Action of a microbial glycerophospholipid:cholesterol acyltransferase on plasma from normal and LCAT-deficient subjects

J. Thomas Buckley,* Roger McLeod,** and Jiri Frohlich**

Department of Biochemistry and Microbiology, University of Victoria, Victoria, British Columbia, Canada V8W 2Y2* and Department of Pathology, University of British Columbia, Vancouver, British Columbia, Canada V6T 1W5**

Abstract The action of a bacterial acyltransferase similar in overall reaction mechanism to the plasma enzyme lecithin:cholesterol acyltransferase (LCAT) has been studied using normal plasma and lipoproteins and plasma from LCAT-deficient patients. The microbial enzyme (GCAT) catalyzed acyl transfer using phosphatidylcholine and cholesterol in all of the lipoprotein fractions, presumably because it has no apolipoprotein cofactor. In addition, the enzyme was capable of hydrolyzing cholesteryl ester in lipoproteins but not in small unilamellar vesicles nor in micellar dispersions containing low amounts of Triton X-100. This suggests that cholesteryl ester is exposed on the surface of lipoprotein particles or that it may be transferred there quickly from the interior. Although considerable interconversion of radiolabeled cholesterol and cholesteryl ester could be demonstrated upon treatment of normal plasma or lipoproteins with the enzyme, there was little change in the actual amount of either steroid. This indicates that the rate of cholesteryl ester formation is very similar to the rate of hydrolysis. The relative proportions of cholesterol and cholesteryl ester in normal plasma are therefore near the equilibrium ratio for the reaction carried out by GCAT, or the ratio is controlled by the properties of the lipoproteins themselves. During reaction with the microbial acyltransferase, the ratio of cholesterol to cholesteryl ester in plasma from LCAT-deficient patients was reduced substantially, suggesting that the enzyme may have some practical applications.—**Buckley, J. T., R. McLeod, and J. Frohlich.** Action of a microbial glycerophospholipid:cholesterol acyltransferase on plasma from normal and LCAT-deficient subjects. *J. Lipid Res.* 1984. **25:** 913–918.

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The microbial glycerophospholipid:cholesterol acyltransferase (GCAT), recently purified and characterized by one of us (1–3), shares several properties with the mammalian plasma enzyme LCAT (lecithin:cholesterol acyltransferase). Both enzymes are capable of catalyzing the transfer of a fatty acid from the two position of phosphatidylcholine to cholesterol and both will act as phospholipases when no acyl acceptor is present. In addition, neither enzyme has an absolute requirement for divalent cations. Normally, PC and cholesterol in high density lipoproteins are the major substrates for the mammalian enzyme (4), perhaps because LCAT is activated by apolipoprotein A-I, which is found in this fraction. The effect of GCAT on plasma lipoproteins has not been studied; however it is known from the results obtained using lipid vesicles as substrates that the enzyme has no absolute apoprotein cofactor requirements (1). This suggests that the enzyme is likely to catalyze acyl transfer in any of the lipoprotein fractions and, if so, it should prove useful as a tool in the study of lipid interactions in plasma lipoproteins. For this reason and because the enzyme has several potential applications as an analogue of LCAT, we have undertaken a preliminary comparison of the action of both enzymes on normal and abnormal human lipoprotein substrates. This communication summarizes the results obtained using plasma and isolated lipoproteins from normal subjects and using plasma from three patients with LCAT deficiency.

MATERIALS AND METHODS

Materials

EDTA plasma was obtained from five apparently healthy volunteers or from LCAT-deficient patients after a 12-hr fast. Two of the LCAT-deficient patients have been described by one of us (5, 6). The third patient is one of three recently diagnosed siblings investigated by Dr. J. Kane of the University of California at San Francisco. [1,2-^{3}H]Cholesterol and cholesteryl [1-^{14}C]oleate were from New England Nuclear. Cholesterol,

egg phosphatidylcholine, and albumin (essentially fatty acid-free) were from Sigma. ApoA-I was purified as previously described (6).

