Effect of the Human Plasma Apolipoproteins and Phosphatidylcholine Acyl Donor on the Activity of Lecithin: Cholesterol Acyltransferase†

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ABSTRACT: The human plasma apoproteins apoA-I and apoC-I enhanced the activity of partially purified lecithin: cholesterol acyltransferase five to tenfold with chemically defined phosphatidylcholine:cholesterol single bilayer vesicles as substrates. By contrast, apoproteins apoA-II, apoC-II, and apoC-III did not give any enhancement of enzyme activity. The activation by apoA-I and apoC-I differed, depending upon the nature of the hydrocarbon chains of phosphatidylcholine acyl donor. ApoA-I was most effective with a phosphatidylcholine containing an unsaturated fatty acyl chain. ApoC-I activated LCAT to the same extent with both saturated and unsaturated phosphatidylcholine substrates. Two of the four peptides obtained by cyanogen bromide cleavage of apoA-I retained some ability to activate LCAT. The efficacy of each of these peptides was approximately 25% that of the whole protein. Cyanogen bromide fragments of apoC-I were inactive. The apoproteins from HDL, HDL_2, and HDL_3, at low protein concentrations, were equally effective as activators of LCAT and less effective than apoA-I. Higher concentrations of apoHDL, $apoHDL_2$, and $apoHDL_3$ inhibited LCAT activity. ApoC and apoA-II were both found to inhibit the activation of LCAT by apoA-I. The inhibition of LCAT by higher concentrations of apoHDL was not correlated with the apoA-II and apoC content.

Cholesteryl esters are formed in human plasma from unesterified cholesterol and phosphatidylcholine (PC)[1] in human plasma lipoproteins by the enzyme lecithin:cholesterol acyltransferase (LCAT, EC 2.3.1.43) (Glomset, 1962). It is thought that the reaction takes place on or within the high density lipoproteins, predominantly the lower molecular weight subfractions (Glomset et al., 1966). A nonenzymic role for LCAT has also been suggested. The cholesteryl ester content of the other lipoprotein species is the result of exchange, stimulated by LCAT, between products of the reaction in HDL and the lipids of the other particles (Glomset et al., 1966; Akanuma and Glomset, 1968). The gross distortion in the normal distribution of the plasma lipiproteins in individuals with an inheritable deficiency of LCAT indicates the importance of this enzyme in the metabolism of plasma lipoproteins (Norum et al., 1972, Glomset and Norum, 1973). However, little is yet known of the mechanism of action of LCAT.

A variety of PC acyl donors can serve as substrates for the enzyme. We have shown that the physical properties of the fluid hydrocarbon region of the substrates, single bilayer vesicles of synthetic PC containing cholesterol, largely determine the rate of transacylation (Soutar et al., 1974). The rate of transacylation increases abruptly at the temperatures which coincide with the gel to liquid-crystalline transition temperatures of the PC acyl donors. The decrease in LCAT activity with increasing cholesterol content also reflects the well-documented (Chapman, 1973) reduction in the mobility of the acyl chains of the PC caused by cholesterol. Furthermore, changes in the activation energy of LCAT occur at the transition temperature of the acyl donor in PC–cholesterol mixtures under conditions such that the transition is no longer detected by pyrene excimer fluorescence. These findings suggest that boundary lipid interacts with LCAT and that the less fluid bulk phase lipid domain is not involved in a lipid–protein interaction. LCAT activity is clearly influenced by the physical state of the boundary lipid and may vary with the heterogeneous acyl donors available for reaction in the native substrate.

Although the two lipid substrates for LCAT are present in all lipoprotein classes, only HDL appears to be a substrate for LCAT. The protein components of the plasma lipoproteins occur in more than one lipoprotein class[2] (Morrisett et al., 1975). ApoHDL activates a partially purified preparation of LCAT when HDL lipids are used as the substrates (Fielding and Fielding, 1971). ApoA-I, the predominant protein species in apoHDL, is an active component, and other apoproteins have been found to be inactive (Fielding et al., 1972). Because these studies used sonic dis-

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[1] Abbreviations used are: LCAT, lecithin:cholesterol acyltransferase; HDL, high density lipoproteins; HDL_2 and HDL_3 are subfractions of HDL isolated between d = 1.063–1.120 and 1.120–1.210, respectively; LDL, low density lipoproteins; VLDL, very low density lipoproteins; PC, phosphatidylcholine; Dip-F diisopropyl fluorophosphate. DMPC, dimyristoylphosphatidylcholine; PPOPC, 1-palmitoyl-2-palmitoleoylphosphatidylcholine; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine.

[2] HDL contain two major protein components, apoA-I and apoA-II, in a molar ratio of 3:1, and a group of small molecular weight proteins designated apoC. VLDL also contain apoC in addition to apoB, the principal protein component of LDL.

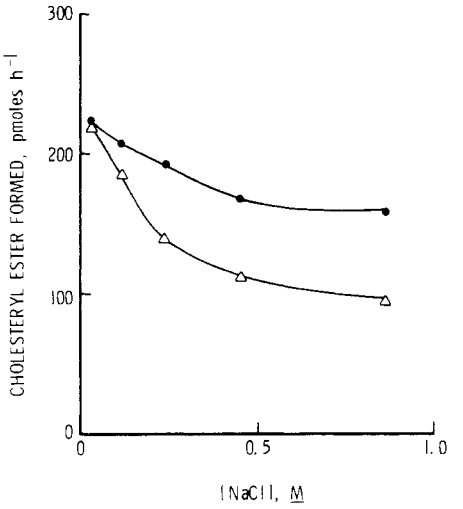

FIGURE 1: Effect of sodium chloride concentration on LCAT activity. Substrate vesicles, prepared by injection of lipids into 50 m*M* potassium phosphate (pH 7.5), containing 0.15 *M* NaCl, were diluted sixfold into 50 m*M* potassium phosphate (pH 7.5) (△), or 50 m*M* Tris-HCl (pH 7.5) (●) containing 10 μM apoA-I and varying concentrations of NaCl, as indicated, in a final volume of 110 μl. After incubation at 37° for 30 min, enzyme, 10 μl containing 12 μg of protein, was added to start the reaction.

