

# Plasma Lipoproteins in Familial Lecithin:Cholesterol Acyltransferase Deficiency: Lipid Composition and Reactivity In Vitro

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**ABSTRACT** Plasma lipoproteins from patients with familial lecithin:cholesterol acyltransferase (LCAT) deficiency have been fractionated by preparative ultracentrifugation and gel filtration and their lipid content and reactivity studied. All of the lipoproteins are abnormal with respect to lipid concentration or relative lipid content. The low density lipoproteins (LDL) and high density lipoproteins (HDL) appear to react normally with partially purified LCAT from normal plasma. Also, the lipids of the very low density lipoproteins (VLDL) and LDL, like those of the corresponding lipoproteins of normal plasma, are indirectly altered by the action of LCAT on normal HDL. Thus, during incubation in vitro VLDL cholesteryl ester is increased and VLDL triglyceride is decreased, as described by others for VLDL from hyperlipemic plasma, and both the unesterified cholesterol and lecithin of the VLDL and LDL are decreased. The patients' VLDL and LDL are abnormal, however, in that they lose unesterified cholesterol and lecithin to normal HDL in the absence of LCAT. Also, the patients' HDL lose these lipids to erythrocyte membranes in the absence of the enzyme.

Our results provide further evidence that the abnormal cholesterol and phospholipid composition of the patients' lipoproteins is caused by the LCAT deficiency. They support the postulate that an excess of unesterified cholesterol and lecithin develops as VLDL are converted to LDL and HDL and suggest that in the absence of LCAT this excess lipid distributes among plasma lipoproteins and plasma membranes. They further suggest

that LCAT normally reduces this excess lipid through a combination of direct and indirect effects.

## INTRODUCTION

An inborn error of metabolism, which may yield important new information about plasma lipoprotein physiology, was recently discovered in Scandinavia (1-3). This disease is characterized by corneal infiltration, anemia, and proteinuria, by several plasma lipoprotein abnormalities, and by absence or near absence of plasma lecithin:cholesterol acyltransferase (LCAT) activity. The disease has been provisionally named familial LCAT deficiency (1) because the lipid abnormalities that seem to be most significant, viz., the unusually low levels of cholesteryl ester and lysolecithin in plasma (1, 3) and the unusually high levels of unesterified cholesterol and lecithin in plasma and erythrocytes (1, 3, 4), appear to be caused by the enzyme deficiency. Nevertheless, many features of the disease remain to be explained or even described at the molecular level. Consequently, inability to synthesize or secrete LCAT is not conclusively established as the primary defect.

One particularly critical question is whether all the plasma lipoprotein abnormalities are directly or indirectly caused by the enzyme deficiency. The possibility has not been ruled out that some primary lipoprotein abnormality underlies the LCAT deficiency. Before this question can be resolved, the lipoprotein abnormalities themselves will have to be carefully defined; not only the lipid composition and reactivity, but also the peptide composition and overall structure of the individual lipoproteins will have to be considered. With this objective in mind we initiated the present investigation of the lipid composition and reactivity toward LCAT of the patient's very low, low, and high density lipopro-

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teins (VLDL, LDL, HDL). We also reasoned that, irrespective of the identity of the primary defect, a study of the abnormalities most directly related to the LCAT deficiency might provide significant information about the normal effects of the LCAT reaction on the composition and structure of plasma lipoproteins.

## METHODS

**Patients.** The three patients studied are females. Two, M. R. (age 21) and A. R. (age 35), were described in the original reports of the disease (1, 2). The third, A. A. (age 42) from a second Norwegian family, was described more recently (5). Except in one series of experiments in which A. R. had been on a high carbohydrate, low fat diet during the week before her blood was withdrawn, the patients were under no special form of treatment.

Plasma was prepared from the blood of the patients and the normal controls after they had fasted overnight. 0.005 M EDTA or acid citrate dextrose solution (U.S.P. Formula A) was used as anticoagulant. Sodium *p*-chloromercuriphenyl sulfonate (PCMPS) was added to the plasma to a final concentration of 0.002 M to facilitate comparison with other experiments with normal plasma, in which PCMPS was always added to inhibit LCAT activity.

Preparative ultracentrifugation of the patients' plasma lipoproteins was performed in Spinco model L or L2 65B preparative ultracentrifuges by a slight modification (6) of the method of Havel, Eder, and Bragdon (7). All lipoprotein fractions were washed at least once by recentrifugation. Duplicate samples were extracted by adding 20 vol of 1:1 chloroform-methanol, and duplicate aliquots of each sample were analyzed. Recoveries of lipoproteins based on analyses of cholesterol, lecithin, or triglyceride usually varied between 85 and 95%.

Gel filtration of the patients' HDL was performed on 4.5 × 150 cm columns of Sephadex G 200 by a method described previously (8) except that the patients' VLDL and LDL were removed by ultracentrifugal flotation at a density of 1.063 g/ml rather than by chromatography on hydroxylapatite. 20-ml fractions of the effluent were collected. In the experiments in which subfractions from the Sephadex columns were rechromatographed or incubated with LCAT, etc., the proteins were concentrated by ultrafiltration through Diaflo filters (Amicon Corp., Lexington, Mass.).

LCAT was prepared from normal plasma as described previously (6). The preparations were usually 40- to 150-fold purified. In all incubation experiments in which the partially purified enzyme was used, the concentration of the enzyme in the incubation medium was adjusted to approximately that of native plasma. This adjustment was based on assays (9) of the activity of the partially purified enzyme and of the freshly obtained plasma used as starting material in preparing the enzyme. These assays, performed 1-2 days before the incubation experiment, were used to calculate the amounts of purified enzyme to be included in the incubation medium. These amounts contained only traces of lipid. In other experiments, advantage was taken of the endogenous LCAT activity present in the  $d > 1.063$  g/ml of proteins of normal plasma. This was done in all cases in which normal HDL were preincubated in the presence of the enzyme and subsequently incubated further with LDL or VLDL. In these experiments  $d > 1.063$  g/ml of proteins were prepared from the plasma by ultracentrifugation, dialyzed against Tris-

EDTA-NaCl<sup>1</sup> for at least 18 hr, and incubated for 18 hr at 37°C (usually in the presence of 0.01 M mercaptoethanol). Subsequently, the enzyme was inactivated by heating at 56-60°C for 30 min or, after removing the mercaptoethanol by dialysis against Tris-EDTA-NaCl, by adding PCMPS to a final concentration of 0.002 M.

