Characterization of proteoliposomes containing apoprotein A-I: a new substrate for the measurement of lecithin:cholesterol acyltransferase activity

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Abstract Proteoliposome vesicles containing apoA-I, lecithin, and cholesterol (including labeled cholesterol) were prepared from various molar ratios of the three components by the cholate dialysis technique. Comparative studies on the sensitivity and efficiency of these proteoliposomes to serve as substrate for lecithin:cholesterol acyltransferase (LCATase) indicated that the proteoliposome with apoA-I:lecithin:cholesterol molar ratio of 0.8:250:12.5 was ideal for assaying LCATase activity of both plasma and purified enzyme. This proteoliposome was shown to be comparable in size by gel filtration (radius, 131.9 ± 4.8 Å, n = 6) and by electron microscopy (radius, 123.4 ± 5.1 Å, n = 100). The proteoliposome preparation was stable as LCATase substrate for at least 3 and 5 weeks, respectively, when stored at 4°C and −20°C, and was a better substrate for the enzyme activity assay than were lecithin-cholesterol liposomes incubated with apoA-I. Under the standardized assay system LCATase activity was a linear function of plasma enzyme added and was independent of the amount of plasma cholesterol added to the proteoliposomes in the range of 3 to 20 μl of plasma. The mean LCATase activity by this method was 95.1 ± 14.0 (range 76.5–122.5) nmol/hr per ml of plasma from fifteen normal human subjects. This method of substrate formation using the cholate dialysis technique permits the preparation of large amounts of stable, efficient, homogeneous, and well-defined substrate that is suitable for measuring low levels of enzyme activity, comparative studies, and large scale investigations of plasma LCATase, as well as studies of the mechanism and regulation of LCATase reaction.—**Chen C-H., and J. J. Albers.** Characterization of proteoliposomes containing apoprotein A-I: a new substrate for the measurement of lecithin:cholesterol acyltransferase activity. *J. Lipid. Res.* 1982. **23:** 680–691.

Supplementary key words lecithin • cholesterol • electron microscopy

Human plasma and that of many other mammals contains an enzyme, lecithin:cholesterol acyltransferase (LCATase, EC 2.3.1.43), that reacts preferentially with high density lipoproteins (HDL) to catalyze the transfer of a fatty acyl group from lecithin to unesterified cholesterol, with the formation of lysolecithin and cholesteryl ester (1–3). A variety of methods using natural and artificial substrates have been employed for estimating the rate of LCATase reaction. The existing assay methods, based on the nature and source of the substrate, are the endogenous self-substrate method and the exogenous common-substrate method. The endogenous self-substrate method, which uses native plasma (the subject's own plasma) as substrate and enzyme source, measures the endogenous cholesterol esterification rate either a) by measuring the rate of conversion of labeled cholesterol to cholesteryl ester after preincubation of fresh plasma with a reversible inhibitor (DTNB) and equilibration with radioactive cholesterol followed by incubation with mercaptoethanol to reactivate the enzyme (4), or b) by directly measuring the decrease in the mass of endogenous unesterified cholesterol after incubation of the plasma sample itself, using gas–liquid chromatography (5) or an enzymic method coupled with various kinds of chromogens (6–9). The measurement of cholesterol esterification rate by the radioassay technique measures both enzyme and substrate effects. However, it may not reflect the true esterification rate, since long preincubation and equilibration may alter the substrate properties of the plasma, and the labeled exogenous cholesterol may not be in complete equilibration with endogenous cholesterol (10, 11). Therefore, the cholesterol esterification rate by the radioassay procedure usually yields lower values than do the rates obtained by direct chemical measurement of unesterified cholesterol (11). On the other hand, the direct measurement of the change in unesterified cholesterol by gas–liquid chromatography (5) or by the enzymic method (6–9) may reflect the initial cholesterol esterification rate, but it is not appropriate for the measurement of the intrinsic en-

Abbreviations: LCATase, lecithin:cholesterol acyltransferase; apoA-I, apolipoprotein A-I; HSA, human serum albumin; DTNB, 5,5′-dithiobis-nitrobenzoic acid; DMPC, L-α-dimyristoylphosphatidylcholine.

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zyme activity because it does not distinguish qualitative or quantitative alterations of the enzyme from changes of the substrate or cofactors of the reaction (10, 11). In addition, the latter method requires a large number of replicates and a highly sensitive method for cholesterol quantification, since it measures the rate of disappearance of substrate cholesterol by the difference between test and control samples (11). Consequently, this method is not suitable for large-scale investigation or for measurement of very low levels of esterification rate. The common-substrate method, which uses heated plasma (12) or artificial liposomes (13, 14) as common substrate, measures LCATase activity by measuring the conversion of radiolabeled cholesterol to cholesteryl ester after incubation of fresh plasma as enzyme source, with heated plasma equilibrated with radioactive cholesterol (12), or with synthetic lecithin-cholesterol liposomes prepared by sonication (13), or by dissolving these lipids in ethanol (14). Although the use of heated plasma as a common substrate can minimize the effect of the substrate of the donors' plasma on the assay of LCATase activity, this method is not ideal; heated plasma is a poor substrate for LCATase, usually resulting in very low levels of LCATase activity (11, 15) and the results vary, depending on the source of substrate (7, 11, 16). The sonicated or ethanolic lecithin-cholesterol liposomes have been used as common substrate for plasma fractions and purified enzyme, but have been used little for the measurement of plasma LCATase activity. In this approach, the plasma constituents could alter the substrate properties of the artificial substrate. Furthermore, an artificial substrate has yet to be standardized or validated for the measurement of plasma LCATase activity. Therefore, existing methods for measuring LCATase activity are inadequate.

In this report, we describe a new method for preparation of a well-defined and standardized common artificial substrate, a proteoliposome containing apoA-I, egg lecithin, and [4-^{14}C]cholesterol, for a sensitive and precise determination of LCATase activity. The validity of this new substrate was evaluated with both plasma and purified enzyme.

MATERIALS AND METHODS

Chemicals

Egg-yolk lecithin (50 mg/ml ethanol solution) was purchased from Applied Science Laboratories, Inc. [4-^{14}C]Cholesterol (sp act, 54.0 mCi/mmol) and [carboxyl-^{14}C]cholic acid (sp act, 52.0 mCi/mmol) were obtained from New England Nuclear Corporation. Cholesterol, cholesteryl oleate, sodium chloride, Tris (hydroxymethyl) aminomethane, sodium cholate, and human serum albumin (essentially fatty acid-free) were supplied by Sigma Chemical Co. Ethylenedinitrilotetraacetic acid was a product of MCB Manufacturing Chemists, Inc. Bovine serum albumin (BSA) was purchased from Armour Pharmaceutical Co. and Sepharose CL-4B from Pharmacia Fine Chemicals.

Tris–HCl buffer solution which contained 10 mM Tris–HCl, 140 mM NaCl, and 1 mM EDTA, pH 7.4, was used throughout this study. All chemicals and proteins unless otherwise specified in this study were made in this Tris–HCl buffer solution, pH 7.4.

