Effect of very low-density lipoproteins on lipid transfer in incubated serum

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SUMMARY Incubation of human serum solutions low in glyceride content for 16 hr resulted in a net increase of cholesterol esters in all three major human serum lipoprotein classes, namely the very low-density lipoproteins of d < 1.006 (VLDL), low-density lipoproteins of d 1.006–1.063 (LDL) and high-density lipoproteins of d 1.063–1.21 (HDL). Transfer of glycerides from the VLDL to the LDL and HDL was also observed during incubation. Reductions in lipoprotein unesterified cholesterol and phospholipids occurred during incubation and indicated the presence of serum fatty acid transferase activity in these solutions.

When serum was incubated in the presence of high concentrations of VLDL lipoproteins, the increase in cholesterol esters was found only in the VLDL fraction. The VLDL apparently took up newly-formed cholesterol esters produced by serum fatty acid transferase activity, and accepted some cholesterol esters initially bound to the LDL and HDL. When the transferase was inhibited with sodium *p*-hydroxymercuribenzoate, some of the cholesterol esters initially bound to the LDL and HDL were still transferred to the VLDL. The HDL class was then the principal contributor of the cholesterol esters. These observations indicate that reciprocal transfer of cholesterol esters for glycerides in human serum lipoproteins can occur.

KEY WORDS serum incubation · lipoproteins · lipid transfer · effect of very low-density lipoproteins · glycerides · cholesterol esters · fatty acid transferase · inhibition · transesterification · ultracentrifugal fractions

An earlier report (1) from this laboratory has described a substantial increase in the glyceride content of high-density lipoproteins of d 1.063–1.21 (HDL) isolated from the lipemic serum of healthy subjects receiving a fat load. Since the serum concentration of the glyceride-rich very low-density lipoproteins of d < 1.006 (VLDL) was also markedly elevated in these subjects, the HDL-glyceride increase may have resulted from a transfer in vivo of glyceride from the VLDL to the HDL. The ability of HDL to bind additional glyceride has been demonstrated by Ashworth and Green (2) in experiments in which HDL species were exposed to Celite-dispersed glyceride. During in vitro incubation experiments in which VLDL were added to serum samples low in glyceride content, Nichols and Smith (3) observed significant transfer of VLDL glyceride to HDL. Furthermore, this study showed a considerable reduction in the amount of cholesterol esters bound to the HDL during uptake of glyceride by the HDL. In the present paper, we report our studies, in detail, on lipid transfer between all major human serum lipoprotein classes, the VLDL, LDL (low-density lipoproteins of d 1.006–1.063), and HDL in sera incubated with large amounts of added human VLDL lipoproteins. In addition, preliminary data are presented on the influence of inhibition of the cholesterol esterifying enzyme of human serum—the serum fatty acid transferase as described by Glomset (4)—on this lipid transfer.

EXPERIMENTAL METHODS

Serum for the incubation experiments was separated by methods previously described (5) from freshly-drawn blood of two fasting female subjects (D.P., age 43, and J.C., age 44) who exhibit low levels of VLDL. The VLDL that were added to the above sera for lipid transfer studies were rapidly separated ultracentrifugally (6) from the sera of two apparently healthy male subjects (R.H., age 53, and D.D., age 40) having elevated levels of these lipoproteins. The sera of subjects D.P. and J.C. were incubated with the VLDL from subjects R.H. and D.D. respectively.

Serum mixtures were incubated with gentle shaking in tightly-capped lusteroid tubes (6 ml) for 0 and 16 hr in a 37° water bath. The experimental protocol con-

sisted of duplicate incubation studies on a total of 4 ml of the following mixtures: 3 ml of serum with 1 ml of a salt solution (d = 1.006); and 3 ml of serum with 1 ml of a salt solution (d = 1.006) containing VLDL. In order to investigate lipid transfer when serum fatty acid transferase activity was inhibited, an additional incubation experiment was performed on a serum mixture, consisting of 3 ml of serum (subject J.C.) and 1 ml of VLDL solution (subject D.D.), which was 0.001 M in sodium *p*-hydroxymercuribenzoate (Sigma Chemical Co., St. Louis, Mo.).