**Lipid transfer experiments.** Unless specifically stated otherwise, lipoproteins from the patients or from normal subjects were incubated for 18 hr at 37°C with normal HDL which had either been pretreated with LCAT or had been protected from action of the enzyme *in vitro*. Control experiments were performed by incubating the lipoproteins separately. After the incubations the lipoproteins were chromatographed in quintuplicate on columns of hydroxylapatite (8). The HDL were eluted with 0.25 M potassium phosphate, the salt was removed by dialysis against distilled water, the fractions were concentrated against Aquacide (Calbiochem, Los Angeles, Calif.), and lipids were extracted by adding 20 vol of chloroform:methanol (1:1). Lipids of LDL or VLDL were directly eluted from the column with approximately 15 times the column volume of chloroform:methanol (1:1). Recoveries of total (HDL + LDL or VLDL) lipid varied from 80 to 100% unless specifically noted in the text. Two types of transfer experiments were performed with the patients' HDL. In one the HDL were incubated with erythrocyte membranes prepared from the blood of normal humans (10). After incubation the erythrocyte membranes were removed by centrifugation for 15-30 min at 20,000 rpm in a Spinco No. 40 rotor followed by filtration through Millipore filters (Millipore Corporation, Bedford, Mass.). In the second type of transfer experiment, the patients' HDL were incubated with normal HDL, which had been coupled to Sepharose 2B (Pharmacia Fine Chemicals, New Market, N. J.) (11), and perincubated with LCAT. The coupling procedure routinely yields preparations that contain about the same amount of HDL per gram wet weight of Sepharose as is normally present per milliliter of human plasma. After preincubation with LCAT, the HDL-Sepharose was washed with Tris-EDTA-NaCl, reacted with 0.1 M *N*-ethylmaleimide for 20 min at room temperature to inactivate residual LCAT activity, and again washed with Tris-EDTA-NaCl. After incubating the HDL-Sepharose with the patients' HDL, the latter were separated from the coupled HDL by filtration.

**Lipid analyses.** Measurements of cholesterol and phospholipid were performed as described previously (8). Triglyceride measurements were performed by the method of Wieland (12) after hydrolysis of material scraped from the neutral lipid thin-layer chromatography plates in 1 N KOH in 95% ethanol for 45 min at 70°C.

Statistical calculations were performed by Student's *t* test or by the *F* test, using a BMD 05 V computer program (13).

## RESULTS

**Plasma lipoprotein lipids.** Plasma lipoprotein lipids of six normal females are compared to those of three female patients with familial LCAT deficiency in Tables I and II. Note that our data comparing the lipids of normal lipoproteins (Table I) support and extend the findings of others (14-17). They show that the following significant relationships exist: the ratio of cho-

<sup>1</sup> 0.01 N Tris HCl, 0.001 M EDTA, 0.14 N NaCl, pH 7.4.

TABLE I  
*Plasma Lipoprotein Lipids of Six Normal Females, Aged 22–35\**

Lipoproteins	UC†	CE/UC‡	PC‡	PC/UC§	PC/S§	TG‡
VLDL	0.084 ±0.074	0.755 ±0.094	0.077 ±0.008	0.826 ±0.151	8.00 ±1.71	0.220 ±0.223
LDL (1.006–1.019)	0.020 ±0.008	1.30 ±0.131	0.010 ±0.005	0.455 ±0.087	5.89 ±1.34	0.009 ±0.003
LDL (1.019–1.063)	0.627 ±0.134	2.16 ±0.153	0.336 ±0.088	0.519 ±0.039	2.21 ±0.395	0.071 ±0.012
HDL	0.514 ±0.085	2.84 ±0.202	1.07 ±0.151	2.11 ±0.253	6.20 ±0.998	0.052 ±0.009

The following abbreviations have been used: UC = unesterified cholesterol; CE = cholesteryl ester; PC = lecithin; S = sphingomyelin; TG = triglyceride; LDL (1.006–1.019) = LDL of densities between 1.006 and 1.019 g/ml; LDL (1.019–1.063) = LDL of densities between 1.019 and 1.063 g/ml.

\* None of the subjects was on oral contraceptive medication.

† Values for lipids given in  $\mu$ moles per ml  $\pm$ SD.

§ The following relationships exist (Student's *t* test):

(a) for CE/UC, VLDL < LDL (1.006–1.019),  $P < 0.005$ ; LDL (1.006–1.019) < LDL (1.019–1.063),  $P < 0.005$ ; LDL (1.019–1.063) < HDL,  $P < 0.005$ .

(b) for PC/UC, VLDL > LDL (1.006–1.019),  $P < 0.005$ ; LDL (1.006–1.019), LDL (1.019–1.063), NS; LDL (1.019–1.063) < HDL,  $P < 0.005$ .

(c) for PC/S, VLDL > LDL (1.006–1.019),  $P < 0.025$ ; LDL (1.006–1.019) > LDL (1.019–1.063),  $P < 0.005$ ; LDL (1.019–1.063) < HDL,  $P < 0.005$ .

lesteryl ester to unesterified cholesterol increases with increasing lipoprotein density; the ratio of lecithin to unesterified cholesterol is significantly higher in the VLDL than in the LDL, although the ratio is highest in the HDL; and the ratio of lecithin to sphingomyelin decreases with increasing density among the VLDL and LDL, but is high in the HDL. Table II shows that all of the patients' lipoproteins are abnormal in relative lipid content and that some of the lipoprotein classes contain increased absolute concentrations of lipid. All of the patients' lipoproteins contain decreased cholesteryl ester relative to unesterified cholesterol, and the ratio of cholesteryl ester to unesterified cholesterol

decreases rather than increases with increasing lipoprotein density. The fact that the lowest ratio is associated with the HDL is consistent with the LCAT deficiency because studies of normal plasma (18) have shown that HDL are preferred substrates of the enzyme. The fact that the highest ratio is associated with the VLDL is consistent with previous evidence (1) that the few cholesteryl esters present in the plasma of the patients are formed in the intestine rather than in the plasma or liver.