Plasma samples

Plasma samples used as enzyme source for LCATase activity study were obtained from adult volunteers without overt disease after a 12–16-hr overnight fast. Blood was drawn into vacutainer tubes containing sodium EDTA (1 mg/ml) as anticoagulant and plasma was obtained after centrifugation at 4°C.

Protein determination

Protein concentration was determined by the method of Lowry et al. (17) using BSA as a standard.

Purification of apoA-I

ApoA-I was isolated from fresh normal human plasma as previously described (18). The final apoA-I preparation was in 10 mM Tris–HCl buffer containing 140 mM NaCl and 1 mM EDTA, pH 7.4.

Purification of LCATase

The enzyme was purified to homogeneity from fresh normal human plasma (in EDTA) by a combination of density ultracentrifugation and chromatography on phenyl-Sepharose, DEAE-Sepharose, and hydroxylapatite as described (3, 19). The final enzyme preparation was stored in Tris–HCl buffer, pH 7.4 (10 mM Tris–HCl, 140 mM NaCl, and 1 mM EDTA).

Preparation of new artificial substrate proteoliposomes

Proteoliposomes were prepared by the cholate dialysis technique similar to that described for reconstitution of membrane proteins into artificial lipid vesicles (20–22). A typical proteoliposome (apoA-I:lecithin:cholesterol molar ratio 0.8:250:12.5), which was found to be an ideal substrate for LCATase in this study, was prepared as follows. For every 40 assays, the substrate was made by pipetting 0.154 ml of 50 mg egg lecithin/ml ethanol solution, 0.116 ml of 1 mg unlabeled cholesterol/ml ethanol solution, and 0.108 ml of 1.8 mg [4-^{14}C]cholesterol/2.5 ml benzene solution (0.25 mCi/2.5 ml benzene solution) into a glass vial. The lipid mixture was carefully evaporated to dryness under a stream of nitrogen at room

temperature. To these dried lipids, 2.50 ml of Tris–HCl buffer solution, pH 7.4, 0.8 ml of 1.10 mg apoA-I/ml in Tris–HCl buffer, and 0.3 ml of 725 mM sodium cholate solution in Tris–HCl buffer were added, mixed on a Vortex mixer for 1 min at room temperature, and then incubated in a 24°C shaking waterbath for 20 min. The mixture was dialyzed extensively against Tris–HCl buffer, pH 7.4, for 20 hr at 4°C to remove cholate. The dialysate was then adjusted to 4 ml using Tris–HCl buffer, pH 7.4. One experiment with [^{14}C]cholate indicated that 99.4% and 99.9% of the cholate was removed, respectively, after 10 and 18 hr of dialysis.

Measurement of LCATase activity with proteoliposomes

For all assays using proteoliposomes as substrate the following procedures described for a typical assay, using the standardized proteoliposome (apoA-I:lecithin:cholesterol molar ratio 0.8:250:12.5) as substrate, were followed unless otherwise specified. The assay was carried out in a screw-capped culture tube (16 × 125 mm) in which 0.230 ml of Tris–HCl buffer, pH 7.4, 0.125 ml of 2% HSA, and 0.1 ml of the proteoliposome were added. The substrate mixture was preincubated at 37°C for 20 min, 0.025 ml of 100 mM mercaptoethanol was added, and this was followed by addition of 0.015 ml of plasma (or purified enzyme). This assay mixture contained 250 nmol lecithin, 7.5 nmol unlabeled cholesterol, 5 nmol [4-^{14}C]cholesterol, 0.8 nmol (22 μg) apoA-I, 0.5% HSA, 5 mM mercaptoethanol, and 0.015 ml of plasma (or purified enzyme) in a final volume of 0.5 ml. The assay mixture was mixed immediately on a Vortex mixer and incubated in a 37°C waterbath for 30 min. The enzymic reaction was stopped, the cholesterol and cholesteryl ester were separated, and radioactivity was counted as previously described (19, 23). LCATase activity was determined from the conversion of [4-^{14}C]cholesterol to labeled cholesteryl ester. Control reactions containing no enzyme were run simultaneously to correct for nonenzymic reaction. The rate of LCATase reaction is expressed as both fractional activity (the percentage of cholesterol esterified per 30 min), and molar activity (nmol cholesterol esterified/hr per ml plasma or per mg purified enzyme). This latter value was obtained by multiplying the fractional activity with the concentrations of cholesterol in the proteoliposomes.

Measurement of LCATase activity with liposomes

Liposomes containing lecithin:cholesterol (including labeled cholesterol) molar ratio of 250:12.5 and 4:1 were prepared in the absence of apoA-I, respectively, by the cholate dialysis technique used in this study and by the method of Batzri and Korn (14) as described (23, 24). The enzyme activity assay was carried out by incubating the liposomes with apoA-I in the assay mixture as previously described (19, 24).

Gel filtration of proteoliposomes

The proteoliposome preparations were chromatographed on a Sepharose CL-4B column (2.2 × 50 cm) according to the procedure of Huang (25). The Stokes' radius of the proteoliposome vesicles was determined by using the equation of Ackers (26) as described by Huang (25). Ovalbumin (Stokes' radius = 27.3 Å) and fibrinogen (Stokes' radius = 110.0 Å) were used to determine the two calibration constants a_0 and b_0 of the column.

Composition analysis of proteoliposomes

Proteoliposomes were analyzed for lecithin, cholesterol, and apoA-I. Lecithin was determined according to the method of Bartlett (27), cholesterol by the enzymic procedure of Nagasaki and Akanuma (7), and protein by the method of Lowry et al. (17).

Electron microscopy of proteoliposomes

Electron microscopy of the proteoliposomes negatively stained with 1% sodium phosphotungstate, pH 7.4, was carried out as previously described (28).

RESULTS

Preparation of proteoliposomes

In order to develop a well-defined, standardized, and effective common artificial substrate for LCATase, proteolipid vesicles containing apoA-I, lecithin, and cholesterol (including labeled cholesterol) were prepared from various molar ratios of the three components by the cholate dialysis technique. The proteoliposomes were then used as substrate for assaying LCATase activity in both plasma and purified enzyme preparations in the presence of 0.5% HSA (unless otherwise specified) and 5 mM mercaptoethanol. A series of studies was carried out to select the relative proportions and absolute concentrations of the three vesicle constituents so that when the substrate was used the enzyme activity would follow zero-order kinetics and be independent of enzyme concentration within a given range, and yet the substrate would still be in excess in the assay mixture.