At the end of all incubation periods, 2 ml of salt solutions (d = 1.006), containing appropriate amounts of sodium *p*-hydroxymercuribenzoate to yield a final concentration of 0.001 M of this compound, were added to each sample. The purpose of adding these solutions was to bring the mixture volumes to 6 ml, as the first step in the ultracentrifugal isolation of the three major lipoprotein classes (7), and to inhibit the cholesterol esterifying activity of the serum fatty acid transferase (4). All salt solutions used in the preparative ultracentrifugal procedures contained 0.10 g/liter of the disodium salt of ethylenediamine tetraacetic acid. Evaluation of possible contamination of the LDL and HDL fractions by VLDL lipids, resulting either from the incubation or ultracentrifugation procedures, was performed by control studies on solutions of VLDL alone. For incubated solutions of VLDL, ranging in glyceride concentrations between 300 and 450 mg/100 ml, the contamination of LDL and HDL fractions by VLDL lipids totaled on the average only 3 mg/100 ml and 1.5 mg/100 ml respectively. The above concentrations of total contaminating lipids, as well as all other lipid concentrations reported in this paper, are calculated for the volume comprising the incubation mixture (4 ml). The magnitude of these contaminations in the separate lipid classes is negligible relative to the concentration changes observed for lipid transfer in this investigation.

Lipids were extracted from the lipoprotein solutions by a modified method of Sperry and Brand (8), and each lipid extract was fractionated by silicic acid chromatography using the elution solvent mixtures described by Hirsch and Ahrens (9). The chromatographed lipids (cholesterol esters, glycerides, unesterified cholesterol, and phospholipids) were quantified by an infrared spectrophotometric method reported by Freeman et al. (10).

RESULTS

Lipid Concentrations in VLDL, LDL, and HDL Fractions Isolated from Incubated Solutions of Serum

Table 1 shows the changes in lipid concentrations of the three major lipoprotein fractions following incubation of the sera with saline solution (d = 1.006).

Cholesterol ester concentrations increased in all three lipoprotein fractions in the incubated sera of both subjects. The largest increases in cholesterol ester concentrations were observed in the LDL fractions.

Glyceride concentrations decreased in the VLDL and increased in the LDL and HDL of both serum samples. The glyceride concentration of the LDL of subject J.C. increased by about 41% following incubation, while the glyceride concentration in subject D.P.'s LDL fraction showed only an 8% increase. This difference in response is probably due to the considerable difference in the initial VLDL glyceride concentration in the sera of these two subjects. The initial glyceride concentration

TABLE 1 CHANGE IN LIPID CONTENT OF ULTRACENTRIFUGAL FRACTIONS ISOLATED FROM INCUBATED SERUM SOLUTIONS

	Lipid Concentration (in mg/100 ml)*					
	VLDL		LDL		HDL	
	Nonincubated	Incubated	Nonincubated	Incubated	Nonincubated	Incubated
			Subject D.P.			
Cholesterol esters	1.0(0.5)†	2.2(0.5)	65.1(2.3)	71.5(2.2)	60.7(1.1)	64.4(2.4)
Glycerides	3.9(0.2)	1.9(0.4)	9.7(0.6)	10.5(0.5)	4.4(0.4)	6.9(0.4)
Unesterified cholesterol	0.4(0.1)	0.5(0.1)	15.1(0.7)	11.9(0.2)	10.0(0.6)	7.3(0.5)
Phospholipids	1.2(0.2)	1.0(0.1)	38.3(2.9)	36.1(1.6)	78.2(1.1)	67.1(2.2)
			Subject J.C.			
Cholesterol esters	11.3(3.2)	16.3(3.5)	139.5(2.8)	150.8(3.0)	55.0(0.0)	60.5(2.5)
Glycerides	32.3(3.8)	16.3(2.0)	21.5(0.9)	36.3(1.5)	11.5(0.5)	13.8(0.9)
Unesterified cholesterol	5.5(1.0)	2.5(0.4)	27.3(1.1)	17.3(0.8)	6.3(0.4)	4.0(0.2)
Phospholipids	13.8(2.3)	8.3(1.6)	75.3(1.5)	69.0(2.0)	70.3(1.5)	57.5(1.2)

* Lipid concentrations tabulated in this and subsequent tables are concentrations of lipid present in the incubated volume. Incubation for 16 hr at 37°.

