papers and notes on methodology

New substrate for determination of serum lecithin:cholesterol acyltransferase

Mitsuhisa Manabe,[1,*] Toshikatsu Abe,[*] Masayuki Nozawa,[*] Akemichi Maki,[*] Miyoshi Hirata,[*] and Hiroshige Itakura[†]

Tokyo Laboratory, Daiichi Pure Chemicals Co., Ltd.,[*] 5-12 Narihira 5-chome, Sumida-ku, Tokyo 130, and the Third Department of Internal Medicine, Faculty of Medicine,[†] University of Tokyo, Hongo, Bunkyo-ku, Tokyo 130

Abstract Serum lecithin:cholesterol acyltransferase (LCAT) was estimated by enzymatically measuring the decrease in unesterified cholesterol after incubation of serum with liposomes. A high-performance liquid chromatography (HPLC) study showed the uptake of the lipids of liposomes by serum high density lipoprotein. Of all the examined liposomes prepared from cholesterol and various synthetic phosphatidylcholines, liposomes with dimyristoylphosphatidylcholine (DMPC) were found to be the most reactive in the LCAT reaction. When serum was used as an enzyme source, addition of purified apolipoprotein A-I, which is known to be an endogenous activator of LCAT, to the assay mixture resulted in a slight decrease in enzyme activity. Using DMPC-cholesterol liposomes as the substrate, the LCAT activities in 120 human sera showed a mean value of 485.4 ± 64.6 nmol/hr per ml (mean ± SD), which is 4.4- to 5.4-fold higher than the values obtained by self-substrate methods. LCAT activity was a linear function of the serum sample volume up to 670 nmol/hr per ml and coefficients of variation (CV) less than 4% were obtained under the standardized conditions. Moreover, when partially purified LCAT was added to various heat-inactivated sera, the activity was efficiently recovered. These results suggest that this method is sensitive, reproducible, and not greatly influenced by serum components. — **Manabe, M., T. Abe, M. Nozawa, A. Maki, M. Hirata, and H. Itakura.** New substrate for determination of serum lecithin:cholesterl acyltransferase. *J. Lipid Res.* 1987. **28:** 1206–1215.

Supplementary key words apolipoprotein A-I • HPLC • high density lipoprotein • substrate specificity

Lecithin:cholesterol acyltransferase (LCAT, EC 2.3.1.43) is known to catalyze the transfer of a fatty acyl group from phosphatidylcholine to unesterified cholesterol, thereby forming lysophosphatidylcholine and cholesteryl ester (1). In human serum, this enzyme is thought to exist on the surface of high density lipoprotein (HDL) and plays a major role in the transport of cholesterol from the peripheral tissues to the liver (2, 3). In addition to the importance of the enzyme in lipoprotein metabolism (4, 5), the serum LCAT activity has been reported to have clinical significance in various diseases (6–8).

Although a variety of methods using a natural (9, 10) or artificial substrate (11–15) have been employed for estimating the serum LCAT activity, technical difficulties have prevented wider use of plasma LCAT assay in clinical laboratories.

Nagasaki and Akanuma (16) reported a simple method for the determination of LCAT activity by enzymatically measuring the change in the concentration of unesterified cholesterol. However, this method is not sensitive due to the low esterification rates.

In this report we describe a simple method for sensitive determination of the serum LCAT activity, using dimyristoyl phosphatidylcholine (DMPC)-cholesterol liposomes as the substrate.

MATERIALS AND METHODS

Reagents

Phosphatidylcholines, palmitoyl lysophosphatidylcholine, cholesterol, glutathione, and heparin were purchased from Sigma Chemical Co. Bovine serum albumin (BSA, essentially fatty acid-free grade) and hydroxyapatite were

Abbreviations: LCAT, lecithin:cholesterol acyltransferase; apoA-I, apolipoprotein A-I; HDL, high density lipoprotein; DMPC, dimyristoylphosphatidylcholine; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetate; Tris, tris(hydroxymethyl)methylamine; CV, coefficient of variation; HPLC, high performance liquid chromatography.

[1]To whom reprint requests should be addressed.

obtained from United Biochemicals Corp. and Behring Diagnostics (California), respectively. DEAE-cellulose and protein-A Sepharose were supplied by Pharmacia Fine Chemicals. Triton X-100, N,N-diethyl-m-toluidine, potassium biphthalate, uric acid, glucose, ascorbic acid, N-ethylmaleimide, and anticoagulants other than heparin were purchased from Kishida Chemical Co. (Tokyo). Cholesterol oxidase (203 U/ml), peroxidase (238 U/mg), and other enzymes were obtained from Toyojozo Co., Ltd. (Shizuoka, Japan). Tris, EDTA disodium salt, 4-aminoantipyrine, bilirubin, rabbit anti-human apolipoprotein A-I (apoA-I) antisera, and ApoA-I plate "DAIICHI",[2] a commercially available kit for the determination of apoA-I, were supplied by Daiichi Pure Chemicals Co., Ltd. (Tokyo).

Specimens

Human sera were obtained from patients admitted to the Third Department of Internal Medicine, Faculty of Medicine, the University of Tokyo, and from apparently healthy volunteers (normal sera) after an overnight fast. Diagnosis was established on the basis of physical examination and the laboratory findings. Hemolyzed specimens were prepared by the addition of hemolyzed erythrocyte solution (in 10 mmol/l phosphate buffer, pH 7.4) to serum. The hemoglobin concentration was determined by the cyanmethemoglobin method (17).

Preparation of liposomes

We used liposomes containing lecithin-cholesterol in a molar ratio of 2.63:1 to avoid interaction of the liposomes with serum apolipoproteins and/or proteins possibly contaminating the commercial preparation of BSA (18). A chloroform-methanol 2:1 (v/v) solution containing 108 μmol of phosphatidylcholine and 41 μmol of cholesterol was evaporated to dryness under nitrogen, and then 10 ml of a 62.5 mmol/l Tris solution (pH 10.5) was added to the dried lipid mixture. After the lipids were allowed to swell at 37°C for 60 min, a suspension containing all the lipid materials was prepared by agitation with a vortex mixer. This suspension was diluted to 80 ml with the 62.5 mmol/l Tris solution (containing 50 μmol of palmitoyl lysophosphatidyl choline) and sonicated for 40 min at 30-40°C using a 300 W ultrasonic disintegrator (model US-300, Nihon Seiki Co., Tokyo). The resulting vesicles were dialyzed against the 62.5 mmol/l Tris solution and filtered through a 0.22-μm Millipore filter. Then the particle sizes were estimated by photon correlation spectroscopy (Particle Sizer, model BI-90, Brookhaven Instruments Corp.).

[2]ApoA-I plate "DAIICHI" is a trade name of Daiichi Pure Chemicals Co., Ltd.