Preparation of substrates

Lipoprotein fractions were isolated using standard sequential ultracentrifugation methods (7). Whole plasma was used within several hours of venipuncture. Unless otherwise specified, in all experiments where GCAT was to be studied, LCAT activity in the plasma substrates was destroyed by heating at 56–60°C for 30 min before incubation. Liposomes were prepared by ethanol injection as in previous experiments (8). Plasma was labeled with radioactive cholesterol as described by Glomset and Wright (9) and with radioactive cholesteryl ester by exchange from phosphatidylcholine liposomes according to a published procedure (10).

Enzyme purification

LCAT was isolated from human plasma by a modification of the Varma and Soloff procedure (11) as reported earlier (6). GCAT was purified from culture supernatants of *Aeromonas hydrophila* according to the method of Buckley, Halasa, and MacIntyre (1). The microbial enzyme preparation contained a single Coomassie blue-staining band upon electrophoresis in polyacrylamide gels with sodium dodecyl sulfate.

Measurement of cholesteryl esterase

Breakdown of cholesteryl ester was determined using the artificial substrate described by Hyun et al. (12).

Lipid analysis

Incubations were stopped by the addition of 20 vol of chloroform–methanol 2:1 (v:v) and the lipids were extracted according to Folch et al. (13). Phospholipids were separated by thin-layer chromatography as described by Skipski, Peterson, and Barclay (14), neutral lipids were separated on plates developed in hexane–ether–acetic acid 90:10:1 (v/v/v). Lipids were recovered from the thin-layer plates according to the method of Arvidson (15). Phospholipids were quantitated by the procedure of Turner and Rouser (16) and cholesterol and cholesteryl ester were quantitated by the method of Huang et al. (17).

RESULTS

The microbial enzyme GCAT was capable of carrying out the acylation of radiolabeled cholesterol in each of the three lipoprotein classes, whereas the amount of transesterification catalyzed by LCAT was highest by far in the HDL fraction (**Fig. 1**). The results in the
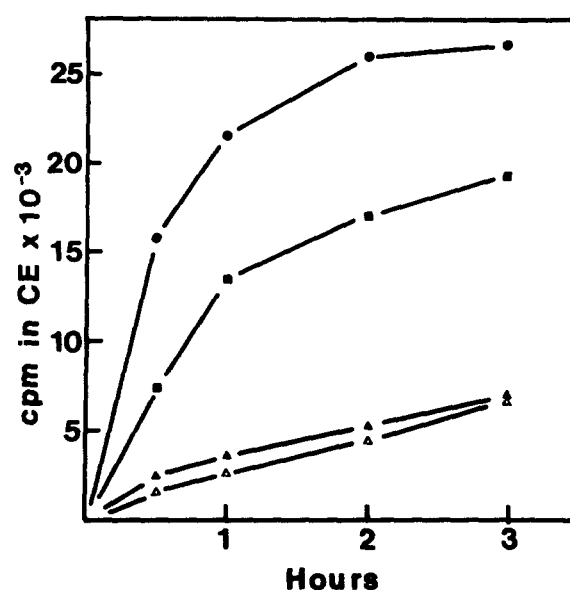

Fig. 1. Plasma lipoproteins as acyl transfer substrates. Action of GCAT on (●) VLDL, (■) LDL, (▲) HDL; (Δ) action of LCAT on HDL. Lipoprotein fractions were isolated from normal plasma, dialyzed against 40 mM phosphate, pH 7.2, labeled with [^{3}H]cholesterol and concentrated to 96 μg of free cholesterol in 0.5 ml. The concentrated lipoproteins (4.8 μg of cholesterol) were incubated with GCAT (20 ng) or LCAT (500 ng) in a total volume of 200 μl for the indicated times prior to extraction and determination of radioactivity in free and esterified cholesterol.

figure also show that the activity of GCAT was much higher than that of the purified plasma enzyme when the substrates were high density lipoproteins radiolabeled with cholesterol, as much less was required to obtain the same amount of esterification of labeled cholesterol as occurred with LCAT.