† Values in parentheses in this and subsequent tables are standard errors of measurement $SE_M = \sqrt{(\Sigma d^2/2k)}$, where d = difference between duplicates, and k = number of pairs of duplicates. These values represent the estimate of the standard error for a single pair of duplicates.

TABLE 2 CHANGE IN LIPID CONTENT OF ULTRACENTRIFUGAL FRACTIONS ISOLATED FROM INCUBATED SERUM CONTAINING ADDED VLDL

Lipid	Lipid Concentration (in mg/100 ml)					
	VLDL		LDL		HDL	
	Nonincubated	Incubated	Nonincubated	Incubated	Nonincubated	Incubated
	Serum (Subject D.P.) Plus VLDL (Subject R.H.)					
Cholesterol esters	71.3(2.5)	124.4(2.3)	65.0(3.6)	53.3(2.3)	58.1(0.2)	35.6(1.0)
Glycerides	440.2(17.5)	365.4(9.0)	11.9(0.2)	39.7(0.2)	4.4(0.2)	38.0(0.8)
Unesterified cholesterol	31.0(4.2)	28.5(5.9)	14.9(0.7)	9.5(0.4)	9.7(0.1)	5.9(0.6)
Phospholipids	98.5(2.9)	82.7(1.8)	39.5(2.5)	38.3(2.4)	78.5(1.8)	66.8(1.3)
	Serum (Subject J.C.) Plus VLDL (Subject D.D.)					
Cholesterol esters	109.5(2.2)	148.8(3.0)	142.0(2.8)	134.5(2.0)	56.5(1.3)	53.0(1.0)
Glycerides	310.8(9.3)	243.8(11.6)	22.8(0.8)	69.8(3.2)	12.8(0.6)	31.0(1.2)
Unesterified cholesterol	36.3(5.4)	25.8(3.6)	28.0(1.0)	25.8(1.5)	5.8(0.9)	4.0(0.6)
Phospholipids	113.8(2.3)	96.5(3.0)	72.0(2.2)	69.5(1.8)	68.3(1.4)	57.5(1.2)

of the VLDL fraction of subject J.C. was about 8 times greater than that of subject D.P.

Unesterified cholesterol concentrations were reduced in all three fractions in the serum of subject J.C., and in the LDL and HDL fractions in the serum of D.P. following incubation. The unesterified cholesterol concentration in the VLDL of subject D.P. was extremely low and from these data it is not possible to determine the direction of its change following incubation.

Phospholipid concentrations decreased in all three lipoprotein fractions in the incubated serum solutions of both subjects.

The reductions in unesterified cholesterol and phospholipid concentrations can be explained as resulting from the activity of a serum fatty acid transferase as described by Glomset (4). The decrement in measured lipoprotein phospholipids results from (*a*) a migration (11) of some of the newly formed lysolecithin from lipoprotein sites to the $d > 1.21$ fraction, and (*b*) from an incomplete recovery of the remaining lipoprotein-bound lysolecithin by the lipid extraction and processing procedures used.

Lipid Concentrations in VLDL, LDL, and HDL Fractions Isolated from Serum Incubated with Added VLDL

Table 2 shows the effects of incubation on the distribution of lipids bound to lipoproteins in serum mixtures of both subjects containing large amounts of added VLDL lipoproteins. Emphasis in this report is on the effect of very high concentrations of VLDL in the incubation media. In the present work, the concentrations of VLDL glyceride in the initial incubation mixtures, after the addition of the concentrated VLDL fractions, were 440.2 mg/100 ml with the serum of subject D.P. and 310.8 mg/100 ml with the serum of subject J.C.