The first step to select the proteoliposomal composition was to examine the effect of lecithin:cholesterol molar ratios on LCATase activity. Assays were carried out using proteoliposomes formed from mixtures of constant apoA-I (1 nmol/assay) and various proportions of lecithin to cholesterol (Figs. 1 and 2). Assay of plasma LCATase activity in proteoliposomes, keeping lecithin

content constant and varying the cholesterol content (**Fig. 1**, top) indicated that increasing the cholesterol from 1 to 5 mol % resulted in only a slight decrease in the fractional LCATase activity. However, increasing the cholesterol content to 10 mol % reduced the fractional LCATase activity to approximately one-half of that obtained with 5 mol % cholesterol. Keeping cholesterol content constant and varying the lecithin content (Fig. 1, bottom) showed that decreasing the lecithin content from 500 to 100 nmol resulted in a slight increase in the fractional LCATase rate; but at a lecithin content of 50 nmol (10 mol % cholesterol), the fractional LCATase activity was reduced to one-half of that obtained with proteoliposomes containing 100 nmol of lecithin. Similar results to those shown in Fig. 1 were obtained in four other plasma. Qualitatively similar trends in LCATase activity were obtained when purified LCATase from three different preparations was used as enzyme source (**Fig. 2**). These preliminary investigations suggested that proteoliposomes with 5 mol % cholesterol would give the desired sensitivity (fractional LCATase activity > 5%) and produce a molar estification rate in the range obtained by

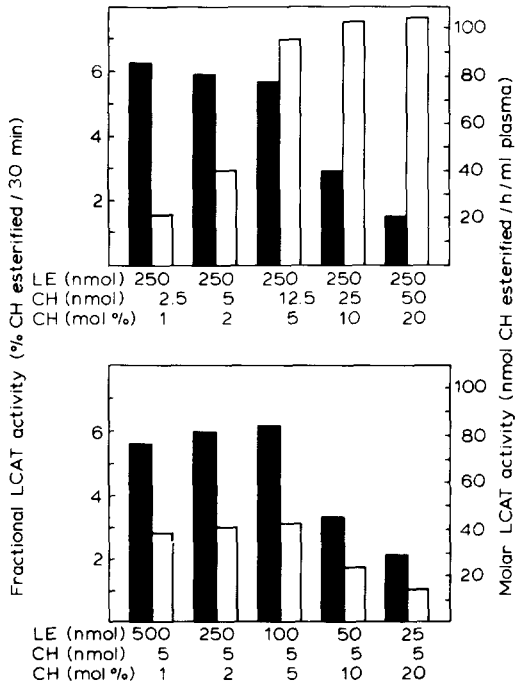

Fig. 1. Effect of various molar ratios of lecithin:cholesterol in proteoliposomes on plasma LCATase activity. Proteoliposomes prepared by the cholate dialysis technique from mixtures of various lecithin:cholesterol molar ratios and constant apoA-I (1 nmol/assay) were used for assay of plasma LCATase activity by incubating the assay mixture at 37°C for 30 min. Each assay mixture (0.5 ml) contained 15 μl of fresh plasma, 0.5% HSA, 5 mM mercaptoethanol, 1 nmol apoA-I, and various amounts of lecithin and cholesterol as indicated. The number under each pair of bars represents amounts of lecithin and cholesterol/assay, and mol % cholesterol. ■, fractional LCATase activity; □, molar LCATase activity. Each value is the mean of six determinations with an average error of less than 5.1%.

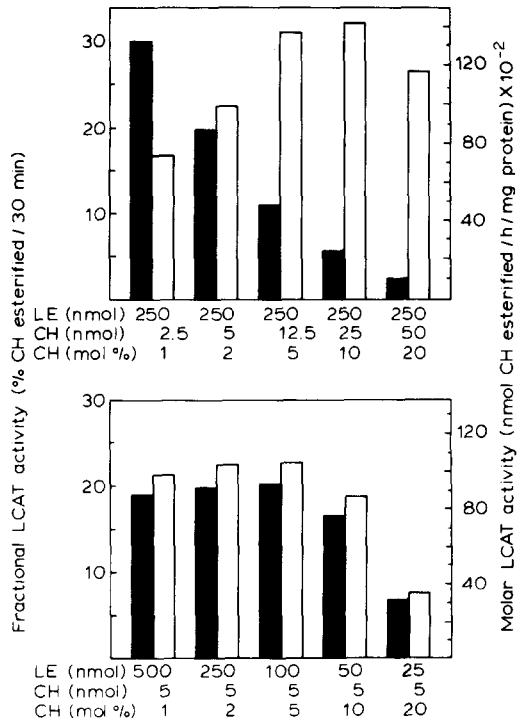

Fig. 2. Effect of various molar ratios of lecithin:cholesterol in proteoliposomes on the activity of purified LCATase. The substrate compositions and assay conditions were the same as in Fig. 1, except that 0.2 μg of purified enzyme was used instead of 15 μl of plasma. The number under each pair of bars represents amounts of lecithin and cholesterol/assay, and mol % cholesterol. ■, fractional LCATase activity; □, molar LCATase activity. Each value is the mean of six determinations with an average error of less than 5.6%.

the endogenous substrate method. Therefore, we further investigated different concentrations of proteoliposomes containing 5 mol % cholesterol (**Fig. 3**). With proteoliposomes containing 250 nmol of lecithin and 12.5 nmol of cholesterol, the fractional LCATase activity of plasma was maximal but it decreased significantly if higher concentrations of lecithin and cholesterol were used (Fig. 3, top). Proteoliposomes containing 125 nmol of lecithin and 6.3 nmol of cholesterol had the highest fractional cholesterol esterification if tested with purified enzyme preparations (Fig. 3, bottom). Similar results were obtained with three other plasma and three different LCATase preparations.

In all previous experiments (Figs. 1–3) the apoA-I was kept constant at 1 nmol and lecithin and cholesterol were varied. Therefore, we next investigated the effect of varying the amount of apoA-I in the proteoliposome while keeping the concentration and molar ratio of lecithin to cholesterol constant (**Fig. 4**). Plasma LCATase activity increased as the apoA-I increased, with maximal activity obtained with 0.8 nmol of apoA-I. Further increases in apoA-I did not change the LCATase activity. Similar findings were obtained with purified enzyme, except that maximal activity was reached at 0.4 nmol

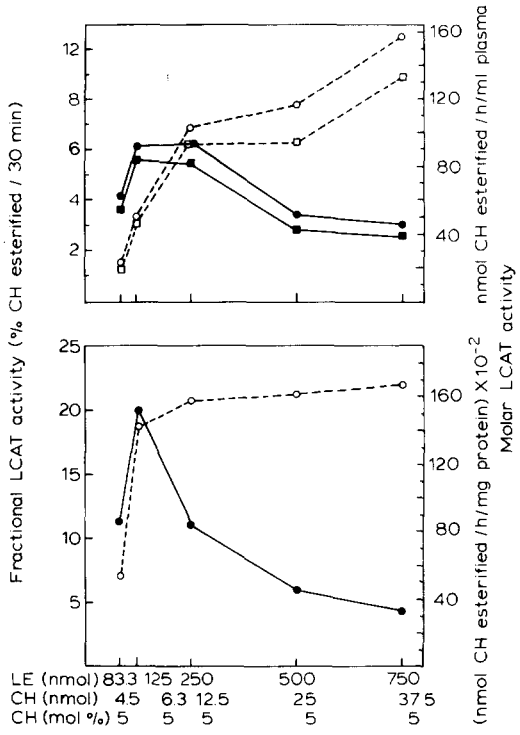

Fig. 3. Effect of various concentrations of lecithin and cholesterol. Proteoliposomes made by the cholate dialysis technique from mixtures of constant apoA-I (1 nmol/assay) and lecithin and cholesterol in a 20:1 molar ratio were used for the assay of enzyme activity. The assay mixture was incubated at 37°C for 30 min. Each assay mixture (0.5 ml) contained 15 μl plasma (top), or 0.2 μg purified enzyme (bottom), 0.5% HSA, 5 mM mercaptoethanol, 1 nmol apoA-I, and various amounts of lecithin and cholesterol as indicated. The number under each set of data points represents the amount of lecithin and cholesterol/assay and mol % cholesterol. Top: ● —— ●, ■ —— ■, fractional LCATase activity from two individual plasma samples; ○ —— ○, □ —— □, molar LCATase activity from the two individual plasma samples. Bottom: ● —— ●, fractional LCATase activity; ○ —— ○, molar LCATase activity. Each value is the mean of six determinations with an average error of less than 5.4%.