persions of HDL lipids as the substrate, the complexity of the assay system may have influenced the cofactor requirements of the reaction. In a related study, Nakagawa and Nishida (1973) observed both activation and inhibition of cholesterol esterification by HDL_3 and $apoHDL_3$. In their experiments the effect was dependent on the PC–cholesterol ratio of the sonicated substrate dispersions.

The purpose of this study was to examine the effects of different lipid and apoprotein components on LCAT specificity. Purified, soluble apoproteins and chemically defined PC substrates with different hydrocarbon chains were used. Our results suggest that there is a relationship between the stimulatory effects of the apoproteins, apoA-I and apoC-I, and the fluidity of the lipid substrate.

Preliminary reports of these data have been presented (Garner et al., 1972; Soutar et al., 1974b).

Materials and Methods

Materials. L-α-Dimyristoylphosphatidylcholine was obtained from Supelco; L-α-1-palmitoyl-2-palmitoleoylphosphatidylcholine, L-α-1-palmitoyl-2-oleoylphosphatidylcholine, and L-α-dioleoylphosphatidylcholine were obtained from Applied Science. The nominal fatty acid composition was that specified by the supplier. Egg yolk PC, prepared by the method of Singleton et al. (1965) and purified as described by Rouser et al. (1963), was a gift from Dr. J. D. Morrisett. All lipids used in this study contained less than 1% impurity as indicated by thin-layer chromatography.

Generally-labeled [^{3}H]cholesterol, specific activity 8.5 Ci/mmol, was obtained from Amersham-Searle.

Urea (ultrapure) was purchased from Schwarz/Mann; solutions of 8 *M* urea were passed through a column of a mixed-bed resin (Dowex, AG 501-X8) shortly before use. All other chemicals were obtained as previously described by Soutar et al. (1974a).

Enzyme Purification. LCAT was isolated from single units of plasma from fasting donors (Blood Services of Houston). The purification procedure previously described (Soutar et al., 1974a) was modified to include DEAE-cellulose chromatography. All procedures were conducted at 4°. The clear central zones (200 ml) between the lipoprotein fraction and the infranatant solution, d = 1.23, isolated by ultracentrifugation, were dialyzed against 10 m*M* Tris-HCl (pH 7.4) containing 1 m*M* EDTA, and applied to a column of DEAE-cellulose (4.5 cm × 60 cm), equilibrated with the same buffer. The column was washed with 500 ml of buffer before the application of a linear salt gradient (0–0.75 *M* NaCl in a total volume of 4 l.). Fractions containing LCAT activity were pooled (approximately 700 ml), precipitated with ammonium sulfate and butanol, and applied to a column of Sephadex G-100 as previously described (Soutar et al., 1974a). The final LCAT preparation was approximately 100-fold purified from the lipoprotein-free plasma. In immunodiffusion experiments with goat antibody prepared against apoA-I, the purified enzyme preparation was compared with known amounts of apoA-I and the small amount of apoA-I was estimated to be about 5–10% by weight of the protein.[3] Protein concentration was determined from the A_{280}/A_{260} ratio (Warburg and Christian, 1941).

Enzyme Assay. LCAT activity was assayed as described previously by Soutar et al. (1974a). For routine assays, the single bilayer vesicles of PC and [^{3}H]cholesterol were incubated in a final volume of 110 μl for 30 min at 37° with the apolipoprotein. The final concentrations of the substrates were 0.35 m*M* PC and 0.035 m*M* [^{3}H]cholesterol (specific activity 100 Ci/mol). Each experimental determination was done in triplicate. Enzyme solution (10 μl containing 5–50 μg of protein) was added to initiate the reaction and the mixture incubated for 60 min. The reaction blank contained no enzyme. The lipids were then extracted and the radioactivity in the cholesteryl ester fraction was determined; enzyme activity is expressed as picomoles of cholesteryl ester formed per hour.

Apoproteins. Plasma HDL fractions were isolated by ultracentrifugal flotation in KBr between densities 1.063 and 1.210 g/ml for whole HDL, densities 1.063 and 1.120 for HDL_2, and densities 1.120 and 1.210 g/ml for HDL_3. The lipoproteins were delipidated with diethyl ether–ethanol (3:1 v/v) at 4° (Gotto and Kon, 1970). ApoA-I and apoA-II were purified to homogeneity from apoHDL as described previously (Baker et al., 1973; Jackson and Gotto, 1972).

ApoC-I, apoC-II, and apoC-III were isolated from VLDL of subjects with type IV and V hyperlipidemia as described by Brown et al. (1969, 1970). The purity of the apoproteins was estimated by analytical gel electrophoresis on polyacrylamide gels containing urea (Davis, 1964), by amino acid analysis, and by specific antiserum prepared against each apolipoprotein. ApoC from HDL used in Figure 6 was isolated from the column shown in Figure 4.

Sources of Variability. In early experiments the magnitude of the effects of the apoproteins on LCAT activity were somewhat variable and certain measures were taken to minimize this problem. When these steps were taken, the overall pattern of activation by the different apoproteins was reproducible with enzyme preparations of different specific activities (Figures 2 and 3) and with various preparations of apolipoproteins.