The ratios of lecithin to unesterified cholesterol in the patients' plasma lipoproteins also show abnormalities. The ratio decreases slightly if at all from the VLDL

TABLE II  
*Plasma Lipoprotein Lipids of Three Patients with Familial LCAT Deficiency\**

Patient	Lipoproteins	UC	CE/UC	PC	PC/UC	PC/S	TG
M. R.	VLDL	0.107	0.514	0.083	0.775	12.1	0.308
	LDL (1.006–1.019)	0.151	0.231	0.095	0.629	7.24	0.142
	LDL (1.019–1.063)	0.934	0.134	0.650	0.696	7.72	—
	HDL	0.402	0.080	0.392	0.975	7.83	0.043
A. R.	VLDL	6.39	0.145	3.45	0.540	8.48	—
	LDL (1.006–1.019)	0.711	0.055	0.390	0.548	6.78	—
	LDL (1.019–1.063)	4.50	0.022	2.56	0.568	7.08	—
	HDL	0.334	0.036	0.302	0.904	7.38	—
A. A.	VLDL	2.06	0.185	1.21	0.583	8.41	4.68
	LDL (1.006–1.019)	0.482	0.131	0.231	0.479	6.60	0.375
	LDL (1.019–1.063)	1.76	0.014	0.910	0.516	6.94	0.366
	HDL	0.506	0.058	0.397	0.784	6.84	0.067

Values for UC, PC, and TG given in  $\mu$ moles per ml.

\* Abbreviations as in Table I.

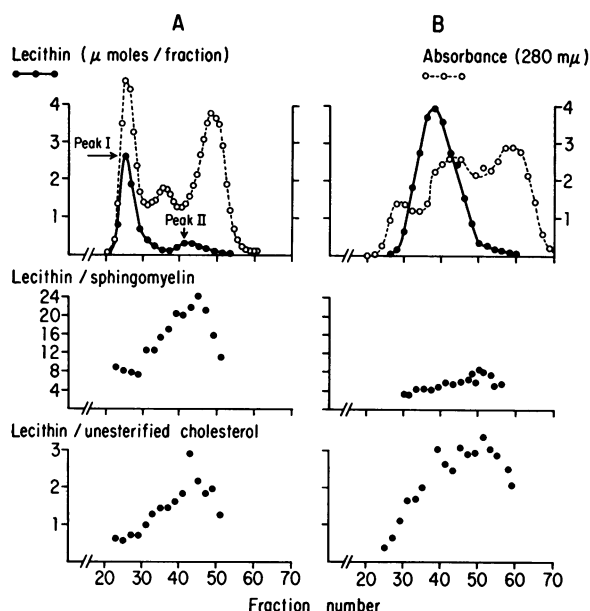


FIGURE 1 (A) Cholesterol and phospholipid compositions of HDL subfractions from patient M. R. proteins of  $d > 1.063$  g/ml from approximately 40 ml of plasma were filtered through Sephadex G 200, and the lipids of the effluent were analyzed. The upper part of the figure shows absorbance of proteins and elution of lecithin. The lower two parts show the ratios of lecithin/sphingomyelin and lecithin/cholesterol. (B) Cholesterol and phospholipid compositions of HDL subfractions from the plasma of a normal female. Redrawn from data published earlier (8). Experimental and figure details as in part A except that VLDL and LDL were removed by chromatography on hydroxylapatite before gel filtration.

to the LDL, and in the HDL the ratio is less than half of normal. The ratio of lecithin to sphingomyelin appears to decrease from the VLDL to the LDL of densities 1.006–1.019 g/ml, but does not decrease further to the LDL of densities 1.019–1.063 g/ml. As a result the ratio is about three times greater than normal in the lipoproteins of this density, a fact which is consistent with the absence of LCAT, since LCAT decreases lecithin but has no effect on sphingomyelin.

Total concentrations of VLDL cholesterol, lecithin, and triglyceride are increased in the plasma of A. R. and A. A., but not in the plasma of M. R., the youngest patient. The increased concentrations of VLDL in the two older patients seem to be associated with increased concentrations of LDL, although total concentrations of unesterified cholesterol and lecithin in the LDL are increased in all the patients. The concentration of HDL unesterified cholesterol is normal or nearly normal, but the concentrations of HDL lecithin and total cholesterol are less than half of normal.

Further abnormalities of the patients' HDL are illustrated by the experiment shown in Fig. 1. In this ex-

periment proteins of  $d > 1.063$  g/ml from the plasma of M. R. were subfractionated by gel filtration on Sephadex G 200. The two lipoprotein subfractions shown in Fig. 1 A (solid curve) emerge in the same positions as those obtained by Torsvik (19). They correspond approximately to the flanks of the single peak always obtained in comparable experiments with normal plasma (Fig. 1 B, solid curve, top panel). They contain about twice as much lecithin relative to sphingomyelin as do normal HDL subfractions that emerge in comparable positions but are similar to normal HDL in that the subfractions of smaller apparent molecular weight contain more lecithin relative to sphingomyelin than do those of higher apparent molecular weight. Note that a slow increase in the ratio of lecithin to unesterified cholesterol occurs with increasing elution volume throughout a large part of the effluent. The rapid decline in the ratios of lecithin to sphingomyelin and lecithin to unesterified cholesterol toward the end of HDL peak II may have been caused by tailing of HDL peak I. In one experiment tailing was observed when the lipoproteins of this peak were concentrated and refiltered through the column, and the material in the tail contained lecithin and sphingomyelin in the same proportion present in the major peak. Results comparable to those shown in Fig. 1, although with less pronounced second peaks, were observed in experiments with the plasma of A. R. and A. A.