of apoA-I. Therefore, the minimal amount of apoA-I in the proteoliposomes needed for maximal plasma LCATase activity is 0.8 nmol. Similar results were obtained with four other plasma and three other LCATase preparations.

Characterization of proteoliposomes

Gel filtration of the proteoliposome vesicles (apoA-I:lecithin:cholesterol molar ratio 0.8:250:12.5) on a Sepharose CL-4B column (2.2 × 50 cm) gave only one symmetric peak which contained all vesicular constituents initially introduced to the column (**Fig. 5**). Compositional analysis of the isolated proteoliposome vesicles from the peak of the column showed that the molar ratio of the three vesicular components was identical with that of the initial mixture (apoA-I:lecithin:cholesterol molar ratio 0.8:250:12.5) from which the proteoliposome was formed (data not shown). These results show that apoA-I, lecithin, and cholesterol in this proteoliposome preparation were fully associated with each other as fairly uniform vesicles; this was confirmed by electron microscopy using proteoliposome preparations negatively stained with sodium phosphotungstate (**Fig. 6**). The apparent vesicle radius of this proteoliposome was found to be 131.8 ± 4.8 Å (mean ± SD, n = 6) and 123.4 ± 5.1 Å (mean ± SD, n = 100) by Sepharose CL-4B column chromatography and by electron microscopy, respectively.

Standardization and validation of the assay system

The addition of apoA-I to the assay mixture that contained the proteoliposomes (apoA-I:lecithin:cholesterol molar ratio 0.8:250:12.5) was examined for its effect on LCATase activity (**Fig. 7**). Addition of low concentrations of apoA-I (0.036 to 0.36 nmol/assay) to the assay mixture containing plasma and proteoliposomes did not affect LCATase activity, but addition of a higher concentration of apoA-I (0.71 to 4.26 nmol/assay) reduced it slightly (2 to 8%). In contrast, addition of apoA-I to the assay mixture containing purified enzyme and proteoliposomes caused a marked decrease in the activity of the purified enzyme, with maximal inhibition at 1.43 nmol of added apoA-I. However, no further inhibition was observed by adding more than 1.43 nmol of apoA-I to the assay mixture. Similar results were obtained with four other plasma and three different LCATase preparations.

The effect of HSA on LCATase activity assay was also examined by adding various amounts of HSA to assay mixture (**Fig. 8**). When plasma (15 μl) was used

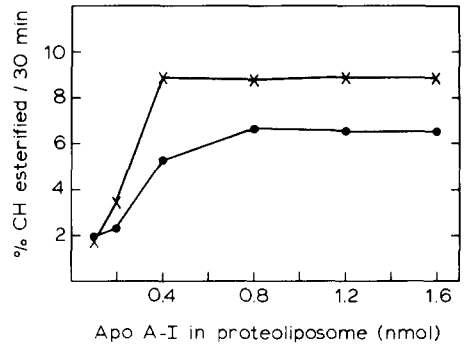

Fig. 4. Effect of apoA-I concentration in proteoliposomes containing a lecithin:cholesterol molar ratio 250:12.5. Proteoliposomes made by the cholate dialysis technique from mixtures of various concentrations of apoA-I and constant concentration of lecithin and cholesterol in a molar ratio 250:12.5 were used for assay of enzyme activity by incubating the assay mixture at 37°C for 30 min. Each assay mixture (0.5 ml) contained 15 μl plasma or 0.2 μg purified enzyme, 0.5% HSA, 5 mM mercaptoethanol, 250 nmol lecithin, 12.5 nmol cholesterol, and various amounts of apoA-I as indicated. ● —— ●, % Cholesterol esterified by plasma; × —— ×, % cholesterol esterified by purified enzyme. Each value is the mean of six determinations with an average error of less than 4.7%.

as enzyme source, a slight increase in LCATase activity was observed with the addition of 0.13–0.5% HSA in the assay mixture; however, no further enhancement of enzyme activity was obtained in the presence of more than 0.5% of HSA. When purified enzyme was used for the assay, the enzyme activity again reached its maximal value at 0.5% HSA, which was approximately double the enzyme activity obtained without HSA. Therefore, the presence of 0.5% HSA in the assay mixture is essential for obtaining maximal LCATase activity using proteoliposomes as substrate.

To validate the assay system, the proteoliposome (apoA-I:lecithin:cholesterol molar ratio 0.8:250:12.5) was incubated with various amounts of plasma or purified enzyme (**Fig. 9**). The plasma LCATase activity was a linear function of plasma enzyme added and was independent of the amount of plasma cholesterol added to the proteoliposomes in the range of 3 to 20 μl of plasma (Fig. 9, top). Similar results were obtained with two normal plasma and two hyperlipidemic plasma. Also, LCATase activity was linear with the amount of purified enzyme up to approximately 2.5 μg/assay or until approximately 40% of the cholesterol was converted to cholesteryl ester (Fig. 9, bottom). Similar results were obtained with three other LCATase preparations. There was excellent agreement between the plasma cholesterol esterification rate and the plasma LCATase activity determined by the proteoliposome method on four plasma samples, two normolipidemic (1 and 2) and two hyperlipidemic (3 and 4) (**Table 1**). Furthermore, the plasma LCATase activity agreed well with the cholesterol esterification rate of the plasma-proteoliposome mixture with the esterification rate of the mixture approximately 9% higher (range 8 to 10%) than the plasma LCATase activity. This result suggests that there is little equilibration between plasma unesterified cholesterol and proteoliposome labeled unesterified cholesterol. Furthermore, it suggests that plasma is not a significant source of substrate for LCATase under conditions of the proteoliposome assay.