For each PC a single preparation of substrate vesicles was used to compare the effects of apoproteins. To ensure that the variations were not due to small changes in substrate concentration, the concentration of substrates was in-

[3] The authors are grateful to Simon Mao for providing the antibody to apoA-I.

creased to 0.6 mM phospholipid and 0.06 mM cholesterol for the comparative experiments shown in Figures 2 and 3.

The rate of esterification catalyzed by LCAT is extremely sensitive to ion concentration; the activity was decreased by an increase in the sodium chloride concentration (Figure 1). The inhibition was more pronounced in phosphate buffer than in Tris-HCl of the same molarity. In the comparative experiments, care was taken to ensure that the salt concentration in the final assay mixture was maintained at a constant value.

The purified apolipoproteins are highly susceptible to aggregation in solution (Morrisett et al., 1975). To minimize this source of variability, solutions of the homogeneous proteins in 10 mM Tris-HCl (pH 8.1) containing 1 mM EDTA and 8 M urea were passed through Sephadex G-10 equilibrated with 10 mM Tris-HCl (pH 8.1) containing 1 mM EDTA. The resultant dilute protein solutions were concentrated by ultrafiltration with an Amicon UM2 membrane to give protein concentrations less than 3 mg/ml. The protein solutions were maintained at 4° and used within 48 hr.

On calibrated columns of Sephadex G-150, the apoproteins, apoA-II, apoC-I, apoC-II, and apoC-III after treatment with urea elute as dimers.[4] ApoA-I elutes predominately as hexamers, with about 20% of the protein appearing after the main peak as tetramers, trimers, and dimers. Otherwise, apoA-I appears entirely as a large molecular weight aggregate in the void volume of the column and the other apoproteins, to a variable extent, appear as higher molecular weight aggregates.

For comparisons between the different apoproteins, plasma from a single donor was used as the source of the proteins used in the experiments shown in Figures 2 and 3.

Since protein–lipid complex formation with apoA-I is rather slow, about 8–15 hr,[5] in each experiment, the apoprotein was incubated with the substrate vesicles for 15 hr at 37°, a temperature above the transition temperature of all PC species studied. This precaution allowed maximum complex formation prior to addition of enzyme and reduced variation that might result from different degrees of interaction between the lipid and apoprotein.

Results

The effect of the purified apoproteins on the rate of transesterification catalyzed by partially purified LCAT with two defined PC substrates, PPOPC and DMPC, is shown in Figure 2. Of the five apolipoproteins studied, apoA-I and apoC-I stimulated the activity of the enzyme; apoA-II, apoC-II, and apoC-III were inactive. The extent of activation by apoA-I and apoC-I was dependent upon the nature of the PC substrate. The addition of apoA-I to the reaction mixture greatly enhanced the activity with PPOPC as the substrate, maximum activation of the transacylation occurring at approximately 5 μM apoA-I (Figure 2a). The range of concentrations of apoA-I giving maximum activation varied from 2 to 7.5 μM with different preparations of either apoA-I or lipid vesicles with PPOPC as the acyl donor. With DMPC as the substrate, the activity obtained was lower (Figure 2b). A higher concentration of apoA-I, approximately 20 μM, was required to attain maximum activation. In some experiments with DMPC as the acyl donor for LCAT, low concentrations of apoA-I appeared to be inhibitory, while higher concentrations activated the enzyme (Figure 2b).

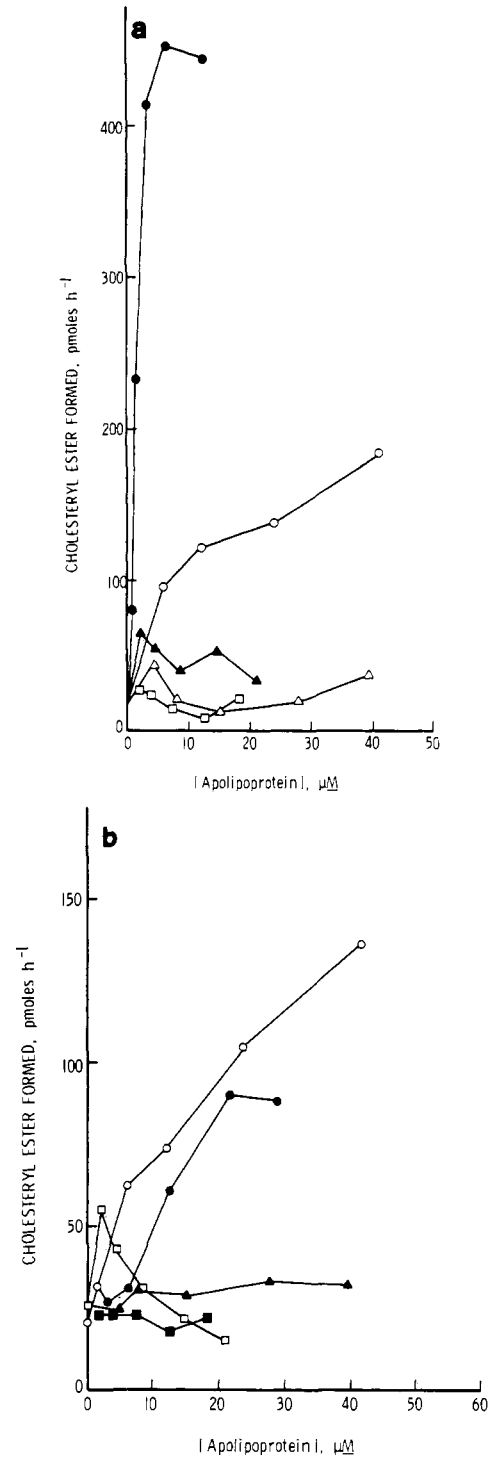

FIGURE 2: Activation of LCAT by apolipoproteins with different phosphatidylcholine acyl donors. Substrate vesicles, containing 0.6 mM PC and 0.06 mM [^{3}H]cholesterol, were incubated with each apolipoprotein at the concentrations indicated in the figure for 15 hr at 37° in a total volume of 110 μl before the addition of enzyme, 10 μl containing 7.5 μg of protein, to initiate the reaction. (a) 1-Palmitoyl-2-palmitoleoylphosphatidylcholine; (b) dimyristoylphosphatidylcholine. ApoA-I, ●; ApoA-II, □; ApoC-I, ○; ApoC-II, ▲; ApoC-III, △.

