainder consisted of VHDL.

When we calculated the changes in lipid composition obtained for methods 1a and 1b found in VLDL, LDL, and HDL, we got surprising results (Figure 4). If plasma was incubated for 24 h at 37 °C in the absence of LCAT, com-

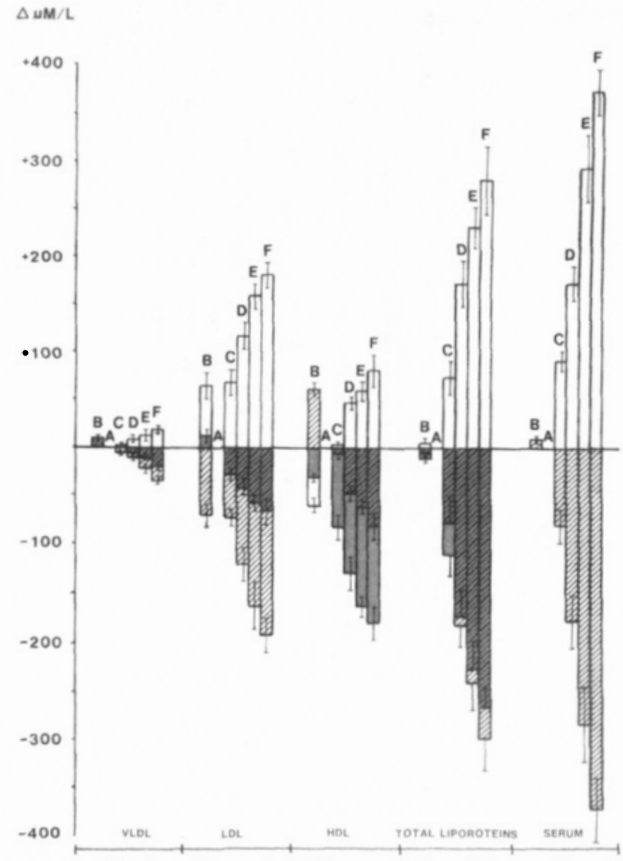

FIGURE 4: Absolute changes in lipid composition of lipoproteins after incubation of serum in the presence or absence of active LCAT. Serum was incubated at 37 °C in the absence (C–F) or presence (B)of 5 mM sodium iodoacetate. As control, one aliquot of the LCAT-inactive serum was stored at 4 °C (A). Duration of incubation for A, B, and F was 24 h; C, D and E were incubated for 3, 6, and 12 h, respectively. The values represent the absolute changes ± the standard deviation in lipid composition ($\Delta\mu M/L$) in relation to the values obtained for the control serum (=base line, A). The original lipoprotein composition was as follows: VLDL, 36 ± 3.6 $\mu M/L$ of FC, 15 ± 2.6 $\mu M/L$ of CE, and 32 ± 5.1 $\mu M/L$ of PL; LDL, 279 ± 33 $\mu M/L$ of FC, 823 ± 135 $\mu M/L$ of CE, and 292 ± 41 $\mu M/L$ of PL; HDL, 146 ± 13 $\mu M/L$ of FC, 734 ± 88 $\mu M/L$ of CE, and 606 ± 93 $\mu M/L$ of PL. The values for the native control serum were 576 ± 96 $\mu M/L$ of FC, 1917 ± 240 μMoL of CE, and 1148 ± 126 $\mu M/L$ of PL. Values were obtained from three experiments and triplicate analyses. White bars, CE; hatched bars, FC; gray bars, PL.

positional changes were obtained in LDL and HDL: the absolute amount of free cholesterol decreased up to 25% in LDL, whereas the absolute amount of both cholesteryl esters and phospholipids increased between 4 and 8%. In contrast, free cholesterol increased in HDL up to 42%, whereas cholesteryl esters and phospholipids decreased between 4 and 9%. In VLDL only a slight increase in free cholesterol and phospholipids seemed to occur. But due to the low content of VLDL in pig serum the results for VLDL must be viewed with caution. Expressed in micromolar per liter as in Figure 4, it is clearly visible that HDL are donors of cholesteryl esters, which are probably all taken up by LDL, and phospholipids. LDL and probably VLDL, in turn, are donors of free cholesterol.