Preparation of substrate solution

Three grams of BSA was solubilized in 80 ml of the liposome solution. This intermediate liposome-BSA solution was stable at −20°C for at least 3 months. Before use, the liposome-BSA solution was adjusted to pH 7.3 by the addition of 1.75 g of EDTA disodium salt powder and diluted to 100 ml with deionized water. The prepared substrate was kept in an ice bath during the experiment and was stable for 4 days at 10°C.

Assay of enzyme activity

Two hundred μl of serum was added to 500 μl of substrate solution in a stoppered test tube and mixed. Two hundred μl of the mixture was placed in a test tube containing 50 μl of a 50 g/l Triton X-100 solution as an inhibitor of the LCAT reaction (S 0 min). This test tube (S 0 min) was incubated for 40 min at 37°C. The remaining mixture was incubated for 40 min at 37°C, and 200 μl of the mixture was taken into another test tube containing the Triton X-100 solution mentioned above (S 40 min). Subsequently, these samples (S 0 min and S 40 min) were mixed with 3 ml of working reagent for cholesterol analysis, which contained 100 μl of cholesterol oxidase, 0.13 mg of peroxidase, 20 μl of N,N-diethyl-m-toluidine, 5.2 mg of 4-aminoantipyrine, 0.2 g of Triton X-100, and 50 mmol/l of potassium biphthalate (pH 6.1) in a final volume of 100 ml. Then the reagent mixtures were allowed to stand for 10 min at 37°C, and the developed color was measured at 545 nm on a Gilford spectrophotometer (model 1220, Gilford Instrument Laboratories Inc.). Simultaneously, the reagent blank and standard values were also measured. For the colorimetric analysis of cholesterol, a 500 nmol/l recrystallized cholesterol solution (in isopropanol) was used as a standard. The molar activity and fractional activity of serum LCAT were calculated by the following equations.

$$\text{Molar activity (nmol/hr per ml)} = \frac{A(0\text{ min}) - A(40\text{ min})}{A(\text{std}) - A(\text{RB})} \times 500 \times 3.5 \times \frac{60}{40}$$

$$\text{Fractional activity (\%/hr)} = \frac{A(0\text{ min}) - A(40\text{ min})}{A(0\text{ min}) - A(\text{RB})} \times \frac{60}{40}$$

where A(0 min) = absorbance of sample before LCAT reaction; A(40 min) = absorbance of sample after LCAT reaction; A(std) = absorbance of standard solution; A(RB) = absorbance of blank test; 3.5 = dilution factor of samples; 500 = cholesterol concentration in standard solution (nmol/ml); and 40 = incubation time for LCAT reaction (min).

In order to compare this method with the endogenous-substrate method, determination of the serum LCAT activity was also performed by the method reported by Nagasaki and Akanuma (16).

Analysis of free cholesterol, total cholesterol, and phospholipid of serum lipoproteins and liposomes

The separation of serum lipoproteins and liposomes was carried out using HPLC (Waters Associates) with columns for gel permeation chromatography (TSK GEL G 5000 PW and G 3000 SW, Toyo Soda Mfg. Co., Tokyo) (19). The quantitation of free cholesterol and phospholipid was performed by enzymatic reactions (19, 20). The enzyme solution was added to the eluate from the column, and the concentrations of free cholesterol, total cholesterol, and phospholipid were measured by absorbance after passage through the reactor (stainless steel tube) at 37°C.

Enzyme preparation

LCAT was purified from human plasma by the procedure of Akanuma and Glomset (21), which consists of DEAE-cellulose column chromatography, precipitation with ammonium sulfate, and hydroxyapatite chromatography. Then, the resulting crude preparation of LCAT was applied to a column containing rabbit antihuman apoA-I immunoglobulin adsorbed to protein-A Sepharose and washed with a 10 mmol/l Tris-EDTA buffer (pH 7.3). The effluent was collected and concentrated. No contamination by apoA-I was seen in the final preparation by means of Ouchterlony immunodiffusion or SDS-polyacrylamide gel electrophoresis. The enzyme activity in each fraction was estimated by our assay method, in which the specimen for the LCAT determination was a mixture of 50 μl of fractionated eluate and 150 μl of heat-inactivated plasma that had been preincubated for 1 hr at 37°C. The protein concentration was determined by the method of Lowry et al. (22), using BSA as the standard. Purification was approximately 100-fold when the activity was measured in the presence of heat-inactivated original plasma.

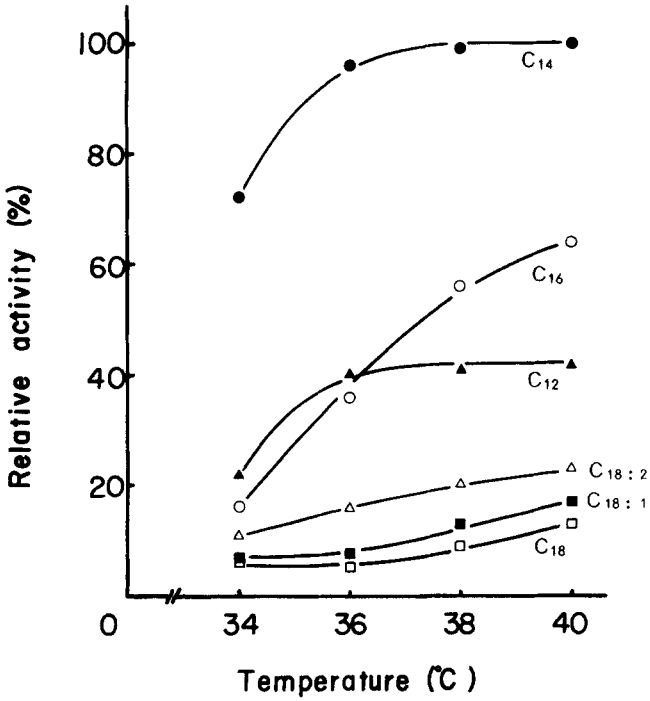

Fig. 1. Relative reaction rate of serum LCAT with phosphatidylcholine-cholesterol liposomes at various temperatures. The reaction rate with DMPC liposomes at 40°C was taken as 100. Each fatty acyl chain of phosphatidylcholines is indicated as dilauroyl (▲), dimyristoyl (●), dipalmitoyl (○), distearoyl (□), dioleoyl (■), and dilinoleoyl (△), respectively.

Purification of apoA-I

ApoA-I was isolated from normal human plasma by the method of Cheung and Albers (23). A single band was observed in SDS-polyacrylamide gel electrophoresis of the final apoA-I preparation.

Determination of lipids and apoA-I

Phosphatidylcholine (24), total cholesterol (25), and unesterified cholesterol (16) were determined enzymatically. Estimation of the apoA-I concentration was performed with the commercially available kit, ApoA-I plate "DAIICHI" (26).