ApoA-II had little effect on the reaction with these substrates, although with a different acyl donor, Fielding et al. (1972) have reported that apoA-II inhibits LCAT.

Of the apoC group of proteins, apoC-I had a stimulatory effect with both acyl donors; the maximum rate of cholest-

[4] J. D. Morrisett, unpublished data.

[5] H. J. Pownall, personal communication.

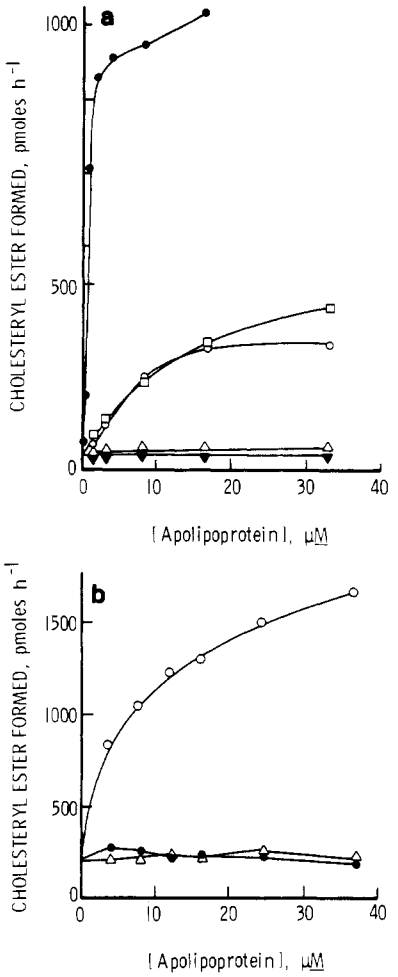

FIGURE 3: Activation of LCAT by cyanogen bromide fragments of apoA-I and apoC-I. (a) Substrate vesicles, containing 0.35 m*M* PC and 0.035 m*M* [^{3}H]cholesterol, were incubated with varying concentrations of apolipoprotein as indicated for 30 min at 37° before the addition of enzyme, 20 μg of protein, to start the reaction. ApoA-I, ●; CNBr fragments of apoA-I: CNBr-I, ○; CNBr-II, □; CNBr-III, △; CNBr-IV, ▲. (b) Substrate vesicles, containing 0.35 m*M* DMPC and 0.035 m*M* [^{3}H]cholesterol, were incubated with varying concentrations of apolipoprotein as indicated for 30 min at 37° before the addition of enzyme, 50 μg of protein, to start the reaction. ApoC-I, ○; CNBr fragments of apoC-I: CNBr-I, △; CNBr-II, ●.

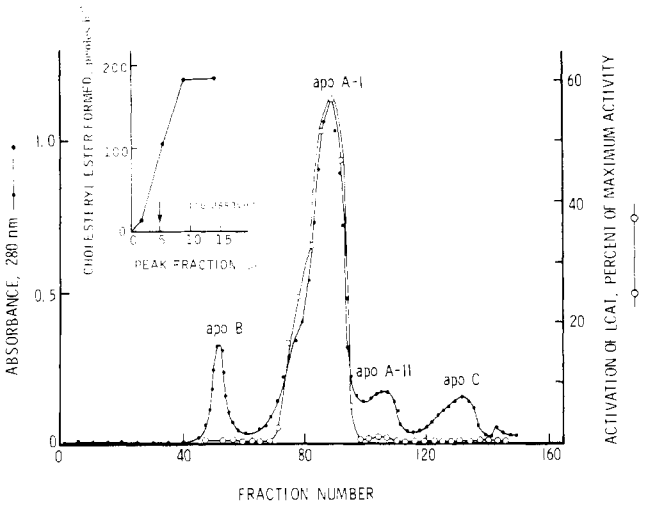

FIGURE 4: Activation of LCAT by fractions obtained from separation of apoHDL by gel filtration on Sephadex G-150. Delipidated apoHDL, 100 mg, was applied to a column of Sephadex G-150, 200 cm × 2.6 cm, equilibrated with 0.1 *M* Tris-HCl (pH 8.0) containing 5.4 *M* urea and 0.01% EDTA, which was eluted with the same buffer. Each fraction was dialyzed extensively against 10 m*M* Tris-HCl (pH 8.1). Substrate vesicles containing 0.35 m*M* egg yolk PC and 0.035 m*M* [^{3}H]cholesterol were incubated with 5.0 μl of each fraction for 30 min at 37° before the addition of enzyme, 7.5 μg of protein, to initiate the reaction. The activation is expressed as the percentage of maximum activity obtained at saturating level of activator protein. This value was determined by assaying increasing concentrations of fraction number 86 for LCAT activation as shown in the insert in the figure.

eryl ester formation and the concentration of apoC-I required to give activation were similar in both cases (Figure 2a and b). Although the LCAT activity with apoC-I was similar to that with apoA-I with DMPC as the substrate, apoA-I was a much more effective activator than apoC-I with PPOPC. Comparable results were obtained with POPC and with egg yolk PC.