The results presented in Fig. 1 indicate that GCAT is quite capable of converting large quantities of cholesterol to cholesteryl ester in plasma lipoproteins; however, when the amount of cholesterol actually esterified was measured in whole plasma, it was found that there was very little mass conversion (**Fig. 2**). This was in spite of the fact that a large portion of the radiolabeled cholesterol was converted to cholesteryl ester in the same experiment (**Fig. 3**). It seemed unlikely that the apparent discrepancy could be accounted for by inadequate mixing of the radiolabeled cholesterol with unlabeled cholesterol in the plasma and that a very small amount of transacylation from the pool of high specific activity was being measured, for in the same experiment there was a large amount of plasma PC breakdown (**Fig. 4**). A similar discrepancy between conversion predicted by radioactivity cholesterol incorporation studies and mass transfer data was observed when the action of GCAT on individual lipoprotein classes was examined. Thus the results in **Fig. 5** show that there was considerable incorporation of label into CE of each lipoprotein class
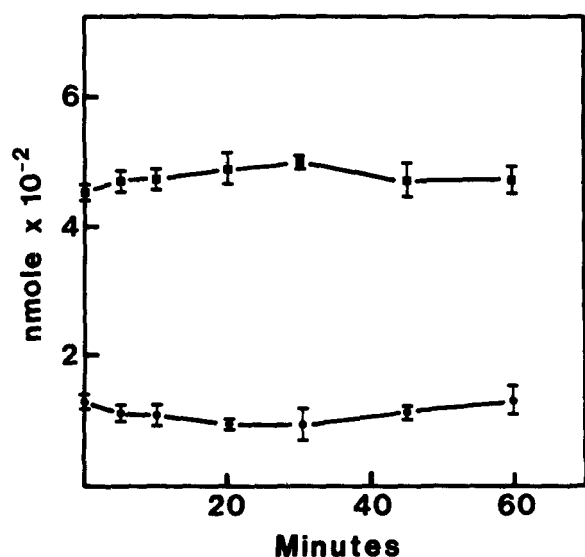
Fig. 2. Changes in cholesterol and cholesteryl ester levels during GCAT action on normal plasma. Each incubation contained 125 μl of plasma from fasting subjects, 1.4% albumin, 40 mM Tris-HCl, pH 7.2, and approximately 500 ng of GCAT in a total of 500 μl. After lipid extraction, samples were taken for measurement of steroid and phospholipid levels as well as for radioactivity measurements. The results are the means ± SD of three determinations. This is one of five very similar experiments with plasma from apparently normal subjects, additional data are in Table 2; (●) cholesterol, (■) cholesteryl ester.

during reaction with the enzyme, yet the proportions of cholesterol and CE did not change appreciably in either LDL or HDL. Only in the VLDL fraction did incorporation of radioisotope into CE lead to a concomitant decrease in unesterified cholesterol mass (Fig. 5).

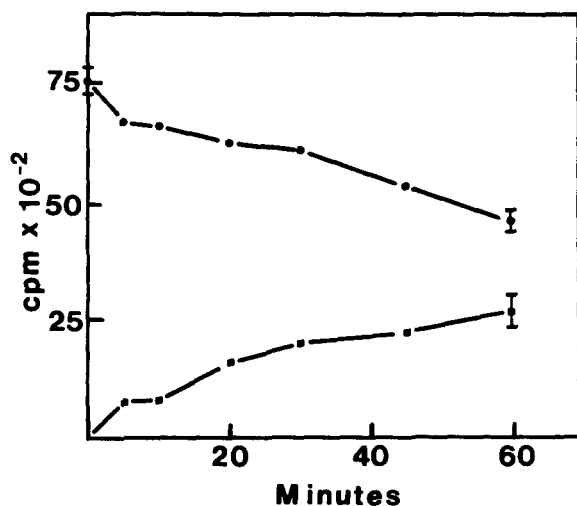These results could be accounted for if the microbial