RESULTS

Effect of the nature of liposomes on LCAT activity

In order to develop an effective substrate for LCAT, liposomes were prepared from various kinds of phosphatidylcholines. The liposomes were then used as the substrate for assaying the LCAT activity in serum samples in the presence of 30 g/l BSA and 50 mmol/l Tris-EDTA buffer. **Fig. 1** shows the relative reaction rates with six diverse substrates, with the activity with DMPC liposomes at 40°C taken as 100; it also shows their dependence on the reaction temperature. The highest rate was obtained with DMPC liposomes, followed in order of decreasing relative activity by dipalmitoylphosphatidylcholine, dilauroylphosphatidylcholine, dilinoleoylphosphatidylcholine, dioleoylphosphatidylcholine, and distearoylphosphatidylcholine. Qualitatively similar trends of thermodependence of the activity were observed with DMPC and dilauroylphosphatidylcholine liposomes. On the other hand, the pattern of the temperature-activity curve with dipalmitoylphosphatidylcholine liposomes was different from the patterns with DMPC and dilauroylphosphatidylcholine; the patterns of the other substrates were unclear due to low availability of the materials for the reaction. The reaction with dipalmitoylphosphatidylcholine liposomes appeared to be the most thermodependent within the examined ranges and required a higher temperature to be optimized in comparison with the behaviors of DMPC and dilauroylphosphatidylcholine.

Subsequently, we examined the effects of DMPC liposomes with different particle sizes on the serum LCAT activity, keeping the DMPC and cholesterol concentrations almost constant. Lipid dispersions obtained by vortexing were sonicated for various periods to give a series of liposomes with various sizes, and the mean diameters of the resulting vesicles were estimated by photon correlation spectroscopy after filtration through a 3.0-μm Millipore membrane filter. As shown in **Fig. 2,** the mean diameters of the liposomes covered a range of 50 to 500 nm, depending on the sonication period. The detected serum LCAT activity increased as the particle size of the liposome substrate decreased, and it reached a plateau when liposomes with a diameter of less than 80 nm were used.

Interaction between serum lipoproteins and liposomes

Fig. 3 shows the elution patterns for liposomes, serum, and the mixture of serum and liposomes before and after incubation. These patterns were obtained by detecting free cholesterol as well as phospholipid using the HPLC system. Incubation of liposomes with serum resulted in a significant decrease in the peak area of free cholesterol (Fig. 3, A-d peak 1+2) and phospholipid (Fig. 3, B-d peak 1) of liposomes and an increase in the peak areas of both lipids of HDL (Fig. 3, A-d peak 5 and B-d peak 5). On the contrary, when serum was incubated with liposomes in the presence of 100 mM N-ethylmaleimide, an LCAT inhibitor, no significant changes in elution pattern were observed when monitored by detecting the free cholesterol. These results clearly demonstrate that free cholesterol and DMPC in liposomes are easily taken up by HDL during the LCAT reaction. The elution patterns monitored by detecting the total cholesterol are shown in **Fig. 4,** where the increase in total cholesterol was observed only in the HDL region after the incubation of serum with liposomes.

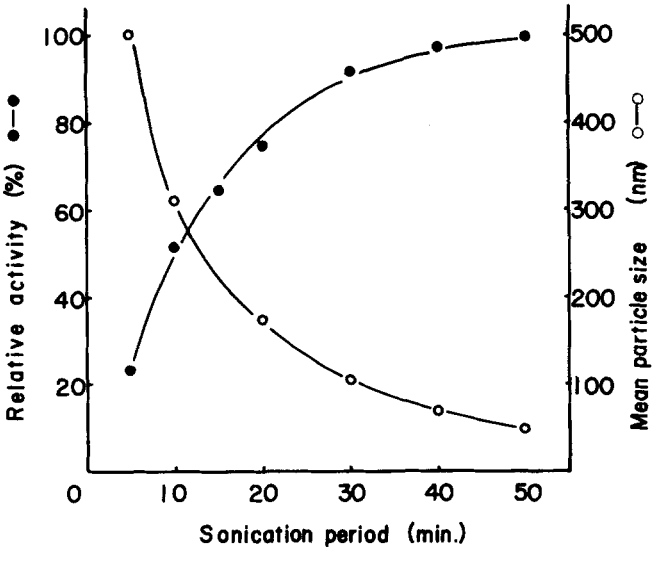

Fig. 2. The relationship between relative reaction rate of serum LCAT when the reaction rate with liposomes possessing a mean diameter of 50 nm was taken as 100 (●) and the particle sizes of DMPC liposomes were estimated by photon correlation spectroscopy (○). A series of liposomes with different sizes was obtained by changing the sonication period.

Optimal conditions for LCAT reaction

The detected serum LCAT activity was dependent upon the liposome concentration in the reaction mixture (**Fig. 5**). When a 30 g/l BSA solution (in 50 mmol/l Tris-EDTA buffer, pH 7.4) was added to serum instead of the substrate, the obtained LCAT activities were extremely low. The optimal concentration, expressed as the concentration of cholesterol in the liposomes, was found to be 0.41 mmol/l or greater with all the examined samples in spite of slight variation in the patterns of the saturation curves, although the samples varied with the concentration of endogenous free cholesterol. We chose a concentration of 0.41 mmol/l, since an excess of liposomes is undesirable for the subsequent colorimetric analysis of cholesterol.

BSA, which has conventionally been used in LCAT assays, was also effective for the reaction in our study. Although the activation was saturated at a concentration of 5 g/l, 30 g/l BSA was selected because of its contribution to the stability of the liposomes during the preservation below 0°C.

The optimal pH of the substrate solution is 7.1–7.4, so we chose a pH of 7.3. At this pH, 50 mmol/l Tris-EDTA buffer was preferable to other possible buffers, such as Good's buffers (BES, HEPES, MES, MOPS, and PIPES), Tris-HCl, Tris-borate, Tris-citric acid, and phosphate buffer.

The addition of purified apoA-I, which is considered to be a potent activator of LCAT, to the assay medium resulted in no further activation under these conditions (**Fig. 6**). The LCAT reaction was inhibited at higher concentrations of apoA-I, independent of the presence of BSA. Based upon this finding, which differs from those of other investigators (11–13, 27), experiments were performed without addition of exogenous apoA-I.

Fig. 7 shows the time course of the decrease in the unesterified cholesterl concentration in the assay medium during the incubation. The time course is essentially linear during about the first 60 min of incubation and subsequently falls off to a lower rate. Hence, for detection of the initial rate of the reaction, we used sampling periods of 40 min.