The results obtained after in vitro incubation of pig plasma in the presence of active LCAT at differing time intervals show that in all three lipoprotein classes the loss of free cholesterol started immediately and was most pronounced in LDL. The same was true for phospholipids; however, it seemed that for the LCAT reaction the major part of phospholipids originated from HDL, since this lipoprotein fraction lost approximately

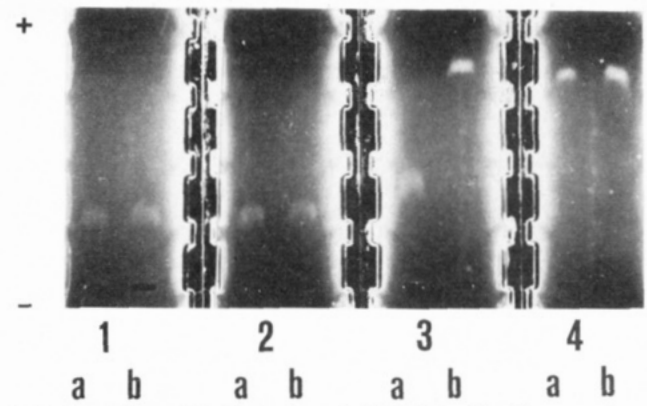

FIGURE 5: Lipoprotein electrophoresis of LDL fractions in comparison to total HDL before and after incubation in the presence of LCAT. Pig serum was incubated in the absence and presence of LCAT for 24 h at 37 °C. As control, one aliquot of the LCAT-inactive sample was stored at 4 °C. Lipoproteins were isolated by a single density gradient ultracentrifugation run according to method 1b. Five microliters of each fraction was applied. (1) (a) LDL-1, (b) LDL-2 stored at 4 °C; (2) (a) LDL-1, (b) LDL-2 incubated at 37 °C in the absence of active LCAT; (3) (a) fused LDL, (b) total HDL obtained after incubation in the presence of active LCAT at 37 °C; (4) (a) total HDL incubated in the absence of LCAT at 4 °C and at (b) 37 °C.

200 $\mu M/L$, whereas VLDL lost about 10 $\mu M/L$ and LDL about 60 $\mu M/L$ after a 24-h incubation. The loss of free cholesterol was practically equivalent to the newly formed cholesteryl esters in all fractions. In contrast, HDL lost more phospholipids than necessary for the amount of newly formed cholesteryl esters in HDL. It is additionally noteworthy that at any time interval studied 60–70% of the newly synthesized cholesteryl esters were found in the LDL fraction. These findings were true for the three experiments performed at different time intervals and the four experiments with 24-h incubations.

To ascertain that the fused LDL fraction did not contain α-lipoprotein-like particles, which may have been responsible for the altered chemical composition of this density region, lipoprotein electrophoresis was performed. The purity of the LDL and HDL fractions isolated by a single gradient ultracentrifugation run before and after incubation was confirmed by this experiment (Figure 5). The fused LDL particles migrated slightly faster than LDL-1 and LDL-2. This was due to the uptake of apoA-I, apoC-II, and apoE by the LDL particles as demonstrated by immunoelectrophoresis. As shown in Figure 6, apoA-I, apoC-II, and apoE were present at the same particle as apoB. Such an uptake of apolipoproteins by LDL during the action of LCAT was previously noticed by others (Abbey et al., 1984; Zechner et al., 1985).

Figures 7 and 8 show the compositional alterations in the LDL and HDL subclasses. Despite their differing concentrations in serum (LDL-1:LDL-2 approximately 2:1), LDL-1 and LDL-2 seem to be equivalent substrates for LCAT in native serum. The total mass of LDL was slighlty increased, probably due to the increase of protein and to the obvious lower loss of phospholipids than needed for the amount of newly formed cholesteryl esters.