To further examine the effect of cholesterol on the assay system, 7.5 μl of heated normal plasma, or 7.5 μl of plasma from each of two subjects (S.F. and D.H.) with no detectable LCATase activity from a Canadian kindred (10), or 7.5 μl of assay buffer was added to the assay system containing the proteoliposomes and 7.5 μl of normal fresh plasma as enzyme source. Normal fresh plasma diluted with heated plasma or LCATase-defi-

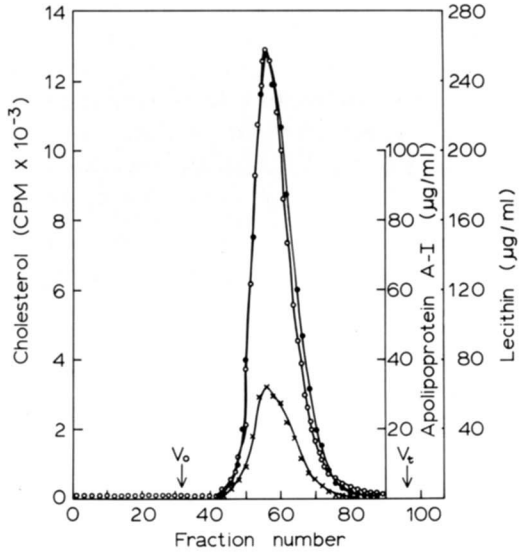

Fig. 5. Sepharose 4B column chromatography of the proteoliposomes (apoA-I:lecithin:cholesterol molar ratio 0.8:250:12.5). Two ml of the proteoliposome preparations containing 0.440 mg apoA-I, 3.850 mg lecithin, and 0.095 mg cholesterol (molar ratio 0.8:250:12.5) was chromatographed on a Sepharose CL-4B column (2.2 × 50 cm) equilibrated with 10 mM Tris buffer, pH 7.4, containing 140 mM NaCl and 1 mM EDTA. The column was eluted with the same buffer, and 2-ml fractions were collected at a flow rate of 30 ml/hr. Each fraction was analyzed for lecithin, cholesterol, and apoA-I as described under Materials and Methods. V_0 and V_t designate excluded column volume and total column volume, respectively. ● —— ●, Lecithin; ○ —— ○, cholesterol; × —— ×, apoA-I.

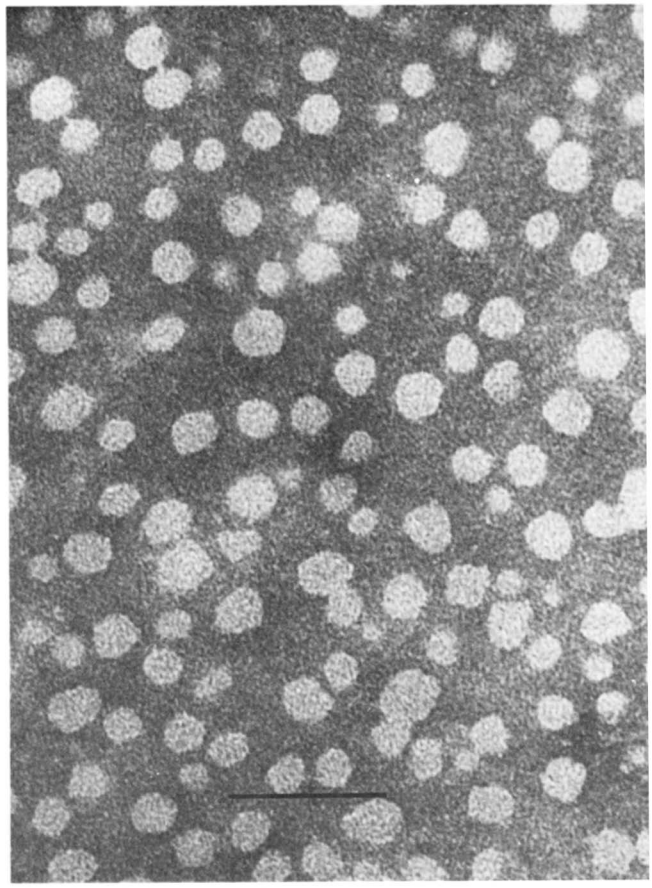

Fig. 6. Electron micrograph of the proteoliposome (apoA-I:lecithin:cholesterol molar ratio 0.8:250:12.5). The proteoliposome preparation was negatively stained with 1% sodium phosphotungstate at pH 7.4. Bar marker represents 1000 Å.

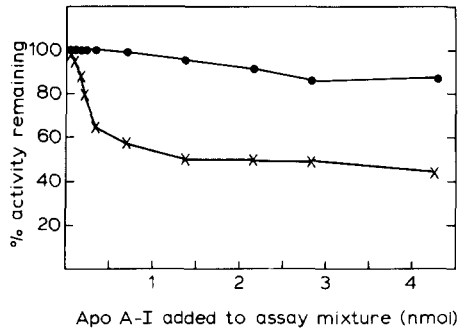

Fig. 7. Effect of addition of apoA-I to assay mixture on LCATase activity. Various amounts of apoA-I were added to the assay mixture, which contained the proteoliposomes, and the enzyme activity was assayed by incubating the mixture at 37°C for 30 min. Each assay mixture (0.5 ml) contained 15 μl plasma or 0.2 μg purified enzyme, 0.5% HSA, 5 mM mercaptoethanol, 250 nmol lecithin, 12.5 nmol cholesterol, and 0.8 nmol apoA-I in proteoliposomes, plus various amounts of added apoA-I as indicated. ● —— ●, % LCATase activity remaining in plasma; × —— ×, % LCATase activity remaining in purified enzyme. Each value is the mean of six determinations with an average error of less than 4.5%.

cient plasma gave the same fractional LCATase rate as plasma diluted with buffer. The assay system was further validated when addition of 5–20 μl of heated normal plasma or 5–20 μl each of plasma from the same two LCATase-deficient subjects to the assay mixture containing 0.2 μg of purified enzyme as enzyme source failed to influence the activity of the purified enzyme (**Table 2**). Furthermore, the addition of 5–20 μl of heated plasma from five other normolipidemic subjects and three hypertriglyceridemic subjects (triglyceride range 600 to 1600 mg/dl) to normal plasma or purified enzyme had no effect on the measurement of LCATase activity using the proteoliposomes.

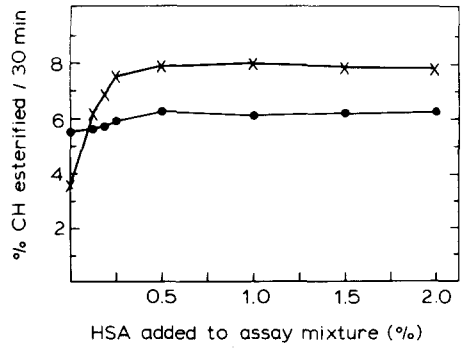

Fig. 8. Effect of HSA concentration in the assay mixture on LCATase activity. Various amounts of HSA were added to the assay mixtures, which contained the proteoliposomes, and the enzyme activity was assayed by incubating the mixtures at 37°C for 30 min as described under Materials and Methods. Each assay mixture (0.5 ml) contained 15 μl plasma or 0.2 μg purified enzyme, 0.8 nmol apoA-I, 250 nmol lecithin, 12.5 nmol cholesterol, and various amounts of HSA as indicated. ● —— ●, % Cholesterol esterified/30 min by plasma; × —— ×, % cholesterol esterified/30 min by purified enzyme. Each value is the mean of six determinations with an average error of less than 5.3%.