*Reactivity of the patients' lipoproteins with LCAT.* The patients' lipoproteins appear to react normally with partially purified preparations of LCAT from normal plasma (Table III, Fig. 2). The patients' VLDL, like normal VLDL (6), do not react significantly with the enzyme, but the patients' LDL do. Fig. 2 shows that the patients' HDL react with LCAT and suggests that the smaller molecular weight HDL (HDL peak II)

TABLE III  
Incubation of Lipoproteins from M. R. and A. R. with Partially Purified LCAT

Subject	Lipoproteins incubated	Cholesteryl esters		Significance*
		0 hr	24 hr	
		$\mu\text{moles/ml} \pm \text{SD}$		
M. R.	VLDL	$0.057 \pm 0.003$	$0.057 \pm 0.002$	NS
	LDL	$0.315 \pm 0.007$	$0.443 \pm 0.015$	<0.0005
A. R.	VLDL	$0.517 \pm 0.020$	$0.531 \pm 0.013$	NS
	LDL	$0.125 \pm 0.019$	$0.227 \pm 0.014$	<0.0005

Lipoprotein concentrations approximately equal to those in native plasma (different plasma samples from those shown in Table II). LCAT prepared from normal plasma. Activity per milliliter incubation medium approximately equal to that present in normal plasma (see Methods). Incubation performed at 37°C in presence of 0.01 M mercaptoethanol. Cholesteryl ester in LCAT preparation ( $0.011 \mu\text{mole/ml}$  of incubation medium) subtracted to obtain values in table.

\* Student's *t* test.

are more reactive than the larger molecular weight HDL (HDL peak I). A similar relation has been observed (8) between the reactivity of smaller and larger molecular weight subfractions of normal HDL. It should be noted that the experiment shown in Fig. 2 was designed to compare the reactivity of the two HDL subfractions on the basis of their initial content of lecithin<sup>2</sup> and that normal erythrocyte membranes were included in the incubation mixture to provide a continuing supply of unesterified cholesterol (20), but that both the lecithin and the unesterified cholesterol of the HDL subfractions decreased in the presence of these membranes (Table IV). This decrease occurred even in the absence of LCAT and also occurred in experiments with HDL from A. R. Since the absolute amounts of lecithin and unesterified cholesterol lost from the HDL peak I subfraction were greater than those lost from the HDL peak II subfraction (Table IV), the two HDL substrates in the experiment shown in Fig. 2 are not strictly comparable on the basis of lecithin content. Nevertheless, the absolute increase in cholesteryl ester in the HDL peak II subfraction was almost three times greater than that in the HDL peak I subfraction, a relation far out of proportion to the difference in lecithin content between the two subfractions. Hence, the HDL peak II subfraction appears to have been a better substrate. Note that LCAT promoted an increase in the total cholesterol of both subfractions compared to controls incubated with erythrocyte membranes in the absence of the enzyme (Table V). A nearly 3-fold increase occurred in the case

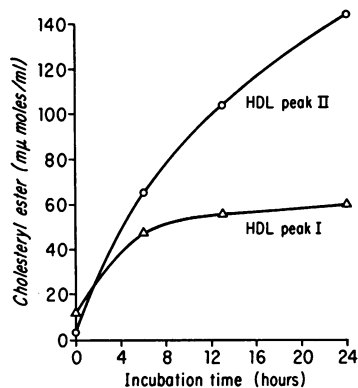


FIGURE 2 Reactivity of HDL subfractions with LCAT. Plasma proteins of  $d > 1.063$  g/ml from M. R. were fractionated by gel filtration as shown in Fig. 1. HDL peaks I and II were concentrated and incubated with normal erythrocyte membranes and LCAT at 37°C. Initially, 0.17  $\mu$ mole of lipoprotein lecithin and 1.9  $\mu$ moles of membrane lecithin were present per ml of incubation medium, and the concentration of LCAT was approximately equal to that of normal plasma.

<sup>2</sup> Evidence (18) exists that lecithin is a limiting factor in the LCAT reaction.

TABLE IV  
Incubation of HDL Peaks I and II from the Plasma of M. R. with Erythrocyte Membranes from Normal Plasma\*

	UC	PC
	<i>μm moles/ml</i>	
HDL peak I		
0 hr	156	124.1
24 hr	77	60.0
HDL peak II		
0 hr	76	111.4
24 hr	62	89.1

Same experiment shown in Fig. 2. Each flask contained erythrocyte membranes and one of the two HDL subfractions in concentrations given in the text of Fig. 2. No LCAT was present. At 0 and 24 hr aliquots were removed, and the lipids of the soluble lipoproteins were measured. Values for control flasks containing membranes and Tris-EDTA-NaCl buffer, but no HDL subfractions, were subtracted to obtain HDL lipid shown in table.

\* Abbreviations as in Table I.

of the HDL peak II subfraction, whereas the increase in total cholesterol of the HDL peak I subfraction was only 1.4-fold.

*Nonenzymic transfer of VLDL lipid.* In experiments with VLDL from patients with familial hyperlipemia, Rehnberg and Nichols (21) and Nichols and Smith (22) showed that LCAT promotes nonenzymic transfers of cholesteryl ester from HDL to VLDL and of triglyceride from VLDL to HDL. Figs. 3-5 indicate that the patients' VLDL can participate in similar reactions. In the experiment shown in Figs. 3 and 4 VLDL from patient A. R. were incubated with proteins of  $d > 1.063$

TABLE V  
Increase in Total Cholesterol of the HDL Subfractions of M. R. upon Incubation for 24 hr with Normal Erythrocyte Membranes and LCAT\*

	Cholesterol			Per cent increase total
	UC	CE	Total	
	<i>μm moles/ml</i>			
HDL peak I + membranes	77.1	6.6	83.7	
HDL peak I + membranes + LCAT	56.9	60.3	117.2	
			$\Delta 33.5$	40
HDL peak II + membranes	62.4	8.0	70.4	
HDL peak II + membranes + LCAT	56.9	146.8	203.7	
			$\Delta 133.3$	190

Same experiment shown in Fig. 2. Values for control flasks containing membranes or membranes + LCAT subtracted to obtain values in table. All values obtained after 24 hr incubation.

\* Abbreviations as in Table I.