ApoC-III had little or no effect on the enzyme reaction with either DMPC or PPOPC, while apoC-II, the activator protein for lipoprotein lipase (LaRosa et al., 1970; Havel et al., 1970; Ganesan et al., 1971) that forms an apoprotein-enzyme complex at the interface (Miller and Smith, 1973) appeared to stimulate the reaction slightly at low concentrations but was inhibitory at higher concentrations with both substrates.

Because of the insolubility of apoB in the absence of detergent its effect on LCAT was not tested.

The stimulatory effect of the lipoproteins on LCAT activity is probably related to their ability to interact with PC and cholesterol. The ability of apoA-I and apoC-I to activate LCAT might require the intact protein molecule, or by analogy with the PC binding sites for synthetic fragments of apoC-III (Sparrow et al., 1973), require only a portion of the primary sequence for activation. Treatment of apoA-I, 244 amino acid residues, with cyanogen bromide produces four peptides (Baker et al., 1973) with the following alignment in the sequence: CNBr-II, NH_2-terminal fragment, 89 amino acid residues; CNBr-IV, 25 amino acid residues; CNBr-III, 36 amino acid residues; CNBr-I, COOH-terminal fragment, 94 amino acid residues (Delahunty et al., 1975; Baker et al., 1975). ApoC-I (57 amino acid residues) yields two peptides when treated with cyanogen bromide, CNBr-I, NH_2-terminal fragment, 38 amino acid residues; and CNBr-II, COOH-terminal fragment, 19 amino acid residues (Jackson et al., 1974a). The two shorter fragments of apoA-I, CNBr-III and CNBr-IV, had no effect on the activity of LCAT with egg yolk PC (Figure 3); however, the COOH-terminal fragment (CNBr-I) and the NH_2-terminal fragment (CNBr-II) both give significant activation of LCAT (Figure 3a). The effects of CNBr-I and CNBr-II were not additive, although neither fragment alone activated as strongly as apoA-I, nor was as effective at low concentrations. The fragments of apoC-I had no effect on the activity of LCAT with DMPC (Figure 3b).

Recent evidence presented by Kostner (1974b) has implicated apoA-III (Kostner, 1974a), also named "thin-line" peptide (McConathy et al., 1973) or apoD (McConathy and Alapovic, 1973), as an activator for LCAT. The "thin-line" peptide is a minor constituent of HDL, appearing predominantly in the HDL_3 fraction (McConathy and Alaupovic, 1973; Kostner, 1974a). To rule out the possibility that the activation obtained with apoA-I was due to contamination by the "thin-line" peptide, individual fractions obtained from chromatography of apoHDL on Sephadex G-150 were examined for LCAT activation. The "thin-line"

Table I: Activation of LCAT by Enzyme Preparation Inhibited with Dip-F.

LCAT (μg/ml)	Dip-F–LCAT (μg/ml)	ApoA-I (μg/ml)	LCAT Activity[a] (pmol of Cholesteryl Ester Formed/hr)
20		32[c]	196
20		65[b]	393
20			16
	350	65	34[d]
20	350	65	439
20	350		230

[a] The assays were carried out essentially as described under Materials and Methods, with PPOPC as the acyl donor. The substrate was incubated 30 min with the indicated concentration of the proteins before the addition of LCAT to start the reaction. [b] Concentration of pure apoA-I giving maximum activation. [c] Concentration of apoA-I that gives activation equivalent to that by 350 μg ml of Dip-F–LCAT. [d] Purified LCAT preparation was incubated at 0° for 1 hr with 10 m*M* Dip-F. Residual inhibitor was removed by gel filtration through Sephadex G-25. The resulting protein solution (Dip-F–LCAT) retained less than 0.6% of the original LCAT in the presence of apoA-I.

peptide, with a molecular weight of 19,000–20,000 (Kostner, 1974a), elutes between apoA-I (mol wt 28,000) and apoA-III (mol wt 17,500) under denaturing conditions. The activation of LCAT appears coincident with apoA-I; the specific activity remains essentially constant throughout the peak (Figure 4). Virtually no cholesteryl ester was formed in the absence of apoA-I, and the volume of each fraction used was such that the peak fraction gave approximately 60% of the maximum activation possible (see insert to Figure 4). The results do not support, but do not exclude, the activation of LCAT by the "thin-line" peptide.

Although the purified enzyme was virtually inactive in the absence of added apoA-I, there was a small amount of apoA-I in the enzyme preparation that could be detected by its reaction with the specific antibody prepared against apoA-I. In order to assess quantitatively the amount of apoA-I and to determine whether this apoA-I, which had not been exposed to denaturing conditions, was more effective as an activator of the enzyme, a portion of purified LCAT was inhibited by treatment with 10 m*M* Dip-F.

The effect of the protein in the inhibited enzyme preparation on LCAT activity is shown in Table I. These data demonstrate that the activation obtained with 350 μg/ml of the inhibited enzyme preparation was equivalent to that obtained with 32 μg/ml of apoA-I. This indicates that approximately 10% of the protein in the enzyme preparation is apoA-I, an estimate identical with that obtained by immunodiffusion. This amount of apoA-I accounts for about 1% of the optimum required to give maximum activation. The apoA-I also appears to be available for interaction. The denaturing conditions employed during the purification of apoA-I had no apparent effect on its ability to activate the LCAT reaction.

Incubation of isolated lipoproteins in vitro with LCAT and analysis of the resulting lipid mixtures have led to the hypothesis that HDL and in particular, HDL_3, is the natural substrate for LCAT (Fielding and Fielding, 1971): the lipids of LDL, VLDL, and HDL_2 are virtually unchanged by incubation with LCAT.