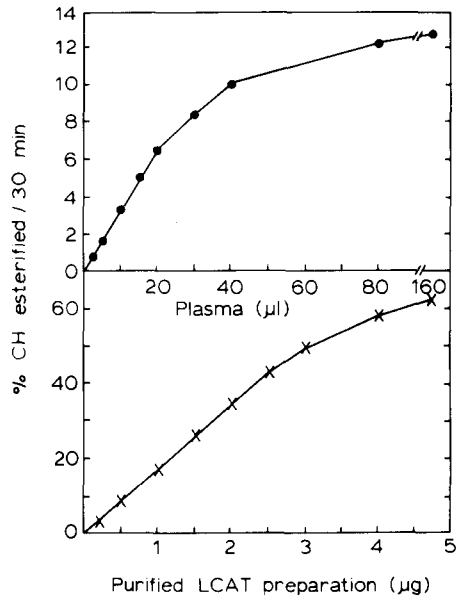

Fig. 9. LCATase activity as a function of plasma volume or amount of purified enzyme assayed. Various amounts of plasma or of purified enzyme were added to assay mixtures which contained the proteoliposomes and the enzyme activity was assayed by incubating the assay mixtures at 37°C for 30 min as described under Materials and Methods. Each assay mixture (0.5 ml) contained 0.8 nmol apoA-I, 250 nmol lecithin, 12.5 nmol cholesterol, 5% HSA, and 5 mM mercaptoethanol, plus various amounts of fresh plasma as enzyme source (top) or various amounts of purified enzyme (bottom). Each value is the mean of six determinations with an average error of less than 5.0%.

The time course of LCATase activity in the presence of either 15 μl of plasma or 0.2 μg of purified enzyme is shown in **Fig. 10.** With all seven plasma samples examined, LCATase activity was linear for up to 60 min, whereas with purified enzyme from three different LCATase preparations it was linear for approximately 120 min.

Effect of storage of plasma on LCATase activity assayed by the proteoliposome method

The effect of storage of plasma at 4°C and −20°C was examined over a period of 5 weeks by using the proteoliposome (apoA-I:lecithin:cholesterol molar ratio 0.8:250:12.5), which was made fresh at each time of assay. **Table 3** shows that there is no visible change in plasma LCATase activity during storage of plasma at either 4°C or −20°C for 5 weeks.

Stability of the proteoliposome

The stability of the proteoliposome (apoA-I: lecithin:cholesterol molar ratio 0.8:250:12.5) was studied by storing the substrate at two temperatures over a period of 5 weeks. At weekly intervals, one portion of the substrate was used for LCATase activity assay, using frozen plasma as enzyme source. It was found that at −20°C the proteoliposome is stable for at least 5 weeks, whereas

TABLE 1. Comparison of plasma LCATase activity determined by the proteoliposome method to plasma esterification rate and to the esterification rate of the plasma-proteoliposome mixture

Plasma	Plasma Unesterified Cholesterol	Plasma LCATase Activity Proteoliposome Method	Plasma Esterification Rate	Plasma-Proteoliposome Esterification Rate
	mg/dl	*nmol cholesterol esterified/hr per ml plasma*		
1	63.70 ± 1.82	97.32 ± 4.06	96.42 ± 6.41	106.32 ± 6.27
2	66.52 ± 1.25	104.20 ± 3.82	108.74 ± 9.38	116.03 ± 8.68
3	104.44 ± 1.65	147.21 ± 4.65	149.00 ± 8.74	159.81 ± 9.78
4	112.51 ± 1.97	151.73 ± 4.34	152.26 ± 7.86	166.57 ± 8.05

The cholesterol esterification rate of plasma and the plasma-proteoliposome mixture was determined by measuring the decrease in unesterified cholesterol in sextuplicate by an enzymic method (7, 11). The determination of plasma LCATase activity by the proteoliposome method was carried out in sextuplicate and calculated as described under Materials and Methods.

at 4°C the substrate is stable for the first 3 weeks, but its substrate efficiency appeared to decrease slightly thereafter (**Table 4**). This slight decrease in LCATase activity after storage at 4°C for a period longer than 3 weeks could not be due to a loss of plasma LCATase activity, because we have shown that plasma LCATase activity does not change if plasma is stored at −20°C for 5 weeks (Table 3).

Comparison of substrate efficiency of the standardized proteoliposome and lecithin-cholesterol liposomes

The efficiency of the standardized proteoliposome (apoA-I:lecithin:cholesterol molar ratio 0.8:250:12.5) was compared with that of lecithin-cholesterol liposomes made in the absence of apoA-I by the cholate dialysis technique and by the Batzri-Korn procedure (14, 19, 23). The liposomes prepared by the cholate dialysis technique had the same lecithin:cholesterol molar ratio (250:12.5) as the standardized proteoliposome, whereas the liposomes prepared by the Batzri-Korn procedure had a lecithin:cholesterol molar ratio of 4:1. Twenty-two μg of apoA-I/assay was added to the assay mixture containing either kind of liposome as substrate, but no apoA-I was added to the assay mixture using the standardized proteoliposome that already contained 22 μg apoA-I/assay. The results show that the standardized proteoliposomes were 6–10 times more efficient for measurement of LCATase activity than were liposomes prepared by the cholate dialysis technique or by the Batzri-Korn procedure (**Table 5**).

DISCUSSION

The existing methods for assaying plasma LCATase activity using the common substrates, heated plasma, or artificial lecithin-cholesterol liposomes have been shown to be far from ideal primarily because of their inferior substrate properties or lack of standardization and validation. If a well-defined common artificial substrate could be made to possess substrate properties similar to those of native plasma substrate, it would be the preferred substrate for the measurement of LCATase activity. Investigations carried out in this study with a new synthetic proteoliposome substrate, in which apoA-I is incorporated into lecithin-cholesterol vesicles by the cholate dialysis technique (20–22), have shown that preparing such a substrate for LCATase is possible. It was found that when apoA-I was kept constant, the LCATase activity varied with the molar ratio of lecithin to cholesterol, and that proteoliposome with 5 mol % cholesterol yielded the desired substrate sensitivity, with fractional activity greater than 5% (Figs. 1–3), and the necessary substrate efficiency with molar LCATase activity approximately

TABLE 2. Effect of addition of heated plasma or LCATase-deficient plasma on LCATase activity of plasma and purified enzyme assayed using the proteoliposome method

Assay Constituents		Fractional LCATase Activity
		percent cholesterol esterified/30 min
Fresh plasma (7.5 μl)		
+ assay buffer	(7.5 μl)	2.27 ± 0.10[a]
+ heated plasma	(7.5 μl)	2.32 ± 0.13
+ S.F. plasma	(7.5 μl)	2.41 ± 0.16
+ D.F. plasma	(7.5 μl)	2.19 ± 0.08
Purified enzyme (0.2 μg)		
+ assay buffer	(20 μl)	14.65 ± 0.43
+ heated plasma	(5 μl)	14.56 ± 0.50
	(10 μl)	14.63 ± 0.62
	(20 μl)	14.60 ± 0.65
+ S.F. plasma	(5 μl)	14.75 ± 0.51
	(10 μl)	14.72 ± 0.46
	(20 μl)	15.03 ± 0.58
+ D.F. plasma	(5 μl)	14.78 ± 0.45
	(10 μl)	14.81 ± 0.63
	(20 μl)	14.92 ± 0.55

[a] Mean ± SD, n = 6.