Fig. 3. Conversion of radiolabeled cholesterol to CE during GCAT action on normal plasma. These data are from the experiment described in Fig. 2. Results are the means ± SD of three determinations (where indicated): (●) cholesterol, (■) cholesteryl ester.
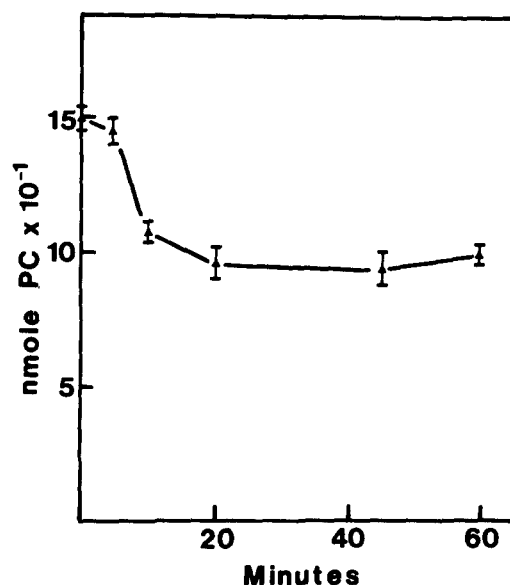

Fig. 4. Changes in PC levels during the action of GCAT on normal plasma. These data are also from the experiment described in the legend to Fig. 2. Results are the means ± SD of three determinations.

enzyme was capable of carrying out the hydrolysis of cholesteryl ester so that changes in the amount of cholesterol due to acylation were counterbalanced by CE breakdown. In order to determine whether cholesteryl ester in the lipoproteins was breaking down, plasma was labeled with CE containing [1-^{14}C]oleate by exchange from liposomes, and the three lipoprotein fractions were isolated. Incubation of each class with GCAT resulted in the hydrolysis of significant quantities of CE (**Table 1**). Nearly all of the label was recovered in free fatty acid. Very little was recovered in PC, indicating that breakdown of CE was not occurring by reversal of the acyl transfer reaction but rather by simple hydrolysis. Very similar results were obtained when unheated plasma was used in the labeling procedure (Table 1). Cholesteryl ester was not hydrolyzed by GCAT when present in small unilamellar liposomes or in Triton micelles, both containing PC (not shown here). Considerable hydrolysis occurred, however, when large amounts of sodium taurocholate were used (**Fig. 6**). As with the lipoproteins, nearly all of the label appeared in free fatty acid.