Since apoHDL and the subclasses $apoHDL_3$ and $apoHDL_2$ contain apoA-I as the major protein species with variable quantities of apoA-II and apoC, we have compared

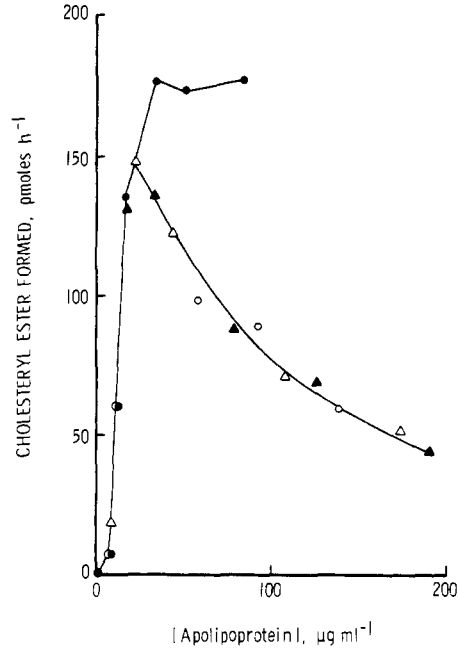

FIGURE 5: Activation of LCAT by apoHDL and apoA-I. Whole HDL (d = 1063–1.21), HDL_2 (d = 1063–1.12), and HDL_3 (d = 1.12–1.21) were delipidated as described by Gotto and Kon (1970) and redissolved in 10 m*M* Tris-HCl (pH 8.0) and 1 m*M* EDTA containing 5 *M* urea. The protein solutions were then filtered through small columns of Sephadex G-10 equilibrated with 10 m*M* Tris-HCl (pH 8.0) containing 1 m*M* EDTA, to remove the urea. The proteins were fully soluble after this treatment. Substrate vesicles containing 0.6 m*M* PPOPC and 0.06 m*M* [^{3}H]cholesterol were incubated with the indicated concentrations of apolipoprotein 30 min at 37° before the addition of enzyme, 7.5 μg of protein, to start the reaction. ApoA-I, ●; apoHDL, ○; $apoHDL_2$, ▲; $apoHDL_3$, △.

the ability of apoHDL, $apoHDL_3$, and $apoHDL_2$ with that of apoA-I to activate LCAT. With egg yolk PC as the phospholipid acyl donor and at low concentrations of apoprotein (<20 μg ml^{-1}), apoHDL, $apoHDL_3$, and $apoHDL_2$ are equally effective as LCAT activators (Figure 5). Maximum activation by apoA-I occurs at protein concentration of 30–40 μg ml^{-1}, at which point LCAT activity is approximately 20% greater than that obtained with optimum concentrations of apoHDL. With concentrations of apoHDL, $apoHDL_2$, or $apoHDL_3$ greater than 20 μg ml^{-1}, LCAT activity was inhibited; 50% inhibition occurred at approximately 100 μg of apoHDL ml^{-1}.

To investigate the cause of this inhibition, the effects of adding purified apoA-II and apoC on the activation of LCAT by apoA-I was examined (Figure 6). ApoA-II caused substantial inhibition of the reaction when apoA-I was added at a concentration of 2.5 μ*M*; 50% inhibition occurred at a concentration of apoA-II of approximately 4.5 μ*M*. ApoC also caused inhibition of the enzyme, although it was less effective than apoA-II.

The maximum activation of LCAT by apoA-I with egg yolk PC as acyl donor was obtained with 2 μ*M* apoA-I and a further increase in concentration to 10 μ*M* had no effect. The possibility that changes in the physical state of egg yolk PC dispersions induced by apoA-I and apoC-I could account for the differences in LCAT enhancement was examined by electron microscopy.[6] Addition of 10 μ*M* apoA-I and 10 μ*M* apoC-I produced no detectable change in the observed structural features of the dispersion. The enhance-

[6] Courtesy of H. F. Hoff.

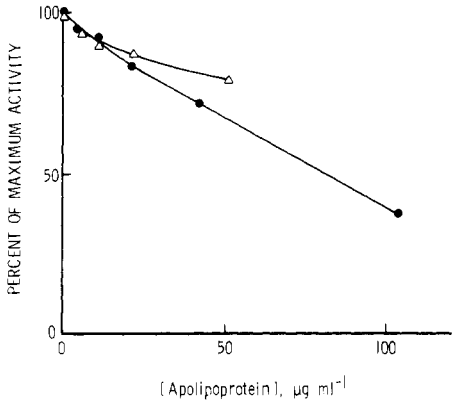

FIGURE 6: Inhibition of apoA-I activation of LCAT by apoA-II and apoC. ApoC and apoA-II were isolated from apoHDL under denaturing conditions as described in Figure 4. The fractions corresponding to apoC and apoA-II were pooled separately, dialyzed against 10 m*M* Tris-HCl (pH 8.0) containing 1 m*M* EDTA, and then concentrated by ultrafiltration with an Amicon UM2 membrane. To minimize aggregation, the protein solutions were made 5 *M* in urea and then passed through columns of Sephadex G-10 equilibrated with 10 m*M* Tris-HCl (pH 8.0) containing 1 m*M* EDTA shortly before use. Substrate vesicles were incubated with apoA-I, 60 μg ml^{-1}, and apoC (△) or apoA-II (●) at the indicated concentrations for 30 min at 37° before the addition of the enzyme, 7.5 μg of protein, to initiate the reaction.