Cholesterol ester concentrations in the VLDL fractions increased markedly in the incubation mixtures for both serum samples. Cholesterol ester concentrations in the LDL and HDL fractions were reduced in both serum samples. Thus, in spite of a definite net increase in the total cholesterol ester concentration in the incubation mixtures (18.9 mg/100 ml with serum of subject D.P. and 28.3 mg/100 ml with serum of subject J.C.), the cholesterol esters of only the VLDL fractions were found to increase. These data show that the VLDL bind not only some of the newly formed additional cholesterol esters, arising from serum fatty acid transferase activity, but also some of the cholesterol esters that were initially bound to the LDL and HDL.

Considerable reductions in the glyceride concentrations of the VLDL fractions were observed following incubation in both serum mixtures. The glyceride concentrations in the LDL and HDL fractions, however, were markedly increased in both serum samples.

Unesterified cholesterol and phospholipid concentrations showed the qualitative changes that indicate the presence of serum fatty acid transferase activity in the incubation systems. Since these two determinations are subject to the recovery limitations previously mentioned, quantitative interpretations of their changes will not be stressed.

Lipid Concentrations in Lipoprotein Fractions after Incubation of Serum Mixtures with Sodium p-Hydroxymercuribenzoate

Table 3 shows the effects of incubation on the distribution of lipoprotein lipids in the serum of subject J.C. containing added VLDL and an inhibitor of serum fatty acid transferase. These results indicate that the transfer of glyceride from VLDL to LDL and HDL, and the transfer of cholesterol esters from the LDL and HDL to the VLDL can proceed in the absence of fatty acid transferase activity. Inhibition of the enzyme activity is demonstrated by the relative constancy of the unesterified

cholesterol and phospholipid concentrations in all three lipoprotein fractions. With inhibitor present, the source of the cholesterol esters of the VLDL fraction appears to be predominantly the cholesterol esters of the HDL fraction, as indicated by the 26% reduction in the cholesterol ester concentration of this fraction. We also find, in agreement with the results in the incubation studies of Minari and Zilversmit (12) on dog chylomicrons, a transfer of phospholipid from the VLDL to the HDL fraction in the absence of serum fatty acid transferase activity.

DISCUSSION

Glomset (11) and Rehnborg and Nichols (13) have described the distribution of cholesterol esters among serum lipoproteins following incubation of sera containing relatively low levels of VLDL lipoproteins. Their data, as well as those presented in this report, show an increase of cholesterol esters in all three lipoprotein classes. These increases are attributed mainly to the activity of a serum fatty acid transferase and indicate that the cholesterol esters produced are bound to serum lipoproteins during incubation. The data of this report, as well as those of Rehnborg and Nichols (13) also show that at relatively low levels of VLDL in the incubated serum there occurs a transfer of glyceride from the VLDL to the LDL and HDL.

At the very high VLDL concentrations used in this study, we find, in agreement with the above observations, both an increased total serum cholesterol ester concentration due to transferase activity and a considerable transfer of glycerides from the VLDL to the LDL and HDL. The distribution, however, of the cholesterol esters among the major classes of lipoproteins is markedly different when elevated concentrations of VLDL are present in the incubation medium, for then the increased concentration of cholesterol esters is found only in the VLDL and the concentrations of cholesterol esters in the LDL and HDL drop below their initial values. These data indicate that VLDL, in high concentration, bind not only some of the transferase-formed cholesterol esters but also take up some of the cholesterol esters initially bound to the LDL and HDL.

When the serum fatty acid transferase is inhibited by addition of sodium *p*-hydroxymercuribenzoate to the incubation system, some cholesterol esters are still taken up by the VLDL and some glycerides are still transferred from them. This experiment shows that net lipid transfer can take place in the absence of transferase activity. In this case, we find for the VLDL a gain of approximately 1 mole of cholesterol esters (assumed molecular weight of 651) for the loss of 1 mole of triglyceride (assumed molecular weight of 885). For the LDL we calculate a transfer roughly of 2 moles of cholesterol esters for 3 moles of triglyceride; and for the HDL we calculate approximately 2 moles of cholesterol esters for 1 mole of triglyceride. It should be emphasized that these calculations for lipid substitution are very approximate and, although the inhibitor has removed possible complications due to transferase activity, the calculations are still subject to other complications in the incubation system. These complications include (*a*) the use of extremely high concentrations of VLDL, (*b*) the presence of all three major lipoprotein classes in the incubation solutions, and (*c*) difficulties in lipoprotein recovery during the ultracentrifugal procedures.