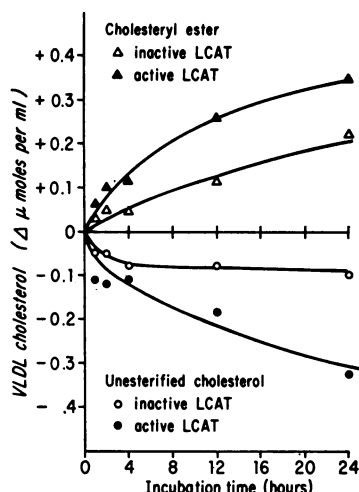


FIGURE 3 Incubation of VLDL from patient A. R. with proteins of  $d > 1.063$  from normal plasma in the presence or absence of LCAT. 0.01 M mercaptoethanol was added to activate LCAT in the appropriate flasks. 0.002 M PCMPS was added to the remaining flasks to inhibit LCAT. At the intervals shown in the figure VLDL and HDL were reisolated by chromatography on hydroxylapatite. The upper part of the figure shows the increase in VLDL cholesteryl ester (starting concentration  $0.39 \mu\text{mole/ml}$ ). The lower part of the figure shows the decrease in VLDL unesterified cholesterol (starting concentration  $1.28 \mu\text{moles/ml}$ ). Corresponding initial values of HDL cholesteryl ester and unesterified cholesterol were 0.51 and 0.16, respectively.

g/ml from normal plasma in the presence or absence of LCAT activity. VLDL cholesteryl ester (Fig. 3, upper part) increased by 90% during the 24 hr incubation in the presence of the enzyme; the corresponding increase in the absence of the enzyme was 58%. During the same period HDL triglyceride (Fig. 4) increased by 240 and 158% in the presence and absence of the enzyme. More important physiologically, in the presence of LCAT the initial rate of increase in VLDL cholesteryl ester was

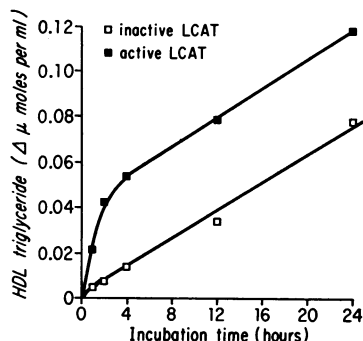


FIGURE 4 Increase in the triglyceride of normal HDL upon incubation with VLDL from patient A. R. Same experiment as shown in Fig. 3. Initial values of VLDL and HDL triglyceride were 2.28 and  $0.05 \mu\text{moles/ml}$ , respectively.

about  $0.05 \mu\text{mole/ml per hr}$ , while the corresponding rate of increase in HDL triglyceride was about  $0.02 \mu\text{mole/ml per hr}$ , about 10 and 40%, respectively, of the initial concentrations of HDL cholesteryl ester and triglyceride.

Fig. 3 (lower part) also shows that the unesterified cholesterol of the VLDL of A. R. decreased and that the change was greater in the presence than in the absence of the enzyme. The changes in VLDL unesterified cholesterol and cholesteryl ester in this experiment probably were indirect effects of the LCAT reaction since LCAT has no effect on the patients' VLDL in the absence of HDL (Table III) and since similar changes occur when VLDL are incubated in the presence of normal HDL that have been preincubated with the enzyme. In the experiment shown in Fig. 5 VLDL unesterified cholesterol decreased and VLDL cholesteryl ester increased significantly even though control values for the total unesterified cholesterol and cholesteryl ester of the mixture (not shown) did not change. Similarly, a significant, although smaller, decrease in VLDL lecithin also occurred in the absence of a change in total (VLDL + HDL) lecithin. However, in this experiment the yields of lecithin from the hydroxylapatite columns were unaccountably low (65%) in both test and control columns. No significant change in VLDL triglyceride

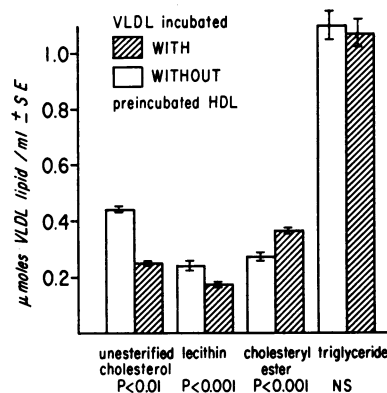


FIGURE 5 Incubation of VLDL from patient M. R. with preincubated HDL from a normal subject. Plasma proteins of  $d > 1.063$  g/ml were preincubated in Tris-EDTA-NaCl buffer for 16 hr at  $37^\circ\text{C}$  in presence of autologous LCAT which decreased the HDL unesterified cholesterol and lecithin by about 90 and 20%, respectively. After preincubation LCAT was inhibited by adding PCMPS (final concentration 0.002 M). One aliquot of the preincubated proteins was incubated with VLDL from M. R. for 18 hr at  $37^\circ\text{C}$  and five equal portions of the mixture were chromatographed on columns of hydroxylapatite. A second aliquot was incubated for 18 hr alone and then chromatographed on five columns of hydroxylapatite with VLDL from patient M. R. which had also been incubated for 18 hr alone. Amount of preincubated HDL per ml corresponded to about  $0.96 \mu\text{mole}$  of cholesteryl ester,  $0.02 \mu\text{mole}$  of unesterified cholesterol,  $0.52 \mu\text{mole}$  of lecithin, and  $0.08 \mu\text{mole}$  of triglyceride.

was demonstrated in this experiment, but the triglyceride of the HDL that had been incubated for 18 hr in the presence of VLDL was 246% of the triglyceride of the HDL control.