The previously homogeneous HDL were converted to HDL-2-, HDL-3- and VHDL-like particles upon incubation with LCAT. Free cholesterol and phospholipids were mainly lost at the expense of the HDL-3 fraction. The newly formed cholesteryl esters seemed to be incorporated first into VHDL, and measurable amounts also in the HDL-2 fraction, after 6 h of incubation. The total lipid mass of HDL was decreased due to the absolute loss of phospholipids. The total amount

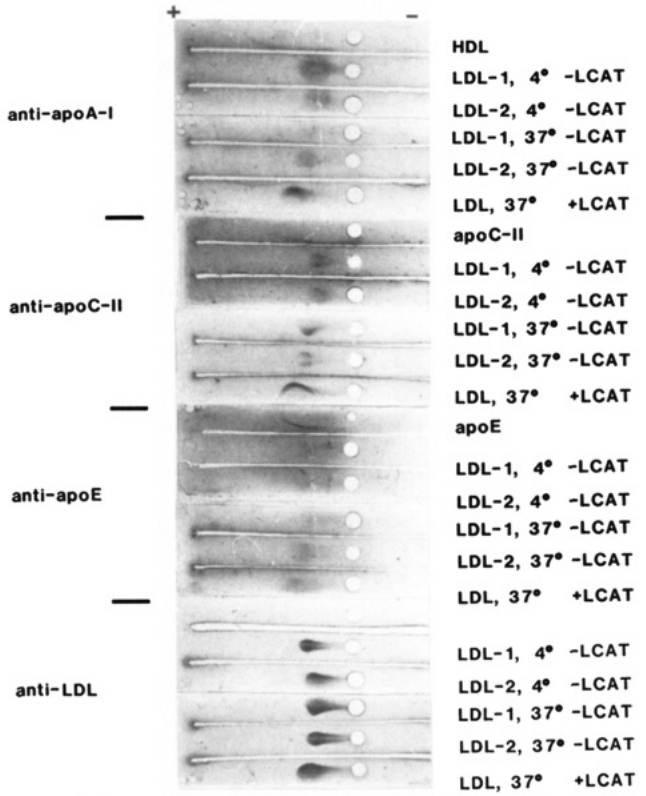

FIGURE 6: Comparison of LDL fractions with HDL or with purified apolipoproteins by immunoelectrophoresis. The experimental design was the same as in Figure 5. HDL, apoC-II, and apoE were used as reference.

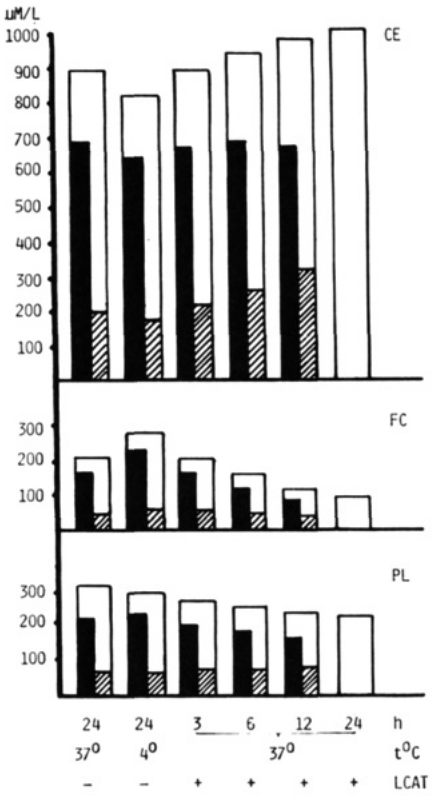

FIGURE 7: Absolute changes in lipid composition of LDL subfractions. Conditions are the same as described for Figure 4. Values are from one single experiment but representative for two others and are given in micromolar per liter. White bars, total LDL; black bars, LDL-1; hatched bars, LDL-2. Since after a 24-h incubation LDL-1 and LDL-2 could not be separated anymore, only the value for total LDL is represented.

of apoprotein could not be exactly determined due to the increase of the albumin concentration in VHDL.

In order to evaluate a possible role of lysolecithin in the fusion of the two LDL fractions, we incubated pig serum in the absence and presence of L-α-lysolecithin in an amount equivalent to the newly formed cholesteryl esters after a 24-h incubation, stimulating the LCAT effect. For comparison, incubations were performed in the absence and presence of the same amount of lysolecithin, but in the absence and presence of the same amount of lysolecithin, but in the presence of active LCAT. As shown in Figure 9, lysolecithin alone did not alter the flotation behavior of LDL-1 and LDL-2. Only in the presence of active LCAT did the fusion of LDL-1 and LDL-2 occur, regardless of whether lysolecithin was added or not. By evaluating the distribution of lysolecithin after incubation by TLC, we found that approximately 98% were present in the bottom fraction.