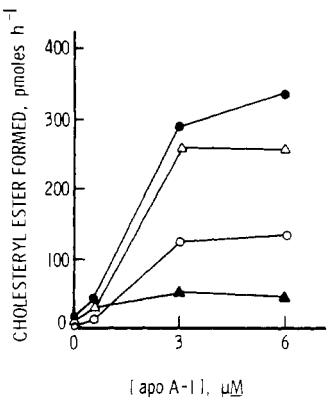

FIGURE 7: Activation of LCAT by apoA-I with different phosphatidylcholine acyl donors. Substrate vesicles, containing 0.6 m*M* PC and 0.06 m*M* [^{3}H]cholesterol, were incubated with varying concentrations of apoA-I as indicated for 30 min at 37° before the addition of enzyme, 7.5 μg of protein, to start the reaction. PPOPC, ●; egg yolk PC, △; POPC, ○; dioleoylphosphatidylcholine, ▲.

ment of LCAT by these apoproteins and the specificity for the acyl donor apparently are not associated with gross alteration of the substrate structure as visualized by electron microscopy.

Since apoA-I was the most effective activator for LCAT, its effect with other phospholipid acyl donors was examined (Figure 7). The concentration of apoA-I, 3 μ*M*, required to activate LCAT maximally, was similar with the PC substrates carrying an unsaturated fatty acid at the 2 position; most naturally occurring PC are of this structure. However, the maximum activity with each acyl donor varied widely, in accordance with other studies on the specificity of LCAT (Sgoutas, 1972).

Discussion

The overall objective of this study is to elucidate the role of the apoproteins in the LCAT-catalyzed reaction in intact lipoprotein particles. The plasma lipoprotein-proteins, or apoproteins, have the unique property of solubilizing and transporting the plasma lipids. These proteins have other functions including the activation of LCAT (Fielding et al., 1972) and of lipoprotein lipase (LaRosa et al., 1970; Havel et al., 1970; Ganesan et al., 1971).

ApoA-I stimulates the activity of LCAT with chemically defined lipid vesicles as the substrate. The degree of activation is dependent upon the nature of the hydrocarbon chains of the phospholipid, in that apoA-I is much more effective with unsaturated than saturated PC substrates. ApoC-I also stimulates LCAT activity. However, in contrast to apoA-I, the activation is similar with both saturated and unsaturated PC. These substrate-dependent differences suggest that the apoprotein exerts its effect on the transesterification reaction initially through an interaction with the substrate vesicles. The greater activation obtained with apoA-I and an unsaturated acyl donor may reflect a difference in the ability of apoA-I to interact with phospholipids of different fluidity, even though the experiments were carried out at a temperature above that of the gel to liquid-crystalline phase transition of the lipids involved.

The binding of an apoprotein to phospholipid vesicles is not sufficient for activation of LCAT. ApoC-III and apoA-II both bind strongly to phospholipid vesicles with simultaneous changes in the conformation of the protein (Pownall et al., 1974; Jackson et al., 1973; Morrisett et al., 1973), yet they were unable to activate LCAT. Also, the peptide fragments obtained from cyanogen bromide cleavage of apoC-I are no longer able to enhance LCAT activity, although they retain to some extent the ability to bind to phospholipid vesicles (Jackson et al., 1974b).

These findings suggest that specific protein-protein interactions between the enzyme and the apoprotein as well as the lipid-protein interactions between the apoprotein and substrate are involved in the activation mechanism. Such interactions at the surface of the vesicle may allow penetration of the enzyme into the phospholipid polar head group region. A similar mechanism has been suggested for the initial step in the hydrolysis of monolayers of phospholipid by phospholipase A (Verger et al., 1973). In the transesterification reaction catalyzed by LCAT with PC-cholesterol vesicles, the water-soluble enzyme would interact with its substrate at the lipid surface. The fluidity of the hydrocarbon chains of the PC substrate would determine the enzyme activity and would account for the discontinuity in the Arrhenius plot of enzyme activity with increasing temperature. This break in the curve corresponds to the transition temperature of the phospholipid (Soutar et al., 1974a). Preliminary studies on the temperature dependence of the activation of LCAT show that the apoproteins activate with unsaturated phospholipids both above and below the transition temperature (T_t) of the phospholipid. However, with saturated PC substrates, neither apoA-I nor apoC-I activates the enzyme below T_t of the substrate (Soutar et al., 1974b). Pownall et al. (1974) have shown that apoC-III binds more rapidly to DMPC vesicles at temperatures above T_t. These studies suggest that increasing the fluidity of the phospholipids promotes penetration of the bilayer by the apoprotein. The role of apoA-I or apoC-I may be the penetration of the interfacial surface and, through subsequent protein-protein interactions with the enzyme, a facilitation of the interaction of the enzyme with its substrate.

Two types of activation of LCAT by the apoprotein may be operative: (1) activation as a cofactor of the enzyme, and (2) activation as an acceptor of the cholesteryl ester

formed.[7] It is conceivable that apoA-I may be enhancing the LCAT reaction in both ways. The small amount of apoA-I in the partially purified LCAT preparation may satisfy the cofactor requirement, while the apoC-I might serve solely as an acceptor for cholesteryl ester formed in the reaction. Additional experiments are required to obtain data which support or exclude the possibility of a dual function of apoA-I and of different roles of apoA-I and apoC-I in the LCAT reaction.