Fig. 6A shows that the unesterified cholesterol and lecithin of VLDL prepared from normal subjects also decrease when the VLDL are incubated with preincubated normal HDL. The design of the experiment was similar to that shown in Fig. 5. The decreases in VLDL unesterified cholesterol and lecithin that occurred in the presence of the preincubated HDL were highly significant; similar results were obtained in two other experiments. Note that in all experiments the decrease in lecithin was less than that in unesterified cholesterol. Although the increase in VLDL cholesteryl ester was of marginal significance in this experiment, highly significant increases occurred in the other experiments. These results appear to reconcile the earlier observation (23) that the unesterified cholesterol and lecithin of VLDL decrease during the incubation of whole human plasma with the more recent finding (6) that LCAT does not attack VLDL directly. Fig. 6B shows that no significant change in either unesterified cholesterol or lecithin occurs when normal VLDL are incubated with normal HDL not previously incubated with LCAT in vitro. The apparent, though not statistically significant, decrease in the lecithin of the VLDL was probably caused by loss during chromatography and subsequent

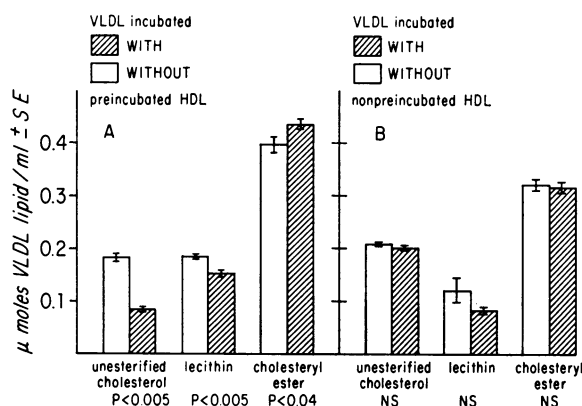


FIGURE 6 (A) Incubation of normal VLDL with preincubated normal HDL. Experimental design similar to the experiment shown in Fig. 5 except that the proteins of  $d > 1.063$  g/ml were preincubated for 18 hr which decreased the unesterified cholesterol and lecithin by 86 and 17%, respectively. Also, LCAT was heat inactivated. The amount of preincubated HDL/ml of incubation medium corresponded to 1.18  $\mu$ moles of cholesteryl ester, 0.05  $\mu$ mole of unesterified cholesterol, and 0.64  $\mu$ mole of lecithin. (B) Incubation of normal VLDL with untreated HDL. Same VLDL and same HDL used as in part A except that the HDL was not preincubated. Amount of HDL lipid per ml: 0.87  $\mu$ mole of cholesteryl ester, 0.31  $\mu$ mole of unesterified cholesterol, and 0.78  $\mu$ mole of lecithin.

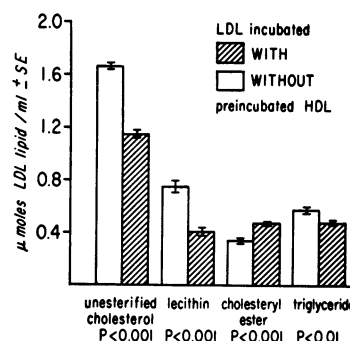


FIGURE 7 Incubation of LDL of  $d = 1.019-1.063$  g/ml from the plasma of M. R. with preincubated HDL from a normal subject. Normal plasma proteins of  $d > 1.063$  g/ml from same pool as in the experiment shown in Fig. 5 were preincubated in the presence of LCAT, incubated with LDL from M. R., and chromatographed on hydroxylapatite.

extraction. The combined recovery of VLDL and HDL lecithin from the aliquots incubated alone was 98%, whereas that for the aliquots incubated together was 90%. (Corresponding values for the experiment shown in Fig. 6A were both 101%.) It can be concluded that the patients' VLDL resemble the VLDL of normal individuals insofar as the lipids of both are similarly affected by preincubated normal HDL but that the patients' VLDL are abnormal in that the unesterified cholesterol and lecithin are altered by incubation with normal HDL not previously incubated with LCAT in vitro.

*Nonenzymic transfer of LDL and HDL lipid.* The patients' LDL of  $d 1.019-1.063$  g/ml participate in nonenzymic lipid transfer reactions in the same general way as do the VLDL of the patients (Figs. 7 and 8). The

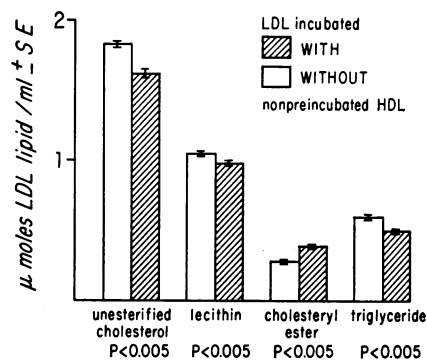


FIGURE 8 Incubation of LDL of  $d = 1.019-1.063$  g/ml from the plasma of A. R. with untreated HDL from a normal subject. Experimental design as in the experiment shown in Fig. 5. Plasma proteins of  $d > 1.063$  g/ml were prepared in presence of 0.002 M PCMPS, dialyzed, and heated for 30 min at 56°C before being incubated with the LDL. Initial HDL lipids: cholesteryl ester, 1.61  $\mu$ moles/ml; unesterified cholesterol, 0.56  $\mu$ mole/ml; lecithin, 1.38  $\mu$ moles/ml; and triglyceride, 0.17  $\mu$ mole/ml.

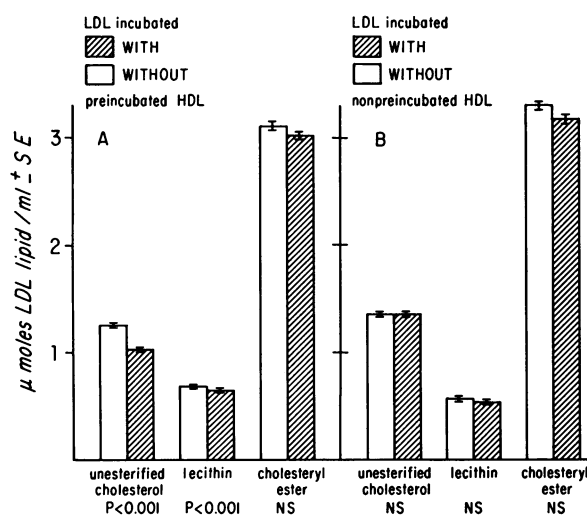


FIGURE 9 (A) Incubation of normal LDL of  $d=1.019$ – $1.063$  g/ml with preincubated normal HDL. Experimental design as in experiment shown in Fig. 5. Plasma proteins of  $d > 1.063$  g/ml were as in experiment shown in Fig. 6 A. (B) Incubation of normal LDL of  $d=1.019$ – $1.063$  g/ml with nonpreincubated HDL. Same LDL used as in part A. Same HDL preparation and same HDL lipid concentration as in experiment shown in Fig. 6 B.

design of the experiments shown in these figures was similar to that of the experiment shown in Fig. 5. Incubation with normal, preincubated HDL increased the LDL cholesteryl ester and decreased the LDL unesterified cholesterol, lecithin, and triglyceride (Fig. 7). Similar, although less pronounced, transfers occurred when the patients' LDL were incubated with normal nonpreincubated HDL (Fig. 8).