Method 2, by which VLDL + LDL were specifically precipitated with anti-apoB after incubation of serum, gave results (two experiments) similar to those of methods 1a and 1b. Free and esterified cholesterol were determined from total plasma and from the supernatant obtained after centrifugation of the precipitate. Even here it was obvious that most of the newly synthesized cholesteryl esters were incorporated in the VLDL + LDL fraction.

Discussion

In this paper we have shown for the first time that a complete fusion of both pig LDL subclasses occurs upon prolonged in vitro incubation of native plasma in the presence of active LCAT (Figures 1 and 3), whereby the lighter LDL-1 floated toward higher and the denser LDL-2 toward lower densities. This fusion processes was time dependent and led to LDL particles of a density intermediate and with molecular sizes in between those of LDL-1 and LDL-2. Concomitantly, a pronounced change in the chemical composition was obtained: in both LDL subclasses the content of cholesteryl esters increased continuously and free cholesterol and phospholipids decreased.

Since Portman and Illingworth (1973) reported that on a molar basis the binding capacity of LDL for lysolecithin is 80 times greater than that of albumin, it might have been possible that the fusion of the LDL particles was due to the formation of lysolecithin in LDL during the LCAT reaction. However, this is not the case. First of all, after ultracentrifugation, more than 98% of the newly formed lysolecithin was found in the lipoprotein-free bottom fraction. We performed further experiments where lysolecithin was incubated with LCAT-inactive serum at 37 °C for 24 h in an amount equivalent to the newly formed cholesteryl esters in total serum, simulating the LCAT effect. No alteration in the flotation profile occurred. On the other hand, a 24-h incubation of the same amount of lysolecithin with LCAT-active serum did not hinder the fusion of LDL-1 and LDL-2 (Figure 9). We have no explanation for the alteration in the flotation behavior or fusion of these LDL subfractions.

Since until today the general opinion prevails that LCAT acts preferentially on HDL or particles that prominently contain apoA-I, we evaluated the compositional changes of various lipoproteins in whole serum upon in vitro incubation with LCAT. The methods applied here allowed a separation of lipoproteins either by a single run using density gradient ultracentrifugation (Figure 3A,B) or by precipitation with anti-apoB. Thus, an absolute quantitative estimation of all changes in lipid composition in the different lipoprotein fractions was possible. We avoided the use of isotopic labeling

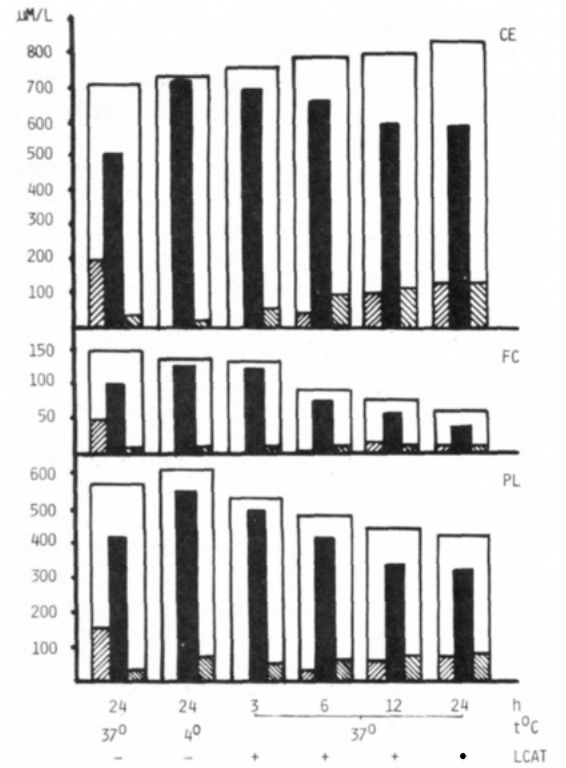

FIGURE 8: Absolute changes in lipid composition of HDL subfractions. Conditions are the same as described for Figure 4. Values are from one single experiment but representative for two others and are given in micromolar per liter. White bars, total HDL; ▨, HDL-2; black bars, HDL-3; ■, VHDL.