Linearity, normal value, precision, and accuracy

The function relating the sample volume included in the assay medium and the activity was linear up to at least 670 nmol/hr per ml, although the activities with serum

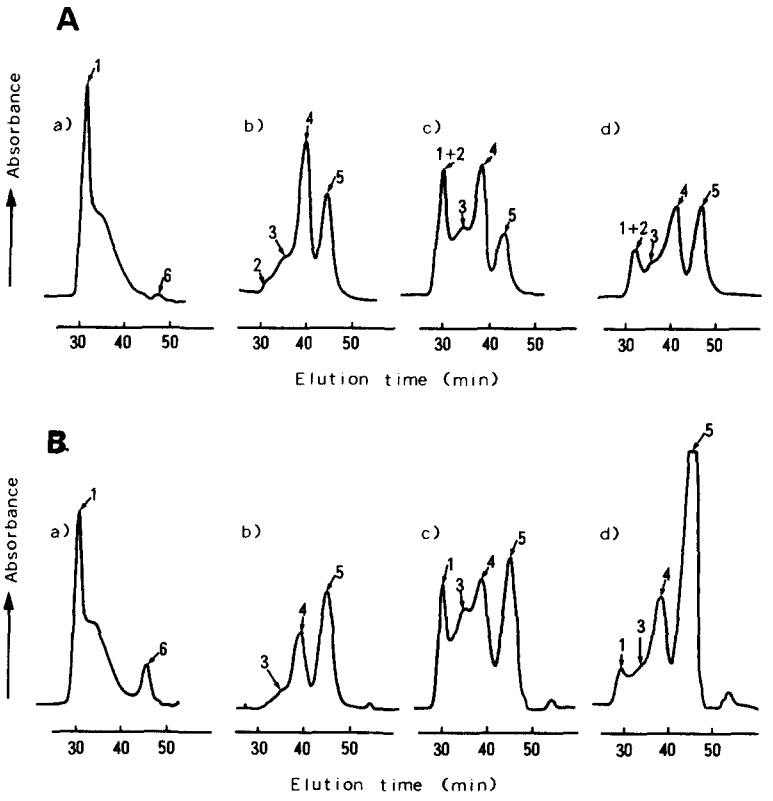

Fig. 3. HPLC elution patterns of free cholesterol (A) and phospholipid (B) for liposomes (a), serum (b), the mixture of liposomes and serum before incubation (c), and after incubation for 3 hr at 37°C (d). Peaks: 1, liposome; 2, chylomicron; 3, very low density lipoprotein; 4, low density lipoprotein; 5, HDL; 6, albumin.

samples diluted more than five times tended to be less than the theoretically expected values (**Fig. 8**).

Using 120 samples from healthy volunteers, determination of the serum LCAT activity by this method gave a mean value and SD of 485.4 ± 64.6 nmol/hr per ml in terms of the molar activity, and the average fractional activity was 21.3%/hr. This value corresponds to a change of 0.088 in absorbance by the enzymatic cholesterol method, indicating that this assay is highly sensitive.

To estimate the day-to-day precision of this method, we made duplicate determinations over a period of 8 days on three sera with different LCAT activities. The CVs for the three sera were 3.4, 2.3, and 1.6%, respectively. The within-run precision was also evaluated for the same three

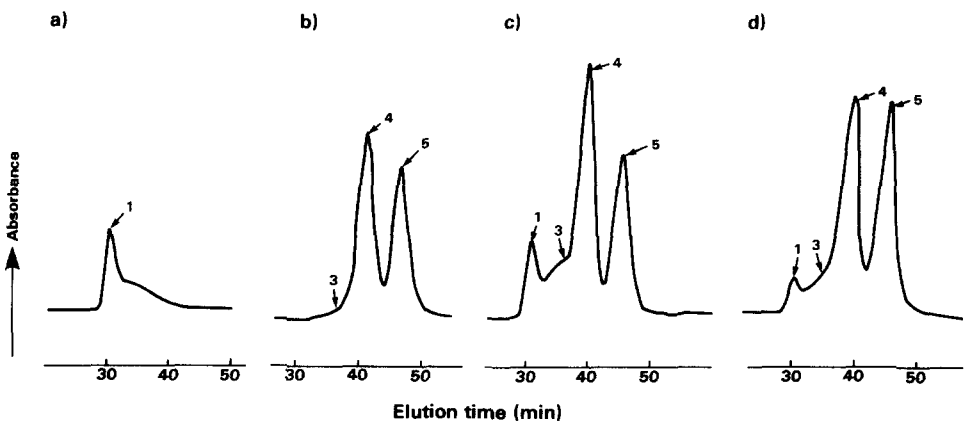

Fig. 4. HPLC elution patterns of total cholesterol for liposomes (a), serum (b), the mixture of liposomes and serum before incubation (c), and after incubation for 3 hr at 37°C (d). Peaks: 1, liposome; 2, chylomicron; 3, very low density lipoprotein; 4, low density lipoprotein; 5, HDL.

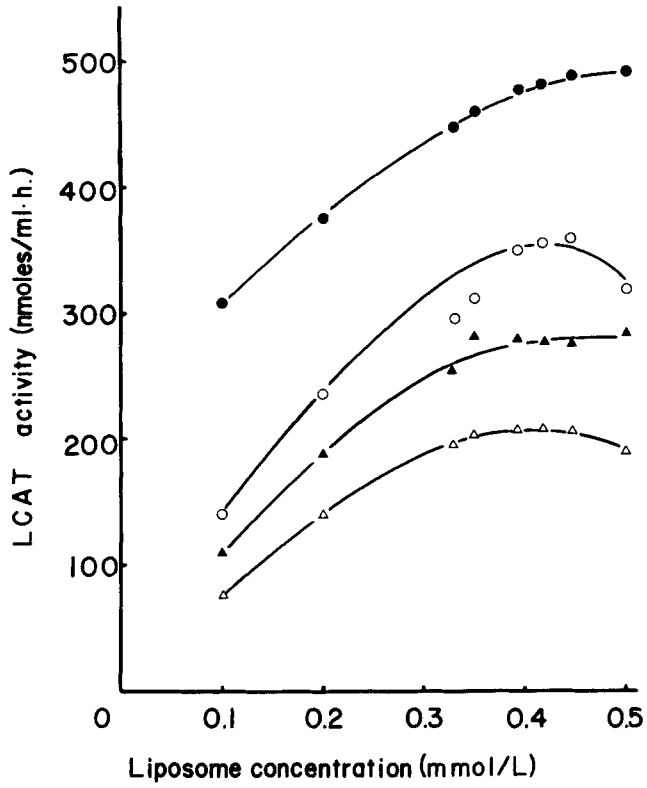

Fig. 5. Activation of serum LCAT by addition of liposomes. Each saturation curve was representative of the results with serum samples from four individuals. The liposome concentration in the substrate was expressed as that of cholesterol in the liposomes.

sera, and 12 replicate determinations showed CVs of 3.7, 2.6, and 1.8%, respectively.