Changes in cholesterol and CE in plasma from LCAT-deficient patients are shown in **Fig. 7.** It may be seen that there is a large initial decline in cholesterol and a parallel increase in CE as a result of enzyme action. Much of the enzyme-catalyzed acylation occurred in the first 10 min and there was little change in cholesterol levels after this time. This was not due to exhaustion of the supply of the acyl donor PC as nearly half of the original PC remained at the end of the incubation (not shown here). A summary comparison of the effect of
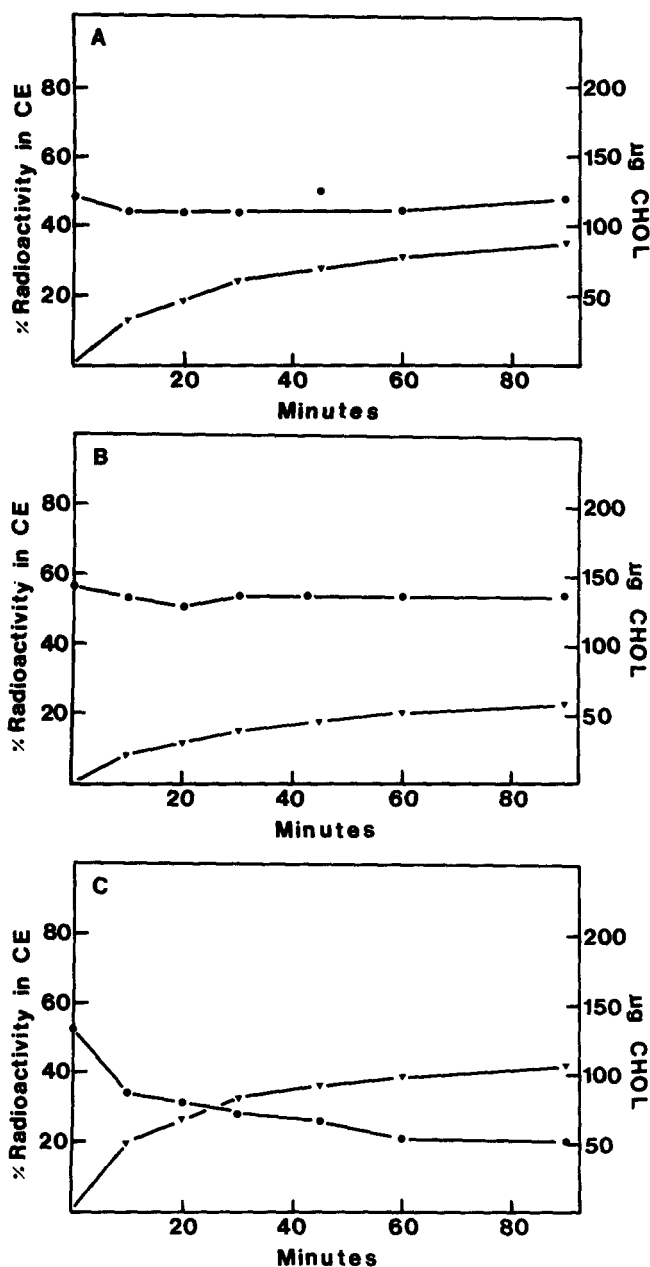
Fig. 5. Comparison of mass transfer and radioactivity incorporation during GCAT action on plasma lipoproteins. A, HDL; B, LDL; C, VLDL; (●) cholesterol mass; (▼) radioactivity in CE. Labeled lipoproteins were prepared as described in Fig. 1. The lipoproteins (125 μg of free cholesterol) were incubated with 500 ng of GCAT in 500 μl.

GCAT on plasma from five normal and three LCAT-deficient subjects is provided by the data in **Table 2.** Ratios of cholesteryl ester to cholesterol in enzyme-deficient plasma were much lower than in normal plasma, and treatment of deficient plasma with GCAT resulted in considerable net esterification of cholesterol so that, after incubation, ratios much closer to normal were reached.

TABLE 1. Action of GCAT on cholesteryl [1-^{14}C]oleate-labeled lipoproteins[a]

Lipoprotein	cpm[b]		
	PC	FA	CE
VLDL, 0 min			2105 (2020)
VLDL, 90 min		1095 (1250)	890 (760)
LDL, 0 min			2100 (1900)
LDL, 90 min		1040 (1230)	850 (650)
HDL, 0 min			2020 (2260)
HDL, 90 min		1325 (2050)	855 (370)

[a] Lipoproteins were labeled with cholesteryl [1-^{14}C]oleate as described in Methods. Each assay contained ^{14}C-labeled lipoproteins (50–57 nmol of phospholipid phosphate) and 250 ng of GCAT in 500 μl of 40 mM Tris-HCl, pH 7.4.

[b] Mean of duplicate determinations which agreed within less than 10%. Values in parentheses are the means of duplicates obtained in a separate experiment in which ^{14}C-labeled lipoproteins from unheated plasma were used in the incubations.