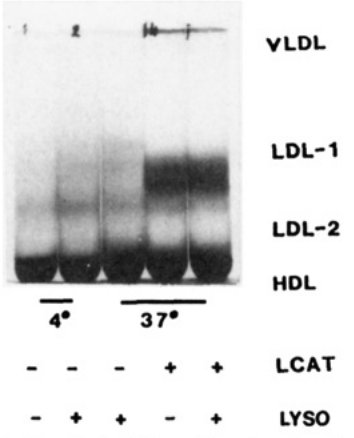

FIGURE 9: Distribution of pig LDL subfractions in a density gradient after ultracentrifugation. Two milliliters of pig serum was incubated at 37 °C in the presence and absence of active LCAT plus and minus 1 μM lysolecithin. As control, LCAT-inactive samples were incubated at 4 °C with and without the same amount of lysolecithin. The LDL fractions were separated according to method 1b.

of cholesterol in whole serum, a method performed in previous studies (Barter, 1983; Rajaram & Barter, 1985), since in preliminary studies (unpublished observation) we found that [^{3}H]cholesterol was unevenly distributed among the different lipoprotein classes; e.g., at zero time and after 1 h of incubation, [^{3}H]cholesterol was mostly found in the HDL fraction. Thus, newly formed [^{3}H]cholesteryl esters must be preferentially found in HDL under those conditions. This assumption is strengthened by the fact that differences in the rate of [^{3}H]cholesteryl ester formation in LDL occurred depending on whether the label was added as a component of LDL or HDL (Barter, 1983). Therefore, after incubation, we preferred to use density gradient ultracentrifugation of pig plasma or precipitation with specific antibodies for separation of lipoproteins and chemical determination of all lipids.

In comparison to control plasma stored at 4 °C, a transfer of free cholesterol to HDL with a reverse transfer of cholesteryl esters to LDL took place upon a 24-h incubation of plasma in the absence of active LCAT at 37 °C. This cholesteryl ester transfer yielded maximally 8% per 24 h and was therefore minimal in comparison to that in human serum (Ha & Barter, 1982; Groener et al., 1986). Whether this transfer of cholesteryl esters is due to an action of a transfer protein or is that amount of cholesteryl esters able to equilibrate freely remains to be investigated. However, the transfer activity in pig serum seems to be negligible with regard to the action of LCAT on the different lipoprotein classes. In the presence of LCAT, the loss of free cholesterol is greatest in LDL. In each individual lipoprotein fraction the loss of free cholesterol is equivalent to the corresponding amount of newly synthesized cholesteryl esters, as demonstrated in Figure 4.

It was surprising that the loss of phospholipids during the LCAT reaction in the particular lipoprotein fractions was not equivalent to the amount of the newly formed cholesteryl esters: VLDL and LDL lost a smaller amount and HDL lost a higher amount of phospholipids than necessary for the synthesis of cholesteryl esters in each lipoprotein fraction.

In contrast, if isolated LDL fractions were incubated in the presence of purified LCAT for 24 h, the loss of phospholipids corresponded to the loss of free cholesterol and to the amount of newly synthesized cholesteryl esters. However, the number of newly synthesized cholesteryl esters was only approximately equivalent to that amount of cholesteryl esters formed in LDL after a 3-h incubation of total serum. Considering that in LDL the molar ratio of phospholipid to free cholesterol is about 1 and Yeagle et al. (1977) already suggested that some of the LDL phospholipids may be immobilized by the intact apoB, it seems intelligible that not all of the phospholipid may be available for the LCAT reaction. Therefore, when isolated LDL are incubated in the presence of active LCAT and no phospholipids from other donors are available, the LCAT reaction slows down after 1–2 h and seems to be totally abolished after approximately 3 h. This may explain the only small alterations in the flotation behavior found with isolated LDL subfractions. However, we found no sound explanation of why even isolated LDL-1 floated toward higher densities and isolated LDL-2 toward lower densities upon incubation with LCAT. In vitro, in whole plasma LDL seem to gain phospholipids from HDL and therefore may act as better substrates for LCAT than LDL preparations, which were artificially enriched with phospholipids (Knipping et al., 1986). Our results in the present paper show that LDL are even better substrates for LCAT than HDL when total plasma, not isolated LDL fractions as done in the former study, is incubated.