Elucidation of the nature of protein–protein interactions in the activation of LCAT by apoA-I or apoC-I and the role of the apoproteins in the enzyme mechanism will involve studies requiring relatively large amounts of pure enzyme not presently available. Specific protein modification reactions may be used to identify portions of the apoproteins that are involved in LCAT activation, however. For example, when the lysine residues of apoC-I are maleylated by treatment of the protein with maleic anhydride (Jackson et al., 1974a) the ability to activate LCAT is abolished.[8] Similarly, Nagakawa and Nishida (1973) showed that, when approximately 95% of the amino groups of HDL_3 were acylated with either succinic anhydride or acetic anhydride, the modified protein no longer had any stimulatory effect on LCAT activity with sonicated dispersions of PC and cholesterol as the substrate. Also, in contrast to the results obtained with apoC-I, when apoA-I is cleaved with cyanogen bromide, the two largest of the four peptide fragments so obtained retain some ability to activate LCAT. Although the enhancement of the reaction by each of these fragments is approximately 25% that obtained with the whole protein, the effects are not additive. These fragments consist of the amino-terminal and carboxyl-terminal portions of the protein and thus it appears that the activation of LCAT by apoA-I as well as by apoC-I is dependent to some degree on the intact secondary and tertiary structures of the molecules. Indeed, it is only the two larger polypeptide fragments of the cleavage products of apoA-I that retain any ability to activate the enzyme, suggesting that a minimum size of protein may be necessary for activation.

ApoA-II does not activate the enzyme-catalyzed reaction, but may prevent activation of LCAT by apoA-I. Ritter et al. (1974) have reported that mixing apoA-II with apoA-I increases the binding capacity of apoA-I for total HDL lipids; this result may not be applicable to interaction between these proteins and the defined PC–cholesterol vesicles used in the experiments described here and does not readily offer an explanation for the inhibition of activation. However, Reynolds and Simon (1974) have shown that when apoA-I and apoA-II are present in equimolar quantities in solution, they behave as a single species with a mol wt 50,000; the ability of this protein complex to bind sodium dodecyl sulfate is reduced significantly from that predicted from simple linear combination of the sodium dodecyl sulfate binding data for the individual proteins. This suggests that hydrophobic binding sites on one or both of the proteins are blocked by interaction between the molecules. It may thus be speculated that masking of sites on apoA-I by apoA-II might be responsible for the interference with the activation of LCAT activity. Alternatively, apoA-I and apoA-II may compete for binding sites on the PC–cholesterol vesicles.

The effects of apoHDL, the subfractions $apoHDL_2$ and $apoHDL_3$, apoA-II, and apoC appear to be somewhat nonspecific. The apoA-II content of apoHDL, $apoHDL_2$, and $apoHDL_3$ are different; however, equal amounts, 100 μg ml^{-1}, of these apoproteins inhibit to the same extent as does 100 μg ml^{-1} of apoA-II. The amounts of the apoC peptides in apoHDL, $apoHDL_2$, and $apoHDL_3$ separated on polyacrylamide gels in 8 *M* urea did not appear to be significantly different. It is possible that the inhibition of apoA-I activation by other apoproteins is an in vitro phenomenon, and not a physiologically important control mechanism.

[7] We are indebted to Dr. John Glomset for this hypothesis.

[8] Anne K. Soutar, unpublished data.

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Synthesis of a New Phosphatidylserine Spin-Label and Calcium-Induced Lateral Phase Separation in Phosphatidylserine–Phosphatidylcholine Membranes†

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ABSTRACT: A new phosphatidylserine spin label with nitroxide stearate attached at the 2 position has been synthesized by the reaction of spin-labeled CDP-diglyceride with L-serine under the catalytic action of phosphatidylserine synthetase. Some structural properties of pure phosphatidylserine (PS) and binary PS–phosphatidylcholine (PC) membranes were studied with the spin label. PS membrane became solidified on lowering solution pH, 50% solidification being attained at pH 3.5. The membrane was also solidified by addition of Ca^{2+}. The effect of Ba^{2+}, Sr^{2+}, and Mg^{2+} was smaller than that of Ca^{2+}. The calcium-induced lateral phase separation in the binary membrane was studied from the side of the calcium-receiving lipid. The results confirmed and extended our previous conclusion drawn with PC spin label. The phase diagram of the binary membrane in the presence of Ca^{2+} was determined. Not all PS molecules were aggregated to form the solid patches but some remained dissolved in the fluid PC matrix. The fluid PS fraction was larger for the membranes containing more PC. The membrane with 10% PS still had a significant fraction of solid phase. The rate of calcium-induced aggregation was greatly dependent on the PS content. The aggregation was almost complete within 5 min in the membrane containing 67% PS, while it was still proceeding after several hours in the membrane with 20% PS. The rate-limiting step was suggested to be in the formation of "stable" nuclei consisting of larger aggregates. The possible biological significance of the ionotropic phase separation was discussed whereby a transient density fluctuation was emphasized.

It has been generally accepted that phospholipid molecules arranged in the bilayer structure act as a two-dimensional fluid matrix for the proteins in biological membranes (see, for example, Singer and Nicolson, 1972). The physical properties of the phospholipid bilayer are not only related to the membrane phenomena taking place in that part of the membrane but also have a deep influence on the functional properties of the membrane proteins (Esfahani et al., 1971; Linden et al., 1973; Overath et al., 1971). Biological membranes contain a variety of classes of lipids and each of them appears to play its own characteristic role. Some provide the two-dimensional matrix while the others are involved more directly in the functions. It has been shown that phosphatidylserine (PS[1]) and phosphatidic acid (PA) responded to Ca^{2+} characteristically (Ohnishi and Ito, 1973, 1974; Ito and Ohnishi, 1974). These phospholipid molecules were aggregated by calcium through intermolecular chelation and segregated from the remaining fluid ma-

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[1] Abbreviations used are: PS, phosphatidylserine; PC, phosphatidylcholine; PA, phosphatidic acid; PS*, PC*, and PA*, spin-labeled PS, PC, and PA, respectively, where acyl chains at the 2 position were replaced with 12-nitroxide stearic acid (4′,4′-dimethyloxazolidine-*N*-oxyl derivative of 12-keto stearic acid).