The serum LCAT activity is considered to be influenced not only by the nature of the substrate but also by endogenous cofactors and the lipid composition. Therefore, the enzyme was partially purified from human plasma and subjected to a preliminary recovery experiment. A 50-μl volume of enzyme preparation was added to each 150 μl of heat-inactivated sera, which varied in lipid composition and apoA-I content, followed by determination of the LCAT activity. Since the suitability of this method for the estimation of purified LCAT activity has not yet been assessed, the percentage recovery could not be calculated. **Table 1** shows, however, that similar activities were recovered with this method, in spite of the differences in the serum components of the specimens.

Interfering substances

The effects of possible interfering substances other than lipids and cofactors were examined. Various substances were separately dissolved in saline, and the resultant saline solutions were added to serum samples not to exceed 20% of the total volume. When these mixtures were used as specimens, no significant inhibition was observed with uric acid, glucose, bilirubin, glutathione, or ascorbic acid up to sample concentrations of 100 mg/l, 5 g/l, 100 mg/l, 500 mg/l, and 40 mg/l, respectively. Anticoagulants also had no effect on the enzyme activity at the concentrations of laboratory use. However, addition of a hemolyzed erythrocyte solution to the assay medium caused considerable inhibition. The enzyme activity was reduced by about 30% at a sample concentration of 10 g/l of hemoglobin. Therefore, we did not apply this method to the hemolyzed specimens.

Correlation between self-substrate and proposed methods

The LCAT activities in 59 serum samples from patients with various diseases were estimated by both the proposed method and the self-substrate method of Nagasaki and Akanuma (16), and the results were subjected to regression analysis. The determined activities lay in a wide range, and discrepancies were often observed between the two methods. The correlation coefficient, slope, and intercept of the regression curve were calculated to be 0.8780, 3.982, and +90.8, respectively, when plotting the results with our method on the ordinate (y-axis).

DISCUSSION

Based upon the nature and source of the substrate, the existing methods fall into two general categories. One is called endogenous self-substrate methods, where the LCAT activity is determined by measuring the rate of

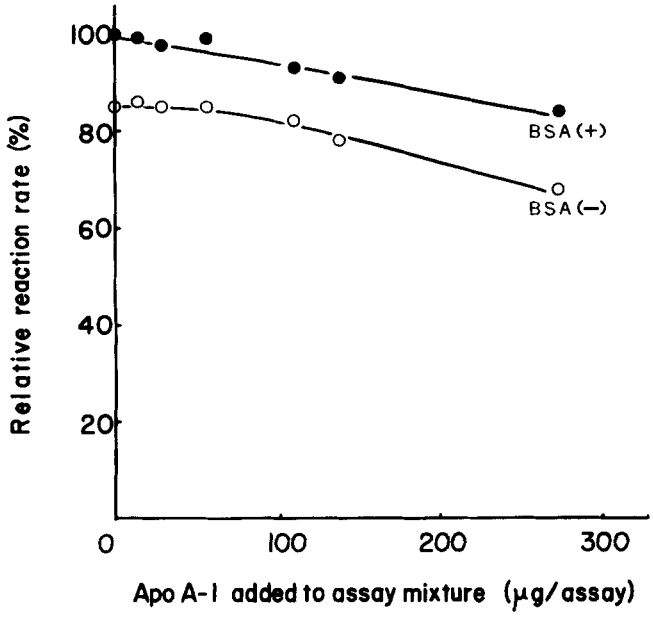

Fig. 6. Effect of apoA-I on relative reaction rate of serum LCAT in the presence (●) or absence (○) of BSA. The reaction rate of serum LCAT free of both BSA and apoA-I was taken as 100.

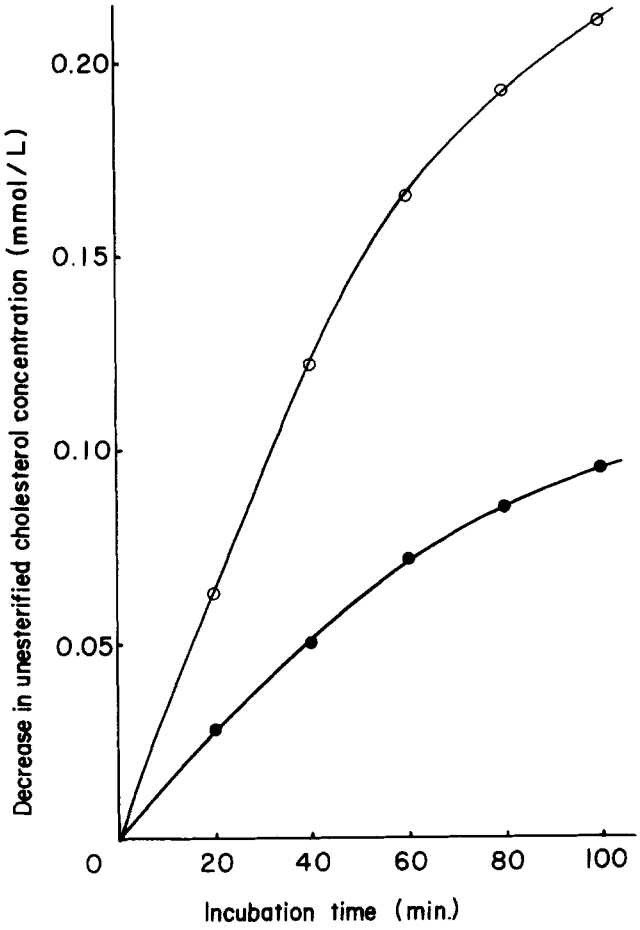

Fig. 7. Time course of cholesterol esterification in assay medium containing substrate and serum, expressed as decrease in unesterified cholesterol concentration; serum 1, (○); serum 2, (●).

enzymatic conversion of labeled cholesterol to cholesteryl ester after equilibration of plasma with radioactive cholesterol (9) or by directly measuring the decrease in endogenous unesterified cholesterol after incubation of plasma itself (16, 28, 29). The second category is the exogenous common substrate methods, which use heat-inactivated plasma (10) or artificial proteoliposomes containing apoA-I, phosphatidylcholine. and cholesterol (12, 13, 15) as the substrate.

In the last few years, artificial substrates have been preferred rather than heat-inactivated plasma because denatured lipoproteins are poor substrates for LCAT and marked variations in the enzyme activity occur when different substrate sources are used (30). The artificial substrate method, however, required purified apoA-I from human plasma and special instrumentation for the radioassay technique.

In earlier studies, Nichols and Gong (14) reported that sonicated dispersions of unesterified cholesterol and phosphatidylcholine were able to serve as the substrate and that the initial rate of esterification was comparable to the results with a heat-inactivated plasma substrate. While phosphatidylcholine-cholesterol liposomes were also utilized in this study, the esterification rates found in normal serum samples were 4.4–5.4 times higher than those in other reports (9, 10, 14, 16). This high reactivity of the proposed substrate may be explained by the apparent LCAT substrate specificity for phosphatidylcholine and by the effect of the physical state of liposomes on the interaction with plasma lipoproteins.