Fig. 9 A shows an experiment in which normal LDL were incubated with normal HDL which had previously reacted with LCAT. The LDL unesterified cholesterol and lecithin decreased but neither the LDL cholesteryl ester nor triglyceride changed. Similar results were obtained in a second experiment. Since the decrease in lecithin in both experiments was considerably less than the decrease in unesterified cholesterol, these results appear to explain why incubation of whole plasma causes a smaller decrement in LDL lecithin than in LDL unesterified cholesterol (23). While direct action of LCAT causes nearly equimolar changes in lecithin and unesterified cholesterol (24), nonenzymic transfers of these lipids subsequently occur from LDL to HDL, and these are not equimolar.

Fig. 9 B shows an experiment in which the same normal LDL used in the experiment in Fig. 9 A were incubated with HDL which had not previously reacted with LCAT in vitro. No significant changes in LDL unesterified cholesterol, lecithin, cholesteryl ester, or triglyceride occurred. It can be concluded that the patients'

LDL are abnormal in that they gain cholesteryl ester and lose triglyceride in the presence of either preincubated or untreated HDL and lose unesterified cholesterol and lecithin in the presence of untreated HDL.

Fig. 10 shows that extensive transfer of lipid occurred when the HDL peak I subfraction from the plasma of A. A. was incubated with normal, preincubated HDL. In this experiment the normal HDL were coupled to Sepharose to facilitate separation from the abnormal HDL (see Methods). Incubation with the preincubated HDL-Sepharose markedly decreased the lecithin and unesterified cholesterol and increased the cholesteryl ester of the abnormal lipoproteins. These results suggest that the patients' HDL peak I lipoproteins behave similarly to the patients' VLDL and LDL. However, they must be qualified in view of the evidence (25) that normal HDL are aggregates comprised of several subunits because exchange of whole subunits as well as transfer of individual lipids may have occurred.

## DISCUSSION

All of the classes of lipoproteins in normal plasma contain high proportions of cholesteryl ester (see, for example, Table I), and most of this cholesteryl ester appears to be formed by the LCAT reaction (18). Presumably, LCAT begins to affect the composition of lipoproteins as soon as they are secreted into the blood and with other enzymes, such as lipoprotein lipase, converts these "precursor" lipoproteins into the classes of lipoproteins commonly recognized in plasma. The details of this conversion are not well understood for several

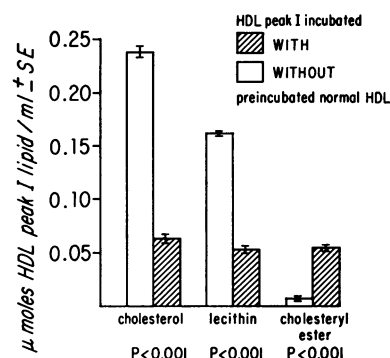


FIGURE 10 Incubation of HDL peak I lipoproteins from A. A. with preincubated normal HDL. HDL peak I lipoproteins prepared as in the experiment shown in Fig. 1 were incubated in quintuplicate for 24 hr at  $37^{\circ}\text{C}$  with 1 g wet weight portions of (normal) HDL-Sepharose or control Sepharose which had been preincubated for 24 hr with partially purified LCAT. Values for the lipid contents of the HDL-Sepharose and preincubated HDL-Sepharose are not available, but incubation with LCAT affects HDL-Sepharose in the same way as it affects uncoupled HDL (Akanuma, Y., and J. Glomset. Unpublished experiments).



reasons, one being the problem of distinguishing newly secreted lipoproteins from the bulk of circulating lipoproteins. The existence of familial LCAT deficiency appears to provide a unique approach to this problem because the abnormal lipoproteins of the patients' plasma may resemble normal "precursor" lipoproteins. Studies of the composition and reactivity of these abnormal lipoproteins may provide useful information about the intravascular events that generate normal lipoproteins. One difficulty with this approach is that the patients' lipoproteins show several abnormalities that cannot be related to LCAT deficiency on the basis of present knowledge. All of the patients have abnormally low concentrations of HDL in the plasma (1) (compare Tables I and II), all have VLDL with abnormally slow electrophoretic mobilities (1),<sup>8</sup> and most of the patients have hyperlipemia (1) (Table II). Furthermore, undiscovered lipoprotein abnormalities not immediately related to the LCAT deficiency may exist. Consequently, the validity of the comparison of the patients' lipoproteins with newly secreted or newly formed normal lipoproteins depends on how well abnormalities directly related to the LCAT deficiency are distinguished from those that are not. We have attempted to make this distinction by focusing attention on aspects of the lipoproteins that studies of normal plasma have shown are related to the LCAT reaction. We have studied the cholesterol and phospholipid composition of the lipoproteins, the ability of the lipoproteins to react with partially purified LCAT from normal plasma, and the ability of the lipoproteins to undergo nonenzymic transfers of lipid promoted by the LCAT reaction.