DISCUSSION

The results presented in this communication point to some clear differences between the microbial and mammalian acyltransferases and at the same time indicate the potential usefulness of GCAT in the study of plasma lipoproteins. Because GCAT has no absolute requirement for apolipoprotein cofactors (1), it was not surprising to find it capable of using the cholesterol and PC in any of the lipoprotein classes as substrates for acyl transfer. It was surprising, however, to discover that in whole
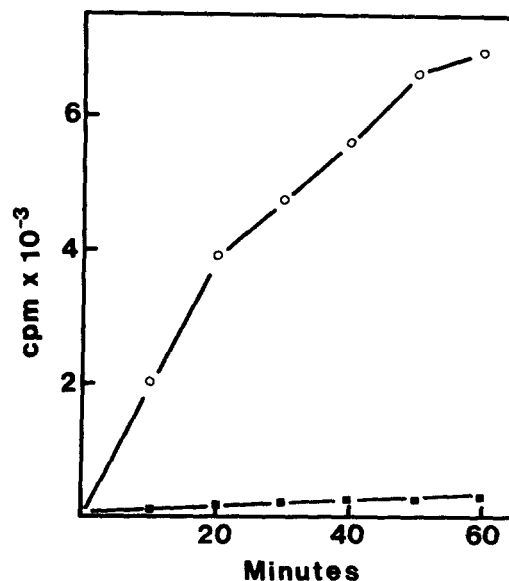

Fig. 6. Breakdown of radiolabeled cholesteryl ester catalyzed by GCAT. Each assay contained 300 μg of PC, 160 μg (25,000 cpm) of cholesteryl [^{14}C]oleate, and 260 μg of sodium taurocholate in 100 μl of 0.1 M Tris, 0.16 M KCl, pH 7.4, containing 500 ng of GCAT: (○) free fatty acid, (■) PC.
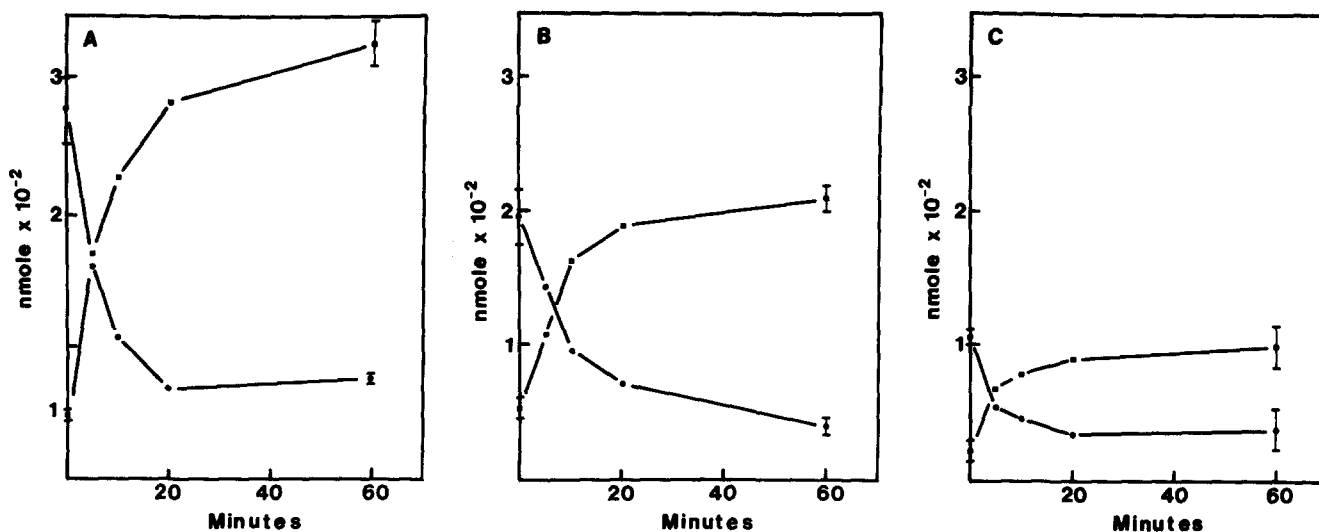
Fig. 7. Action of GCAT on LCAT-deficient plasma. Assay conditions were similar to those used in Fig. 3. Each point is the mean of triplicate determinations ± SD: A, D_1; B, D_2; C, D_3; (●) cholesterol, (■) cholesteryl ester.