It is well known that DMPC liposomes are susceptible to disruption by apolipoproteins, thereby forming discoidal lipid-apolipoprotein complexes (31). However, this effect of apolipoproteins depends greatly upon the cholesterol content of the liposomes (18). The liposomes we employed are relatively rich in cholesterol (molar ratio of DMPC-cholesterol, 2.63:1), so that modification of the liposomes by endogenous apolipoproteins is not likely to occur. In fact, the incubation of liposomes with serum, in the absence of LCAT activity, resulted in no significant changes in the elution pattern monitored by detecting free cholesterol, indicating that small vesicles of lipid-apoA-I complexes could not be formed. On the other hand, in the presence of LCAT activity, a significant increase in free cholesterol in the HDL region was observed. When the elution patterns were monitored by detecting total cholesterol, an increase in the peak area was also seen in the

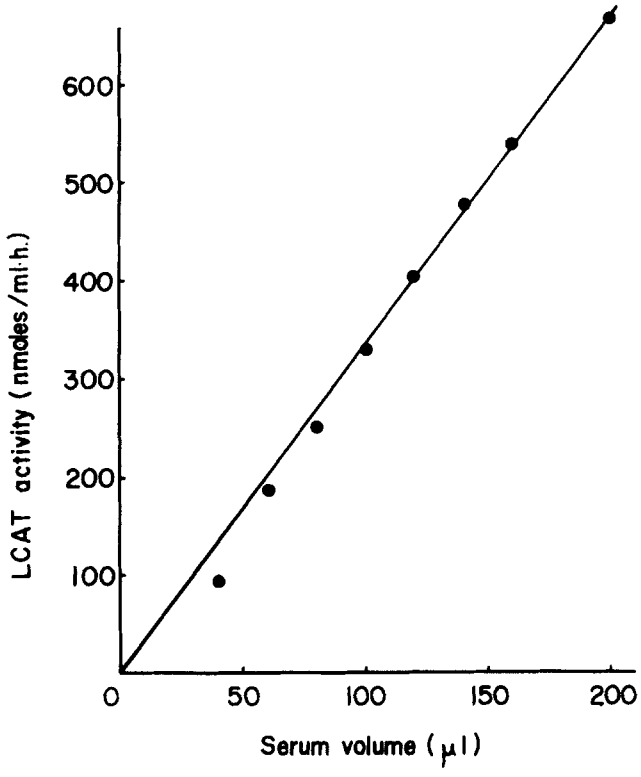

Fig. 8. Serum LCAT activity as a function of serum volume. Various volumes of fresh serum were diluted to a final volume of 200 μl with saline and used as specimens for LCAT determination.

TABLE 1. Activities of prepared enzyme in the presence of heat-inactivated sera from patients with various diseases

Patient Diagnosis	Laboratory Test Results for Serum before Inactivation					Measured LCAT Activity
	ApoA-I	T-Cho	F-Cho	PL	LCAT	
	mg/dl					*nmol per hr per ml*
1 Normal	150	240	60	280	528	318
2 Liver cirrhosis	113	147	45	185	204	303
3 Liver cirrhosis	84	188	57	201	376	325
4 Chronic hepatitis	148	228	60	262	438	295
5 Chronic hepatitis	91	167	49	188	292	307
6 Hyperlipidemia	182	317	104	321	472	318
7 Hyperlipidemia	206	280	73	407	625	295

T-Cho, total cholesterol; F-Cho, unesterified cholesterol; PL, choline-containing phospholipids.

HDL region after incubation of liposomes with serum. This result possibly means that the cholesteryl esters formed by the LCAT reaction, which proceeds on the surface of HDL, might be accumulated within the HDL particles. Another possible explanation is that LCAT might react with interfacial lipids on the surface of liposomes saturated with endogenous apolipoproteins, and the resultant cholesteryl esters are transferred to HDL. However, the latter explanation is less likely, since it is hard to believe that cholesteryl esters would be transferred to HDL rather than to LDL by the lipid transfer system. On the basis of these findings, liposomes added to serum seem to be rapidly taken up by HDL in the presence of LCAT activity and subsequently serve as the substrate for LCAT on the surface of HDL.

A similar activating effect of liposomes on the acylcoenzyme A:cholesterol acyltransferase reaction was investigated by Mathur and Spector (32). They suggested the formation of a complex from liposomes and microsomal extracts containing the enzyme (32). Likewise, the apparent activity of LCAT can be enhanced by changes in the lipid composition on the surface of HDL, with which the enzyme is associated, through the uptake of lipid molecules from liposomes (33).

If the interactions between liposomes and HDL result from collisions, as reported by Chobanian, Tall, and Brecher (34), the enzyme activity would be greatly affected by not only the lipid composition but also the particle size of the liposomes. Indeed, the extent to which serum LCAT was activated showed a dependence on the particle size of the liposomes used as the substrate.

Consequently, with respect to the phospholipid fatty acyl composition, serum LCAT was most active when the added liposomes were prepared from DMPC, although it is believed that phosphatidylcholines with unsaturated fatty acyl chains at the *sn*-2 position were preferable substrates for LCAT (3, 35). However, Pownall, Pao, and Massey (36) recently prepared LCAT substrates (reassembled HDL) from apoA-I, the nonhydrolyzable diether analog of phosphatidylcholine, test phosphatidylcholines and a trace of [^{3}H]cholesterol, and they showed that DMPC was a good phosphatidylcholine substrate. Also Yokoyama, Murase, and Akanuma (37) reported that DMPC was more available for the LCAT reaction than dilinoleoylphosphatidylcholine or dipalmitoylphosphatidylcholine when phosphatidylcholines were incorporated into HDL. Their results are in accordance with ours with regard to the usefulness of DMPC–cholesterol liposomes for estimating the LCAT bound to HDL, since the liposomes in our method are considered to serve as the substrate after being incorporated into HDL. Then it is logical to surmise that the different findings for apparent substrate specificities in other studies are possibly due to differences in the liposome preparations and/or different enzyme sources.

The observed substrate specificities of LCAT in the serum might be closely connected with the transition temperature of phosphatidylcholine. This is because dipalmitoylphosphatidylcholine, with a transition temperature higher than 37°C, was less utilized by LCAT below this temperature, while the activity with DMPC, whose transition temperature is at 23.9°C (31), reached a maximum at about 36°C.