Heated normal plasma or plasma from two LCATase-deficient subjects (S.F. and D.F.) was added to the assay system containing the proteoliposomes and fresh plasma or purified enzyme as enzyme source. The assay was carried out in sextuplicate as described under Materials and Methods.

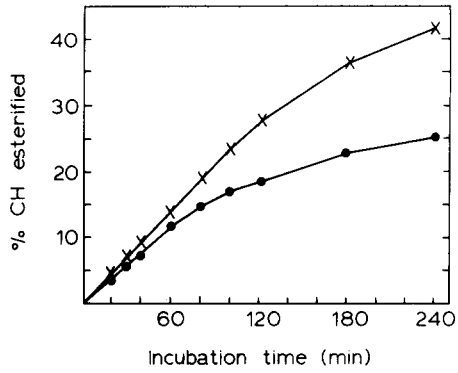

Fig. 10. Time course of LCATase activity assayed by the proteoliposome method. Plasma of purified enzyme was assayed for LCATase activity using the proteoliposomes as substrate. The assay mixtures were incubated at 37°C and the enzymic reaction was stopped at various time intervals as indicated. Each assay mixture (0.5 ml) contained 0.8 nmol apoA-I, 250 nmol lecithin, 12.5 nmol cholesterol, 0.5% HSA, and 5 mM mercaptoethanol, plus 15 μl plasma or 0.2 μg purified enzyme. ● —— ●, % Cholesterol esterified by plasma; × —— ×, % cholesterol esterified by purified enzyme. Each value is the mean of six determinations with an average error of less than 5.8%.

95 ± 14 nmol/hr per ml plasma for 15 normal human subjects, which was comparable to the plasma cholesterol esterification rate of 97 ± 15 nmol/hr per ml by direct measurement of the decrease of unesterified cholesterol (11). LCATase activity was linear with the amount of plasma added as enzyme source up to 20 μl/assay (Fig. 9, top) and with purified enzyme up to 2.5 μg/assay (Fig. 9, bottom). Within this range, the plasma constituents, other than LCATase, have little effect on the proteoliposome substrate or on the measurement of LCATase activity (Tables 1 and 2). On the average, the plasma LCATase activity by the proteoliposome method is nearly identical to the plasma molar esterification rate determined by measuring the decrease in the mass of endogenous unesterified cholesterol. On the other hand, in our previous study (11), plasma LCATase activity in 25 adult volunteers, determined by the older method of Glomset and Wright (12) was only 25 ± 8% of the molar esterification rate. Also, the plasma esterification rate by the radioassay method of Stokke and Norum (4) was only 59 ± 9% of the molar esterification rate determined by enzymic measurement of the decrease of the mass of unesterified cholesterol. Thus, the proteoliposome method, with its better sensitivity, reproducibility, and substrate efficiency is far superior to the Glomset and Wright method.

It should be emphasized that the measure of the plasma LCATase activity by this exogenous common substrate method is not synonymous with a measure of the endogenous cholesterol esterification rate of plasma. LCATase activity as measured by the proteoliposome method is an index of the amount of active enzyme in plasma and is largely independent of the nature or amount of the LCATase substrates or cofactors in the plasma. In contrast to this common substrate method which uses plasma as only the enzyme source, the endogenous self-substrate method of measurement of the LCATase reaction, usually referred to as the plasma cholesterol esterification rate, is an index of not only the amount of LCATase but also the nature and amount of substrate and cofactors in the plasma. We recommend, therefore, that the term "LCATase activity" refer only to the exogenous common substrate method. Although our preliminary studies (11) suggest that most of the variation in plasma esterification rate among individuals is due to variation in LCATase levels, two plasma could have identical LCATase activity, e.g., identical levels of

TABLE 3. Effect of storage of plasma on plasma LCAT activity measured by the proteoliposome method

Plasma Storage Time	Fractional LCATase Activity			
	Plasma A[a]		Plasma B	
	4°C	−20°C	4°C	−20°C
weeks	*percent cholesterol esterified/30 min*			
0	5.68 ± 0.25[b]	5.81 ± 0.14	5.67 ± 0.22	5.67 ± 0.29
1	5.50 ± 0.20	5.85 ± 0.26	5.56 ± 0.14	5.60 ± 0.19
2	5.41 ± 0.17	5.70 ± 0.18	5.53 ± 0.29	5.58 ± 0.25
3	5.53 ± 0.28	5.79 ± 0.30	5.70 ± 0.19	5.64 ± 0.24
4	5.53 ± 0.23	5.79 ± 0.12	5.54 ± 0.29	5.59 ± 0.17
5	5.48 ± 0.26	5.83 ± 0.17	5.60 ± 0.30	5.63 ± 0.22

[a] Plasmas A and B denote two individual plasma samples.
[b] Mean ± SD, n = 6.
A pool of fresh plasma sample was subdivided into 0.2-ml portions and stored at either 4°C or −20°C in sealed vials. At weekly intervals a portion of plasma at each storage temperature was assayed for LCATase activity using freshly made proteoliposomes (apoA-I:lecithin:cholesterol molar ratio 0.8:250:12.5) as substrate. The assay was carried out in sextuplicate as described under Materials and Methods.

TABLE 4. Stability of the proteoliposome as LCATase substrate

Substrate Storage Time	Fractional LCAT Substrate at 4°C	Activity Substrate at −20°C
weeks	*percent cholesterol esterified/30 min*	
0	5.70 ± 0.23[a]	5.70 ± 0.23
1	5.66 ± 0.29	5.65 ± 0.16
2	5.64 ± 0.09	5.72 ± 0.26
3	5.62 ± 0.21	5.61 ± 0.22
4	5.50 ± 0.26	5.70 ± 0.19
5	5.40 ± 0.23	5.64 ± 0.17

[a] Mean ± SD, n = 6.

A pool of freshly made proteoliposomes (apoA-I:lecithin:cholesterol molar ratio 0.8:250:12.5) was subdivided into 0.5-ml portions and stored at either 4°C or −20°C in sealed vials. At the same time, a pool of fresh plasma was subdivided into 0.2 ml-portions and stored frozen at −20°C as an enzyme source. At weekly intervals for a period of 5 weeks, substrate from one portion stored at each temperature was used for assaying LCATase activity using freshly thawed plasma from one portion of the frozen plasma. The assay was carried out in sextuplicate as described under Materials and Methods.

active LCATase, but significantly different cholesterol esterification rates if the substrates of the plasma differ significantly (see reference 11).