That LCAT may act directly on LDL was already suggested in publications from Barter's group (Barter, 1983; Barter & Hopkins, 1983; Rajaram & Barter, 1985). To study the effects of LCAT on the different human lipoprotein classes, they incubated [^{3}H]cholesterol labeled lipoproteins in the absence or presence of CETP and/or LCAT. In the absence of CETP, but in the presence of LCAT, 73% of the newly synthesized [^{3}H]cholesteryl esters were found in HDL, but only 25% in LDL and 1% in VLDL. The difference in the amount of newly synthesized cholesteryl esters found in LDL between their and our studies may be due to the different methods used.

Fielding and Fielding (1980) predicted that in human serum there may exist a cholesteryl ester transfer protein (apoD)–LCAT–apoA-I complex, which may equally interact with all

lipoproteins and thus may be responsible for the synthesis of cholesteryl esters in each lipoprotein class. Both authors (Fielding & Fielding, 1981) found that in human serum the VLDL + LDL fraction lost the greatest amount of free cholesterol during the action of LCAT. They suggested from their studies that this free cholesterol is delivered from the VLDL + LDL fraction to HDL for esterification by LCAT. The authors detected further that the mass of free cholesterol supplied by VLDL and LDL was almost the same as that taken back as cholesteryl esters. Additionally, they found it noteworthy that little cholesteryl ester in whole human plasma is taken up by HDL. However, they attributed this finding to the action of CETP. On the other hand, Barter and Hopkins (1983) observed that in the absence of LCAT the presence of CETP only marginally increased the proportion of the esterified cholesterol incorporated into LDL in human plasma, whereas the transfer of cholesteryl esters from HDL to VLDL was 10 times higher and was positively correlated with plasma triglyceride levels (Rajaram & Barter, 1985).

In contrast to human serum, pig serum contains no or only very low cholesteryl ester transfer activity (Barter et al., 1981) and very low levels of VLDL. Pig VLDL contain approximately half the amount of cholesteryl esters as human VLDL. Thus, the catabolism of pig VLDL cannot be totally responsible for the amount of cholesteryl esters incorporated in LDL. Furthermore, Aigueperse et al. (1983) excluded a VLDL origin for LDL cholesteryl esters by cholesterol-turnover studies in pigs. Since the fatty acid composition of pig LDL cholesteryl esters is similar to that of HDL (Nöthig-Laslo & Knipping, 1984, 1985), the mechanism of cholesteryl ester synthesis should be the same.

In a recent report from this laboratory it was demonstrated that purified LCAT derived from either human or pig plasma may produce cholesteryl esters in isolated LDL from both species up to a value of 38–44% of that obtained for HDL. After enrichment of LDL with phosphatidylcholine to a molar phosphatidylcholine/free cholesterol ratio >3:1, these LDL particles were nearly as good as substrates as HDL (Knipping et al., 1986). We have further demonstrated that LDL contain small amounts of apoA-I, sufficient to activate LCAT, and that this apoA-I must be bound to the particle itself; otherwise, LDL do not serve as good substrates. We additionally showed that removal of apoA-I alone from LDL did not totally abolish the esterification of free cholesterol in isolated LDL by either pig or human LCAT, although the esterification rate became higher after readdition of purified apoA-I. However, if all non-apoB proteins were removed from the LDL particle, no esterification of free cholesterol in LDL was obtained upon incubation with purified LCAT and readdition of apoA-I to such samples could not restore LCAT activity. In this study we now show that during in vitro incubation of pig serum in the presence of LCAT the LDL fractions take up apoC-II, apoA-I, and apoE, the latter two known to activate LCAT. Such uptake of LCAT cofactor proteins may also occur in vivo and thus facilitate the LCAT reaction on apoB-containing lipoproteins.