The effect of endogenous substrates seems to be minimized in our method since similar saturation curves were observed with sera from four individuals, irrespective of the endogenous lipid concentrations, when various concentrations of liposomes were added to the sera. Additionally, in the absence of liposomes, the LCAT activities in the sera diluted with a 30 g/l BSA solution are considered to represent the LCAT activities with endogenous substrates under our assay conditions, and they were extremely low. Thus, it is surmised that liposomes could be a more effective substrate pool for the LCAT reaction than the endogenous lipoproteins, so that the LCAT activities are independent of the endogenous substrate's effect.

In contrast to many other reports (11–13, 27, 38), addition of apoA-I to the assay medium caused a slight decrease in enzyme activity. This discrepancy seems to derive from the difference in the reaction mechanism be-

tween our method and the other apoA-I proteoliposome methods. In our method, the LCAT reaction proceeds upon the surface of native HDL particles, into which the lipid molecules of liposomes were transferred, depending on the enzyme activity. On the other hand, the purified LCAT used in the other studies reacts with the interfacial lipids of the apoA-I proteoliposomes, with which the enzyme has been associated. Since the formation of a stable apoA-I-liposome complex possibly inhibits the uptake of the lipid molecules from liposomes and reduces the enzyme activity in our method, the inhibitory effect of apoA-I, shown in Fig. 6, may indicate the occurrence of a weak interaction between liposomes and an excess amount of exogenous apoA-I. An alternative explanation for the absence of an activating effect of apoA-I is that the liposomes are already saturated with endogenous apoA-I or contaminating apoA-I originating from BSA. However, the latter explanation is less likely, because our observations in the HPLC study negate the possibility of formation of an endogenous apoA-I-liposome complex. Also, the effect of BSA in this experiment was negligible, since the inhibition of LCAT activity by apoA-I was observed to be independent of the presence of BSA.

BSA is considered to minimize product inhibition by binding lysolecithin and to stabilize the enzyme during incubation (15, 39). Therefore, in order to avoid possible interference, the commercial preparation of BSA should be purified when the contaminating apolipoproteins are not negligible.

Since our liposomes are not capable of association with LCAT and the LCAT reaction proceeds upon the native lipoproteins, one may assume that the LCAT activity obtained by this method should depend on the endogenous apoA-I content of the serum. However, apoA-I does not seem to be an absolute activator of LCAT because the laboratory findings of patients with HDL deficiency suggested the presence of an alternative lipoprotein which promotes the LCAT reaction in the patient's serum (40). From this suggestion, it is possible to speculate that the concentration of apoA-I in serum does not exert any apparent effect on the LCAT in our method.

With regard to the effect of the concentration of endogenous substrate, if the lipoproteins associating with the enzyme are able to incorporate the lipid molecules of the liposomes, the LCAT reaction may proceed irrespective of the endogenous substrate concentration. These considerations led us to examine the variability in the recovered activity of prepared enzyme added to various heat-inactivated sera, including samples from patients with liver cirrhosis, chronic hepatitis, and hyperlipidemia. As shown in Table 1, in spite of the differences in LCAT activity before inactivation, and the apoA-I and endogenous substrate concentrations in the samples, the observed activities in those samples were almost constant. This result means that interference due to an abnormal lipoprotein composition in a pathological state can be largely avoided.

The absence of an effect by the endogenous cofactors in our method is supported by other findings in our experiments. First, our HPLC study showed that the lipid molecules of liposomes are transferred into HDL, depending on the LCAT activity. This lipid uptake by HDL does not seem to be mediated by apoA-I. Second, the insensitivity of our method to quantitative changes in the endogenous cofactors was shown in Fig. 8, where a linear relationship was observed between the LCAT activity and the serum volume in the assay medium. This result means that the LCAT activity is scarcely influenced by the concentration of endogenous cofactors in the assay medium. On the basis of these findings, it is surmised that apoA-I could not be directly involved in the LCAT reaction with liposomes, and that the added liposomes must be the most important rate-limiting factor in the reaction.

Therefore, the discrepancy between the results with our method and those with the Nagasaki and Akanuma method (16) may be due to the fact that self-substrate methods measure both enzyme and substrate effects.

On the other hand, enzymatic analysis of the cholesterol content of liposomes was highly quantitative, even in the presence of serum components (data not shown). The present method has the advantages of being less time-consuming, very sensitive, and highly reproducible. A comparative study of our new method and the conventional methods is now being carried out.

The authors gratefully acknowledge the valuable technical assistance of Kanako Goto. We would also like to thank Yasuo Sakai for help with the photon correlation spectroscopy studies. Nobuyoshi Ebata kindly supplied apoA-I for our experiments.

Manuscript received 24 November 1986 and in revised form 21 April 1987.

REFERENCES

1. Glomset, J. A. 1962. The mechanism of the plasma cholesterol esterification: plasma fatty acid transferase. *Biochim. Biophys. Acta.* **65:** 128–135.
2. Akanuma, Y., and J. A. Glomset. 1968. A method for studying the interaction between lecithin:cholesterol acyltransferase and high density lipoproteins. *Biochem. Biophys. Res. Commun.* **32:** 639–643.
3. Glomset, J. A., and K. R. Norum. 1973. The metabolic role of lecithin:cholesterol acyltransferase. *Adv. Lipid Res.* **11:** 1–65.
4. Glomset, J. A. 1970. Physiological role of lecithin:cholesterol acyltransferase. *Am. J. Clin. Nutr.* **23:** 1129–1136.
5. Marcel, Y. L., and C. Vezina. 1973. Lecithin:cholesterol acyltransferase of human plasma. Role of chylomicrons, very low and high density lipoproteins in the reaction. *J. Biol. Chem.* **248:** 8254–8259.
6. Chan, M. K., L. Ramdial, Z. Varghese, J. W. Persaud, R. A. Baillod, and J. F. Moorhead. 1982. Plasma lecithin: cholesterol acyltransferase activities in uraemic patients. *Clin. Chim. Acta.* **119:** 65–72.