The physical transfer of cholesterol esters and glycerides between human serum lipoproteins is apparently a basic property of these macromolecules. Lindgren et al. (14) have observed a strong positive statistical correlation between serum VLDL concentrations and the HDL-glyceride contents in the serum of healthy male subjects. These observations, together with the preliminary data from our fat ingestion studies (1), suggest that similar physical transfer of lipid may occur in vivo. From the available data, it is not possible to determine whether the suggested in vivo transfer is restricted only to the blood compartment or if other cellular or tissue compartments are also involved. If other compartments are involved, then the lipid transfer reaction might play an active role in the transport of lipids from one compartment to another. Furthermore, this type of lipid

TABLE 3 CHANGE IN LIPID CONTENT OF ULTRACENTRIFUGAL FRACTIONS ISOLATED FROM INCUBATED SERUM (SUBJECT J.D.) CONTAINING ADDED VLDL (SUBJECT D.D.) PLUS SODIUM *p*-HYDROXYMERCURIBENZOATE

	Lipid Concentration (in mg/100 ml)					
	VLDL		LDL		HDL	
	Nonincubated	Incubated	Nonincubated	Incubated	Nonincubated	Incubated
Cholesterol esters	101.3(2.0)	118.8(2.5)	142.9(2.3)	137.3(2.5)	56.7(1.2)	41.8(1.3)
Glycerides	303.6(6.1)	278.3(5.2)	21.3(0.6)	32.0(1.1)	11.0(0.7)	21.3(1.7)
Unesterified cholesterol	35.0(5.0)	32.8(4.3)	27.8(1.1)	26.3(1.3)	6.0(0.9)	6.0(0.8)
Phospholipids	116.0(2.1)	108.8(2.2)	77.5(1.6)	77.0(2.0)	73.5(2.5)	78.8(2.0)

transfer reaction also could contribute to the accumulation of cholesterol esters in tissue.

Minari and Zilversmit (12) found an increase in free cholesterol and a decrease in phospholipid in dog chylomicrons following incubation with serum. These were 3-hr incubations and no transferase activity was detected. The glyceride concentrations in their incubation mixtures were considerably lower than those used in the present study. Because of these differences in the incubation times and glyceride concentrations, it is reasonable to say that lipid transfer as described here would probably not be observed in their system. Furthermore, dog lymph chylomicrons possibly may not exhibit the same lipid transfer properties as human VLDL. Thus, in our studies, we have observed a markedly lower lipid transfer with a coconut oil emulsion (Ediol, Schenlabs Pharmaceuticals, Inc., New York, N.Y.), which of course resembles chylomicrons only roughly, than with human VLDL or egg yolk lipoproteins when studied at comparable glyceride concentrations. It is very possible that considerable differences in lipid transfer properties may exist between lipoprotein species of $S_f > 400$ and those of S_f 20–400. This question is currently under investigation.

Rowen (15) has reported that HDL are specifically degraded, and their cholesterol esters are set free, when serum is incubated for 2–6 hr with a streptococcal extract. This degradation is associated with an increase in opalescence of the incubation mixture. The material, predominantly cholesterol esters, responsible for the opalescence could be floated away from the parent solution upon preparative ultracentrifugation of the mixture. In these experiments the VLDL species were removed from the serum prior to incubation with the streptococcal extract. The mechanism whereby the extract degrades the HDL was not established in these investigations. More recently, Rowen and Martin (16) have observed an enhancement of transferase activity in serum mixtures containing the extract. They suggest that disruption of HDL structure exposes lipids to the transferase and thus enhances its activity. Their observation of cholesterol ester release by some form of lipoprotein degradation is of interest here since it is apparently another mechanism for initiating lipid transfer. It should be emphasized, however, that in all the experiments on lipid transfer reported by us in this paper, there was no observable increase in opalescence nor any evidence of HDL degradation. The data in the present report describe a lipid transfer process which occurs in incubated serum solutions between essentially intact lipoproteins.

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