plasma, and during the reaction with both HDL and LDL, there was little net cholesteryl ester formation; rather, the results show that the enzyme was effectively catalyzing an exchange of cholesterol by virtue of its ability to carry out the hydrolysis of cholesteryl ester. Only in the VLDL plasma lipoprotein fraction, which normally has a significantly higher cholesterol to CE ratio than the other lipoprotein classes (18), was there appreciable esterification of cholesterol to form CE. Thus it would appear that in the reaction catalyzed by GCAT, cholesterol and CE in HDL and LDL and in plasma are near equilibrium proportions or steady state levels which are maintained as long as there is a sufficient supply of PC to permit continued formation of CE. The proportions of cholesterol and CE in HDL and LDL

may be a result of the properties of the lipoproteins themselves or of the action of LCAT in vivo. It may, however, simply be fortuitous that the microbial enzyme recognizes these proportions as equilibrium values. It was especially interesting to note that after incubation with GCAT the proportions of cholesterol and cholesteryl ester in LCAT-deficient plasma were much closer to those of control plasma.

The observation that GCAT could carry out CE hydrolysis was unexpected, as in sonicated dispersions of PC and cholesterol and in Triton-lipid micelles we have been unable to detect significant CE breakdown. Apparently, in the artificial system used here and in the lipoproteins, conditions are more favorable, perhaps because the CE is more accessible to the enzyme. It is known that only a very small amount of CE will be at the surface of a cholesterol-PC liposome (19) and it seems probable that this will also be true in the Triton micelles, which have a much smaller proportion of detergent to lipid when compared to the taurocholate-solubilized substrate which the enzyme could degrade.

The observation that breakdown of CE in lipoproteins and lipid suspensions does not proceed via acyl transfer to LPC, that is, as the reverse of the forward reaction, was not unexpected. Complex formation between acyl donor and acceptor appears to be required for acyl transfer to occur (3) and it seems most unlikely that CE and LPC would have any affinity for each other. To our knowledge, breakdown of CE by LCAT has not been reported although it has been suggested by Jones et al. (20) to account for net cholesteryl ester hydrolysis in human plasma. The reaction seems quite possible, however, if the enzyme is as capable of approaching CE in lipoproteins as is GCAT. It may be that CE is the

TABLE 2. Changes in plasma cholesteryl ester and in cholesteryl ester to cholesterol ratios as a result of GCAT action on plasma from normal and LCAT-deficient subjects

Subject	nmol Cholesteryl Ester[a]		Cholesteryl Ester/ Cholesterol Ratio[b]	
	0 min	60 min	0 min	60 min
N_1	161 ± 8.8	164 ± 3.8	2.6	2.8
N_2	203 ± 7.3	209 ± 3.4	3.5	4.0
N_3	144 ± 3.3	146 ± 3.0	3.5	3.6
N_4	180 ± 5.0	185 ± 4.1	3.3	3.6
N_5	140 ± 2.0	148 ± 6.0	2.8	2.9
D_1	47.3 ± 5.4	334	0.2	4.4
D_2	52.7 ± 3.5	208 ± 4.3	0.3	6.1
D_3	21.3 ± 7.0	99.7	0.3	2.2

[a] Nanomoles in 50 μl of plasma. Normally, means of three determinations ± SD; otherwise, mean of two very similar determinations. Some of these data (for N_4 and for the LCAT-deficient patients) are taken from Figs. 2 and 7.
[b] Nanomol/nmol.

unidentified acyl donor in the LCAT-catalyzed acylation of lysolecithin which has been reported recently by Subbaiah et al. (21). If LCAT can, in fact, carry out a reversible acylation of cholesterol, its activity could more clearly account for the rather constant ratio of cholesterol to CE observed in normal plasma.

Because GCAT will use at least three of the lipids in each lipoprotein class as potential substrates (PC, cholesterol, and CE), it should be a useful tool in future studies on the relationships of lipids in plasma lipoproteins. In addition, because of its ability to modify cholesterol/cholesteryl ester ratios in LCAT-deficient plasma, it may prove useful as an LCAT substitute in vitro. Clearly, however, the conditions required to control its reaction and the consequences of its action on the structures of the lipoproteins and on cell membranes remain to be established.▪

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