Our results indicate that the patients' lipoproteins all contain abnormally low proportions of cholesteryl ester and that several contain abnormally high concentrations of unesterified cholesterol and lecithin. They indicate also that these abnormalities are not caused by inability of the lipoproteins to react with LCAT or participate in nonenzymic lipid transfer reactions. Consequently, they suggest that the cholesterol and phospholipid abnormalities are directly related to the LCAT deficiency. Although the patients' lipoproteins undergo some abnormal lipid transfer reactions, these are also probably related to the LCAT deficiency. The transfer of unesterified cholesterol and lecithin from the patients' VLDL or LDL to untreated, normal HDL is probably analogous to the transfer of the same lipids from normal VLDL or LDL to preincubated HDL. In both cases the transfer reactions presumably depend on the ability of unesterified cholesterol and lecithin to exchange and reach an equilibrium distribution among lipoproteins (26, 27) and on the ability of the LCAT

reaction to perturb this equilibrium. Fresh samples of normal plasma presumably contain lipoproteins that are near equilibrium. Therefore, net redistribution of lipid does not occur unless the equilibrium is perturbed by the separate or preferential action of LCAT on HDL. The patients' lipoproteins probably are also at or near equilibrium. However, when they are incubated with lipoproteins from normal plasma, redistribution of lipid presumably occurs because the patients' lipoproteins contain proportionately more lecithin and unesterified cholesterol than the lipoproteins of normal plasma, i.e., incubation of normal HDL with LCAT *in vitro* is unnecessary because the normal HDL have already been reacting with LCAT *in vivo*.

The net transfer of cholesteryl ester from normal HDL to the patients' LDL probably also depends on the LCAT deficiency. The cholesteryl esters of HDL and LDL appear to be at or near equilibrium since transfer from HDL to LDL can be demonstrated by measuring radioactivity (6) but not by measuring mass (Fig. 9 A and B). However, the patients' LDL contain so little cholesteryl ester that transfer *from* the LDL to normal HDL is probably much smaller than transfer from HDL *to* the LDL. Instead, triglyceride, which is present in considerably greater amounts, transfers from the LDL as it does from VLDL.

Our results support the observations of Rehnborg and Nichols (21) and Nichols and Smith (22) that the LCAT reaction promotes exchange of HDL cholesteryl ester for VLDL triglyceride. They indicate also that the reaction indirectly promotes removal of VLDL unesterified cholesterol and lecithin. Consequently, if these reactions occur *in vivo* and if removal of large amounts of VLDL triglyceride by peripheral lipolytic reactions is also taken into account, the net effect is probably to increase considerably the relative content of cholesteryl ester in the VLDL.

The fact that two HDL subfractions can be clearly distinguished in the patients' plasma suggests that two precursors of normal HDL may exist. The smaller subfraction (HDL peak II, Fig. 1 A) appears to correspond to the lower molecular weight HDL subfractions obtained on gel filtration of normal HDL (Fig. 1 B). The lower molecular weight subfractions from both types of plasma contain relatively large proportions of lecithin and appear to be better substrates of LCAT than higher molecular weight HDL subfractions. These properties of the normal HDL and the fact that the cholesteryl esters of corresponding subfractions from baboon plasma rapidly become labeled after the *in vivo* injection of radioactive mevalonate formed the basis of a postulate (8) that the lower molecular weight HDL subfractions contain recently secreted lipoproteins. The HDL peak II subfraction of the patients' plasma may

<sup>8</sup> Norum, K. R. Unpublished experiments.

contain recently secreted lipoproteins for similar reasons.

The main HDL subfraction (HDL peak I, Fig. 1) is actually comprised of HDL and not contaminating LDL because the lipoproteins behave like HDL when chromatographed on hydroxylapatite (19), because they migrate faster than  $\beta$ -lipoproteins on electrophoresis (19),<sup>8</sup> and because they cross-react with anti- $\alpha$ -lipoprotein serum and not with anti- $\beta$ -lipoprotein serum (19). It is possible that the HDL peak I lipoproteins, which have ratios of lecithin to sphingomyelin and of lecithin to unesterified cholesterol so similar to the VLDL (in the case of A. R. and A. A.) and the LDL (in all of the patients), may have arisen from the VLDL. Evidence (28) exists that HDL are formed during the degradation of VLDL by lipoprotein lipase; and high molecular weight subfractions of normal HDL are known to contain peptides in common with VLDL (29). We are currently exploring the possibility that HDL with properties similar to those of normal HDL may be formed when the two subfractions of the patients' HDL are incubated with normal LCAT.

It is of interest to compare the concept (30) that the role of the LCAT reaction is to reduce the excess unesterified cholesterol and lecithin of lipoproteins that have reacted with lipoprotein lipase to the concept (18) that the reaction regulates the cholesterol content of plasma membranes and serves in the transport of cholesterol from peripheral tissues to the liver. We believe that the concepts are not mutually exclusive and that they should be combined. The fact that net transfers of unesterified cholesterol and lecithin occur from the patients' VLDL and LDL to normal HDL suggests that an excess of these lipids indeed develops in the absence of LCAT. Also, it seems likely that this excess distributes among plasma lipoproteins and plasma membranes and that this accounts for the abnormally large amounts of unesterified cholesterol and lecithin associated with the patients' erythrocytes (4). Our results suggest that LCAT normally reduces this excess through a combination of direct and indirect effects. The enzyme does not react directly with VLDL but is able to decrease indirectly VLDL unesterified cholesterol and lecithin by promoting nonenzymic transfers to HDL. Similarly, LCAT promotes nonenzymic transfer of unesterified cholesterol and to a lesser extent lecithin from LDL to HDL, although it also acts directly on LDL. These indirect effects can be presumed to occur in vivo because LCAT reacts preferentially with HDL (8) and because this preferential action would be expected to have a relative effect on HDL unesterified cholesterol and lecithin analogous to that of the separate HDL-LCAT preincubation step in our in vitro experiments (see Figs. 6 A and 9 A). It is worth noting that because of

the nonenzymic transfers much of the HDL unesterified cholesterol that reacts with LCAT is probably derived from VLDL and LDL, whereas much of the cholesteryl ester formed probably transfers to VLDL and may ultimately become the major lipid component of LDL (30-32). Seen from a wider perspective, the net effect of the LCAT reaction could be to prevent unesterified cholesterol and lecithin originally derived from VLDL from excessively accumulating in plasma membranes. For reasons outlined previously (18), it seems also likely that the LCAT reaction indirectly affects cholesterol synthesized or pathologically deposited in peripheral tissues, which introduces the possibility that the transport of cholesterol from peripheral tissues to the liver may compete with the removal of excess VLDL cholesterol, i.e., that the rate of VLDL turnover may affect the rate of removal of tissue cholesterol.

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