Characterization and properties of the proteoliposome preparation

The proteoliposome preparation was shown by both Sepharose CL-4B column chromatography (Fig. 5) and electron microscopy (Fig. 6) to be fairly uniform in size. ApoA-I, lecithin, and cholesterol in the proteoliposome are fully associated with each other in the form of a stable vesicle, since all the protein and lipids are co-eluted in a single symmetric peak from the column (Fig. 5). The composition of the proteoliposome isolated from the column was identical to that of the initial proteoliposome mixture. The vesicle radius obtained by gel filtration (131.8 ± 4.8 Å) and by electron microscopy (123.4 ± 5.1 Å) are comparable. However, this vesicle size is slightly smaller than that of pure lecithin vesicles, which have a 150 Å radius when prepared by solubilizing the lecithin with 30 mM cholate and removing the detergent by gel filtration (29). Other investigators have prepared stable complexes of apoA-I–DMPC–cholesterol either by vortexing (30) or by sonicating (31) the protein-lipid mixture in a buffer solution.

Batch-to-batch variation of egg lecithin and apoA-I did not lead to variation in the substrate efficiency of the proteoliposome preparation. When a freshly made proteoliposome preparation was used at each time of assay, the plasma LCATase activity in plasma stored at either 4°C or −20°C for 5 weeks was observed to be stable (Table 3). This observation is in accord with the result of Heller, Desager, and Harvengt (32), who have recently reported that plasma LCATase activity is not altered by storage of the plasma for 17 weeks at −25°C. Furthermore, the proteoliposome preparation was shown to be stable as an LCATase substrate for at least 3 and 5 weeks, respectively, when stored at 4°C and −20°C (Table 4). The ability to prepare stable proteoliposomes is especially useful for comparative studies and large scale investigations of plasma LCATase activity.

Standardization and validation of the proteoliposome assay system

The proteoliposome contains sufficient apoA-I for maximal enzyme activity and the presence of additional apoA-I in the assay mixture is not necessary. Although

TABLE 5. Comparison of substrate efficiency of the proteoliposomes and lecithin-cholesterol liposomes

	Fractional LCATase Activity[a]		Molar LCATase Activity[b]	
Substrates	Plasma	Purified Enzyme	Plasma	Purified Enzyme
Proteoliposomes	5.80 ± 0.22[c]	29.34 ± 1.72	96.72 ± 3.67	14670 ± 860
Liposomes[d]	1.03 ± 0.07	4.53 ± 0.24	17.18 ± 1.17	2265 ± 120
Liposomes[e]	0.58 ± 0.06	1.80 ± 0.12	15.48 ± 1.60	1440 ± 96

[a] Percent cholesterol esterified/30 min.
[b] nmol Cholesterol esterified/hr per ml plasma or mg protein of purified enzyme.
[c] Mean ± SD, n = 6.
[d] Made by cholate dialysis technique.
[e] Made by Batzri-Korn procedure (14).

The proteoliposomes (apoA-I:lecithin:cholesterol molar ratio 0.8:250:12.5) were prepared by the cholate dialysis technique. Liposomes containing lecithin:cholesterol in molar ratios of 250:12.5 and 4:1 were prepared in the absence of apoA-I, respectively, by the cholate dialysis technique and the Batzri-Korn procedure (14) as described (23, 24). For LCATase activity assay, using both kinds of liposomes, an amount of 22 μg apoA-I was added to each assay mixture; no apoA-I was added to the assay mixture when the optimized proteoliposome, which contains 22 μg apoA-I assay, was used. The assay was carried out in sextuplicate as described under Materials and Methods. One-half μg of purified enzyme was used per assay.

addition of apoA-I to the assay mixture did not cause any significant effect on plasma LCATase activity, it markedly reduced the activity of purified enzyme (Fig. 7). The reason for this difference is unclear, but the purified "destabilized" enzyme may be more sensitive to altered conformation of the apoA-I activator. We showed in other studies that apoA-I added to liposomes is a less effective activator than is apoA-I incorporated into the liposome by the cholate dialysis procedure.

When the proteoliposome was used as substrate, the presence of a 0.5% concentration of HSA in the assay mixture is essential for maximal LCATase activity (Fig. 8). The contribution of HSA from 15 μl plasma added as enzyme source is approximately 1 mg (33), which yields a final concentration of about 0.2% in an assay volume of 0.5 ml. This amount of HSA from the plasma sample is therefore not sufficient to obtain the maximal enzyme activity. Addition of HSA to the assay mixture is essential for optimal measurement of LCATase activity. These observations are in agreement with our previous findings using artificial lecithin-cholesterol liposomes as substrate (19, 23, 24). It is possible that HSA enhances apoA-I-stimulated LCATase activity by stabilizing the enzyme or, alternatively, by binding lysolecithin or cholesteryl ester and thereby minimizing product inhibition.

The ability of the proteoliposomes to serve as substrate for LCATase activity was assessed by comparing their efficiency to that of lecithin-cholesterol vesicles incubated with apoA-I. The liposomes were made in the absence of apoA-I either by the cholate dialysis technique used in the present study or by the Batzri-Korn procedure (14). We found that the proteoliposome used in this study is 6-fold and 10-fold more efficient than the cholate liposome and the Batzri-Korn liposome, respectively. This suggests that cholate permits apoA-I to associate with lecithin and cholesterol in a specific arrangement which serves as a superior substrate for LCATase. The cholate dialysis technique has been used extensively to reconstitute membrane proteins with lipids in order to regenerate their biological activity (20, 21, 34, 35).

This method of substrate formation using the cholate dialysis technique permits the preparation of large amounts of stable, efficient, homogeneous, and well-defined proteoliposome vesicle substrate for a rapid and precise determination of plasma or serum LCATase activity. The availability of this method will permit comparative studies and large-scale investigations of plasma LCATase activity to be carried out as well as studies of the mechanism and regulation of the LCATase reaction. Recent application of this proteoliposome substrate to the measurement of plasma LCATase activity has enabled us to demonstrate that the LCATase activity of heterozygous carriers of the LCATase deficiency is approximately half-normal (10). Earlier studies using conventional methods for measuring LCATase activity failed to distinguish between normal subjects and heterozygotes (36–38). In addition, the proteoliposome substrate has also enabled us to demonstrate that familial LCATase deficiency in the four Norwegian kindreds is due to the presence of low levels of a functionally defective enzyme (39). Thus the proteoliposome prepared by the cholate dialysis method promises to be an effective tool in understanding LCATase regulation, metabolism, and genetic control.

We thank students Chris Chapman and Sue Rafchiek for expert technical assistance. We also thank Dr. Henry Pownall of the Baylor College of Medicine, Houston, Texas, for initially recommending the use of an apoA-I-containing proteoliposome prepared with cholate for the determination of LCATase activity. This research was supported, in part, by grant HL-15263, contract N01-HV1-2157-L from the Lipid Metabolism Branch, NHLBI, and a grant from R. J. Reynolds, Inc. This work was done during the tenure of an Established Investigatorship from the American Heart Association (Dr. Albers) and with funds contributed in part by the American Heart Association of Washington.

Manuscript received 2 October 1981 and in revised form 29 January 1982.

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