7. Cucuianu, M., and D. Tapaiaga. 1978. Similar behavior of lecithin:cholesterol acyltransferase and pseudocholinesterase in liver diseases and hyperlipoproteinemia. *Clin. Chim. Acta.* **85:** 73–79.
8. Norum, K. R., and E. Gjone. 1967. Familial plasma lecithin:cholesterol acyltransferase deficiency. Biochemical study of a new inborn error of metabolism. *Scand. J. Clin. Lab. Invest.* **20:** 231–243.
9. Stokke, K. T., and K. R. Norum. 1971. Determination of lecithin:cholesterol acyltransferase in human blood plasma. *Scand. J. Clin. Lab. Invest.* **27:** 21–27.
10. Glomset, J. A., and J. L. Wright. 1964. Some properties of a cholesterol esterifying enzyme in human plasma. *Biochim. Biophys. Acta.* **89:** 266–276.
11. Morin, R. J., and U. Piran. 1981. A lipoprotein independent assay for human serum lecithin:cholesterol acyltransferase. *Clin. Chim. Acta.* **111:** 211–218.
12. Yamazaki, S., T. Mitsunaga, Y. Furukawa, and Y. Nishida. 1983. Interaction of lecithin:cholesterol acyltransferase with human plasma lipoproteins and with lecithin-cholesterol vesicles. *J. Biol. Chem.* **258:** 5847–5853.
13. Jonas, A., and H. T. McHugh. 1983. Reaction of lecithin: cholesterol acyltransferase with micellar complexes of apoprotein A-I and phosphatidylcholine, containing various amounts of cholesterol. *J. Biol. Chem.* **258:** 10335–10340.
14. Nichols, A. V., and E. L. Gong. 1971. Use of sonicated dispersions of mixtures of cholesterol with lecithin as substrate for lecithin:cholesterol acyltransferase. *Biochim. Biophys. Acta.* **231:** 175–184.
15. Chen, C., and J. J. Albers. 1982. Characterization of proteoliposomes containing apoprotein A-I: a new substrate for the measurement of lecithin:cholesterol acyltransferase activity. *J. Lipid Res.* **23:** 680–691.
16. Nagasaki, Y., and Y. Akanuma. 1977. A new colorimetric method for the determination of plasma lecithin:cholesterol acyltransferase activity. *Clin. Chim. Acta.* **75:** 371–375.
17. International Committee for Standardization in Haematology of the European Society of Haematology. 1965. Recommendations and requirements for haemoglobinometry in human blood. *Scand. J. Clin. Lab. Invest.* **17:** 617–620.
18. Guo, L. S. S., R. L. Hamilton, J. Goerke, J. N. Weinstein, and R. J. Havel. 1980. Interaction of unilamellar liposomes with serum lipoproteins and apoproteins. *J. Lipid Res.* **21:** 993–1003.
19. Okazaki, M., Y. Ohno, and I. Hara. 1981. Rapid method for the quantitation of cholesterol in human serum lipoproteins by high performance liquid chromatography. *J. Biochem.* **89:** 879–887.
20. Okazaki, M., N. Hagiwara, and I. Hara. 1982. Quantitation method for choline-containing phospholipids in human serum lipoproteins by high performance liquid chromatography. *J. Biochem.* **91:** 1381–1389.
21. Akanuma, Y., and J. A. Glomset. 1968. In vitro incorporation of cholesterol-^{14}C into very low density lipoprotein cholesteryl esters. *J. Lipid Res.* **9:** 620–626.
22. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
23. Cheung, M. C., and J. J. Albers. 1977. The measurement of apoprotein A-I and A-II levels in men and women by immunoassay. *J. Clin. Invest.* **60:** 43–50.
24. Takayama, M., S. Itoh, T. Nagasaki, and I. Tanimizu. 1977. A new enzymatic method for determination of serum choline-containing phospholipids. *Clin. Chim. Acta.* **79:** 93–98.
25. Allain, C. C., L. S. Poon, C. S. G. Chan, W. Richmond, and P. C. Fu. 1974. Enzymatic determination of total serum cholesterol. *Clin. Chem.* **20:** 470–475.
26. Sakai, Y., K. Itakura, T. Kanada, N. Ebata, K. Suga, H. Aikawa, K. Nakamura, and T. Sata. 1984. Quantitation of apoprotein A-I in pooled human serum by single radial immunodiffusion and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Anal. Biochem.* **137:** 1–7.
27. Yokoyama, S., T. Murase, and Y. Akanuma. 1978. The interaction of apoproteins with lecithin: cholesterol acyltransferase. *Biochim. Biophys. Acta.* **530:** 258–266.
28. Marcel, Y. L., and C. Vézina. 1973. A method for the determination of the initial rate of reaction of lecithin:cholesterol acyltransferase in human plasma. *Biochim. Biophys. Acta.* **306:** 497–504.
29. Patsch, W., S. Sailer, and H. J. Braunsteiner. 1976. An enzymatic method for the determination of the initial rate of cholesterol esterification in human plasma. *J. Lipid Res.* **17:** 182–192.
30. Blomhoff, J. P. 1974. In vitro determination of lecithin:cholesterol acyltransferase in plasma. *Scand. J. Clin. Lab. Invest.* **33:** 35–43.
31. Pownall, H. J., J. B. Massey, S. K. Kusserow, and A. M. Gotto, Jr. 1979. Kinetics of lipid-protein interactions: effect of cholesterol on the association of human plasma high-density apoprotein A-I with L-α-dimyristoylphosphatidylcholine. *Biochemistry.* **18:** 574–579.
32. Mathur, S. N., and A. A. Spector. 1982. Effect of liposome composition on the activity of detergent-solubilized acylcoenzyme A:cholesterol acyltransferase. *J. Lipid Res.* **23:** 692–701.
33. Nichols, A. V., E. L. Gong, T. M. Forte, and P. J. Blanche. 1978. Interaction of plasma high density lipoprotein HDL_{2b} (d 1.063–1.100 g/ml) with single-bilayer liposomes of dimyristoylphosphatidylcholine. *Lipids.* **13:** 943–950.
34. Chobanian, J. V., A. R. Tall, and P. I. Brecher. 1979. Interaction between unilamellar egg yolk lecithin vesicles and human high-density lipoprotein. *Biochemistry.* **18:** 180–187.
35. Assmann, G., G. Schmitz, N. Donath, and D. Lekim. 1978. Phosphatidylcholine substrate specificity of lecithin-cholesterol acyltransferase. *Scand. J. Clin. Lab. Invest.* **38:** 16–20.
36. Pownall, H. J., Q. Pao, and J. B. Massey. 1985. Acyl chain and headgroup specificity of human plasma lecithin:cholesterol acyltransferase. *J. Biol. Chem.* **260:** 2146–2152.
37. Yokoyama, S., T. Murase, and Y. Akanuma. 1977. Phosphatidylcholine substrate specificity of lecithin:cholesterol acyltransferase in high-density lipoproteins and in lipid dispersions. *J. Biochem.* **82:** 719–724.
38. Fielding, C. J., V. G. Shore, and P. E. Fielding. 1972. A protein cofactor of lecithin:cholesterol acyltransferase. *Biochem. Biophys. Res. Commun.* **46:** 1492–1498.
39. Yao, J. K., S. C. S. Chan, R. V. Ryan, and P. J. Dyck. 1980. Serum lipoproteins and albumin in the lecithin:cholesterol acyltransferase reaction. *Biochem. Biophys. Res. Commun.* **95:** 738–744.
40. Ohtaki, S., H. Nakagawa, N. Kida, H. Nakamura, K. Tsuda, S. Yokoyama, T. Yamamura, S. Tajima, and A. Yamamoto. 1983. A Japanese family with high density lipoprotein deficiency. *Atherosclerosis.* **49:** 79–88.