Besides the fact that pig serum does not contain apoD, other LCAT complexes could not explain how some of the phospholipids of HDL were used for synthesis of cholesteryl esters in LDL. We do not suppose that LCAT acts primarily with HDL phospholipids, which then in part should become transferred to LDL. From our studies we deduce that in vitro LCAT binds primarily to available surface lipids of all lipoprotein classes and the reaction then starts. During the consumption of these available phospholipids, LDL and probably also VLDL absorb more phospholipids from HDL, at least in vitro. In vivo, this may be valid only for LDL because of their longer residence time in plasma. However, this uptake of phospholipids from HDL by other lipoproteins in vitro has to be fast, since more than 60% of the newly formed cholesteryl esters were found in LDL and much less in HDL, irrespective of the incubation time. It may be that depletion of VLDL and LDL from phospholipids during the LCAT reaction accelerates the process of phospholipid transfer from HDL to other lipoproteins. Whether in this process a phospholipid transfer protein is of importance remains to be demonstrated.

Against our results stand those obtained by using different methods, which indicated that only small amounts of LCAT mass could be directly detected on LDL (Albers et al., 1981; Chung et al., 1982; Park et al., 1984). However, some of the methods used may not have been suitable to investigate this problem, and dissociation of LCAT from apoB-containing lipoproteins, e.g., by ultracentrifugation, may have occurred.

How otherwise can most of the cholesteryl esters in pig plasma be transported by LDL if LCAT would not act on these particles and considering a slow cholesteryl ester transfer? Our conclusion is that in pig plasma LCAT acts significantly—if not preferentially—on apoB-containing lipoproteins even in the presence of physiological amounts of HDL. Whether or not similar reactions occur in human plasma remains to be investigated.

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Partial Purification and Characterization of β-Mannosyltransferase from Suspension-Cultured Soybean Cells†

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ABSTRACT: The β-mannosyltransferase that catalyzes the synthesis of Man-β-GlcNAc-GlcNAc-PP-dolichol from GDP-mannose and dolichyl-PP-GlcNAc-GlcNAc was solubilized from microsomes of suspension-cultured soybean cells by treatment with 1.5% Triton X-100 and was purified about 700-fold by chromatography on DEAE-cellulose, hydroxylapatite, and a GDP affinity column. The purified enzyme was reasonably stable in the presence of 20% glycerol and 0.5 mM dithiothreitol. The enzyme required either detergent (Triton X-100 or NP-40) or phospholipid for maximum activity, but the effects of these two were not additive. Thus, either phosphatidylcholine or Triton X-100 could give maximum stimulation. In terms of phospholipid stimulation, both the head group and the acyl chain appeared to be important since phosphatidylcholines with 18-carbon unsaturated fatty acids were most effective. The purified enzyme had a sharp pH optimum of 6.9–7.0 and required a divalent cation. Mg^{2+} was the best metal ion with optimum activity occurring at 6 mM, but Mn^{2+} was reasonably effective while Ca^{2+} was slightly stimulatory. The K_m for GDP-mannose was calculated to be 1.7×10^{-6} M and that for dolichyl-PP-GlcNAc-GlcNAc about 9×10^{-6} M. The enzyme was inhibited by a number of guanosine nucleotides such as GDP-glucose, GDP, GMP, and GTP, but various uridine and adenosine nucleotides were without effect. The purified enzyme was apparently free of α-1,3-mannosyltransferase (and perhaps other mannosyltransferases) and dolichyl-P-mannose synthase since the only product seen from dolichyl-PP-GlcNAc-GlcNAc and GDP-mannose was Man-β-GlcNAc-GlcNAc-PP-dolichol. No activity was observed when dolichyl-P-mannose replaced the GDP-mannose.

It is now well established that lipid-linked saccharides play an intermediate role in the biosynthesis of the oligosaccharide chains of the asparagine-linked glycoproteins of eucaryotic cells (Struck & Lennarz, 1980; Elbein, 1979). Thus, a large number of studies have been done with membrane preparations from various cells and tissues in order to follow the incorporation of GlcNAc, mannose, and glucose into the various

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